Faheem A. Sheikh *Editor*

Engineering Materials for Stem Cell Regeneration

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I want to dedicate this book to my Ph.D. supervisor Prof. Hak Yong Kim of Jeonbuk National University, South Korea. Furthermore, some persons, such as Prof. Javier Macossay of the University of Texas Rio Grande Valley, USA, Prof. Chan Hum Park of Hallym University, South Korea, Gilson Kang of Chonbuk National University, South Korea, and last but not least great Prof. Hern Kim of Myongji University, South Korea, taught me how to be a good person and motivated me to help society by doing excellent research. I am also grateful to Nasser A M Barakat, who gave me professional and spiritual training. I also want to dedicate this book to my wife, Er. Iqra Shafi, and my son, Sheikh Mohmmad Sabik, for helping me to complete this book.

Preface

In the scientific literature, we can find many reviews that are preferably written after investigating materials used in diverse applications while using approaches based on principles of materials sciences. This book, which I published under the title *Engineering Materials for Stem Cell Regeneration*, will enable the reader with the most recent advancements in material science and stem cells. This book's prime focus is to help inspire researchers to understand the interface between biomaterial creation and stem cells focusing on the needs to raise in the twenty-first century. This book comprises 24 uniquely written chapters, with each of them doing critical analyses on different aspects of stem cells regarding techniques, discussing the problem, and addressing the issues. For instance, Chap. 1 covers the prospect of stem cell therapy and the revolution of stem cell nanotechnology. This chapter describes how nanomaterials' structural properties can affect stem cells' proliferation and differentiation. Nanomaterials, such as magnetic nanoparticles, carbon nanotubes, and quantum dots that are developed to deliver drugs or genes to stem cells, are discussed. Chapter 2 introduces the physicochemical and biological properties of scaffolds and different techniques that are used to fabricate them ideally for growing stem cells. Chapter 3 sheds light on various stem cells used for bone regeneration and their osteogenic differentiation capabilities while using appropriate signaling and growth factors. The mechanical stimulation, nanofiber alignment, and scaffold morphology affecting the cellular differentiation were briefly highlighted. Chapter 4 identified the role of nanofibers as the potential candidates for the differentiation of induced pluripotent stem cells into cardiomyocytes. Chapter 5 demonstrates how physics regulates stem cells' fate while interacting with biomaterials, such as magnetic nanoparticles, graphene derivatives, and titanium dioxide-based materials. Chapter 6 gives an overview of the use of dental stem cells and their interaction with polycaprolactone polymers: moreover, how the polycaprolactone scaffolds will be fabricated and how these scaffolds can be used in seeding the dental stem cells. Chapter 7 deals with the use of injectable biomaterials that can be used for alveolar bone regeneration. Chapter 8 describes the natural and synthetic biomaterials that are used and the newer material that have a potential application for dental tissue regeneration and their clinical implications. Chapter 9 aims to show the practical applicability of fabricating the wound dressing materials using 3D printing technology using the lanolin-based sodium alginate scaffolds. Chapter 10 describes how different biomaterials and techniques can be

used to mimic the complex internal structure of testis. Furthermore, using spermatogonial stem cells might help to provide structural support for the spatial distribution of cells to create a niche for in vitro spermatogenesis. Chapter 11 describes stem cell sources, synthetic biomaterials, and scaffold fabrication methods for cardiac tissue engineering. Chapter 12 outlines stem cell therapy strategies applied in various dental/tooth/orofacial organs and tissue regeneration to provide a theoretical basis for clinical workflow.

Chapter 13 brings to light tissue engineering with the latest approaches that can potentially accelerate the clinical application of the technology. The use of chitosan-based scaffolds for healing in the light of tissue engineering and current stem cell approaches are described in Chap. 14. Chapter 15 brings our attention to the dynamic interaction between stem cells and biomaterials, allowing us to understand how stem cell mechanobiology influences tissue engineering. Chapter 16 highlights hydrogel-based therapies affecting cardiovascular diseases and addresses challenges faced in clinical progress in this area. Chapter 17 concerns the decellularized extracellular matrix and the created biomaterial from this technique for culturing stem cells. Chapter 18 describes solvent casting, electrospinning, emulsification, lyophilization, 3D printing, gelation, and crosslinking techniques useful for chitosan so that they are used as biomaterials. Chapter 19 talks about the different chitosan-based hydrogels that are currently used in tissue engineering applications. Chapter 20 finds its place by describing the advantages of hydroxyapatite and bioactive glass for using them as an efficient candidate for bone tissue engineering and regeneration. Chapter 21 draws attention to how the hydrogels are suitable for 3D cultures setup while regenerating different tissues, such as skin, bone cartilage, vascular, cardiac, and neural tissue regeneration. Chapter 22 shows how the interaction of chitosan-based material will affect the cartilage, bone, cardiac, vascular, and neural tissue functioning. Chapter 23 provides a detailed introduction to existing processes in implementing decellularized extracellular matrix with stem cells to alleviate skin, cartilage, heart, kidney, liver, lung, and dental pulp injuries. Finally, Chap. 24 describes the freeze-drying process, solvent casting, particle leaching, gas foaming, 3D bioprinting, electrospinning, and thermal-induced phase separation to create the desired biomaterial used for regenerating tissue and possibly the differentiation of stem cells.

I am thankful to all the contributing authors from all parts of the globe for trusting me to publish their work in this book. It was not an easy task to find the potential authors and their specialized fields, which will match the scope of this book. I would also like to thank the reviewers for their efforts to improve the quality of the chapters. Furthermore, I would like to thank all corresponding authors for enhancing the quality of the chapters while addressing the reviewer comments. This was a fantastic journey; I hope all these chapters will significantly impact the scientific community and students. I also want to thank Dr. Bhavik Sawhney, the publishing editor of biomedical sciences of Springer Nature, for his guidance, suggestions, discussion,

and pushing hard for the completion of this manuscript. My appreciation goes to Mr. Selvakumar Rajendran, the production editor of Springer Nature, and he has helped organize the book contents. This work would not have been possible to publish without their support.

Srinagar, Jammu and Kashmir, India Faheem A. Sheikh

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I am eternally thankful to all the contributors who, without any hesitation, participated and followed my instructions to shape this book. Although writing a book was more complicated than I thought, this was much more rewarding. All such efforts could set an example in our university and globally will catch the attention of researchers who are working in the area of stem cells and biomaterials for the betterment of society. My appreciations go to Dr. Bhavik Sawhney Editor—Biomedicine and Mr. Selvakumar Rajendran, Production Editor at Springer Nature. Furthermore, I would like to acknowledge the financial support provided by Science and Engineering Research Board (SERB) research grants (CRG/2020/000113) and Council of Scientific & Industrial Research (CSIR) sponsored project (22(0846)/20/EMR-II).

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About the Editor

Faheem A. Sheikh is an Assistant Professor in the Department of Nanotechnology at the University of Kashmir, India. He served as an Assistant Professor in the Department of Biotechnology at the Central University of Kashmir, India (2015–2016); Assistant Research Professor at the Department of Energy Science and Technology, Myongji University, South Korea (2014–2015); Assistant Professor Research at the Nano-Bio Regenerative Medical Institute Hallym University, South Korea (2012–2014); Post-Doc/Research fellow at the University of Texas Rio Grande Valley, Texas, USA (2010–2012); and Research Professor at Myongji University, South Korea (2010). His research mainly focuses on fabricating nanomaterials used in tissue engineering.

Currently, he is heading a lab, which works on creating different nanomaterials that can be used in various biological applications; this includes cell culturing and microbial assays. He has considerable expertise in the fabrication of polymeric, ceramic, and metal oxide nanofibers using electrospinning, as well as the production of porous scaffolds by solvent casting, salt leaching, 3D printing, gas forming, sol-gel synthesis, phase separation, freeze-drying, and particulate leaching and self-assembly for hard and soft tissue engineering. He believes that we will always need new and intelligent materials to address the different challenges faced in tissue engineering science. He has more than 15 years of research experience in nanotechnology, focusing on tissue engineering and drug delivery. He has published more than 100 peer-reviewed articles and book chapters.

Prospect of Stem Cell Therapy and Nanotechnology

1

Rumysa Saleem Khan, Taha Umair Wani, Anjum Hamid Rather, Touseef Amna, Mushtaq A. Beigh, and Faheem A. Sheikh

Abstract

This chapter brings light to the recent advances made in stem cell nanotechnology. The introductory chapter emphasizes that stem cells can go on dividing and multiplying unlimitedly. In the early stages of embryo development, the embryonic stem cells are formed and are the most potent. In contrast, adult stem cells are present throughout life with less potency. Subsequently, induced pluripotent stem cells are adult stem cells that are reprogrammed to revert to a stem cell analogous to embryonic stem cells. The advancement of stem cell therapy has developed as a very assuring and advanced research subject. This has recently produced treatment plans that have tremendous prospects. The interplay of nanomaterials and stem cells has led to significant discoveries that have made it an innovative technology in regenerative medicine and material science. Furthermore, this chapter describes how nanomaterials' structural properties can affect stem cells' proliferation and differentiation. Some of the nanomaterials, such as magnetic nanoparticles, carbon nanotubes, and quantum dots, are developed to deliver drugs or genes to stem cells and are briefly discussed. The diversity of opportunities associated with stem cell therapy and biomaterials makes them ideal candidates, offering treatment for terminal disorders.

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Stem cell therapy · Pluripotent stem cells · Nanomaterials · Stem cell therapy

1.1 Introduction

The primary cells that produce cells in all body organs are called stem cells. These are characterized to have self-renewal ability giving rise to differentiated cells (Morrison et al. 1997). These exist both in embryos and adult cells but have somewhat distinct characteristics in each (Bongso and Lee 2005). One example of stem cells is an egg cell of a female fertilized by a sperm from a male (Messinis et al. 2016). The aforementioned single cell, termed a zygote, is the principal cell in human beings (Harper 2013). They are different from progenitor cells, which are unable to divide endlessly. Further, the blast cells are bound to differentiate toward one lineage (He et al. 2009). During the blastocyst stage of embryonic development, about 50–150 cells build up the inner cell mass with stem cell capability (Pittenger and Kerr 2014). They differentiate into all of the body's cell types and are called embryonic stem cells (ESCs) (Bissels et al. 2013). Adult stem cells are located in only selected niches such as bone marrow or gonads, whose function is to renew rapidly lost cell types (Bosio et al. 2011). These cells include hematopoietic stem cells (HPSCs), which replenish blood and immune cells; basal cells which repair the skin epithelium; and mesenchymal stem cells which maintain bone, cartilage, muscle, and fat cells (Jagannathan-Bogdan and Zon 2013). A vital process of the body, hematopoiesis, starts with the stem cells (Hoggatt and Pelus 2013). It is the lifelong process of constant formation and turnover of blood cells to meet everyday requirements and respond to increased demand at the time of injury or infection. Both stochastic and instructive mechanisms carry out the regulation of hematopoiesis (Hoggatt and Pelus 2013). A minority of adult stem cells are among the progenitor cells and terminally differentiated cells that they differentiate into (Atala and Lanza 2013). Stem cells present an opening to examine the mechanisms that coordinate embryonic development, cellular differentiation, and organ maintenance. Because of their proliferation and differentiation abilities, stem cells have great potential for the development of novel cell-based therapies (Daley et al. 2003; Keller 2005) (Fig. 1.1). Recent studies have proposed that dysregulation of stem cell properties may cause certain types of cancer (Reya et al. 2001; Dalerba et al. 2007). The different streams of science have combined to form a newly emerging field, stem cell nanotechnology, referring to the applications of stem cell research and development (Weissman 2002; Solanki et al. 2008). Continuous research will create different nanomaterials that can be efficiently used in stem cell-based therapies for degenerative diseases and injuries (Fig. 1.2) (Dong et al. 2021). In particular, the properties of nanomaterials affect the differentiation and proliferation of stem cells and have become a curious research topic in regenerative medicine (Dong et al. 2021).

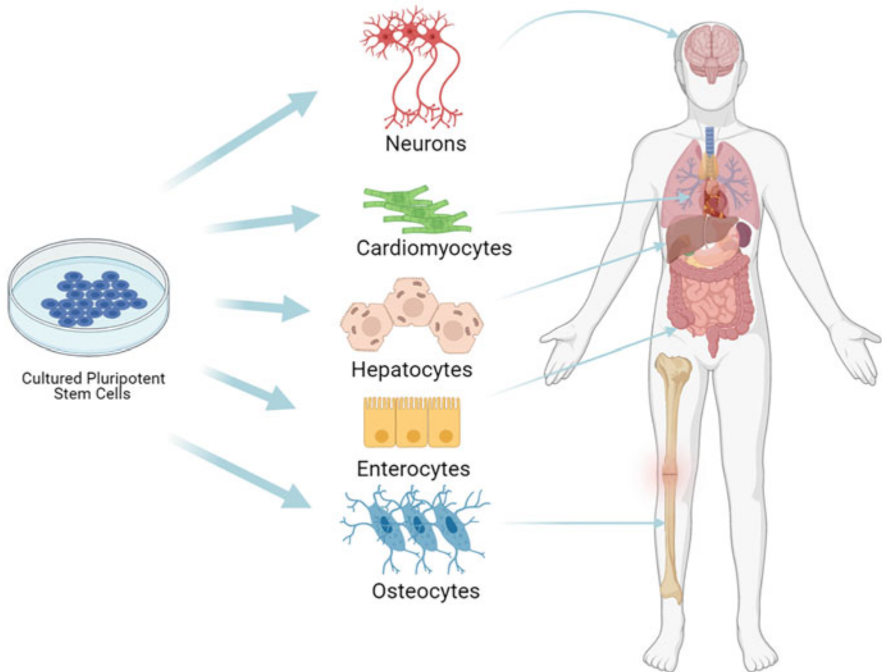

Fig. 1.1 Potential applications of therapy. The pluripotent cells are cultured and stimulated for differentiation into various cells as per the requirement. (Created with (BioRender n.d.))

1.2 Origin of the "Stem Cell"

Historically, the term stem cell emanates in the literature in 1868 from the renowned German biologist Ernst Haeckel (Ramalho-Santos and Willenbring 2007). He was a significant supporter of Darwin's theory of evolution. Given this, Haeckel applied the term "Stammzelle" (German for stem cell) to define the ancestor unicellular organism, which he believed all multicellular organisms have emerged from (Haeckel 1834–1919 1874). In his updated third edition book, *Anthropogenie*, Haeckel went from evolution (phylogeny) to embryology (ontogeny) and asserted that the fertilized egg also be called a stem cell (Ramalho-Santos and Willenbring 2007). The segregation of the germplasm to germ cells from one generation to the next occurs early during an embryo's development, as stated by August Weismann. These cells are different from somatic cells. Valentin Hacker and Theodor Boveri identified the most primitive germ cells in mammalian embryos that they assumed would carry the germplasm with this inspiration.

Further on, Boveri investigated *Ascaris* cell lineages and described them as tree diagrams that he called Stammbäume (Ramalho-Santos and Willenbring 2007). In 1892, Boveri defined stem cells as the fertilized egg and described germ cells as stem

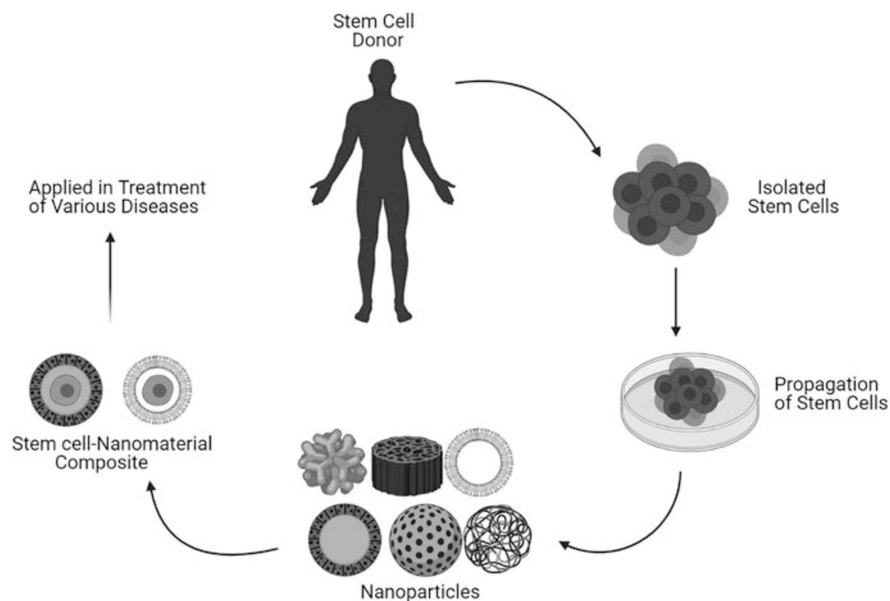

Fig. 1.2 Stem cells can be isolated and propagated easily in the lab. Later on, the different types of nanoparticles can be used to form stem cell-nanocomposite and applied in the treatment of various diseases. (Created with (BioRender n.d.))

cells mentioned by Haeckel. Later on, at the beginning of 1892, Hacker identified a large cell in crustacean cyclops that became internalized upon gastrulation (Ramalho-Santos and Willenbring 2007). He called this cell a stem cell and discerned that it experienced asymmetric division. Hacker also defined stem cells as cells that later develop oocytes in the gonad.

Consequently, these early studies referred to stem cells as the germline lineage, primordial germ cells, and germline stem cells. Later on, after 4 years, Edmund B. Wilson expanded the term "stem cell" in the English language by reconsidering Hacker's and Boveri's work in his book, *The Cell in Development and Inheritance* (Wilson 2011). His manuscript was inspiring to embryologists and geneticists at that time, especially in the United States. Moreover, he is commonly credited to have cited the term stem cell (Shostak 2006; Fangerau 2009).

1.3 The Characteristic Properties of Stem Cells

Any cell to be termed as stem cell should have the following characteristics:

- **Self-renewal:** The cell goes over multiple proliferation cycles, although retaining the undifferentiated state. Two mechanisms maintain stem cell population, viz.,
  - **Asymmetric cell division:** A stem cell divides into two different cells; one is identical to the original stem cell, referred to as mother cell, and another is daughter cell, which undergoes differentiation. The stem cell self-renews without disturbing the undifferentiated state (He et al. 2009).
  - **Stochastic differentiation:** In this type of differentiation, a stem cell matures and divides into two daughter cells, both of which undergo differentiation. In comparison, another stem cell undergoes mitosis giving rise to two stem cells. To extend the cell division limit (the Hayflick limit), stem cells use telomerase to restore telomeres, protecting their DNA against nuclease limit (Cong et al. 2002).
- **Potency:** A cell can differentiate into specialized cell types.

1.4 The Classification of Stem Cells

The stem cells are classified into pluripotent, totipotent, oligopotent, multipotent, and unipotent cells based on potency (Shiffman and Low 2020). The cells that divide and differentiate into any cell of the whole organism, including the extra-embryonic structures such as the umbilical cord and placenta, are totipotent (Shiffman and Low 2020). For example, the zygote is a totipotent cell. After creating the zygote, it divides a few times and becomes an early-stage development called a blastocyst (Messinis et al. 2016). The embryogenic stem cells (ESCs) originate inside a blastocyst. These are the pluripotent stem cells (PSCs), which implies these can get converted to all germ layers but not the extra-embryonic structures (Bosio et al. 2011). In the lab, ESCs are provided by unwanted embryos formed during in vitro fertilization (Arrighi 2018) (Fig. 1.3). Such in vitro fertilization is used to assist a female in becoming pregnant. In the process of pluripotency, the cells start from completely pluripotent cells to become less potent multi-, oligo-, or unipotent cells (Laplane and Solary 2019). With the help of teratoma formation assay, the activity and spectrum of cultured PSCs can be assessed (Bongso and Lee 2005).

Multipotent stem cells have a smaller scope of differentiation if compared to PSCs. However, they can be converted into different cells of specific cell lineages (Can 2008). They can grow into a few distinct types of cells linked to the organs they develop into (Bosio et al. 2011). For example, the skin adult stem cells can divide to produce new skin adult stem cells or grow into more specialized skin cells to replace the cells lost due to damage or cell aging (Bissels et al. 2013). Another example of adult stem cells is HSC, which gives rise to other blood cells (Hoggatt and Pelus 2013). After differentiation, the abilities of an HSC are confined to cells of its lineage and grow to become oligopotent (Laplane and Solary 2019). These are called induced PSCs (iPSCs) (Zakrzewski et al. 2019), developing into any cell in the body. The iPSCs are highly assuring for present and future applications in regenerative medicine (Daley et al. 2003).

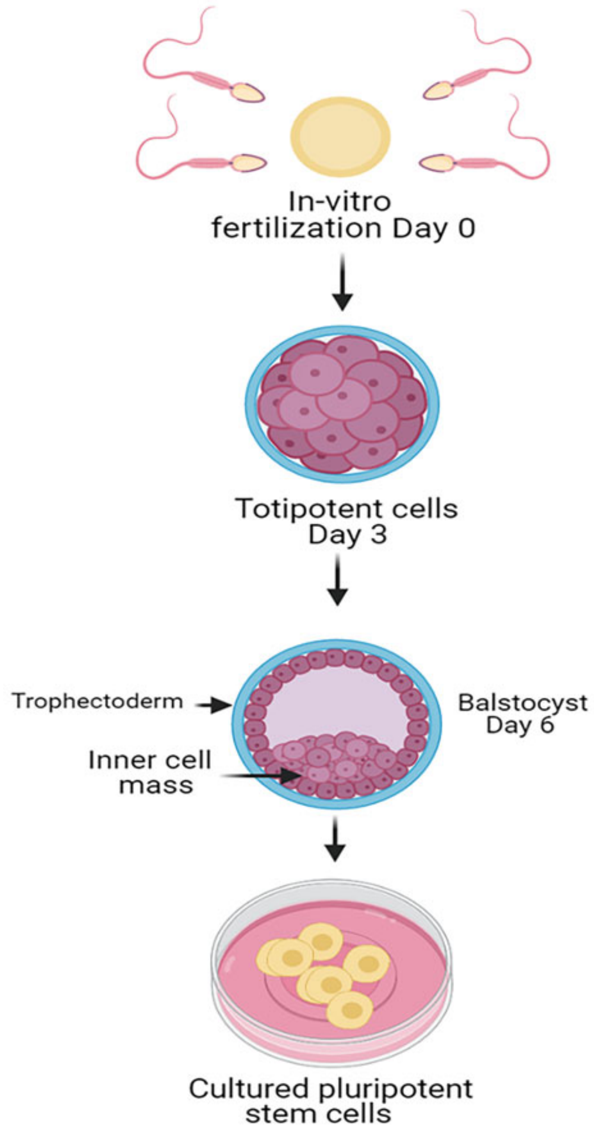

Fig. 1.3 Culturing of PSCs in vitro: a sperm fertilizes the egg leading to the formation of totipotent cells after 3 days. On day 6 of fertilization, the blastocyst's inner cell mass is formed, which is the foundation of PSCs. These are isolated and transmitted onto the culture dish. (Created with (BioRender n.d.))

On the other hand, oligopotent stem cells differentiate into several but not all cell types. For example, a myeloid stem cell that divides into white blood cells cannot divide into red blood cells (Bosio et al. 2011). The unipotent cells have the highest restricted capability of differentiation and have the property of repeatedly dividing (Bissels et al. 2013). They are capable of forming one cell type only, e.g., dermatocytes.

1.5 The Biology of Stem Cells

After a blastocyst is formed post-fertilization, its inner wall constitutes short-lived stem cells, the ESCs (Messinis et al. 2016). Blastocysts consist of two distinct cell types: the inner cell mass (ICM), which continuously grows into epiblasts and induces the development of the fetus, and the trophectoderm (TE) (Zakrzewski et al. 2019). TE proceeds the differentiation to form a functional support structure, but the ICM cells stay undifferentiated, pluripotent, and proliferative (Sukoyan et al. 1993). During embryogenesis, the aggregations of cells form germ layers, endoderm, mesoderm, and ectoderm (Fig. 1.4), ultimately giving rise to differentiated cells and tissues of the fetus and eventually the adult organism (Larijani et al. 2012). After the differentiation of human ESCs into one of the germ layers, these become multipotent stem cells. Their differentiation ability is limited to only the germ-layer cells (Bissels et al. 2013). After that, PSCs occur everywhere in the organism as undifferentiated cells, which proliferate by forming the subsequent generation of stem cells and differentiate into specialized cells under defined physiological conditions (Pittenger and Kerr 2014).

Various signals guide the stem cell specialization process and are divided into external, physical contact between cells or chemical secretion by surrounding tissue, and internal, which are signals regulated by genes (Ogaki et al. 2013). They also act as internal repair systems of the body. As long as the organism is alive, the body continues to replenish and form unlimited new cells (Zakrzewski et al. 2019). Stem cell activity is influenced by the organ they reside in; for example, their division is constant in the bone marrow. On the other hand, in the pancreas, the division only occurs under specific physiological conditions (Sukoyan et al. 1993).

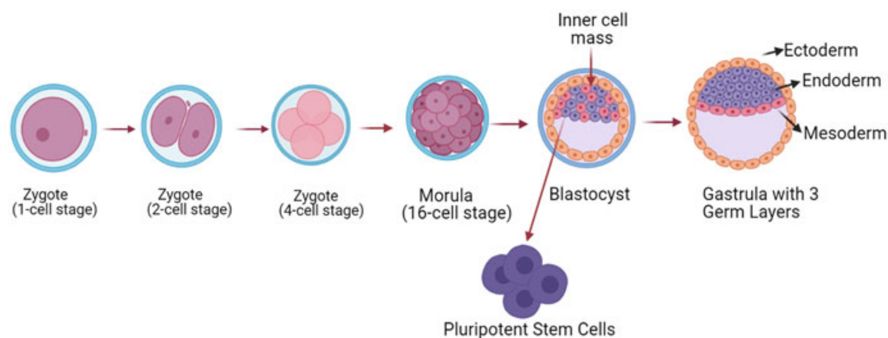

Fig. 1.4 Schematic showing the blastocyst formation: after fertilization, the zygote is formed, which goes through certain stages until it forms the blastocyst. The ICM of the blastocyst is the source of PSCs. Further on, the development leads to the formation of the gastrula and then the embryo. (Created with (BioRender n.d.))

1.6 The Cell Cycle Control of Stem Cells Is Different from the Proliferating Somatic Cells

ESCs undergo unlimited division and also retain their pluripotency because of the specialized mechanisms of cell cycle control (Koledova et al. 2010b). They have unique cell cycle properties different from somatic cells, e.g., fast cell division due to brief G1 phase, absent G0 phase, and modifications in cell cycle checkpoints, leaving the cells mainly in the S phase (Koledova et al. 2010b; Barta et al. 2013). They have a brief doubling time (8–10 h), and somatic cells have nearly 20-h-long doubling time (Zaveri and Dhawan 2018). On differentiation of stem cells, these properties change; the duration of G1 and G2 phases increases and causes longer cell division cycles. From this, it can be inferred that a specific cell cycle structure has a role in establishing pluripotency (Koledova et al. 2010b). ESCs have a short G1 phase; to maintain undifferentiated phenotype as in the G1 phase, cells have high sensitivity for differentiation (Barta et al. 2013). The proteins that control the progression of cells through cell cycle are cyclin, cyclin-dependent kinases, cyclin-dependent kinase inhibitors, retinoblastoma proteins, and other accessory factors (Zaveri and Dhawan 2018).

A highly shortened G1 phase in stem cells permits cells to alternate between the M and S phases rapidly. The oscillatory activity of Cyclin-Cdk complexes in the somatic cell cycle controls the unidirectional transitions between phases: Cyclin D and Cdk4/6 work in the G1 phase, while cyclin E and Cdk2 during the late G1 phase and S phase. Cyclin A and Cdk2 work during the late G1 phase and S phase, Cyclin A and Cdk2 in the S phase and G2, while Cyclin B and Cdk1 are active in G2 and M phases (Zaveri and Dhawan 2018). In ESCs, this ordered oscillatory working of Cyclin-Cdk complexes is not present. Instead, the Cyclin E-Cdk2 complex is functional throughout the cycle and keeps retinoblastoma protein (pRb) hyperphosphorylated and thus inactive. A direct transition from the M phase to the late G1 phase occurs and creates an absence of D-type cyclins and a shortened G1 phase (Barta et al. 2013). The downregulation of Cdk2 increases G1 phase progression, sets a somatic cell-like cell cycle, and causes the expression of differentiation markers (Koledova et al. 2010a). Another aspect of ESCs is the non-functionality of the G1 checkpoint. ESCs do not stop in G1 to repair DNA damages but, instead, depend on S and G2/M checkpoints or undergo apoptosis. The cells with damaged DNA are removed, hence avoiding potential mutations from inaccurate DNA repair (Koledova et al. 2010b; Zaveri and Dhawan 2018).

1.7 iPSCs as the Game-Changers in Disease Modeling

Recently, a new technology existed to program the cell resulting in the formation of pluripotent (iPSCs). These cells can transform into functional potent stem cells. These are the somatic cells reprogrammed to produce cells with pluripotent abilities and not adult stem cells (Shiffman and Low 2020). By applying genetic reprogramming with protein transcription factors, PSCs with ESC-like features

have been derived (Frank-Kamenetsky et al. 2002; Cai 2014; Banda and Grabel 2015). Yamanaka and his colleagues at Kyoto University led the first demonstration of iPSCs (Oshima et al. 2010). They reprogrammed mouse fibroblasts into pluripotent cells with transcription factors Oct3/4, Sox2, c-Myc, and Klf4 (Osakada et al. 2009; Kshitiz et al. 2012; Banda and Grabel 2015). Other scientists like Junying Yu, James Thomson, and their colleagues at the University of Wisconsin-Madison studied other factors, such as Oct4, Sox2, Nanog, and Lin28, and used cells from the human foreskin (Amps et al. 2011; Banda and Grabel 2015). Some similarities in properties exist between iPSCs and ESCs, like their potential of differentiation and pluripotency. Moreover, the expression of pluripotency genes, epigenetic factors, embryoid body, and teratoma formation are also prevalent (Osakada et al. 2009; Oshima et al. 2010). Nevertheless, some differences in these characteristics do exist. For instance, in iPSCs, the chromatin is more closed or methylated than ESCs (Osakada et al. 2009, Oshima et al. 2010). Furthermore, there are distinct origins of gene expression patterns among ESCs and iPSCs (Oshima et al. 2010). They provide therapeutic advantages because of their pluripotency (Oshima et al. 2010). iPSCs offer medical applications in treating several disorders as they have given the power to create in vitro models to investigate toxins and pathogenesis (Zakrzewski et al. 2019). A PSC line for individual patients can be developed from iPSCs (Hewitson et al. 2016). The frozen samples of blood are used as precious sources of iPSCs (Daley et al. 2016). Patient-specific stem cells have enabled the screening for side effects before drug treatment and the controlled risk of transplantation rejection (Hewitson et al. 2016).

1.8 Hematopoiesis: The Process Leading to Blood Cells from a Stem Cell

In humans, postnatal hematopoiesis takes place in the bone marrow. It starts with the HSC, which is self-renewable and can differentiate into red blood cells, responsible for carrying oxygen to tissues, and leukocytes and/or white blood cells, for combating infections. This helps in maintaining hemostasis (Fig. 1.5). The development of blood cells in vertebrates comprises two waves of hematopoiesis: the primitive wave and the definitive wave (Galloway and Zon 2003). The primitive wave includes an erythroid progenitor and produces erythrocytes and macrophages during early embryonic development (Palis and Yoder 2001). Its purpose is to produce erythrocytes that can promote tissue oxygenation as the embryo experiences accelerated growth (Orkin and Zon 2008). The appearance of these progenitor cells in mammals and avians for the first time occurs in the aggregates of blood cells called blood islands found in the extra-embryonic yolk sac early in development (Paik and Zon 2010). The primitive wave is just temporary, and these progenitors are not pluripotent and are not able to replenish, whereas, on the other hand, definitive hematopoiesis takes place in later development. The transient wave of definitive hematopoiesis occurs in blood islands and generates progenitors called erythroid-myeloid progenitors (EMPs) (Bertrand et al. 2007; McGrath et al. 2011).

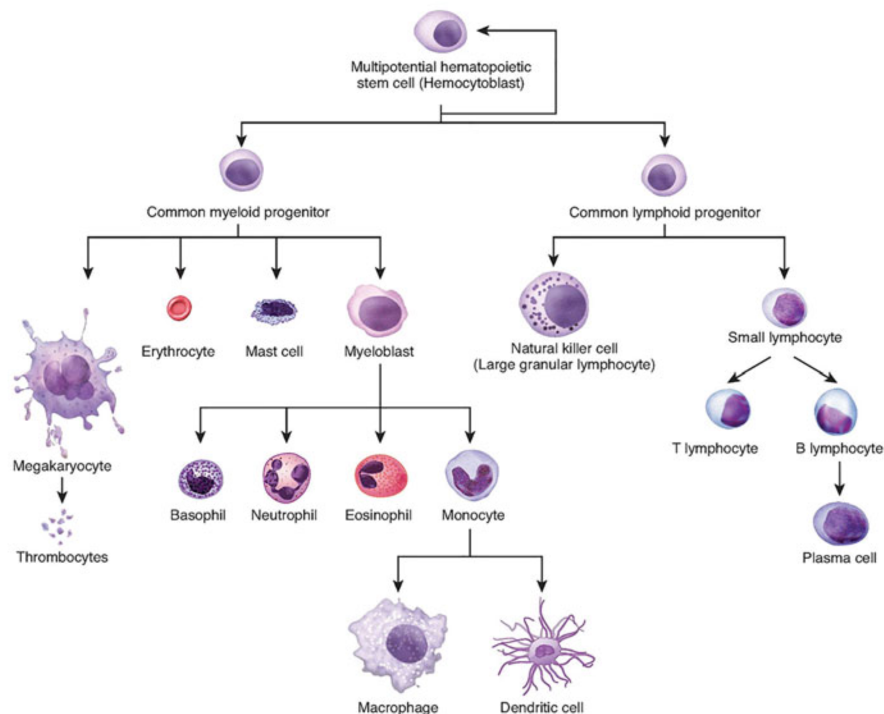

Fig. 1.5 The multipotential hematopoietic stem cells can give rise to all types of blood cells. This is all possible in the presence of a system existing in the bone marrow, liver, and spleen. (Reproduced from Wikimedia)

Later definitive hematopoiesis involves HSCs, which are multipotent and can produce all adult organisms' blood lineages. The developing embryo has an aorta-gonad-mesonephros (AGM) region where definitive HSCs are born; they migrate to the fetus's liver and later to bone marrow (Cumano and Godin 2007). Hematopoiesis in humans rises within the yolk sac and momentarily transitions toward the liver before establishing definitive hematopoiesis in the bone marrow and thymus. Hematopoiesis in children occurs in the bone marrow of the long bones, such as the femur and tibia. Simultaneously, in adults, it takes place chiefly in the pelvis, cranium, vertebrae, and sternum (Haschek et al. 2013).

HSCs are present in the medulla of the bone marrow. They give rise to different blood cell types (Birbrair and Frenette 2016). HSCs divide asymmetrically and leave some daughter cells to remain as HSCs so that the pool of cells is not exhausted (Morrison and Kimble 2006). Heterogenous progenitors are divided into two groups, viz., long-term self-renewing HSC and just transiently self-renewing HSC, also known as short-terms (Morrison and Weissman 1994).

Blood cells are divided into three lineages (Jagannathan-Bogdan and Zon 2013).

- *Erythropoiesis*: The process of formation and release of erythrocytes or RBCs (oxygen-carrying cells) into the blood is known as erythropoiesis.
- *Lymphopoiesis*: Lymphopoiesis is the process of formation of lymphocytes which are the chief cells in the adaptive immune system. This comprises of T and B lymphocytes and NK cells. They are stemmed from common lymphoid progenitors.
- *Myelopoiesis*: Myelopoiesis is the process of formation of myeloid lineage cells, that is, macrophages, megakaryocytes, and granulocytes originate from myeloid progenitors. Their function is providing innate immunity and blood clotting. The other types of blood cell formation processes are granulopoiesis (hematopoiesis of granulocytes) (Haschek et al. 2013) and megakaryocytopoiesis (hematopoiesis of megakaryocytes).

1.9 Stem Cell Therapy

Healing of an injury or wound is a stem cell-mediated event because all tissue repair in the body is initiated by stem cells (Maxson et al. 2012). The reason behind non-healing can be a shortage of stem cells in that area or cells are not functioning optimally (Atala and Lanza 2013). However, stem cells from the other regions of the body are taken where they are plentiful, like bone marrow or adipose tissue, and transplanted in their short supply areas. This results in a tremendous amount of healing which is the essence of regenerative medicine (Duscher et al. 2016). The other sources of stem cells are the umbilical cord or the amniotic tissue of the healthy baby that is utilized to stimulate tissue healing and repair (Luo et al. 2010; Kucharzewski et al. 2019). This method where we use stem cells to treat a disease or condition (Biehl and Russell 2009) is known as stem cell therapy. It is employed to treat diseases and cancers of the blood also (Eppert et al. 2011). For example, in leukemia, the patient's bone marrow produces many unusual blood cells that do not adequately fight infection (Horton and Huntly 2012). Once the abnormal cells are eradicated, the doctor transplants the healthy HSCs from the patient's tissue samples. These healthy stem cells produce new blood cells, including normal white blood cells, which allow the body to fight off infections (Tian et al. 2015) (Fig. 1.6).

Our natural healing system includes the mesenchymal stem cells (MSCs) (Fig. 1.7), which orchestrate the healing process and are referred to as the injury-specific drug store by using signaling compounds that mediate healing naturally (Pittenger et al. 2019). They travel to the injured area, survey it, and respond by releasing various drug-like molecules that help to heal and repair (Pittenger et al. 2019). When MSCs are injected into a damaged knee, these produce different drug molecules compared to if injected into an inflamed lung or a damaged liver. They exist in the universal stem cell niche, where all tissue healing and repair occur (Gazit et al. 2011).

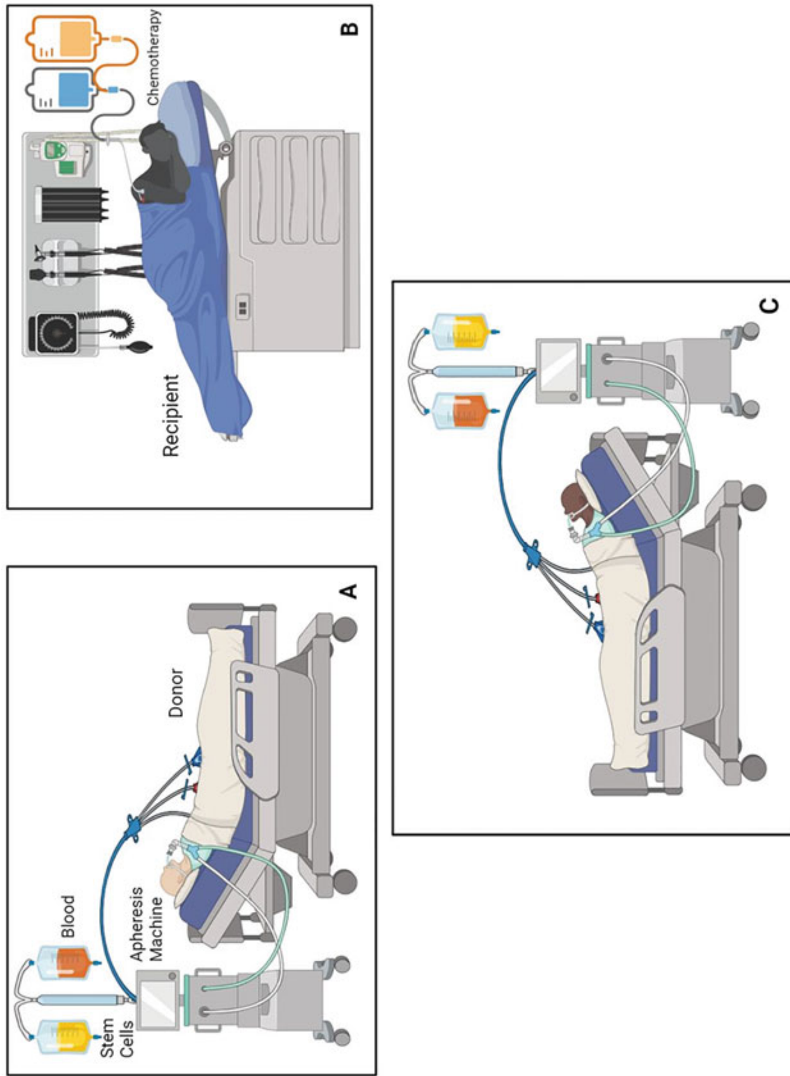

Fig. 1.6 Stem cell transplantation. (**a**) Step 1: From the arm of the donor, blood is taken. The donor could be the patient himself or another person. The stem cells are being removed from the blood of the donor by an apheresis machine, and the blood is returned to the donor through a vein in the other arm. (**b**) Step 2: The patient receives chemotherapy that kills blood-forming cells. (**c**) Step 3: The patient receives the stem cells through a catheter placed into a blood vessel in the chest

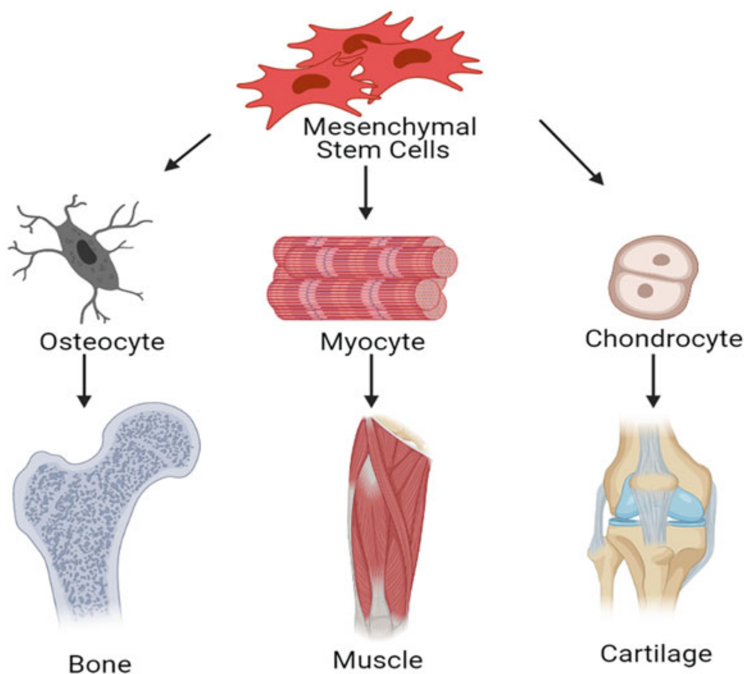

Fig. 1.7 Mesenchymal stem cells orchestrate the recovering processes in the bone, muscle, skin, and cartilage. They use certain signaling compounds that mediate healing. (Created with (BioRender n.d.))

1.9.1 Promises and Dangers of Stem Cell Therapies

Stem cells exist within a relatively large spectrum, starting with ESCs, go through pluripotent adult stem cells and up to iPSCs. However, ESCs possess great differentiation potential and can be replicated to something useful to us given the right cue (Pittenger and Kerr 2014). But, the excellent differentiation potential also means the subject of significant risk wherein if the stem cells go unregulated, they can lead to cancer (Afify and Seno 2019). The adult stem cells employ a cluster of different molecular mechanisms, genetic transfers, transfer of mitochondria, secretions of anti-inflammatory proteins, and secretions of growth factors that modulate critical systems in our body (Christ et al. 2017). Stem cells are available in every part of our body and are used to heal any damaged tissue of the body, e.g., the adipose tissue (Dykstra et al. 2017). In an attempt to heal adipose tissue, one can have stem cell treatment and at the same time have liposculpture. In countries like the United States of America, the number of liposculpture cases recorded every year is 300,000 (Ahmad et al. 2011). Also, since the stem cells act differently with different individuals, the stem cells taken from one individual and transplanted into others may behave differently compared to how they behaved in the donor. In the current scenario, controlling the proliferation of stem cells is not entirely possible yet can

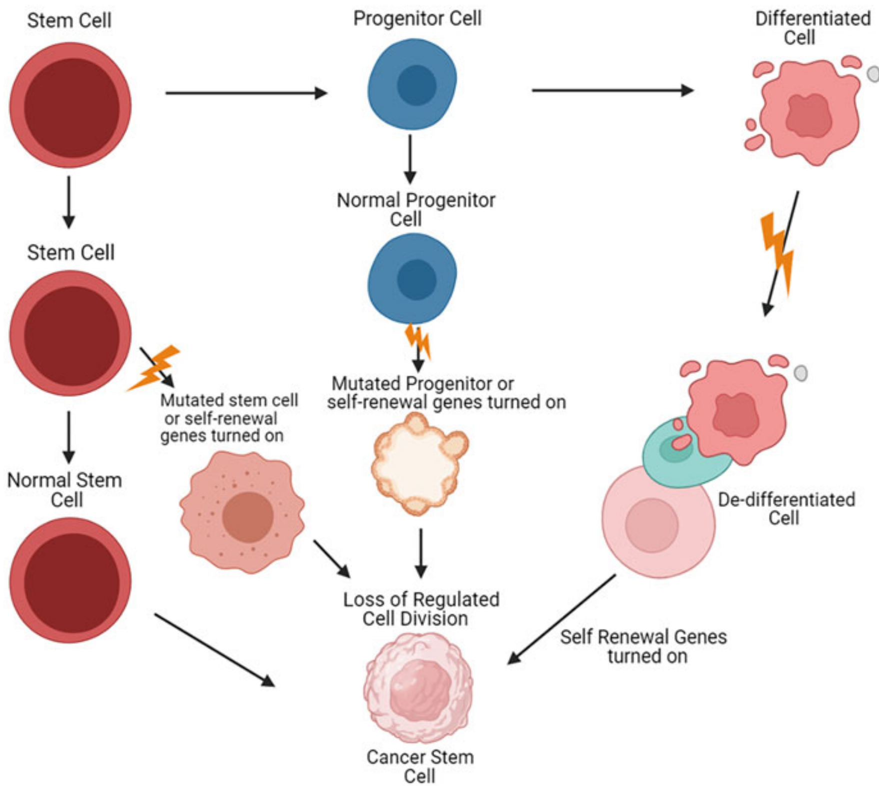

Fig. 1.8 Schematic showing how stem cells can turn into cancerous cells. Due to the longer life of stem cells than normal cells, they have a high chance of accumulating genetic mutations. The cell loses control over its self-renewal and growth; along with transformations, they finally can turn into cancerous cells. (Created with (BioRender n.d.))

lead to cancer (Afify and Seno 2019). Some potential reasons behind how stem cells turn into cancer cells are shown in Fig. 1.8. A failed attempt of stem cell therapy was reported 13 years ago with a woman who wanted to get her face lifted with stem cell therapy (Kota 2017). The physicians decided to inject the stem cells into the patient's face around the eyes, and after 2 weeks, the patient heard a click every time she blinked. The rationale of this sound produced is because the stem cells in her face had turned into bones, and then she needed to have them surgically removed. Another case of stem cell therapy failure was reported when three elderly patients with genetic defects in the eyes underwent stem cell transplants as the treatment option. The physicians went for direct injection of stem cells into their eyes. A few weeks later, they all went blind (Kota 2017). Therefore, this research needs more effort to be placed before the stem cells are clinically used to treat different ailments.

1.9.2 Clinical Trials

In the past and much recently, they were using stem cell therapy for treating multiple sclerosis, rheumatoid arthritis, osteoarthritis, autism, spinal cord injury, asthma, aging, and cancer (Smith and Gomez 2017; Shroff 2018; Lopez-Santalla et al. 2020). A successful example of clinical stem cell therapy is explained by Neimark et al. when they implemented it in a patient who was experiencing pain clicking in the hip. They injected about eight million MSCs into the patient's hip under ultrasound guidance. The procedure lasted about 15 min, and thus a hip replacement surgery was avoided. After 5 or 6 months, there was no pain in his hip, and he started walking normally (Neimark 2019).

Another case explained by Neimark et al. is that of 96 patients with critical limb ischemia (low blood flow to the feet) who were diabetic and were at risk of amputation. The patients were given stem cells taken from their bone marrow and injected along the leg and the foot ulcer base in the first group. In this group, 79% of patients healed entirely by the end of 90 days, while 20% still required amputation. In contrast, in the second group, 44% of patients required amputation. The angiogram below the foot showed massive angiogenesis in the stem cell-administered patients (Neimark 2019).

In another case by Neimark et al., he explains that a patient in the USA was paralyzed after having a catastrophic accident by falling headfirst into the ocean floor and broke his neck at many places. To treat the patient using stem cell therapy, a small amount of MSCs were taken from the patient's belly fat, and these cells were expanded in a culture setup. Once the cells reached 100 million cells, they were injected into the patient's lumbar spine, from where the cells migrated toward the highest area of inflammation, that is, the site of spinal cord injury. The cells helped to regenerate the damaged tissue and augmented his healing, and the patient was able to move and walk normally again (Neimark 2019).

Stem cell therapy has also proven beneficial for treating autism that affects social, mental, and behavioral development (Muhle et al. 2004). Nevertheless, the actual cause of autism is still unknown. However, researchers believe that faulty and/or inadequate connections within the brain may be the cause. Evidence suggests some children with autism have dysfunctional immune responses, affecting the nervous system development and function (Meltzer and Van De Water 2017). Some steroids and anti-inflammatory drug treatments have shown promise in improving symptoms of autism (Golla and Sweeney 2014; Pacheva and Ivanov 2019). Building on this, the researchers have shown that a newborn's umbilical cord blood stem cells can regulate the immune system and show promise in stimulating neurological repair. A new FDA-regulated clinical study evaluates whether the infusion of a child's cord blood stem cells can directly or indirectly potentially modify the nervous system through immune modulation to improve language and behavior in children with autism (NCT02847182 2016).

1.10 Advances in Stem Cell Nanotechnology

Evans et al. (Evans and Kaufman 1981) for the first time isolated ESCs, and since then, stem cells have turn out to be the center of focus of research for regenerative medicine (Fig. 1.9). The stem cell-based therapy for various human injuries and degenerative diseases has gained a lot of attention due to the pluripotent properties of ESCs, which allow differentiating into cell types of all three primary germ lineages (Rao and Stice 2004; Heino and Hentunen 2008). The success of iPSCs has attained great heights in the field of regenerative medicine as they have paved the way for the formation of controllable human-made stem cells (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007). With nanotechnology's development, new hopes in the research and development of stem cells have been brought into the light because of nanomaterials' novel physical, chemical, and biological properties (Pan et al. 2007a; Cui 2007). They exhibit some unique effects like the effects due to their

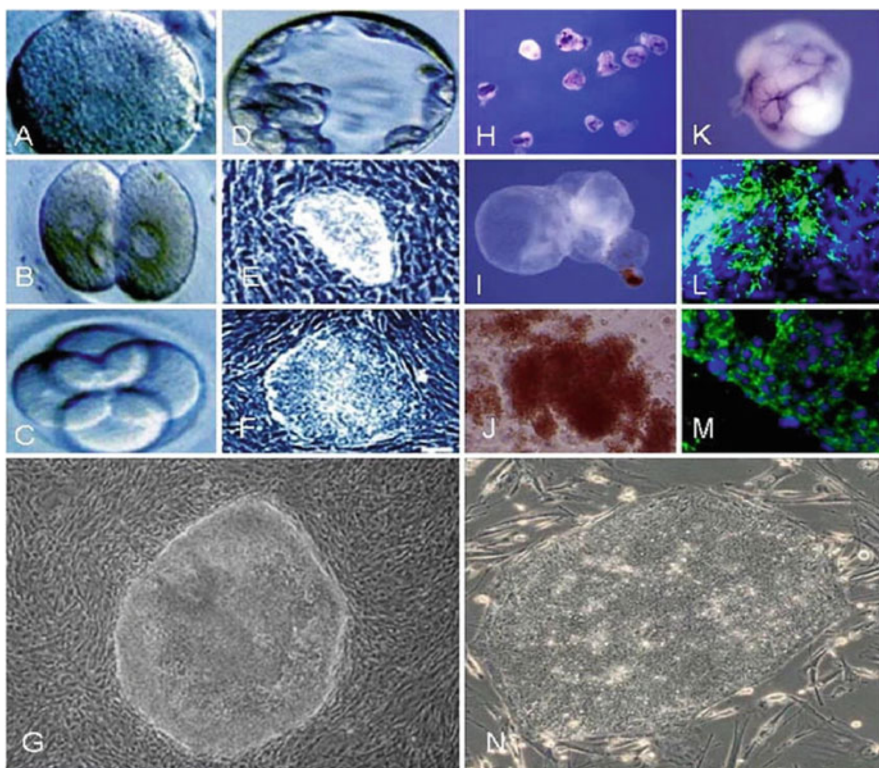

Fig. 1.9 The development of human ESCs (hESCs). (**a**–**f**) The very early stages of human embryo development, (**g**) developed hESCs, (**h**) hESCs derived embryoid bodies showed by the in vitro differentiation towards hepatocytes, (**i**, **j**) hematopoietic differentiation, (**k**) endothelia differentiation, (**l**) neuron differentiation, (**m**) hepatocytes differentiation, (**n**) induced pluripotent stem cells. (Adapted with copyrights permission from (Wang et al. 2009))

small size, surface effects, quantum size effects, and tunnel effects, leading to novel technological opportunities and challenges (Ao et al. 2006; Pan et al. 2007c; Cui et al. 2008). With the advancement in nanomaterials development, nanotechnology has been applied to stem cell research. It has become a fascinating option in solving the present problems met by stem cell research and development. Nanomaterials' structural properties affect stem cells' proliferation and differentiation and have become the latest interdisciplinary, cutting-edge research in regeneration medicine and material science (Washburn et al. 2004). This field has advanced over the past few years, and various approaches have been designed to improve the use of nanomaterials for delivering stem cell therapy.

The use of magnetic nanoparticles (MNPs) is a recent achievement of nanotechnology in stem cell technology (Jing et al. 2007). The CNTs (Cui et al. 2019), the fluorescent CNTs (Shi et al. 2006), and fluorescent MNPs (You et al. 2007), etc. are used to deliver drugs or genes into stem cells. Molecular imaging to trace stem cells is done using quantum dots (Ohyabu et al. 2009). For the speedy development of stem cells, various novel nanostructures are designed to control proliferation and differentiation.

1.10.1 MNPs in Isolation of Stem Cells

Magnetism-engineered iron oxide nanoparticles (Fig. 1.10) are developed (Lee et al. 2007). The superparamagnetic property of these nanoparticles has potential applications in hyperthermia (Kim et al. 2005), magnetic resonance imaging

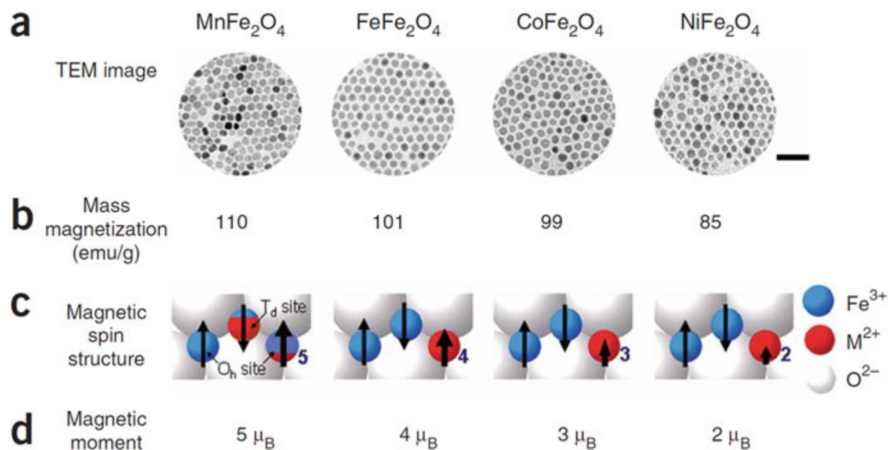

Fig. 1.10 Engineered Fe-based magnetic nanoparticles. (**a**) TEM images of different forms of Fe-based nanoparticles. The size of these nanoparticles was ~12 nm. Scale bar, 50 nm. (**b**). Mass magnetization values of MFe_2O_4 nanoparticles. (**c**) The magnetic spin structure. (**d**) The magnetic moment value is of $MnFe_2O_4$ nanoparticles. (Reproduced with copyrights permission (Wang et al. 2009))

(MRI) (Lee et al. 2007), immunoassay (Sincai et al. 2005), tissue repair (Ito et al. 2005), drug and gene delivery (Morishita et al. 2005), cell separation (Jing et al. 2007), etc. MNPs are used to isolate stem cells from cell mixture economically and in a fast and easy manner for stem cell therapy. Research has shown that MNPs are able to label stem cells which are then isolated by magnetic force or flow cytometry. An example of the same is the work done by Jing et al. (Jing et al. 2007), in which they reported that the combination of MNPs with Cd34 antibody was successfully isolated.

1.10.2 Nanoparticles in Imaging and Tracing of Stem Cells

Different nanoparticles such as MNPs, quantum dots, and gold nanorods can be employed (Medintz et al. 2005; Syková and Jendelová 2006; Lee et al. 2007; Yang and Cui 2008; Ohyabu et al. 2009). Quantum dots have unique properties and are employed in immunostaining assays, optical barcoding, cellular imaging, and DNA hybridization (Han et al. 2001; Huang et al. 2006; Bakalova et al. 2007). In a work by Ohyabu et al. (2009), it was observed that the conjugation of quantum dots with an antibody against mortalin protein formed QD composites that MSCs internalize. These MSCs, which were labeled with QDs, differentiated into adipocytes, chondrocytes, and osteocytes in vitro and in vivo, making it clear that QDs have potential applications in the in vivo imaging and tracing of stem cells. Also, QDs can be constructed as nanoprobes with multiple functions that can be changed with various biomolecules like liposome, polyethylene glycol, liposome, peptides, or antibody (Fig. 1.11) (Hoshino et al. 2005). Other nanoparticles used in molecular imaging and tracing of stem cells include MNPs (Syková and Jendelová 2006; Lee et al. 2007). An example of MNPs is superparamagnetic iron oxide nanoparticles (SPIO) which are used for labeling the stem cells; they are also used in MRI and tracking of stem cells that are being transplanted (Maxwell et al. 2008). The functionalized dextran with iron oxide nanoparticles was covalently linked to fluorescent probes for characterization of HSCs labeling to monitor the process of engraftment (Coyne et al. 2006). The fluorescent MNPs (FMNPs), with their superparamagnetic properties, when internalized into stem cells, are employed in isolating stem cells using the magnetic field for easy detection (He et al. 2007). QDs coated with CNTs are also used in labeling and tracing stem cells (Cui et al. 2019).

1.10.3 Nanoparticles in Gene Delivery Systems for Stem Cells

A study by Cui et al. indicated polyamidoamine dendrimer-functionalized fluorescent MWCNTs were able to enter into mice embryonic stem cell line CCE with high efficiency (Cui et al. 2019). Dendrimers are a unique class of organic molecules that can be modified by changing their functional groups by specific chemical reactions. These contain large cavities in their interior and can be used to carry several genes and drugs (Abbasi et al. 2014). Polyamidoamine (PAMAM) dendrimer-modified

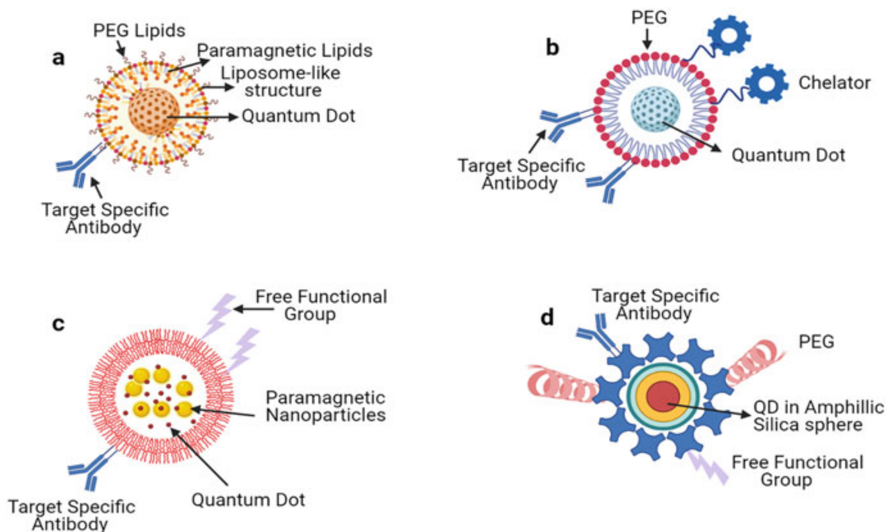

Fig. 1.11 Fibrication of QD probes. (**a**, **b**) Different molecules are attached to QDs for specific interaction to target, for nuclear spin labeling paramagnetic lipids, and chelators are attached to the surface. (**c**) QDs and paramagnetic nanoparticles within the silica sphere and target-specific groups on the outside. (**d**) The QD probe. (Created with (BioRender n.d.))

MNPs have been efficient in enhancing the accuracy of gene delivery (Pan et al. 2006, 2007b; Lee et al. 2006). Recent advancement in the molecular delivery system is the use of atomic force microscopy (AFM) and nanoneedle for the gene transfer into living cells (Han et al. 2005). Han et al. have developed a low-invasive method of gene delivery that employs an etched AFM tip which is also called a nanoneedle of diameter 200 nm and 6μm in length. It is inserted into the nucleus of the cell without damaging it. Gene delivery efficiency of about 70% was attained with a green fluorescent protein insert and the primary cultured single human MSCs. Further, these nanotools change the forces that regulate the performance of stem cells. These nanoscale patterning systems increase the emergence of stem cell mechanobiology and cellular mechanotransduction (Kumar and Gulia 2020) (Fig. 1.12).

1.10.4 CNTs and Stem Cell Proliferation and Differentiation

Because of the unique characteristics of CNTs, their potential applications are ever-increasing, including molecular electronics, chemistry, medicine, and biomedical engineering (Guo et al. 1998; Hafner et al. 2001; Gao et al. 2003; Cui et al. 2004a, b; Warheit et al. 2004; Bharali et al. 2005; Obataya et al. 2005; Liu et al. 2005). With different modifications, CNTs can be improved to make them more biocompatible (Lu et al. 2004; Singh et al. 2005). If CNTs are conjugated with proteins, they can

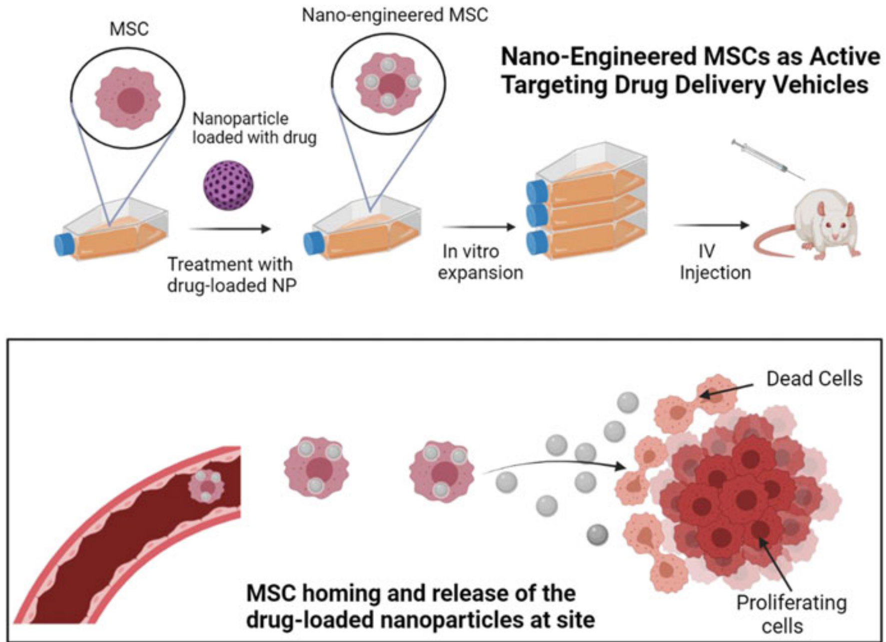

Fig. 1.12 Treating atherosclerosis using nano-engineered mesenchymal stem cell (MSC). (Adapted and modified with copyrights permission from (Kumar and Gulia 2020))

cross a cell membrane and reach the cytoplasm and nucleus of the cell (Kam and Dai 2005; Ghosh et al. 2010). DNA and peptides can be filled in CNTs and carried between the cells for drug and gene deliveries for molecular therapies (Cui et al. 2007). CNTs have the ability to inhibit cell growth by stimulating apoptosis and by decreasing cellular adhesion. This was shown by Cui et al., wherein the effect of SWCNTs was investigated on human embryonic kidney cell lines (Cui et al. 2005).

1.10.5 3D Nanostructures in Stem Cell Tissue Engineering

As already mentioned, using stem cells for tissue engineering applications has developed stem cell-based therapeutic methods for various diseases. Until now, numerous nano-fabrication tools have been developed to design 3D biodegradable scaffolds to guide stem cell differentiation (Lu and Chen 2004; Gabay et al. 2007). The cells deposit their own matrix inside these 3D scaffolds. When the degradation of scaffold occurs, the 3D tissue structure mimicking the natural extracellular matrix of the body is formed by the cells. In a study (Gelain et al. 2006), a 3D nanofiber scaffold using a peptide nanofiber was fabricated to create a 3D cell culture system for mouse neural stem cells. They used 18 varying peptides, incorporating different motifs leading to additional functions such as promoting cell adhesion,

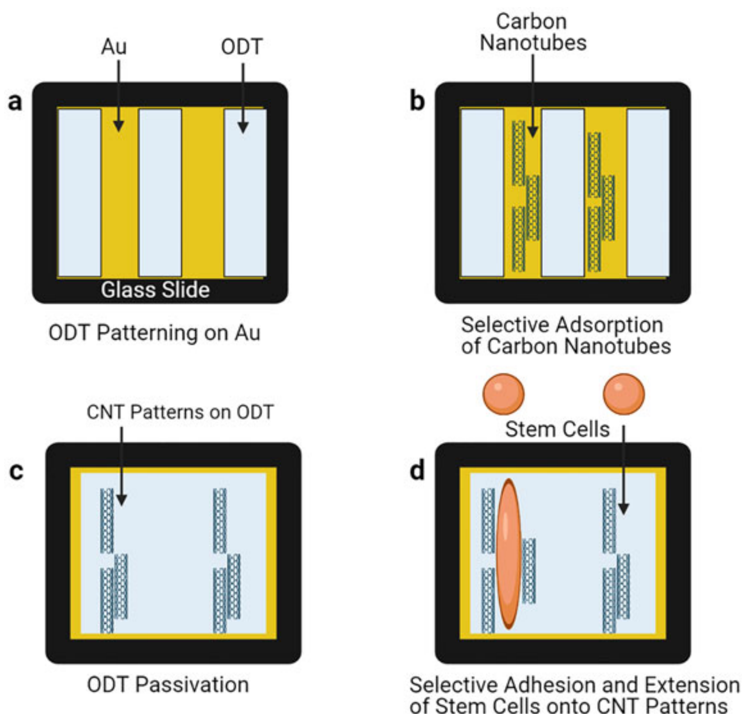

Fig. 1.13 Schematic illustration of MSC on CNT. (**a**) Patterned non-polar 1-octadecanethiol (ODT) self-assembled monolayer and area for gold (Au) is left bare. (**b**) Adsorption of CNTs on Au. (**c**) Passivation of Au surface with ODT. (**d**) Directed growth of MSCs on CNTs. (Created with (BioRender n.d.))

differentiation, and bone marrow homing activities. Apart from this, CNTs are also used to guide the growth and alignment of MSCs (Park et al. 2007) (Fig. 1.13).

1.11 Conclusion

With research going on for decades, stem cell therapy is becoming influential for modern medicine. Ongoing research has diversified the applications of stem cells; however, many obstacles are yet to overcome. Currently, with stem cell therapy, e.g., iPSCs that have allowed the use of patient's cells, there is a considerable opportunity for non-curable diseases like neurodegenerative diseases to become curable. The combination of nanotechnology with stem cells has attracted technological prospects and is advancing in developing novel stem cell technologies. The nanomaterials, such as QDs, CNTs, and MNPs, have been explored in imaging, tracing, drug delivery, and tissue engineering to regulate stem cells' proliferation and differentiation. This has further advanced stem cell therapy applications, e.g., stem cell-based 3D scaffolds for tissue regeneration and wound healing.

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2 Fabrication of Nano-Scaffolding Materials Using Different Techniques and Their Biomedical Applications

Vinitha Rani, Jayachandran Venkatesan, and Ashwini Prabhu

Abstract

Scaffolds are defined as biocompatible materials that are used to deliver drugs into the biological system. Apart from targeted drug delivery, these materials have also gained wide applications in tissue engineering, bone replacement therapies, ligament replacement, repair of nasal, auricular malformations, and joint pain inflammation treatment. Therefore, they have gained attention among researchers from the past decades for their vivid applications. Scaffolds can be synthesized using various natural and synthetic raw materials implanted into the biological system to achieve targeted delivery. Scaffolds can be easily synthesized using simple raw materials and laboratory conditions that do not require sophisticated equipment or modern gadgets. This book chapter deals with the fabrication and applications of scaffolds in drug delivery and various therapeutic applications. It also details multiple pre-clinical trials carried out using scaffolds and their outcome in clinical applications.

Keywords

Scaffolds · Fabrication · Tissue regeneration · Drug delivery · Therapy

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2.1 Introduction

Scaffolds are defined as implants and/or injects that play a vital role in tissue engineering, which are used to deliver drugs, cells, or genes into the body. They are said to function by directing the cell growth within the porous structure of the scaffold and migrating to the affected tissue (Garg et al. 2012). One of the vital scaffolding requirements is a suitable substrate for cell attachment, differentiation, migration, proliferation, and transportation of nutrients. If this requirement is not fulfilled, it may also result in declined cytocompatibility. The matrix material used in the synthesis of scaffolds should be biodegraded at a controllable rate, which is similar to the rate of natural tissue regeneration. It is always suggested to use materials that provoke minimal immune or inflammatory response in vivo, which can readily colonize with the pre-existing cells and transmit physical and chemical growth signals to ensure proper tissue growth (Garg et al. 2012; Romagnoli et al. 2013). Scaffolding biomaterials are synthesized in a collaborative effort by the chemists, physicists, biologists, engineers, and clinicians and have almost been used for the past 60 years in drug delivery systems. These materials have improvised the efficacy of pharmaceutical agents such as antibodies, peptides, and enzymes in drug delivery systems (Romagnoli et al. 2013; Fenton et al. 2018). The need for biomaterial fabrication for controlled drug release aroused the problems associated with conventional-dose delivery methods. In therapeutic drug delivery, it is evident that drugs always required multiple and/or repeated doses of administration, resulting in the high variability of circulating drug concentration during the treatment period, probably leading to toxic side effects whenever the drug concentration is raised above the safe level. In some cases, it was also noted that when repeated doses were failed to be administered, it led to a rapid decrease in drug concentration in the body, which failed the therapeutic approach. These issues resulted in tremendous wastage of drug material and subjected the patients to the immense risk of toxic side effects. To overcome this barrier in the drug delivery system, efforts were made to design materials that could satisfy the need for the slow release of drugs to the targeted site. These formulations consisted of the desired therapeutic drug coated in the form of capsules which aimed at achieving sustained drug release when administered orally (Fenton et al. 2018).

2.2 Physicochemical and Biological Properties of Scaffolds

Scaffolds are synthesized using biodegradable materials such as ceramics, polymers (natural and synthetic), and sometimes composites which are made using combinations of ceramics and polymers. Natural polymers used in the synthesis of biomaterials include cellulose, pectin, and collagen, whereas synthetic polymers make use of polyethylene derivatives, polylactic acid (PLA), polyglycolic acid (PGA), and polyhydroxy butyrate. Before using a suitable polymer to fabricate scaffolds, it is necessary to understand the physicochemical and biological properties of the polymer to ensure productive and effective fabrication (Sultana 2018). One of the significant properties that the scaffold should possess is its compatibility with

biological systems. When administered into the body, the material should not elicit any harmful or toxic response. The scaffolds should be nontoxic and possess the ability of easy biodegradation. If the synthesized biomaterial is toxic to the biological systems, there is a higher chance of rejection from the body, which may also result in the death of the surrounding normal tissues around the area of implantation. Another factor that influences the quality of the scaffolds is their mechanical strength. It is always better to synthesize scaffolds possessing the mechanical strength that matches the tissues where the scaffolds have to be implanted. Porosity, another factor affecting the efficiency of scaffolds, plays a role in determining the interaction of the material with the cells. Tiny pores inhibit the penetration of the cells within the scaffolds, whereas larger pores prevent cell attachment. The pore size also determines the loading capacity of the drug. The designed scaffold should possess maximum loading capacity, and the drug should be released homogeneously throughout the scaffold (Garg et al. 2012; Mooney et al. 1996; Lyons et al. 2008). Studies have also reported that scaffolds with a highly interconnected porous structure are ideal for treating bone defects. Tricalcium phosphate is one of the most widely used bioceramics in bone tissue engineering due to its favorable properties such as biocompatibility, resorbability, and osteoconductivity. However, due to their poor mechanical strength and rapid uncontrolled degradation, these materials have restricted applications in bone replacement therapy (Feng et al. 2014). Hydroxyapatite, another biomaterial, has been widely explored as an effective material in fabricating scaffolds. Kim et al. carried out a study to synthesize artificial bone scaffold using hydroxyapatite by free gel casting technique to improve the mechanical strength. The fabricated scaffolds consisted of 81.9% porosity and compressive strength of 3.23 MPa (Kim et al. 2019).

2.3 Biomaterials Used for Scaffold Preparation

Choosing the right biomaterial for the synthesis of scaffolds is a challenging task in its fabrication. The biomaterial selected should fulfill the basic requirements such as biocompatibility, stability, flexibility, easy biodegradation, and sustained release of drug materials with appropriate physicochemical properties that can be easily implanted in the biological system without induction of any form of the immune response. In the process of fabrication of such scaffolds, both natural and synthetic polymers are widely used (Deb et al. 2018). These biomaterials can be broadly classified into four categories: Metallic-based scaffolds are made up of alloys and stainless steel, which are commonly used in dental and orthopedics fields. In contrast, ceramic-based scaffolds possess desirable thermal and chemical stability, making them a suitable material for surgical implants. Polymer-based scaffolds provide high mechanical strength and porosity and are ideal for regenerative tissue implants. Composite-based scaffolds are prepared by combining two or more raw materials and show biocompatibility in both animals and humans (Kumar et al. 2018; Hutmacher 2001; Cheung et al. 2007). Apart from these, biomaterials can also be classified as natural and synthetic polymers based on their origin. Natural polymers are obtained from natural sources and further subjected to various

treatments to synthesize scaffolds. The use of natural materials to synthesize scaffolds emerged from the old idea from Egyptians wherein they used multiple materials such as coconut shells, wood, and ivory in body implantation to treat defected or injured parts of the body. Natural biomaterials can be further classified into various categories based on their source of origin, such as polysaccharide biomaterials (cellulose, alginate, chitin, agarose), protein biomaterials (collagen, fibrin, silk), and glycosaminoglycans (chondroitin sulfate, hyaluronic acid, heparan sulfate, keratan sulfate) (Brovold et al. 2018). Biodegradable synthetic polymers such as polyglycolic acid, polylactic acid, polydioxanone, trimethylene carbonate, and glycolide have been gaining importance in the past few decades in fabricating scaffolds to deliver drugs and seem to be a promising carrier in therapeutic applications (Gunatillake and Adhikari 2003).

2.4 Different Strategies of Scaffold Fabrication

Scaffold fabrication can be broadly classified into two categories, namely, conventional or rapid prototyping methods. Conventional synthesis is used to design porous scaffolds with simple cell adhesion properties. However, it is challenging to synthesize complex biomaterials using this method. Rapid prototyping scaffold fabrication is widely used in tissue engineering (Eltom et al. 2019). Figure 2.1 indicates the diagrammatic presentation of the most significant fabrication techniques used to make scaffolds using biomaterials. In detail, the most commonly used fabrication techniques include the following.

(a) *Solvent casting and particle leaching:* This technique involves the process of leaching out of solid particles from the polymer solution. The solution is then subjected to evaporation using any one of the techniques such as air-drying, vacuum drying, and/or freeze-drying as a result of which salt particles remain embedded in the polymer matrix and, when this matrix is immersed in water, the salt particles are leached out leaving a porous structure in the scaffolds (Janik and Marzec 2015). Solvent casting and particle leaching techniques directly affect the shape and size of the pores of scaffolds. Salt addition in the synthesis of scaffolds is a critical step since the scaffold’s structure depends on the size and amount of salt content. If the salt added is not sufficient, the polymer solution will tend to aggregate, and isolated pores begin to form. If the salt content added is too high, the geometric packing of the scaffold tends to be too close and stiff (Janik and Marzec 2015; Grenier et al. 2007).
(b) *Thermally induced phase separation (TIPS):* This technique consists of a series of steps that involve the homogenization of polymer solution at high temperature by melting and continuous blending to form a solid diluent. After that, the polymer solution is cast into the desired shape and cooled to induce phase separation and subjected to solidification by the application of thermal energy to remove the excess diluent by the solvent extraction process. Finally, the resultant material forms a microporous structure. The polymeric membrane synthesized by this technique possesses narrow pore size distribution, desirable

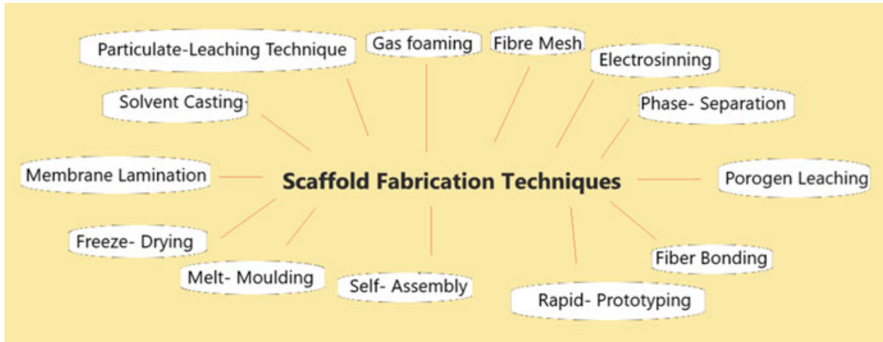

Fig. 2.1 Techniques in practice for scaffold fabrication. The image depicts the various techniques employed in the synthesis of scaffolds

mechanical strength, and high porosity (Liu et al. 2016). A study carried out by Chen et al. suggests that TIPS synthesis is favorable for the synthesis of porous scaffolds and renders several advantages in drug delivery system. Furthermore, the synthesized scaffolds using this technique possess desirable features such as good mechanical strength and easy biodegradation (Chen et al. 2020).

(c) *Freeze-drying:* This technique is popularly called lyophilization. This is employed in the pharmaceutical industry to improve the stability of the drugs and thus to be preserved for several years without hampering their quality. This method possesses an upper hand over the other techniques. When a lyophilizer is used for freeze-drying due to the closed environmental conditions, the process does not allow any external impurities into the sample, and thus, an additional purification step is not needed. Therefore, the scaffolds and biomaterials synthesized can be extensively used in bioengineering and drug delivery (Qian and Zhang 2011). Apart from lyophilization, freeze-drying can also be carried out by the following steps, which include freezing, primary drying, and secondary drying. In the first step, the aqueous polymer solution is subjected to freezing until it reaches a solid state, after which the sample is immersed in liquid nitrogen by placing it on a cold plate of controlled temperature. However, this technique is usually not followed because the scaffolds synthesized usually tend to have large pores (Chen and Wang 2007).

(d) *Rapid prototyping (RP):* Commonly called solid freeform fabrication (SFF), this method is used to produce complex scaffolds directly using a computer model. This technique plays a vital role in the medical field in guiding surgical procedures to place the implant materials. The ability of this technology to coordinate the overall micro- and macroscopic shape of the implants makes rapid prototyping an excellent tool in tissue engineering to synthesize scaffold and implant it in the desired site (Yang et al. 2002). Compared to other techniques, rapid prototyping aims to build porous scaffolds and reduce efforts in interactive modeling, thus allowing successful implantation in biological systems (Armillotta and Pelzer 2008).

2.5 Applications of the Scaffolds in Targeted Therapy

(a) *Bone tissue engineering:* Compared to the traditional autograft and allograft surgeries, bone tissue engineering using natural and synthetic scaffolds has been a promising approach in bone replacement therapies. This new technology has eliminated the most common problems faced before surgery, such as donor scarcity, pathogen transfer, and immune rejection, which are the common drawbacks faced in donors and recipients (Liu and Ma 2004). Biodegradable scaffolds tend to serve the purpose of the temporary skeleton, inserted or placed in the region of the defective site in the biological systems, which play the role of support forming material stimulating bone tissue regeneration and degrade gradually when new bone tissue is being developed (Li et al. 2005). A study was carried out by Wu et al. to fabricate biocompatible and biodegradable akermanite scaffold using the polymer sponge technique. The biodegradable potency was evaluated by soaking the scaffolds in Ringer's solution. In addition to this, the hydroxyapatite formation ability of akermanite scaffolds in simulated fluid and its effects on osteoblasts were also studied. The results of the study revealed that the scaffolds showed a porosity of 63.5–90.3% bearing a compressive strength between 1130 and 530 kPa. The biodegradable test carried out suggests that when the scaffolds were soaked in Ringer's solution, the weight of the scaffolds decreased gradually, resulting in the increase of ionic content in the solution suggesting possible degradation activity. The ionic products formed stimulated osteoblast proliferation, thus proving its efficient bioactivity (Wu et al. 2006).

(b) *Skin tissue engineering:* This is an emerging therapeutic strategy in medical applications which makes use of 3D biofunctional material to synthesize scaffold, which serves the purpose of cell adhesion and attachment to produce neo-tissue with an extracellular matrix that guides the proliferation of newly growing cells. Collagen is a natural polymer that is present in abundance in all vertebrates and also tends to provide mechanical support for cell attachment (Gaspar et al. 2011). Adekogbe and Ghanem carried out a study to fabricate scaffolds from chitosan for skin tissue engineering wherein they used chitosan with 80%, 90%, and 100% degree of deacetylation crosslinked with dimethyl 3–3, dithio bis' propionimidate and compared it to the uncrosslinked scaffolds. The results of the study showed that the tensile strength of the scaffolds, which was synthesized from 100% degree of deacetylation, was higher than that of the scaffolds made from 80% and 90% degree of deacetylation. However, all the samples showed water vapor transmission rate and pore size distribution which is suitable for skin tissue engineering. It was also noted that the biodegradation rate of scaffolds did not differ in the samples and showed consistent results irrespective of crosslinking or no crosslinking (Adekogbe and Ghanem 2005).

(c) *Cardiovascular tissue engineering:* Cardiovascular disease is the primary cause of death worldwide, accounting for 29% of deaths worldwide. However, successful cardiovascular therapies are limited and very expensive. An alternative solution for this problem is achieved through tissue engineering strategy by

implanting scaffolds, which degrade without leaving any form of remnants in the body (Generali et al. 2014). Mombini et al. developed chitosan-PVA-CNT nanofiber scaffolds for cardiovascular tissue engineering. The nanofiber scaffolds were fabricated using polyvinyl alcohol, chitosan, and varying carbon nanotubes produced using electrospinning. The synthesized nanofiber scaffolds showed mechanical strength of about 130 ± 3.605 MPa and electrical conductivity of 3.4×10^{-6} S/Cm, whereas the water uptake and cell viability were greater than 80%. The fabricated scaffolds also showed cardiac differentiation properties. The thigh bone marrow of young male rats was separated, and undifferentiated mesenchymal stem cells were isolated and washed with phosphate buffer saline, and the cells were collected after centrifugation. The cells were then cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100µg/mL streptomycin and 100 IU/mL penicillin. The cells were passaged to obtain homogenous mesenchymal stem cells. Its evaluation to differentiate into cardiomyocytes was carried out. The cells were treated with the synthesized scaffold nanofiber followed by gene profiling for the samples. The results of gene expression profiling suggest the upregulation of Nkx 2.5, troponin I, and β–MHC cardiac markers which are responsible for cardiac differentiation and electrical stimulation in the development of cardiovascular tissue and seem to be a promising candidate in tissue engineering (Mombini et al. 2019).

(d) *Cartilage tissue engineering:* In orthopedic practices, the repair of cartilage and ligament defects in the knee and joint regions of individuals remains the most commonly observed problem since these defects have limited ability to heal and most of the time tend to progress and cause osteoarthritis which causes severe pain and swelling in the affected region (Iwasa et al. 2009). Scaffolds used in cartilage tissue engineering can be synthesized in an injectable form to treat arthritis, and the main aim of this strategy is to recapitulate the normal organogenesis to form normal functioning tissue (Lu et al. 2001). Whu et al. synthesized chitosan-gelatin scaffolds for cartilage tissue engineering wherein gelatin was crosslinked with water-soluble carbodiimide after testing its efficiency. The chitosan-gelatin scaffold complex was able to initiate proliferation in chondrocytes and secrete the extracellular matrix required for tissue regeneration. When this material was implanted in defective rabbits, it caused the upregulation of $C1G1_{WSC}$ that played a vital role in cartilage regeneration (Whu et al. 2013). A similar study was carried out by Maciulaitis et al., in fabricating 3D microstructured scaffolds by using hybrid organic-inorganic material SZ2080 for cartilage tissue engineering. The material possessed a size of about $2.1 \times 2.1 \times 0.21$ mm^3 and was tested in vivo by implanting them into rabbits of about 1, 3, and 6 months of age, which were subjected to allogenic rabbit chondrocytes. Histological examination of the samples suggests that the pre-growing chondrocyte injected before scaffold implantation improved the activity of the scaffold (Mačiulaitis et al. 2015). This study thus signifies the importance of chondrocytes, scaffolds, and their dual effect in tissue engineering. Some of the significant in vitro studies carried out using scaffolds and their potential outcome is represented in Table 2.1.

Table 2.1 Scaffold based pre-clinical studies and their potential outcome

Sl. no.	Scaffold conjugate	Highlights of the study	Outcome	Reference
1.	Chitosan-collagen-hyaluronic acid scaffold conjugate with nano-hydroxyapatite	The scaffolds were synthesized using a blend of chitosan-collagen-hyaluronic acid, which was conjugated with nano-hydroxyapatite by using the freeze-drying method. The scaffolds were tested on male white rabbits. The animals were anesthetized. A vertical 1-cm incision was made with the scalpel between the 7th and 8th rib. The sterile scaffolds were placed subcutaneously into the targeted region, and the pockets were sutured, and the skin of the animals was sealed. An identical surgery was also performed on the right side of the subcapsular region, and the scaffolds were inserted separately, i.e., (1) Chitosan/collagen supplemented with 2% hyaluronic acid and 80% hydroxyapatite, (2) Chitosan/collagen supplemented with 5% hyaluronic acid and 50% hydroxyapatite, and (3) Unmodified scaffolds without hydroxyapatite. After the surgeries, the animals were housed in separate cages to minimize the risk of infection. After 6 months of implantation, blood samples were collected for hematological and biochemical studies. The animals were euthanized, and skin samples from the implanted region were collected for further analysis	The microscopic observation of the tissue samples suggests that the postoperative wounds healed well with no complications. The tissues did not show any signs of inflammation or swelling. Even the animals did not show any signs of illness or health complications during their survival period. The histological assessment of the samples showed the presence of angioblasts, fibroblasts, elastin, and collagen fibers. The scaffolds synthesized from chitosan-collagen and 5% hyaluronic acid showed fast degradation because of its low stability. In contrast, the scaffolds synthesized with the addition of hydroxyapatite was still visible at the site of implantation even after 6 months, and the morphology of scaffolds was similar to that of the surrounding tissue. Hydroxyapatite also played a vital role in the formation of blood vessels and capillaries that was necessary during tissue regeneration	Kaczmarek et al. (2018)

2.	Carboxymethyl chitosan-gelatin-laponite nanoparticle scaffold complex	The scaffolds were synthesized using carboxymethyl chitosan-gelatin-laponite nanoparticles by freeze-drying method. The scaffolds were subjected to sterilization to make them free from any sort of contamination and further seeded with rat bone marrow-derived mesenchymal stem cells (rBMSCs). About 12-week-old male Sprague-Dawley rats were used for the study. A sagittal incision of about 2 cm was made on the scalp region, and the calvarium was exposed, after which a bilateral critical-sized defect was made in every animal and grouped according to the treatment, i.e., (1) gelatin-chitosan scaffold with rBMSCs, (2) gelatin-chitosan-laponite 5% scaffold, and (3) Gelatin-chitosan-laponite 10% scaffold. Sterile scaffolds were successfully implanted into the defective area of the animal. The animals were observed for few hours after surgery to monitor any form changes. After 8 weeks of surgery, the animals were sacrificed by air embolization, and the skulls were removed and fixed in 4% paraformaldehyde for further analysis	After 4 weeks of implantation, the micro-CT imaging suggests that no new bone formation was observed in the gelatin-chitosan scaffold complex group, whereas only a small area of new bone formation was observed in the gelatin-chitosan-laponite 5% group, and clear bone regeneration was observed in gelatin-chitosan-laponite 10% group. The new bone volume to tissue volume ratio was higher in gelatin-chitosan-laponite 10% group (34.80% ± 2.53%) when compared to the other two groups (gelatin-chitosan scaffold complex, 4.60% ± 1.50%; gelatin-chitosan- laponite 5%, 18.64% ± 2.18%). The study also concluded that laponite incorporated scaffolds were successful in rectifying the defected margin area and the nearby region of the calvarial bone defect	Tao et al. (2017)

(continued)

Table 2.1 (continued)

Sl. no.	Scaffold conjugate	Highlights of the study	Outcome	Reference
3.	Starch-gelatin scaffold complex	Scaffolds were synthesized from starch-gelatin through the crosslinking method. Human dermal fibroblasts and human epidermal keratinocytes were then seeded onto the scaffolds to develop a stratified epidermal layer. The study was carried out using 15 Sprague Dawley rats which were grouped into 3 categories, i.e., (1) animals with skin injury, (2) animals treated with blank scaffolds, and (3) animals treated with scaffolds seeded with cells. After anesthetizing the animals, the dorsal hair was removed, and a full-thickness excision of 1.2 cm diameter was made using a sterile biopsy punch. The scaffolds of similar size and shape as that of the wound were cut and rinsed in saline and used to cover the wound, and the area was sealed with a silicone splint using wound dressing tape. After the completion of the procedures, the animals were shifted into separate cages and were given gentamycin and tramadol for 5 days to suppress pain and any kind of infection	After 1 week of scaffold implantation, it was observed that the animals which were treated with scaffold seeded cells promoted wound healing and tissue regeneration and showed a visible, prominent layer of the epidermis. In contrast, the skin injury animal group showed a lack of epidermis. The cell density in the wounded region of the blank scaffold group and scaffold treated with cells group was much higher when compared to the skin injury group suggesting that the implanted scaffolds provide a hostile environment for the surrounding cells for their growth. In the skin injury group, the formation of the epidermis was initiated on the 14th day and lacked the presence of a well-defined stratum basal layer compared to the other two groups	Chhabra et al. (2020)
4.	Silk scaffold	Silk is a well-known biomaterial that is suitable for ligament tissue engineering. In this study, the potency of silk scaffolds in treating anterior cruciate ligament regeneration using mesenchymal stem cells along with silk scaffolds in pig models was	After 6 months of observation, the histological studies from the sample collected suggest that the mesenchymal stem cells were distributed throughout the targeted region, and the samples showed the presence of the cells with fibroblast morphology.	Liu et al. (2009)

		evaluated. The scaffold was synthesized by incorporating the microporous silk sponges into a knitted mesh. The mesenchymal cell-scaffold conjugate was implanted into the pig model to regenerate the anterior cruciate ligament after which the pigs were maintained to observe any behavioral changes	Growth factors such as collagen I, collagen III, and tenascin-C were detected in the samples. These growth factors play a vital role in ligament regeneration, and thus, silk scaffolds seem to have potential in future clinical applications	
5.	Hydroxyapatite sponge scaffolds	This study signifies the importance of the extracellular matrix in tissue regeneration. Hydroxyapatite was incorporated with extracellular matrix by implanting sponge replica scaffolds subcutaneously in rats, and these implants were tested for the potency of bone regeneration. New Zealand rabbits of 4–8 weeks age bearing 11 mm defects were implanted with decellularized scaffolds to study the effects of the deposited extracellular matrix	The results of the study suggest that the animals showed no severe inflammation with increased cell infiltration. The new bone formation occurred in the 8th week. The outcome of the study reveals that the extracellular matrix when incorporated into the scaffolds and implanted in the biological system can potentially improve bone regeneration	Ventura et al. (2015)

2.6 Role of Scaffolds in Organ Regeneration

From the past decades, tissue regeneration and regenerative medicine have gained a lot of attention worldwide to treat chronic life-limiting conditions and manage end-stage organ failure. Figure 2.2 represents a brief introduction about the scaffolds used in tissue engineering applications. Cadaveric organ transplantation is widely used as a treatment option for end-stage disease, but this strategy limited success due to the shortcomings in donors. This drawback can be overcome by the in vitro generation of transplantable tissues or organs. These biocompatible tissues can be used in regenerative medicine. The cells used in this process tend to form replacement parts that regulate and differentiate into the respective organ and eventually carry out the normal functions. However, in the absence of these scaffolds, the cells cannot form the cellular and vascular network needed for organ regeneration (Faulk et al. 2014; Yesmin et al. 2017). The organ regeneration process requires a large number of cells that ought to be accommodated in the scaffold. For this purpose, highly porous scaffolds are used, which provides a large volume for the transplanted cells to be seeded. It is always favorable to use scaffolds having a pore size larger than the diameter of the cell so that they can be easily accommodated into the scaffold. If scaffolds with smaller pore sizes are used, then there is a possibility of the cells not being able to penetrate the scaffold (Thomson et al. 1995). A study was carried out by Versteegden et al. to synthesize tubular collagen scaffolds for urethra

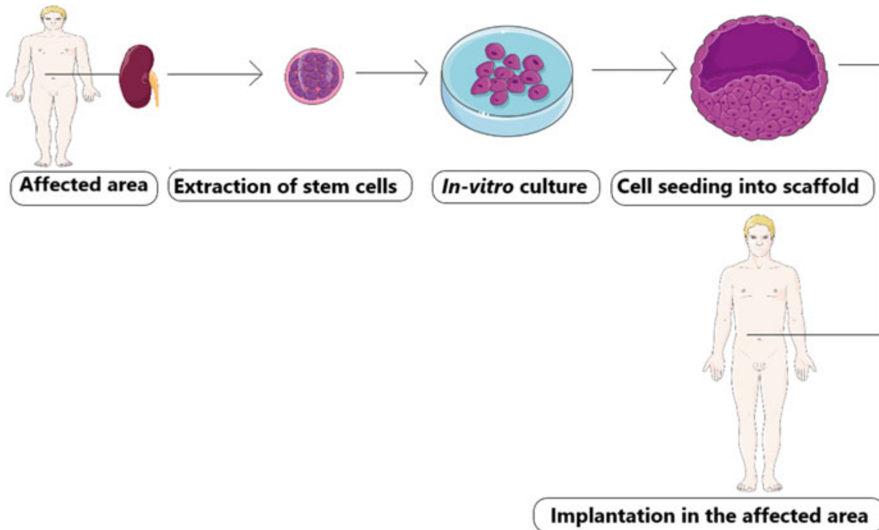

Fig. 2.2 Applications of the scaffolds in organ regeneration. Scaffolds play a vital role in the regeneration of affected organs and can be a promising tool in the field of medicine. The stem cells from the affected organs can be extracted and grown in vitro in normal sterile laboratory conditions. After the complete growth of these cells, they can be seeded onto the scaffolds and incubated at appropriate conditions to ensure their proliferation. The cell-seeded scaffolds can be implanted in the affected area, wherein they differentiate to form the tissue and aid in organ regeneration

regeneration that exhibited radial elasticity. The scaffolds were constructed by compressing the fibrillar collagen around a star-shaped mandrel that mimicked the lumen's folds. When the luminal pressure was increased, the scaffolds expanded in size; when the pressure was released, the scaffolds formed a star-shaped conformation. The scaffolds were then seeded with human epithelial cells, which were then cultured in a bioreactor under controlled conditions mimicking the rate of urination. The synthesized scaffold was efficient and showed promising results in regenerative medicine for the development of tubular organs (Versteegden et al. 2017). A similar study was carried out by Remuzzi et al. to understand the potency of organ regeneration by scaffold recellularization. Experimental rat kidneys were completely decellularized through SDS infusion by applying perfusion pressure. The organs were then observed under optical microscope to confirm decellularization. To understand the recellularization pattern, the scaffolds were orthotopically implanted into the Lewis recipients. The renal artery and veins of the recipients were successfully anastomosed, followed by uniformity in blood flow throughout the scaffold. The implanted scaffolds were biocompatible and well-tolerated in animals. The experimental animals were sacrificed on days 3, 7, and 21 after scaffold implantation. The animals sacrificed on days 3 and 7 showed significant clotting, and partial reabsorption was observed in animals of 21 days treatment group. The microscopic studies from animals sacrificed after 21 days of implantation showed a decrease in inflammatory cells, indicating the potential benefit of the scaffold in organ regeneration (Remuzzi et al. 2017).

2.7 Challenges in Clinical Applications and Their Solutions

To realize the potential applications of scaffolds in clinical applications, the scaffolds have to be well designed and should not possess any toxic immune response in the human body. The study should be carried out in a large animal model to confirm its potential therapeutic benefits that can ultimately be applied to future clinical transplantations. 3D scaffolds synthesized from porcine organs are widely used in fabricating non-immunogenic transplantable organs with wide applications in organ regeneration and repair (Zhou et al. 2015). However, even though scaffolds have shown promising results in many in vitro and in vivo studies, the translation of scaffold-based bone tissue therapy to clinical applications remains a failure. However, when talking about clinical applications, we need to understand the challenges and then formulate the strategies to address them. The other obstacles that one needs to clear to make scaffolds a promising tool in clinical applications involve the technical challenges that include designing and manufacturing these materials (Hollister and Murphy 2011).

The main two barriers which a researcher and clinicians have to face in scaffold transition include technical and business barriers. Technical barrier involves the four fundamental needs (form, function, formation, and fixation) that an ideal scaffold should have. The fabricated scaffold should easily fill in the defects and guide the tissue to match the original 3D anatomy and provide temporary support to perform

day-to-day activities at ease. The scaffold must also enhance tissue regeneration through its interaction with the biological system. When these scaffolds are introduced into the body in the implantation process, they should readily implant and attach to the targeted region (Hollister 2009). However, it is also recommended that the scaffolds should not be overdesigned by adding too many raw materials, which will result in them being too stiff that tend to cause adverse effects in biological systems leading to tissue resorption (Hutmacher 2001; Hollister 2009). The business barrier includes the challenges such as regulatory approval and fetching suitable funding for the development and commercialization of the product. If the researcher is interested in translating the scaffolds into the commercial world, then he/she must face these issues individually (which is rare), or they can even hand over the product in the form of license to other authorized agency who will tackle such issues and resolve them (Hollister 2009; Bone Anchoring System Guidance Document n.d.). However, scaffold translation from concept to the clinic for tissue engineering and drug delivery is a complicated and time-consuming task. In the practical application of scaffolds in clinical trials; the planar organs and tissue act as a considerable barrier. The requirement to synthesize scaffold for connective tissues, nervous tissue, bone, and muscle is different and, owing to its ability to expand in the biological system, also varies. The scaffolding system to repair complicated organs and tissues such as kidney, liver, and pancreas is still a significant challenge at the present date (Webber et al. 2015). However, to surpass these hurdles, all these aspects need to be addressed, which begins with a robust scientific foundation for synthesizing the scaffold and its patent strategy. The fabricated scaffolds have to be tested in vitro and must also be well established in pre-clinical trials. The animals used in pre-clinical trials should be either one among the following: guinea pigs, rabbits, dogs, or mouse models. The researcher should be dedicated to carrying out the work. In contrast, the funding agency must be secure enough to fund generously the study and have a clear vision of bringing the product forward for commercialization (Langer 2013).

2.8 Conclusion and Future Perspectives

Natural and synthetic scaffolds are gaining importance in medical therapy for the past decades due to their enormous benefits. These scaffolds can be easily fabricated using simple laboratory techniques and readily available raw materials. These therapeutic biomaterials have shown promising results in their in vitro and in vivo studies and are presently subjected to clinical trials. These cost-effective and affordable biomaterials have been fetching importance in various therapeutic applications such as bone tissue engineering, skin regeneration, cardiovascular engineering, and cartilage and ligament replacement therapy. However, when talking about the clinical implications of these novel biomaterials, it is imperative to understand the allergic immune responses caused by these agents in the biological systems since they possess major drawbacks in making the clinical trials a successful study. It is indispensable to design the fabrication of these materials considering the basic

properties such as biocompatibility, biodegradation, adherence property, mechanical strength, and porosity. The degradation rate of these materials should be the same as that of the regeneration of the new tissues. If this is achieved, then these materials are said to be perfect for tissue engineering therapy. These biomaterials should not cause any harm to the pre-existing cells nor cause any form of allergic reactions to the system, thus making them an ideal biomaterial in drug delivery and tissue engineering techniques. These materials seem to have a good effect in future medical therapy in replacing damaged tissues and thus have to be widely explored by researchers and clinicians to extract the possible benefits from them.

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3 Nanofiber-Mediated Stem Cell Osteogenesis: Prospects in Bone Tissue Regeneration

Taha Umair Wani, Rumysa Saleem Khan, Anjum Hamid Rather, Abdalla Abdal-hay, Touseef Amna, and Faheem A. Sheikh

Abstract

Tissue engineering and regeneration, over the years, have evolved as a prospective means of treatment for bone defects as an alternative to auto−/allograft techniques. The stem cells have a strong potential to differentiate into various lineages under artificial conditions. This has led to extraordinary regenerative medicine progression. Studies have demonstrated that stem cells from many sources could be guided for differentiation into specific tissue through biochemical and mechanical stimulation. This has paved the way for developing a considerable number of biomaterials/methods mediating osteogenic differentiation. These cells' potential can be used to form bones in various bone defects/fractures using different materials. Nowadays, the biomaterials used for the regeneration purpose are made up of nanofibers that interact precisely with the physiological milieu to facilitate high cell proliferation and osteogenic differentiation. However, this process requires fine-tuning the features, such as nanofiber diameter, scaffold porosity, topography, etc., seeding technique, and signaling factors. This

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chapter gives a brief account of the various stem cells used for bone regeneration and their osteogenic differentiation capabilities using appropriate signaling and growth factors. Typical components of tissue engineering scaffold, the procedure for seeding the cells into the scaffold, and the mechanism of induction of cell differentiation are briefly discussed in this chapter. Furthermore, the factors such as mechanical stimulation, nanofiber alignment, and scaffold morphology that affect cellular differentiation have also been highlighted.

Keywords

Mechanical stimulation · Nanofiber · Signaling factors · Osteogenic differentiation

3.1 Introduction

Tissue engineering has emerged as a versatile technique in the field of medicine for the treatment of various diseases/defects, offering replacement or restoration of tissues. From the past several years, investigations into nanofibers' use as biomaterial scaffolds for tissue engineering have been mostly in place (Bianco and Robey 2001). Nanofibers are thin fibrous polymeric string-like structures with diameters of the range of nanometers generally prepared by electrospinning technique under high voltage. The other techniques used for nanofiber preparation are phase separation, template synthesis, self-assembly, drawing, etc. (Lim 2017; Nemati et al. 2019; Abdal-hay et al. 2012, 2013a, 2014a, b, c, 2015a, b, 2018). However, electrospinning is the most commonly used technique for the purpose. The applications of nanofibers in tissue engineering are enormous like wound healing, bone regeneration, patches for cardiac tissue, neural engineering, etc. (Kumar et al. 2018). This is owed to the remarkably porous structure in addition to the extremely high surface area to volume ratio of nanofibers, which enables efficient cell adhesion, migration, differentiation and proliferation of cells (Dahlin et al. 2011). Yet another outstanding property of nanofibers is their biomimetic nature, making them unique for tissue engineering applications. Nanofibrous scaffolds are better than other types of scaffolds, such as microporous/microfibrous scaffolds due to the fact that the former resembles the extracellular matrix (ECM). The ECM is a complex network of extracellular components, such as fibrous proteins, proteoglycans, glycoconjugate, and glycosaminoglycans, which along with cell adhesion proteins (i.e., fibronectin and laminin) is the defining framework of tissues (Alberts et al. 2002; Abdal-hay et al. 2013b; Pant et al. 2013) (Fig. 3.1).

ECM is primarily applauded for maintaining tissue homeostasis, acting as a sensor for signal transmission across the cell membrane, thus playing a central role in tissue formation and repair (Lutolf and Hubbell 2005). Nanofibers resembling the ECM thus mimic the structural and functional roles of ECM, making these excellent for tissue replacement therapy. In addition to acellular materials/implants, nanofibers offer substrate for stem cell seeding options for tissue regeneration. In such

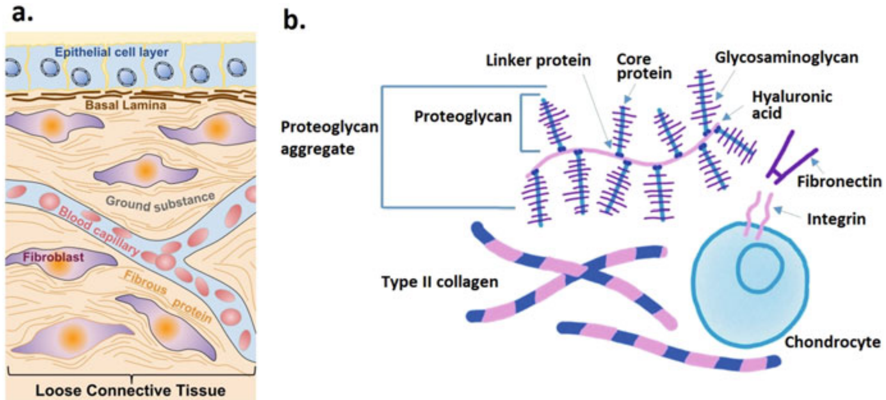

Fig. 3.1 Structure of extracellular matrix. (**a**) ECM embedded with fibroblasts and blood capillaries and surrounded by epithelial cell layer; (**b**) components of ECM. (Figures adapted from Wikimedia)

cell-embedded nanofiber scaffolds, potent stem cells, capable of rebirth into a specific type of cells and tissues, are seeded into the nanofibrous materials (O'brien 2011; Hutmacher 2000). Stem cells are taken from the patient's body and grown in a culture media under appropriate conditions. The cells are then seeded onto a suitable scaffold with growth factors and signaling substances and allowed to culture. Thus the process involves cells grown into tissues in vitro using polymeric/metallic/ceramic scaffolds under the optimum cellular differentiation conditions by using biochemical factors or mechanical stimulation. The scaffold is then implanted at the site of damage. Alternatively, the tissue regeneration can be carried out in vivo as well (Fig. 3.2). Applying tissue engineering approaches for bone regeneration presents an excellent alternative for autografts and allografts practiced as frequent procedures for bone replacement during fractures due to trauma or certain bone defects, e.g., osteogenesis imperfecta (Aro and Aho 1993; Laurencin et al. 2006). An essential element of a bone tissue engineering material is the supporting substrate known as a scaffold that holds various components like those of extracellular matrix (ECM). Nanofibers mediate bone regeneration within the scaffold that offers excellent interactions with the physiological milieu of a tissue due to their small size and interaction with the cellular functions (Venugopal et al. 2008). This is mediated through the increase in the absorption of cell adhesion proteins like collagen, vitronectin, laminin, fibronectin, etc. (Dutta and Dutta 2009).

Many stem cells are capable of osteogenic differentiation finally resulting in the growth of bony structures under artificial conditions. These stem cells broadly fall under two categories, i.e., adult stem cells and embryonic stem cells. Osteogenic differentiation of stem cells is mediated through a range of transcription factors and signaling pathways, including transforming growth factor-β, insulin-like growth factor, and fibroblast growth factor signaling. Some of the critical transcription

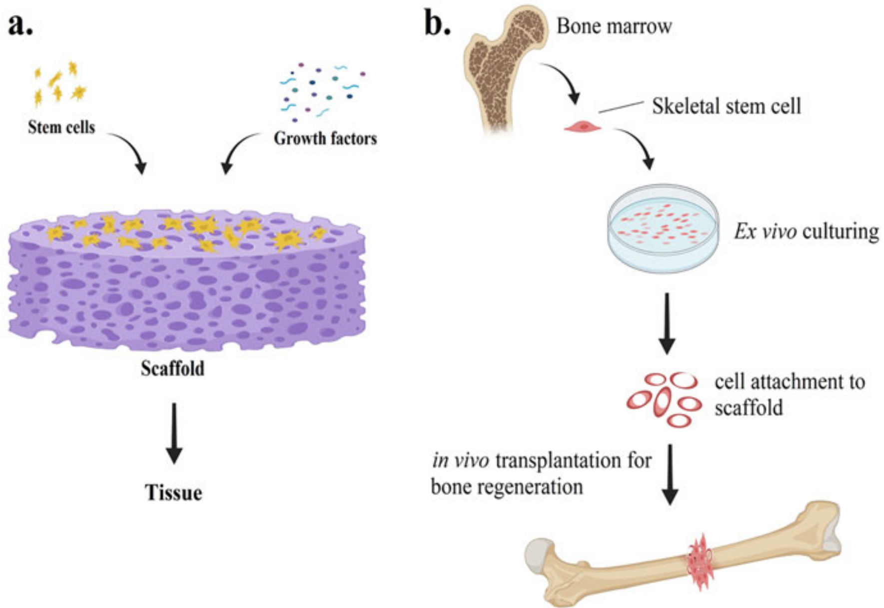

Fig. 3.2 Basis of bone tissue engineering, (**a**) in vitro tissue regeneration using stem cells and growth factors together seeded on scaffold, (**b**) in vivo bone tissue regeneration using stem cells isolated from the bone and seeded into scaffolds to be implanted at a defected bone

factors involved in osteogenic differentiation are bone morphogenetic protein, Smad proteins, β-catenin, Runx2, osterix, etc. Transcription factors are proteins that activate the expression and/or suppression of genes in a differentiating cell and change the cell's proteome, thus altering its structural or functional characteristics and directing it to a specific phenotype. The following discussion in this chapter will provide an overview of the stem cells used in bone regeneration, essential techniques for efficient stem cell seeding, mechanism of osteogenic differentiation, and some factors critical in osteogenic differentiation.

3.2 Structure of Stem Cell-Seeded Nanofibers

A tissue engineering material or scaffold consists of three major components, viz., cells, scaffold, and cell signaling substances; these are highlighted underneath.

3.2.1 Stem Cells

Nowadays, stem cells are considered the central component of modern tissue engineering. Specific stem cells capable of differentiating into desired tissues are obtained from proper cell sources, such as embryonic stem cells, adult stem cells, or

progenitor cells, and seeded onto a suitable substrate known as scaffolds (Lim and Mao 2009). When placed in an appropriate medium of culture, these cells can differentiate into specific full-sized tissue.

3.2.2 Scaffolds

To develop properly into a tissue, the stem cells are grown on substrates known as scaffolds. Scaffolds are the materials that harbor cell growth factors and signaling substances and act as a mechanical support for cell proliferation. With precise engineering, scaffolds provide the prospect for desired cellular interactions and development into specific tissue with enormous biomedical applications, such as tissue regeneration (in organs like the skin, bones, cardiac tissues, liver, etc.), wound healing, implants, etc. A scaffold can be a synthetic or a natural polymer (e.g., collagen, gelatin, chitosan, polylactic acid, etc.), metal (titanium, tantalum, magnesium, etc.), or ceramic (hydroxyapatite, calcium phosphate, etc.) substance depending upon the intended use (Abdal-hay et al. 2014b, d, e, 2015b, 2018, 2020; Hosseinkhani et al. 2014; Iravani and Varma 2019). The typical roles of scaffolds are the following:

- Cell attachment, migration, and growth.
- Harbor and deliver biochemical factors.
- Diffusion of essential cell nutrients.
- Modification of cellular interactions by altering their mechanical and biological behavior.
- Transplantation for in vivo treatment.

3.2.3 Signaling Factors

As already mentioned, the scaffolds contain certain signaling substances to exert behavioral alterations in cells. These behavioral alterations by scaffolds are realized through protein or mechanical stimulations, which initiate signals to communicate with the cells. Some examples of the signaling factors used in osteogenic differentiation are transforming growth factor β, platelet-derived growth factors, β-catenins, fibroblast growth factor, insulin-like growth factors, bone morphogenetic proteins, vascular endothelial growth factors, Hedgehog, etc.

3.3 Stem Cell Seeding

Cell seeding in nanofibrous scaffolds is performed by culturing the cells in a medium. This process is simple in 2D membranes wherein the cells settle down under their weight and undergo adhesion to the nanofiber surface. However, in 3D scaffolds, additional care is required during cell seeding since the adherence to the

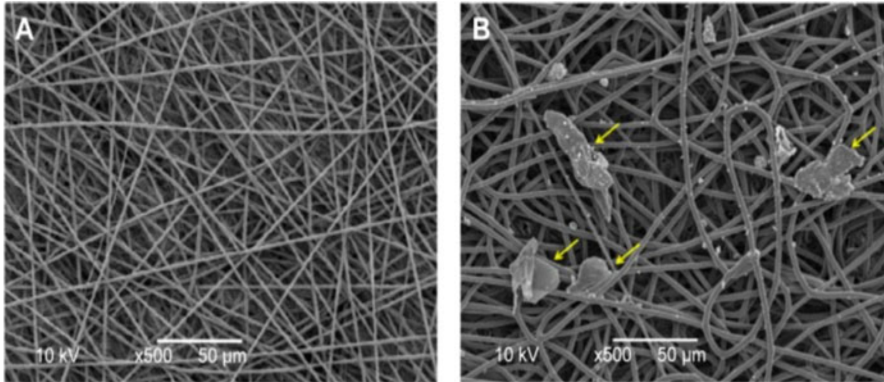

Fig. 3.3 SEM images of electrospun nanofiber scaffolds. (**a**) Non-seeded nanofibers. (**b**) Cell-seeded nanofibers. (Reprinted with permission (Braghirolli et al. 2015). Copyright (2015), Dove Press)

surface only is not sufficient, and the cells need to reach the depth of the scaffold. There are several techniques used for the seeding of cells into 3D scaffolds, including static (or passive) and dynamic approaches. A thorough description of such techniques has been given by Villalona et al. in their review (Villalona et al. 2010). Apart from these usual cell seeding methods, a number of other approaches have evolved to improve the efficiency of seeding and enhance the cell distribution within the scaffolds. One such technique used to improve the stem cells' distribution in the scaffold is carrying out the electrospinning and cell seeding simultaneously. Cells are seeded by spraying the cell suspension, a process known as electrospraying, onto the electrospun nanofiber layer on a solid substrate. For this purpose, a device that possesses two parallel running infusion needles is used wherein one needle ejects the polymer solution for nanofiber formation. In contrast, the other sprays the stem cells onto the nanofibers. Both fibers and cells are finally collected onto a solid collector. Both the processes (electrospinning and electrospraying) employ simultaneously at the same voltage and result in an even distribution of cells in the nanofibrous scaffold. This process has already been employed by many researchers, e.g., Braghirolli et al. (2015). These researchers used simultaneous electrospinning and electrospraying for incorporation and uniform distribution of mesenchymal stem cells into poly(lactide-co-glycolide) nanofibrous scaffolds. The process of electrospinning was carried out at 0.54 mL/h and 15 kV, with the distance between the needle and the collector being 7.5 cm. On the other hand, the cells were sprayed under the same voltage at a rate of 2.60 mL/h and 4 cm distance between the needle and the collector. The uniform distribution of cells was ensured by rotating the electrospraying collecting drum for every 5 min during the process. The SEM images (Fig. 3.3) and confocal microscopy of the scaffolds showed several cell adhesion points and distribution of cells throughout the nanofiber scaffold on day 1, which further improved until day 15.

Another method for simultaneous electrospinning and cell seeding is layer-by-layer seeding of the cells. In this method, the electrospinning and cell seeding processes do not run in parallel, instead, a layer of nanofibers is first cast onto the substrate, followed by spraying of cells followed by another layer of nanofibers, and so on. In this way, the cells adhere to every layer, and a uniform cell distribution is achieved. This method was employed by Yang et al. for the development of multilayered nanofibrous scaffold for tissue engineering applications (Yang et al. 2009). Human dermal fibroblasts were seeded into the poly(ε-caprolactone)/collagen nanofibrous scaffolds by alternate electrospinning of nanofibers and seeding of the cells on coverslips or aluminum foils. An illustration of this method is given in Fig. 3.4. However, the only problem with the system is getting contaminations while layering nanofibers and cells together.

One of the earliest methods used for the seeding of cells into scaffolds is by using a tube running through the middle of the scaffold. In this technique, a hole-bearing catheter is inserted in the center of a multilayered porous 3D scaffold. The stem cells are injected into the catheter, which ultimately travels through the catheter's holes and gets distributed throughout the scaffold. This technique has been used by Wald et al. to seed hepatocytes in a multilayered 3D poly(lactic acid) scaffold using a silicone catheter running through the middle layer of the device (Wald et al. 1993). The catheter contained two holes on its opposite sides, either at the center of the scaffold or 1 cm apart and facing toward the scaffold's edges. The cell suspension, injected at a standard rate of 0.5 mL/min, showed excellent cell distribution throughout the device. Such a low rate of injection was suggested to be crucial in order to prevent the hepatocytes from damaging.

3.4 Mechanism of Cell Differentiation in Bone Regeneration

Cell signaling mechanisms operating in bone regeneration are very complex, and a detailed account of this is out of the scope of this chapter; however, a brief overview is provided as follows. The leading indicators of osteogenic differentiation are gene expression, mineral deposition, and alkaline phosphatase activity. The best way of elucidating a pathway of stem cell differentiation is to evaluate cells' gene expression at different stages of their journey to tissue formation. Such analysis has revealed that the genes related to the division and proliferation of cells are the ones that are most upregulated in the early stage of cell differentiation. In the later stages of cell differentiation, the genes that govern the biological functions appropriate for developing a complete tissue are upregulated. In the case of bone regeneration also, the gene expression concerning the biological function is regulated in the later stages of osteogenesis. Several cell-cell communication systems control the differentiation of cells and the formation of final tissue in engineering scaffolds. The primary mechanism of osteoblast differentiation is through the activation of transcription factors (Ohata and Ozono 2014). One of the most critical pathways followed during osteogenic differentiation is the transforming growth factor-β (TGF-β) pathway. The most prominent member proteins of this pathway are the

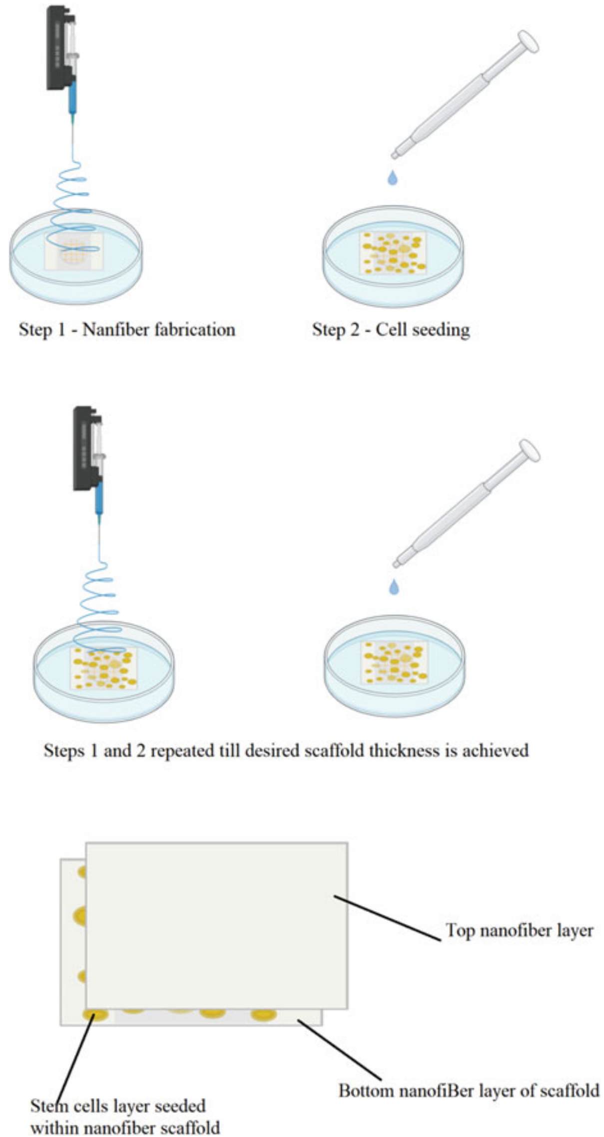

Fig. 3.4 Stem-wise illustration of layer-by-layer nanofiber fabrication and cell seeding process on nanofiber scaffolds for achieving uniform cell distribution

bone morphogenetic proteins and Smad proteins (Arnsdorf et al. 2010). Bone morphogenetic proteins have regulatory functions in the early formation of the skeletal structure, among other embryonic developments. The binding of these proteins with morphogenetic receptors activates the signal transducer family of proteins known as Smad proteins which function as transcriptional modulators. The Smad, after undergoing phosphorylation, is translocated into the nucleus where it activates various transcription factors which interact with the target genes

to regulate the expression and production of factors governing the phenotype of the osteoblasts.

Another signaling pathway that comes into play during osteoblast differentiation is the insulin-like growth factor family proteins (Chen et al. 2010; Al-Kharobi et al. 2014). These proteins stimulate collagen and non-collagenous proteins, which are essential for the formation, progression, and maintenance of bone. Another process in the stem cells' osteoblast differentiation is by preventing β-catenin degradation through the inactivation of glycogen synthase kinase 3 (Yang et al. 2011). β-catenin binds to the T-cell transcription factor in the nucleus resulting in the augmented expression of Runx2. This transcription factor acts as an osteogenic promoter for osteoblast differentiation favoring both endochondral and intramembranous ossification. Runx2 is considered very important in osteogenesis because specific inhibition of Runx2 leads to the inhibition of bone formation ultimately. Another transcription factor, osterix, is associated with the regulation of the late-stage bone formation through Col1a1 gene activation responsible for collagen construction (Baglìo et al. 2013). Osteocalcin secretion is also augmented through gene expression by osterix which leads to the deposition of calcium. Besides specific transcription factors, some other systemic factors that regulate a cell's energy metabolism can have osteoblastic differentiation functions as well. For example, dlk1/FA1 protein has been found by Abdallah et al. as a regulatory protein for differentiation of adipocytes and osteoblasts through pro-inflammatory cytokine expression (Abdallah et al. 2007). Similarly, apart from increasing the calcium deposition, osteocalcin has been found to regulate adiponectin levels, an adipokine that controls glucose and fatty acids levels. Finally, the fibroblast growth factors and the signaling proteins play an essential role in ossification, osteoblast maturation, and essentially arresting previously differentiated osteoblasts. Figure 3.5 gives the overview of the mechanism of signal transduction through transcription factors during osteogenic cell differentiation.

3.5 Stem Cells Used in Bone Regeneration

3.5.1 Adult Stem Cell-Seeded Scaffolds

The most reliable and efficient bone regeneration technique requires the introduction of cells at the site of injury. There are two significant sources of stem cells, viz., adult stem cells and embryonic stem cells. A prominent example of adult stem cells is the mesenchymal stem cells (MSCs) which are the multipotent cells nearly capable of expression into mesenchymal tissues, such as the bone, cartilage, and fat. A thorough description of MSCs as tissue regeneration progenitors can be found in the following references (Ding et al. 2011; Caplan 1991; Minguell et al. 2001; Short et al. 2003; Zomorodian and Baghaban Eslaminejad 2012; Iaquinta et al. 2019). The excellent differentiating property of the MSCs has mostly led to these cells' use for bone regeneration applications. Generally present in almost all tissues, these cells are isolated from the bone marrow, periosteum, endosteum, etc. and cultured in vitro

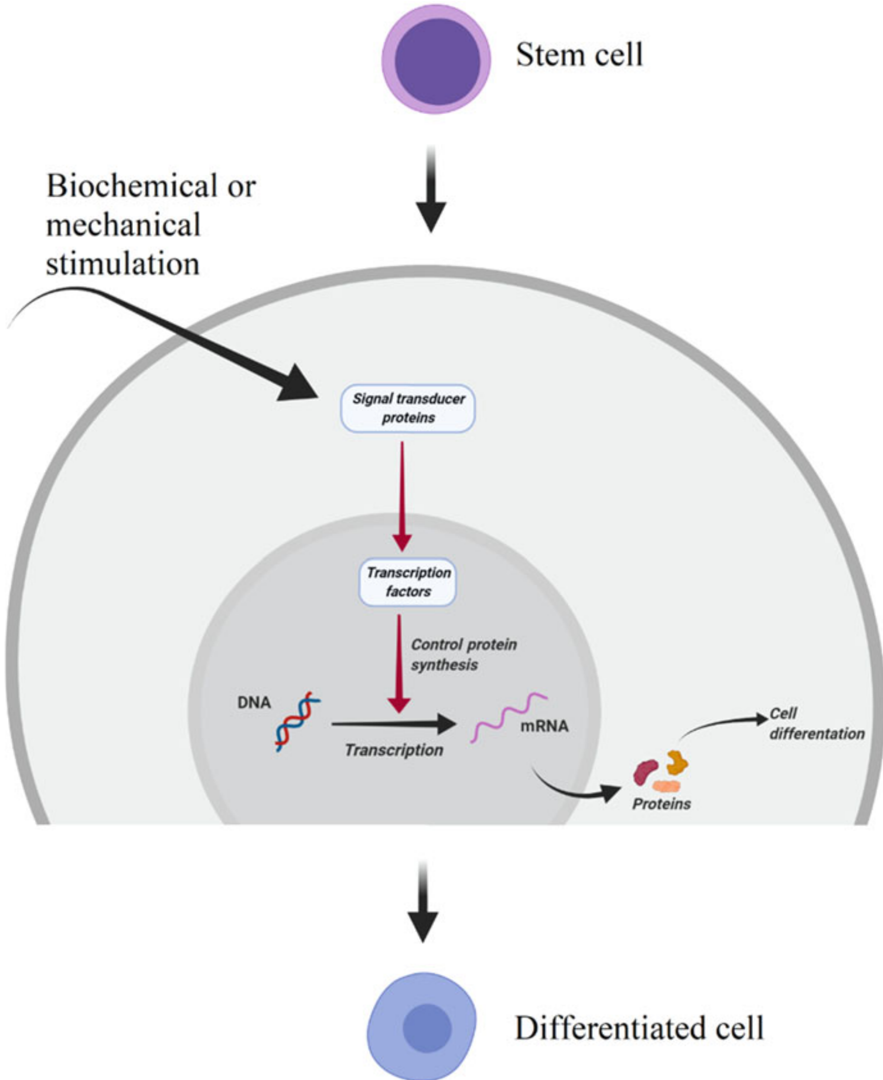

Fig. 3.5 Mechanism of signal transduction through transcription factors during osteogenic cell differentiation. The external biochemical or mechanical stimulation stimulates signal transducer proteins (like Smad) which translocate and activate transcription factors in the nucleus. The transcription factors then take control of the transcription of the cell's DNA to mRNA and direct it to manufacture proteins that decide the phenotype of the final differentiated cell

through adherent cell cultures. Other essential characteristics of MSCs are their anti-inflammatory, antimicrobial, and immunomodulatory effects and differentiation into almost any kind of cell lineages (Fig. 3.6). These cells have been widely used for the fabrication of bone components through osteogenic induction and bone regeneration. The one remodeling role of MSCs can be realized from Fig. 3.7. These cells'

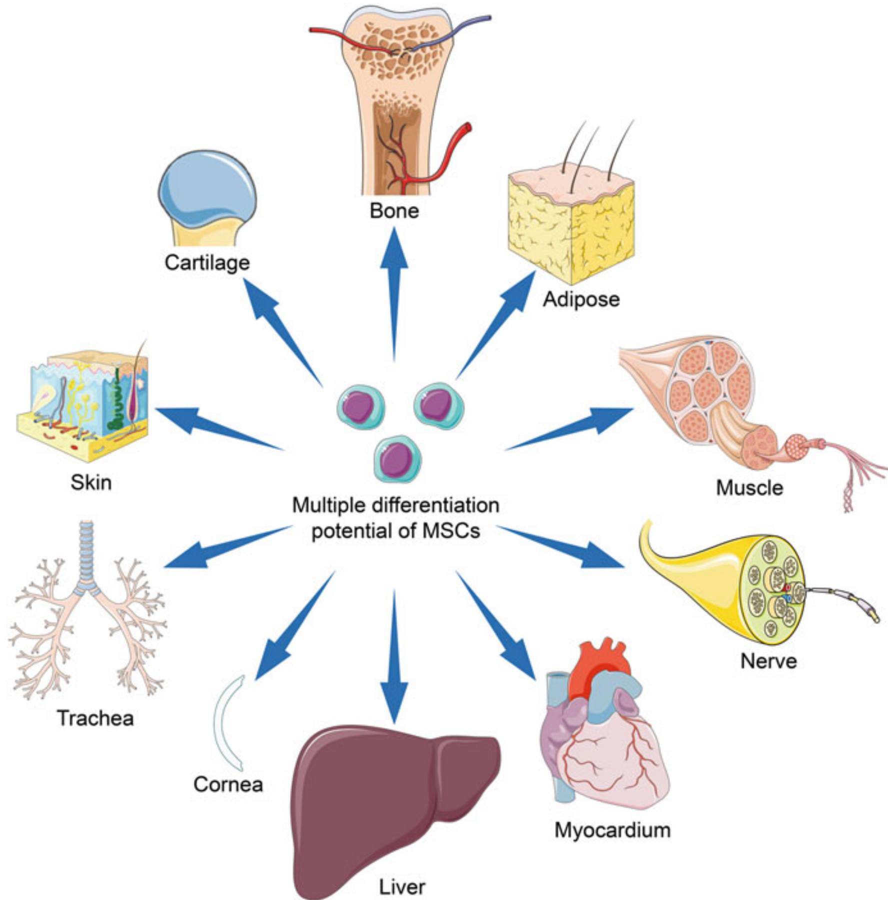

Fig. 3.6 Regeneration potential of MSCs into various tissues, including soft to hard tissues. (Reprinted with permission from (Han et al. 2019). Copyright (2019))

osteogenic properties are confirmed when the cells express mRNAs of bone matrix proteins and there is deposition of hydroxyapatite-rich ECM and interim expression of alkaline phosphatase. These activities within the MSCs approve their bone-forming potential and their use in vivo for bone healing. The cells are then seeded onto a proper substrate/scaffold and implanted at the regeneration site. Extensive research has been dedicated to using MSCs for bone regeneration, e.g., Li et al. have developed MSC-seeded 3D porous nanofiber scaffold for tissue engineering of cartilages. The scaffolds were prepared using poly(ε-caprolactone), a biodegradable synthetic polymer. The nanofibers were randomly oriented and had a diameter of 700 nm, with its structural integrity retained over 21 days in the culture medium. While culturing in the presence of TGF-β1, the gene expression specific to cartilages and the presence of cartilage ECM proteins were the evidence for MSC differentiation into chondrocytes (Li et al. 2005a).

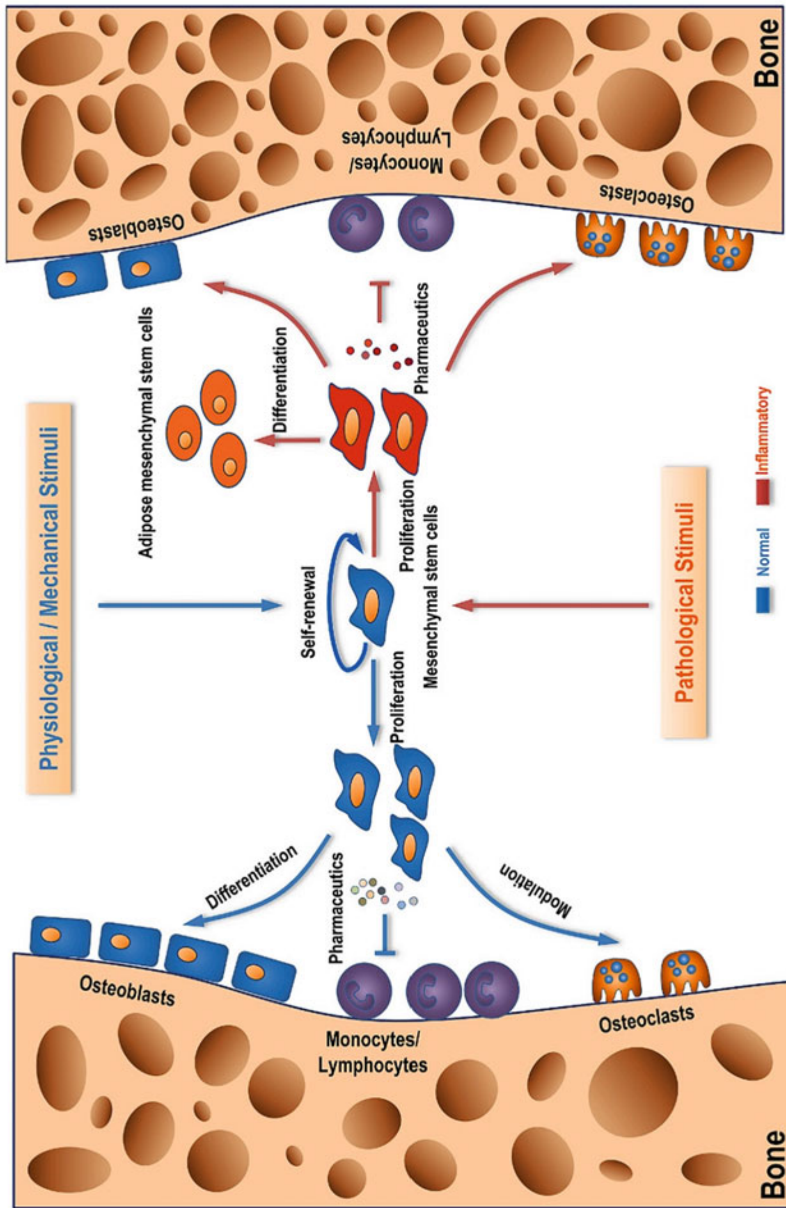

Fig. 3.7 Role of MSCs in osteogenesis, bone remodeling, pathogenesis, and differentiation into bone cells. (Reprinted with permission from (Shang et al. 2020). Copyright (2020), Elsevier BV)

In yet another study, Li et al. showed that human MSCs seeded onto nanofibrous scaffolds of poly(ε-caprolactone) could differentiate into osteogenic lineage in addition to adipogenic and chondrogenic lineages (Li et al. 2005b). Donzelli et al. developed an MSC-embedded collagen scaffold for osteogenic differentiation in vitro (Donzelli et al. 2007). The MSCs were isolated from the bone marrow of adult rats and cultured on collagen scaffolds using plastic dishes in the presence of osteogenic substances. The cultured cells could express osteocalcin and osteopontin, which are considered specific lineage biomarkers of osteogenic cell differentiation. Osteocalcin is a protein hormone secreted by osteoblasts essential for augmenting the concentration of calcium in bones. Osteopontin is an extracellular phosphoprotein that exhibits strong calcium-binding ability and a role in mineralization and bone resorption and remodeling. Increased alkaline phosphatase activity is responsible for the hydrolysis of inorganic pyrophosphate, mineralization of bones, and hydroxyapatite synthesis (Szulc and Bauer 2013).

Another example of adult stem cells used for bone regeneration is dental pulp stem cells. These are the pluripotent stem cells derived from dental pulp. These cells can differentiate into multiple lineages and are considered to have a higher proliferation rate than bone marrow stem cells (Shi et al. 2001). This is attributed to the less maturity of dental pulp stem cells than some other types of stem cells. With the use of a proper culture medium, dental pulp stem cells have great potential for osteogenic differentiation through transcriptional changes.

3.5.2 Embryonic Stem Cell-Seeded Scaffolds

Embryonic stem cells can offer one of the most versatile sources of cells for bone tissue regeneration owing to their extraordinary regenerative properties. The process of bone development from embryonic stem cells has been extensively studied, and comprehensive knowledge of genes, growth and transcription factors, and related conditions is well documented. This has led researchers to understand the osteogenic differentiation of embryonic stem cells into complete bone structures and paved the way for the use of these flamboyant cells in bone tissue engineering (Liu et al. 2014; Bielby et al. 2004). In comparison to adult stem cells that generate limited amounts of cells, embryonic stem cells can source countless cells that can be adequately used for tissue regeneration purposes. The first step in the differentiation of embryonic stem cells into specific cell types is the development of embryoid bodies, which are 3D pluripotent cell aggregates capable of differentiating into endoderm, mesoderm, and ectoderm (Nickolls et al. 2020). Embryonic stem cells have been used in osteogenic differentiation for a while now. Rutledge et al. developed poly (lactic-co-glycolic acid) scaffolds for osteogenic differentiation of human embryonic stem cells using natural bone ECM for improved bone formation. The scaffolds were initially seeded with osteoblasts, which led to bone ECM components' deposition in the scaffold. The scaffolds were later decellularized to remove the cells while keeping the bone ECM intact, including collagen, alkaline phosphatase, and calcium, as revealed by specific staining techniques. The human embryonic stem cells

were seeded onto the scaffold cultured in a culture medium containing appropriate osteogenic differentiation factors. The scaffold supported excellent cytoskeletal formation as revealed by the F-actin staining. The osteogenic differentiation markers, such as RUNX2 and bone gamma-carboxyglutamate protein genes, osteocalcin expression, and calcium deposition, were used to determine the differentiation of human ESCs. RUNX2 and bone gamma-carboxyglutamate protein expressions were augmented by the use of decellularized scaffolds (Rutledge et al. 2014).

Marolt et al. developed osteoconductive scaffolds to form compact bone grafts from human embryonic stem cells in the osteoinductive medium using perfusion bioreactors. The embryonic stem cells were first differentiated into mesenchymal progenitors and were seeded in the scaffolds for specific bone tissue differentiation. The authors noted that the undifferentiated embryonic stem, when used directly for implantation in mice, resulted in the formation of teratomas. The prior differentiation of the embryonic stem cells into mesenchymal progenitors solved this problem. When implanted in mice, the scaffolds revealed that the embryonic stem cell differentiation was well controlled using this protocol, and there was no evidence of any other germ cell layers or tumor at the site of implantation (Marolt et al. 2012).

3.6 Some Important Factors Affecting Stem Cell Differentiation

The scaffold porosity, cell attachment, fiber diameter, and alignment in the scaffold are important factors governing the success of stem cell differentiation. Nanofiber interaction with the cells can control their osteogenic differentiation through specific pathways. An example of this is the study carried out by Chang et al. They showed that single bone marrow stem cells were grown on a specially micropatterned matrix to eliminate any possibility of osteogenic stimulation through intercellular communications. The nanofibers could impart excellent adhesion and guide the single-cell differentiation through the FAK/RhoA/YAP1 pathway (Fig. 3.8). These workers observed that small-sized micropatterns halted the cells' commitment for osteogenic lineage leading to a small cell spreading area. The nanofibers led to better cell morphology, fewer stress fibers, and higher alkaline phosphatase activity than flat film fiber (Chang et al. 2018). Some important nanofiber/scaffold properties affecting osteogenic differentiation of cells are discussed as follows.

3.6.1 Mechanical Stimulation

Like chemical stimulation, stem cell differentiation can also be triggered by mechanical stimulation (Arnsdorf et al. 2010). Mechanical stimulation is induced by the application of an exogenous mechanical force, e.g., tensile loading, shear stress, ultrasound, electromagnetic stimulation, etc. Such stimulation by these forces is believed to generally work by induction of conformational changes in the functional

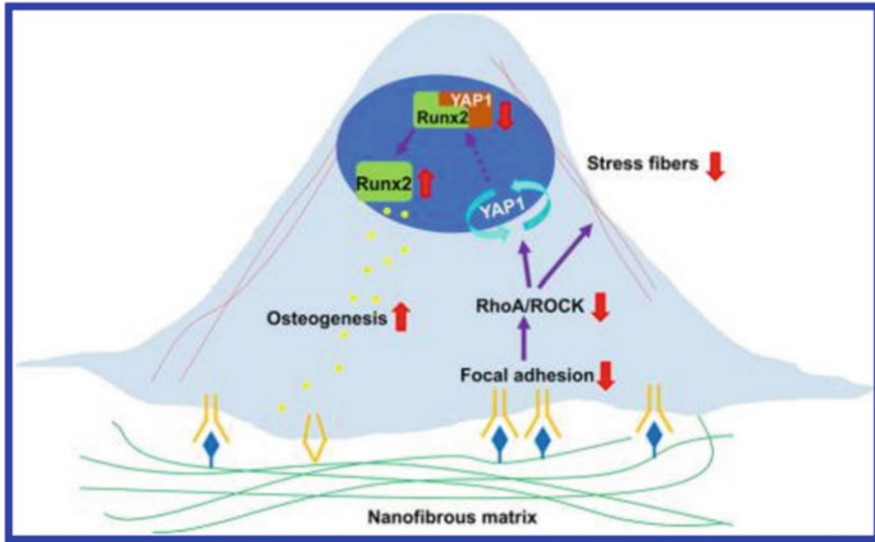

Fig. 3.8 Osteogenic transcription factors' function in osteogenic differentiation of bone marrow stem cells as described by Chang et al. (Reprinted with permission from (Chang et al. 2018). Copyright (2018), American Chemical Society)

proteins, ion channels, and receptors or activate phosphorylation, etc. This leads to the active zones' exposure to the target molecules, thus triggering a series of biochemical changes in the cells deciding the cell differentiation's fate. One good example of mechanical stimulation is the binding of vinculin to talin molecules after the latter is stretched by the application of external force. Vinculin and talin are the two cytoskeleton proteins that are very significant in cell signaling carried out through membrane integrins that integrate talin with the ECM (Del Rio et al. 2009).

Altman et al. showed the differentiation of mesenchymal bovine bone marrow cells into ligament cells by applying external multidimensional strain for 21 days using a bioreactor (Altman et al. 2002). The mechanical stress resulted in the formation of collagen fibers aligned in the same direction as that of the applied strain. Specific patterns of collagen banding and mRNA expression for biomarkers, including collagen type I and III, fibronectin, and tenascin-C, were observed in the mechanically strained cells. At the same time, the biomarkers for other cell lineages were not observed. Graysona et al. developed temporomandibular joint condylar bone from human MSCs using a decellularized trabecular bone scaffold in a human temporomandibular joint-shaped bioreactor. During the tissue differentiation, an interstitial flow of the culture medium was maintained in the bioreactor. In the first week of the culture, these researchers observed 7.5 times rise in the DNA content, which meant an extensive cell proliferation. The matrix formation, tissue growth, and mineral deposition were excellent throughout the scaffolds' entire volume when a bioreactor was used compared to the static conditions. The microcomputer tomography images revealed mineral deposits in the pores of the scaffold and significant

growth in the bone volume (Grayson et al. 2010). Subramony et al. used a combined strategy of chemical and mechanical stimulation for the differentiation of human MSCs for ligament regeneration. These workers combined the effects of necessary fibroblast growth factor stimulation of the cells with the tensile loading using a bioreactor. The application of the external mechanical force was observed to increase cell proliferation and collagen deposition significantly. The expression of type I and III collagen also increased considerably in the case of mechanically loaded scaffolds compared to the unloaded ones (Subramony et al. 2014).

3.6.2 Nanofiber Topography or Substrate Alignment

The orientation of the fibrils in the ECM is a defining factor in the determination of bone anisotropy. Researchers have implicated that cellular differentiation is sensitive to the nanofiber topography in the scaffolds. The nanofiber scaffolds' topography largely determines the expression of cellular biomarkers and can even stimulate complete cell differentiation. Jiang et al. demonstrated the effect of tissue culture polystyrene surface and randomly oriented and aligned nanofibers on the differentiation of MSCs. The aligned nanofibers had the best topography among the three (Jiang et al. 2012). When grown on tissue culture polystyrene or randomly oriented nanofibers, the cells attained polygonal shape. They exhibited random orientations compared to the cells grown on the aligned nanofibers that showed elongated and aligned cell nuclei. The topographical stimulation of cell differentiation operates via actin-intermediate filament system mediating gene expression regulation. The tissue culture polystyrene was also found to induce higher levels of actin stress fibers in the cells attributed to its stiff topography that stimulates the formation of excessive focal adhesion leading to high cytoskeleton stress.

In the case of osteogenic differentiation of stem cells, cell alignment plays a vital role. Panday et al. have provided a comprehensive study on the effect of the aligned and non-aligned nanofibers on the osteogenesis of MSCs derived from canine adipose tissue. Poly(ε-caprolactone) was used as the polymer for the preparation of nanofibers. Using real-time-PCR, the levels of COL1A1 and osterix (osteocyte markers) were seen elevated. The proliferation and calcium deposition of the cells were also increased. The high proliferation was attributed to the parallel alignment of the nanofibers and high mechanical strength leading to better cell attachment with the individual fibers and even more cell-cell contact sites (Pandey et al. 2018). Martins et al. designed patterned nanofiber meshes consisting of differently oriented nanofibers with areas of parallel and randomly oriented poly(ε-caprolactone) fibers (Martins et al. 2011). The osteogenic differentiation of human bone marrow stem cells on the nanofiber meshes was carried out to evaluate the nanofiber orientation effect. The alignment of the nanofibers had a great impact on the growth and differentiation of the cells. In the case of randomly oriented nanofiber meshes, the cells depicted an undifferentiated phenotype of MSCs and random cell polarity. On the other hand, the cells grown on the aligned nanofiber meshes exhibited excellent nanofiber attachment and proliferation. The expression of alkaline phosphatase

established the osteogenic differentiation of the MSCs on the aligned nanofibers in addition to osteocalcin bone sialoprotein and osteopontin. The osteogenesis-related proteins or transcription factors like Runx2, alkaline phosphatase, and Osterix were also found in elevated levels after 21 days of cell culture.

3.6.3 Scaffold Porosity and Diameter

Nanofiber characteristics like diameter and porosity significantly impact the differentiation of stem cells, gene expression, cell shape, and matrix formation. Porosity and pore sizes within the scaffolds are suggested to influence the cells' attachment, proliferation, and migration (Karageorgiou and Kaplan 2005). However, optimum pore size is essential to maintain a balance between the attachment and the migration processes. While large pore size is necessary to facilitate the smooth migration of cells within the scaffold, pore size should be small enough to enable adequate cell attachment owing to the high specific surface area associated with small pore size. The high porosity of the scaffolds is believed to enhance the supply of oxygen and nutrients to the cells leading to high cell proliferation (Cho et al. 2009). Then again, proper cell differentiation requires restricted cell proliferation, thus requiring less porous scaffolds. Researchers have suggested a scaffold pore diameter of 100–500μm as optimum for cell proliferation and differentiation (Hulbert et al. 1970; Wake et al. 1994). Takahashi et al. showed the effect of pore size within the polyethylene terephthalate scaffolds on the proliferation of rat femur-derived MSCs. They reported high cell proliferation on high-porosity scaffolds after 21-day cell culturing. Conversely, the increased cell differentiation was noted in cells grown on low-porosity scaffolds, as demonstrated by the high osteocalcin content and alkaline phosphatase activity (Takahashi and Tabata 2004). Gomes et al. studied the influence of the porosity of starch-poly(ε-caprolactone)-based scaffolds on the proliferation and osteogenic differentiation of rat bone marrow stromal cells derived from femora and tibia. High scaffold porosity was reported to significantly influence cell proliferation and differentiation when cultured under static conditions or in a bioreactor (perfusion cultures). Highly porous scaffolds enable extensive growth of cells and matrix formation in addition to the excellent osteogenic differentiation as demonstrated by enhanced alkaline phosphatase activity and calcium deposition under perfusion conditions ascribed to the increased cell number and density (Gomes et al. 2006).

Nanofiber diameter is another critical factor influencing cell proliferation and differentiation. This determines the efficiency of cell attachment within the scaffold, with large diameters offering enough surface area for ensuring efficient contact with the cells and vice versa. Further, the large fiber diameter enables larger inter-fiber spaces and thus increased porosity. A more efficient cell attachment results in commendable cellular responses and cell proliferation. The nanofiber scaffolds have been found to exhibit a fiber diameter-dependent efficiency in cellular differentiation as described by Takahashi et al., who evaluated MSC differentiation on polyethylene terephthalate nanofibers of varying diameters of 2.0 to 42.0μm and

porosity (Takahashi and Tabata 2004). The workers observed varying cell shapes, morphologies, cell attachment, and expression of osteogenic biomarkers. The shapes of the MSCs attached on the smaller diameter 2μm nanofibers were spherical, which became flattened as the nanofibers' diameter increased. The cell attachment and proliferation were found to be highest for fibers of diameter 22 and 42μm, and the increase was statistically significant than the smaller diameter nanofibers. Biomarker expression also depended on nanofiber diameter with the intermediate diameters 9–12μm, leading to the highest alkaline phosphatase activity and osteocalcin deposition. Badami et al. have also studied the dependence of cell proliferation and differentiation on the nanofiber diameter. The nanofiber scaffolds were prepared from poly(ethylene glycol) and poly(lactic acid) by electrospinning with a nanofiber diameter of 0.141 to 2.14μm. These workers reported increased cell density with the increase in the diameter of the nanofibers (Badami et al. 2006).

3.7 Conclusion and Future Perspectives

In conclusion, stem cells have great potential for osteogenic differentiation in artificial conditions using biochemical or mechanical stimulation. Researchers have successfully used adult and embryonic stem cells for osteogenic differentiation and developed complete bone cell phenotypes. Several signaling pathways and transcription factors involved in the osteogenic differentiation of stem cells have been identified. Thus, stem cell-seeded tissue engineering scaffolds have applications as regenerative medicine for treating bone defects and fractures, and research has shown that such scaffolds can be efficiently used to develop complete bone tissues from these stem cells. Much research has been dedicated to the fabrication of scaffolds favoring efficient osteogenic differentiation. However, there is still a lot of research required in realizing nanofiber-based stem cell therapy as efficient regenerative medicine for clinical use. Efforts in devising techniques for efficient cell seeding, uniform cell distribution, and preclinical in vivo studies are some of the areas that require careful consideration.

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4 Using Nanofiber Scaffolds for the Differentiation of Induced Pluripotent Stem Cells into Cardiomyocytes: The Latest Approaches in Tissue Engineering

Rumysa Saleem Khan, Taha Umair Wani, Anjum Hamid Rather, Mushtaq A. Beigh, and Faheem A. Sheikh

Abstract

Organ and tissue replacement therapies are complicated by immune rejection that restricts the long-term effectiveness of implanted devices. With advancements in nanotechnology and tissue engineering, its applications in the biomedical field have gradually increased. To increase the immunologic acceptance of these devices and to mitigate diseased conditions, stem cells have arisen as a suitable choice. The heart is known to recover its function after myocardial infarction with stem cell transplantation. The cardiomyocytes (CMs) to be used can be generated and applied in regenerative medicine by creating tissue-engineered cardiac patches with the evolution of human-induced pluripotent stem cell (hiPSC) technology. Several novel 3D scaffolds have been introduced as stem cell carriers with favorable surface morphologies. Electrospinning-mediated fabrication of tissue engineering scaffolds is considered a method of choice as it can make fibers that best mimic the extracellular matrix of the heart. Stem cells combined with nanofiber carriers to regenerate cardiac tissues show a vast potential to treat cardiac diseases. This chapter gives insights into the production of hiPSC-derived CMs on nanofibrous scaffolds and how these biomaterials can improve stem cell function in the cardiac tissues with potential applications in cardiac regenerative medicine.

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Keywords

Cardiomyocytes · Nanofibers · 3D scaffolds · Extracellular matrix · Human-induced pluripotent stem cell

4.1 Introduction

Cardiovascular disease (CVD) is a heart and blood vessel disease group and is a significant reason for deaths in the USA. Millions of people have coronary heart disease, and more than 0.7 million new cases are registered of myocardial infarction (MI) every year in the USA (Benjamin et al. 2018). Some of the well-known CVDs are given in Fig. 4.1. When blood flow to the heart is blocked because of damage to the heart muscle, it results in MI and causes necrosis of cardiac tissue (Fig. 4.2). Scar formation and defective responses after MI cause a decrease in function of the left ventricle and, eventually, complete heart failure (Lloyd-Jones et al. 2009; Prabhu and Frangogiannis 2016) (Fig. 4.3). If the heart cannot supply sufficient blood to prepare the needs of the body, it causes problems in heart functions (Kemp and Conte 2012). For the failure of the heart, the fundamental reason is high blood pressure (Nabel and Braunwald 2012). In the past, trials to repair cardiac function using therapeutic delivery did not benefit the pumping function of the heart (Dimmeler et al. 2008). After MI, a quarter of heart cells vanish, and a considerable cell supply is needed for its regeneration (Kajstura et al. 1998; Yoon et al. 2006). To

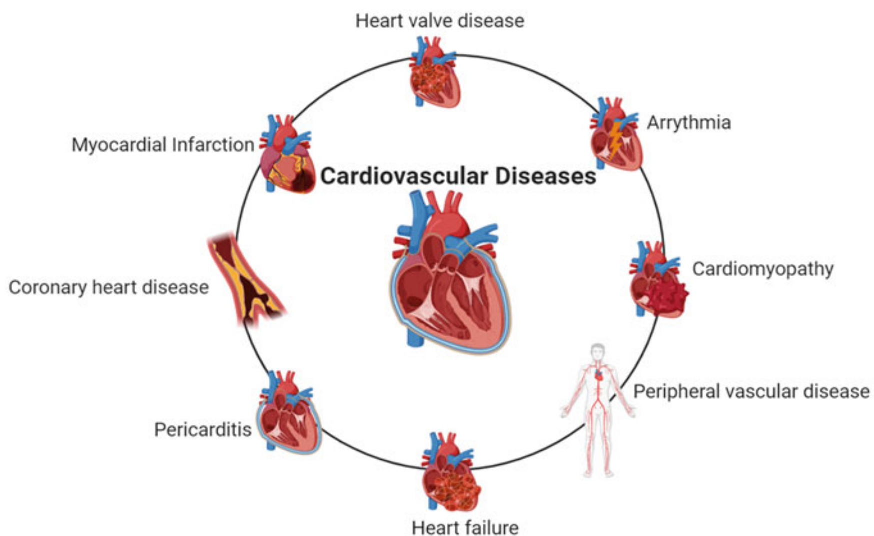

Fig. 4.1 CVDs—heart and blood vessel diseases. These constitute heart valve disease, arrhythmia, cardiomyopathy, peripheral vascular disease, heart failure, pericarditis, coronary heart disease, and MI

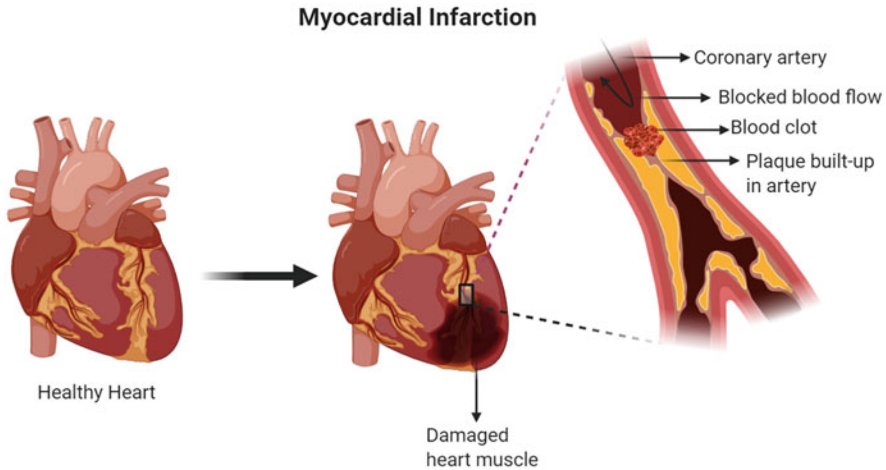

Fig. 4.2 MI occurs on the accumulation of atherosclerotic plaque in the inner lining of a coronary artery, which then bursts, leading to thrombus formation; blood flow to the heart is blocked, resulting in necrosis of cardiac tissue. (Image created using BioRender)

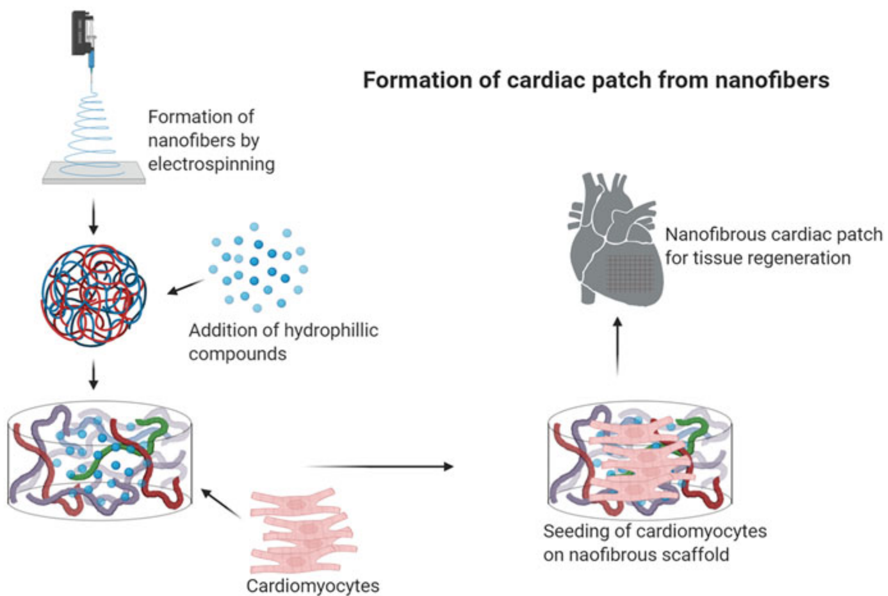

Fig. 4.3 MI refers to the death of the heart muscle tissue by lack of oxygen in myocardial tissue, leading to subsequent consequences. (Image created using BioRender)

repair cardiac tissue and treat heart failure, cardiac regeneration is seen as the best option (Laflamme and Murry 2011). To treat MI, fabricated cardiac tissue-like constructs have been established by culturing cardiomyocytes (CMs) on nanofibers

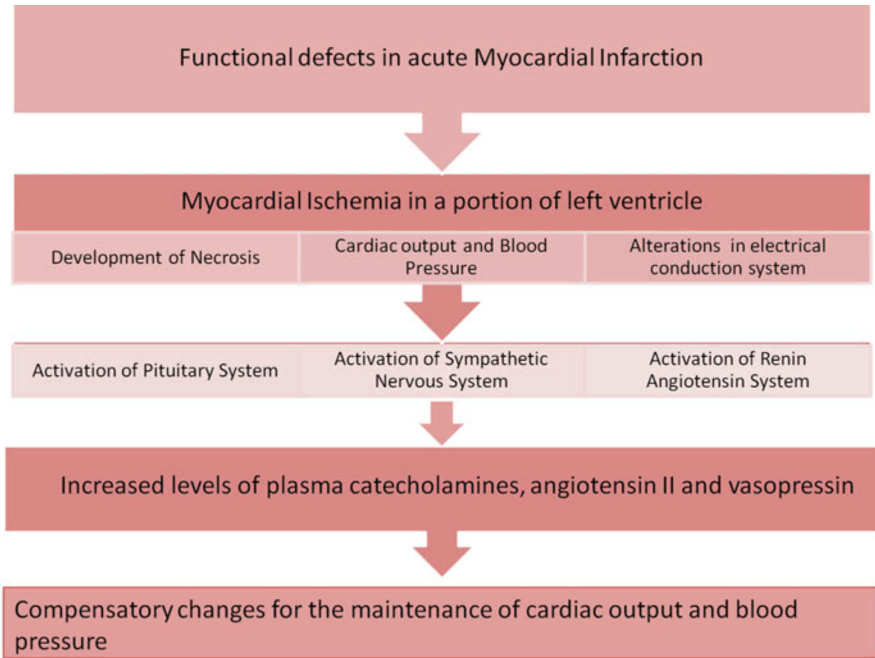

Fig. 4.4 Fabrication of cardiac patch using electrospun nanofibers: Cardiac patches are made by suspending the CMs in a scaffold of a biomaterial fabricated to resemble the ECM. Patches produced with aligned scaffolds are more easily vascularized by the circulatory system of the host

(Fig. 4.4) (Radisic and Christman 2013; Martins et al. 2014; Li et al. 2017a; Gao et al. 2018). The therapeutic potential of embryonic stem cells (ESCs) is enormous, but their use is limited on account of immunological rejection by the host (Boheler et al. 2002). Yamanaka and colleagues discovered a novel approach to induce stemness in fibroblasts by incorporating genetic factors and named them induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006), which are like ESCs in appearance and differentiation potential. New myocardium and enhanced cardiac function in rats, mice, pigs, and primates have been formed by transplantation of ESCs- or iPSCs-derived cardiac cells and patches (Plowright et al. 2014; Breckwoldt et al. 2016; Foo et al. 2018).

There are mainly parallel-aligned CMs in the cardiac muscle, interspersed with parallel-aligned microvessels (Kaneko et al. 2011). Investigations have been done on the consequences of anisotropic myocardial fabricated tissues on primary (Engelmayr et al. 2008; Bian et al. 2014; Kai et al. 2014; Lin et al. 2014) or stem cell-derived CMs (Parrag et al. 2012). ECM is the essential component of engineered tissues, and it provides signals to cells. Parallel-aligned (anisotropic) scaffolds reflect the native cellular organization and give directions for the cell rearrangements. This leads to the elongated CM morphology (Kai et al. 2011; Orlova et al. 2011; Kharaziha et al. 2014; Khan et al. 2015; Ruan et al. 2016; Li et al. 2017a; Lemoine

et al. 2017). Spatially oriented electrospinning is an approach to fabricate anisotropic scaffolds (Zong et al. 2005). When CMs are cultured on these spatially fabricated scaffolds, these arrange the cytoskeleton according to the alignment of fiber (Zong et al. 2005; Parrag et al. 2012; Wanjare et al. 2017; Allen et al. 2019). As the energy consumption of myocardium is high, an uninterrupted blood supply from capillaries is needed for its sustenance (Parker and Ingber 2007). The CM and vascular endothelial cell interaction is necessary in order to establish contact with host vasculature (Sekine et al. 2008; Zamani et al. 2018; Huang et al. 2018). Endothelial cells in engineered heart tissue lead to angiogenesis after myocardial injury because of the presence of endothelial cells within them (Sekine et al. 2008; Gao et al. 2018). Regarding this, stem cell transplantation has drawn enormous attention with the discovery of iPSCs (Takahashi and Yamanaka 2006). Due to the improvement in the direct differentiation of hPSCs, now there is a certainty of producing stem cell-derived CMs and endothelial cells from ESCs or iPSCs with better effectiveness (Burridge et al. 2014). The induced CMs and endothelial cells resemble their appearance and functions to native cells and are tested in animal models for heart tissue regeneration (Rufaihah et al. 2011; Nakayama et al. 2018; Ishida et al. 2019). Many stem cells have the potential to differentiate into cardiac cells, like mesenchymal stem cells (Müller-Ehmsen et al. 2002; Orlic et al. 2003), ESCs (Caplan and Dennis 2006), iPSCs (Lahti et al. 2012), and CPCs (Miyahara et al. 2006), so a lot of attention has been drawn toward PSCs (Braam et al. 2010; Minami et al. 2012; Liang et al. 2013; Navarrete et al. 2013; Mathur et al. 2016). CM constructs that resemble tissues are necessary, not the non-organized clusters of cells (Braam et al. 2010; Matsa et al. 2011; Liang et al. 2013; Navarrete et al. 2013).

The ESCs are taken from the inner mass of cells of the blastocyst (Fig. 4.5). The inner cell mass grows to form ectoderm, endoderm, and mesoderm of the embryo proper, in vivo (Kingham and Oreffo 2013). Because ESCs are mainly produced from preimplantation embryos (Olson 2006; Kattman et al. 2011; Noseda et al. 2011) and iPSCs are generated from somatic cells (Lian et al. 2012; Zhang et al. 2012), these have gained more attention. Recently, there has been a strong surge in the production of hiPSC using fibroblasts under defined factors (Jaffe 2008). Also, there is an increasingly sophisticated capacity of iPSCs to easily differentiate into cell types related to diseases such as CMs (Burridge et al. 2012; Mordwinkin et al. 2013; Matsa et al. 2014).

4.2 Gene Expression and Signaling Pathways in CM Differentiation

Many studies have been performed on model organisms that demonstrate that the signaling pathways like Wnt, BMP, and Activin/Nodal/TGF-β play essential roles in establishing the cardiovascular system (Olson 2006; Evans et al. 2010; Noseda et al. 2011). From a mixed population of iPSCs, purification of CMs is attained by non-genetic methods, for example, cell-surface markers (Kattman et al. 2011; Lian et al. 2012; Zhang et al. 2012; Abilez et al. 2014; Sanchez-Freire et al. 2014),

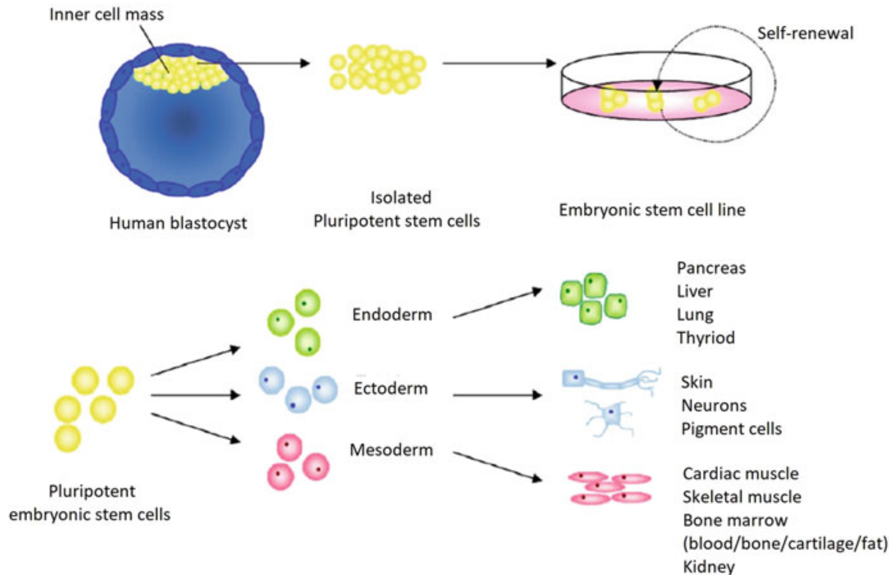

Fig. 4.5 Differentiation of human ESC lines: ESCs are derived from the cells of the blastocyst. With their maintenance in culture, they experience self-renewal and proliferation and retain their stem cell state. (They are adapted with permission from (Kingham and Oreffo 2013))

mitochondria-specific cells (Uosaki et al. 2011), fluorescent probes (Ban et al. 2013), and glucose deprivation (Tohyama et al. 2013). iPSC differentiation toward CMs at the molecular level is coordinated by the patterned expression of various genes at certain steps, which include genes for the establishment of mesoderm, mesoderm for cardiogenesis, cardiac-specific progenitors, and genes for muscle-related proteins of CMs, respectively (Kattman et al. 2011; Liang et al. 2013; Abilez et al. 2014). The ion channel genes of cardiac tissues are in the left ventricle, such as sodium, potassium, and L-type calcium channels (Liang et al. 2013). iPSC-CMs express genes for Ca^{2+} cycling machinery, such as inositol triphosphate receptor, sarcoplasmic reticulum Ca^{2+} ATPase, ryanodine receptor, calsequestrin 2, junctophilin 2, calreticulin, phospholamban, sodium exchanger, and triadin (Itzhaki et al. 2011; Jung et al. 2012a; Rao et al. 2013). Mitochondrial complexes I–V and genes for cholesterol metabolism and genes against apoptotic and oxidative stress processes are expressed in iPSC-CMs (Rana et al. 2012). ROCK signaling pathway, which downregulates cell migration and cell-cell adhesion, is often targeted in cardiac engineering (Riento and Ridley 2003). On screening a diverse compound library using hPSC-CPC, it was found that inhibitor of Wnt pathway signaling (XAV939); bone morphogenetic proteins; a dorsomorphin inhibitor of AMP-activated kinase, RepSox, which acts as an inhibitor of TGF-β type 1 receptor; ALK5; or other inhibitors of ALK5 (Drowley et al. 2016) enhance differentiation of hPSC-CPC.

4.3 Electrospinning Is a Preferable Method for Nanofiber Fabrication

Among many methods employed for tissue engineering, electrospinning has attracted the most attention as it produces nonwoven meshes in the form of scaffolds that structurally resemble the ECM of the heart (Ali et al. 1993; Czyz and Wobus 2001). For the fabrication of fibers, electrospinning is a well-known nanotechnology technique that utilizes electrically charged polymeric solution and is widely used to produce biomaterials for tissue engineering (Ali et al. 1993; Dorfman et al. 1998; Xie et al. 2009) (Fig. 4.6). Electrospun nanofibers produce nanoscale structures that are highly porous and interconnective and have high surface area to volume ratio, imparting the properties of attachment with the cells, better proliferation, and, lastly, their differentiation (Han et al. 2016). It is also a successful technique to fabricate anisotropic scaffolds (Barnes et al. 2007) rather than other conventional methods like soft lithography, microfluidics, photolithography, and two-photon initiated polymerization (Kim et al. 2010; Ma et al. 2014; Xiao et al. 2014).

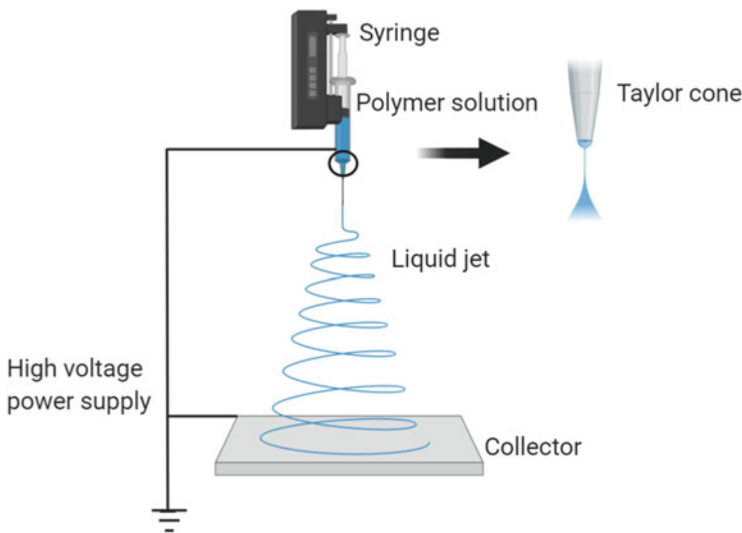

Fig. 4.6 Electrospinning is a versatile micro-nanofiber production method that uses electric force to form threads of the polymer solutions of a hundred nanometers. On applying a high voltage, the liquid is charged due to the electrostatic repulsion and surface tension; the droplet gets stretched, and then at a point, a stream of liquid flows from the surface. This point is where the Taylor cone formation occurs. The liquid jet, which is now produced, gets elongated by a whipping process due to electrostatic repulsion. Finally, it is deposited on the collector, and uniform fibers of nanometer-scale diameters are perfectly formed. A brief review of the studies done on the differentiation of stem cells into CMs using different nanomaterials is given in Table 4.1.

Table 4.1 Differentiation of CMs from stem cells on different nanomaterials

S. no.	Polymer	Application	Reference
1	Gelatin	Crosslinked gelatin nanofibers supported by poly(ethylene glycol) diacrylate honeycomb frame prevent heterogeneous cardiac clusters and larger cardiac clusters	Tang et al. (2016b)
2	Gelatin	Gelatin nanofibers are suitable for the long-term expansion of human pluripotent stem cells under the feeder and serum-free culture conditions	Yu et al. (2019)
3	Polycaprolactone	The 3D-aligned polycaprolactone nanofiber scaffolds show improved CM differentiation of hiPSC-CPCs	Ding et al. (2020)
4	Polycaprolactone	There is a predominant topography role over endothelial culture in cell survival, angiogenesis, and vessel formation as the aligned scaffolds provide the directions to the formation of anisotropic vessels	Willerth et al. (2019)
5	Polycaprolactone	3D polycaprolactone nanofibrous scaffolds directly promote CM differentiation, which might be mediated by the activation of canonical Wnt/β-catenin signaling during early differentiation	Chen et al. (2015)
6	Polycaprolactone	Electrospun anisotropic fibrous scaffolds induce efficient alignment of hPSC-CMs	Han et al. (2016)
7	Immobilized fibronectin on polycaprolactone	The implantation of umbilical cord blood-derived mesenchymal cells with fibronectin on polycaprolactone causes a reduction in MI size and fibrosis and causes a rise in thickness hence an effective carrier for stem cell transplantation to treat MI	Kang et al. (2014)
8	Chitosan-polycaprolactone	hESCs on aligned chitosan-polycaprolactone substrates enhance their myogenic differentiation and show MyoD expression better than those on collagen or polycaprolactone alone, hence showing the importance of ECM topology in directing the myogenic differentiation of hESCs	Leung et al. (2013)
9	Polydimethylglutarimide	There is an increment in the expression of cardiac maturation markers in CMs cultured on polydimethylglutarimide-aligned fibers compared to the control	Li et al. (2016)
10	Poly(lactic-co-glycolic acid)	Poly(lactic-co-glycolic acid) scaffold raises the hydrophilicity and biodegradability of scaffold and finally	Torabi et al. (2020)

(continued)

Table 4.1 (continued)

S. no.	Polymer	Application	Reference
		leads to better CM differentiation potential of hiPSCs	
11	Poly(lactic-co-glycolic acid)	Culturing ESCs on poly(lactic-co-glycolic acid) with collagen nanofibers shows better differentiation to CMs than poly(lactic-co-glycolic acid) nanofibers	Prabhakaran et al. (2014)
12	Poly(lactic-co-glycolic acid)	Efficient quality cardiac tissue-like constructs are formed by culturing hiPSCS-CMs on grounded and aligned poly(lactic-co-glycolic acid)	Li et al. (2017b)
13	Polyaniline/ polyethersulfone	Biocompatible polyaniline/ polyethersulfone scaffolds conduct electricity and, just like the bundles in heart pacemakers, help in delivering electrical pulses to the cells. Using these aligned electroactive nanofibrous scaffolds, a large increase in differentiation of hiPSCs to CMs is observed	Mohammadi Amirabad et al. (2017)
14	Hydrogel based on polyethylene glycol	Hydrogel injections enhance heart functioning post-MI and do not disrupt normal heart functions	Chow et al. (2017)
15	Hydrogel based on RAD16-I	The hydrogel in the patch made of RAD16-I hydrogel with hiPSCs acts on the cells in a pro-cardiogenic manner. It improves cell distribution in order to assist in the uniformity of colonization of the membrane pores and assist in differentiating progenitor cells to CMs	Puig-Sanvicens et al. (2015)
16	Chitosan hydrogel	Temperature-responsive chitosan hydrogel is used in delivering stem cells to the damaged myocardium and increasing graft size	Lu et al. (2009)
17	Polydimethylsiloxane	The substrate of intermediate elasticity causes the cell-substrate and cell-cell interaction, which enhances embryoid body-like formation, and its elasticity is the same as that of the native tissue	Wang et al. (2019)
18	Atelocollagen	Electrospun atelocollagen scaffold seeded with hiPSC-CMs is feasible for the stabilization of the dilated cardiomyopathy with potential for its clinical use in the future	Joanne et al. (2016)
19	Fibrin-collagen microthreads	hiPSC-CMs are capable of attaching to fibrin microthreads; however, a collagen IV protein coating improves their attachment to fibrin microthreads	Hansen et al. (2018)
20	Collagen	The vascular grafts on aligned nanofibers can amend the arrangement of primary	Nakayama et al. (2015)

(continued)

Table 4.1 (continued)

S. no.	Polymer	Application	Reference
		and iPSC-derived vascular lineages and restrain the inflammatory reaction of primary endothelial cells and iPSC-derived endothelial cells	
21	RAD/PRG and RAD/KLT peptide nanofiber	The co-transplantation of mesenchymal stem cells with RAD/PRG promoted their localization and survival in the infarcted myocardium. The therapeutic effect was improved with either RAD/PRG or RAD/KLT	Li et al. (2017c)
22	PA-RGDS Peptide nanomatrix	PA-RGDS enhances the survival of endothelial stem cell-derived CMs and enhances heart functioning after MI	Ban et al. (2014)
23	Polyurethane	Pre-differentiation of mesenchymal stem cells into CMs before injection results in better cardiac regeneration than only injecting undifferentiated mesenchymal stem cells into the heart	Guan et al. (2011)
24	Polyethylene terephthalate	hiPSC-CMs grown on polyethylene terephthalate textiles with gelatin coating demonstrate superior structural properties like rod-shaped structure and enhanced sarcomere orientation	Pekkanen-Mattila et al. (2019)

4.4 Polymers and Bioactive Agents Used in Tissue Engineering

Nanofibrous scaffolds with a diameter range of nanometer to a few microns are utilized for heart muscle generation more than other scaffold types, like sponge scaffolds (Prabhakaran et al. 2011). Whether natural or synthetic, numerous polymers have been successfully used in the production of mammalian culture suitable nanofibers. However, due to the close resemblance of natural polymers to structural proteins of ECM, they are widely used, e.g., collagen, laminin, and gelatin (Jung et al. 2012b; Boccaccini et al. 2015). Synthetic polymers, such as polycaprolactone, poly-lactic-co-glycolic acid, and polyurethane, are mostly used in the production of biocompatible scaffolds (Khil et al. 2005; Vasita and Katti 2006; Song et al. 2008). Other synthetic polymers include chitosan, polyaniline, polyethersulfone, polydimethylglutarimide, peptide amphiphile, and some other peptide nanofibers. Polymers are combined to create co-polymers to be used in the making of scaffolds of desired properties. Other than polymers, e.g., synthetic hydrogels, sometimes based upon polyethylene glycol, are utilized as a scaffold for drug delivery and cell culture (Lin and Anseth 2009). They are biocompatible and have excellent safety records and have established well in the medical field (Van Tomme et al. 2008; Hoffman 2012; Frey et al. 2014). Bioactive agents, like hormones, growth factors, and other small molecules, are introduced within the

scaffold matrix to make an ECM-like environment for the proliferation of cells to enhance cell survival, proliferation, integration, and differentiation (Laflamme et al. 2007). Erythropoietin is an example of a bioactive agent successful in clinical studies to reduce cell death and remodel post-MI (Brines and Cerami 2008). A crucial ECM molecule for stem cell differentiation and adhesion is fibronectin (Tate et al. 2002; Wijelath et al. 2004; Van Dijk et al. 2008), which is expressed in the normal heart also. Platelets are the growth factors that are involved in blood clotting, immune response, angiogenesis, and recovery of the damaged tissues in the body. This has attracted the attention of physicians in damaged engineering tissues because the probability of a reaction is near to the ground due to the use of personal blood (Boswell et al. 2012; Lang et al. 2018).

4.5 Differentiation of CMs from Stem Cells Using Nanofibers

4.5.1 Gelatin Nanofibers

PSCs demand more acclimated 3D cellular microenvironments than conventional 2D surfaces to keep their pluripotency or differentiation homogeneity. Matrigel (gelatinous protein compound of mice tumor cells) (Hughes et al. 2010) recombinant proteins like laminin (Rodin et al. 2014) or vitronectin (Rowland et al. 2010) were introduced for iPSC growth and differentiation. Additional substrates have also been used, like oxygen plasma etched plates (Mahlstedt et al. 2010), porous materials (hydroxyapatite scaffolds) (Kim et al. 2007a), and electrospun nanofibers (Kumar et al. 2015; Li et al. 2017c), which have textured surface morphology. Without improvement to conventional approaches, these will not overcome the risks of genetic instability and tumorigenicity (Okita and Yamanaka 2011; Liyang et al. 2013). These methods do not show the production of rigid and thick cardiac sheets because these have a limitation of cardiac diffusion (Shimizu et al. 2006). For the differentiation of hiPSCs to motor neurons, a patch culture method has been proposed, which has shown an enhanced upregulation of gene expression of the neurons and smooth maturation of motor neurons (Tang et al. 2016a). Tang et al. extended the patch process to culture and differentiated hiPSCs toward functional CMs. A crosslinked monolayer of gelatin nanofibers backed by a poly(ethylene glycol) diacrylate honeycomb frame consisted of the patch. Poly(ethylene glycol) diacrylate, a derivative of polyethylene glycol, could be utilized for multiple drug delivery and tissue engineering-based purposes. UV-based molding method was used for the preparation of the poly(ethylene glycol) diacrylate frame and gelatin nanofibers, electrospinning was used (Fig. 4.7). On the poly(ethylene glycol) diacrylate frame gelatin, nanofibers were electrospun. Due to the wide-sized pores of the patch and natural polymer used, the crosslinked gelatin nanofibers minimized the exogenic substance contact of hiPSCs. Vitronectin or additional extracellular matrix proteins can be used to coat the culture patch if required for more functional cell-nanofiber pairing. As the culture patch was within off-ground conditions, the crosslinked monolayer nanofibers allowed significantly enhancing the exposure field

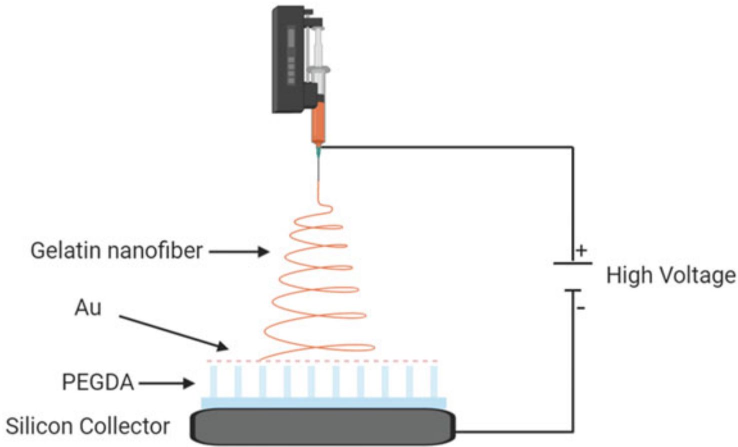

Fig. 4.7 Nanofibers of gelatin formed by electrospinning on honeycomb frame made of polymer poly(ethylene glycol) diacrylate

of hiPSCs upon the culture medium. They had also shown in their previous work that preferentially, cells remained confined in the nano-patterned region due to intensified distribution of nutrients and cell uptake (Hu et al. 2010; Tang et al. 2016b). Due to the increased exposure area with the help of monolayer of crosslinked nanofibers supported, cell metabolites could more efficiently be diffused, and in this manner, the culture patch is favorable over other 3D substrate types (Hu and Li 2007). Due to good mechanical stability and weak in-plane resistance to cardiac contraction, the honeycomb structure was chosen (Nishikawa et al. 2003; Arai et al. 2008; Engelmayr et al. 2008), and to aid hiPSC clustering, poly(ethylene glycol) diacrylate was used. Poly(ethylene glycol) diacrylate has been popularly adopted to outline the cell culture surfaces for their weak cell adhesivity and inadequate protein absorption and smooth chemical alteration (Moon et al. 2009). Colonies of ~250μm diameter (~1300 cells) refer to the most suitable size of hiPSC colonies for cardiac differentiation, and a honeycomb structure patch size of 500μm was chosen to generate the hemisphere colonies of this size (Dahlmann et al. 2013). In this study, ROCK inhibitor Y-27632 was utilized to adjust the impact of vitronectin, promoting the integrin-mediated cell adhesivity to the substrate. The cell-substrate interaction decreased due to low vitronectin surface coating and a short interval Y-27632 application given to the cells. It also supports cell to cell adhesion and then augments the cell grouping and development of hemispheric hiPSC colonies. As Y-27632 lasted for 4h or longer, flat hiPSC colonies were generated. However, when the application was restricted to 1–2h, hemisphere colonies with a diameter of around 225μm were obtained. These colonies were selected for cardiac differentiation due to the morphological similarity of hemisphere colonies with embryoid bodies. These are more resembling to in vivo development process of embryos and can assist in CM induction (Mummery et al. 2002, 2003). Poly (ethylene glycol) diacrylate honeycomb compartment prevents the formation of

heterogeneous cardiac clusters because of necessary fusion until the later stage of cardiac differentiation.

Cells belonging to the same population exhibit a considerable heterogeneity degree (Janes et al. 2010; Wilson et al. 2015). The determination of the fate of stem cells is also influenced by their inherent heterogeneity (Warren et al. 2006; Musina et al. 2006; Franco et al. 2010). Variations among donor cells result in operative variability and diverse differentiation potentials among other hPSC lines (Adewumi et al. 2007; Kim et al. 2007b; Cahan and Daley 2013). Also, those obtained from different types of tissues, for example, various germ layer tissues, show lineage bias as they go through directed differentiation (Kim et al. 2011), suggesting that hPSCs may hold on to distinct memory about their origin. Furthermore, reprogramming is a complicated and multi-step procedure that adds additional modifications (Liang and Zhang 2013). Building single cell-derived clones could diminish this heterogeneity (Narsinh et al. 2011; Lecault et al. 2011; Smallwood et al. 2014). Leqian et al., in their study (Yu et al. 2019), developed a single hPSC separation and culture platform of gelatin nanofibers. These nanofibers are appropriate for the long-term development of hPSCs supporting the feeder and serum-free culture environments. They established a single cell-derived sub-clone that proved to possess a discrete morphology related to other sub-clones. When this clone was used for differentiation toward CMs, it demonstrated much greater differentiation capability, maturation, and more substantial beating than those obtained from the other sub-clones. These observations present a suitable approach for single-cell separation and culture and illustrate those disparities in differentiation biases among sub-clones belonging to a cell line (Fig. 4.8).

4.5.2 Polycaprolactone Nanofibers

Polycaprolactone is a high-molecular-weight synthetic polymer, which is recognized by the US Food and Drug Administration for therapeutics (Kuppan and Sethuraman 2013). Moreover, the 3D-polycaprolactone nanofibrous scaffolds fabricated by electrospinning are employed for tissue engineering based on stem cells because of their good mechanical and biodegradable characteristics (Hashemi et al. 2009; Lim et al. 2009). These scaffolds provide a possibility to design scaffolds of micro- to nanoscale topography with a significant porosity similar to that of native ECM (Sill and von Recum 2008). Ding et al. (2020) examined the influence of 3D-aligned polycaprolactone nanofiber scaffolds upon cardiac differentiation of hPSC-CPCs. Cells treated with Wnt signaling inhibitors on 3D-aligned nanofiber scaffolds displayed enhanced CM differentiation of hPSC-CPCs. A notable rise in cTnT-positive cells on the 14th day in the 2D culture of differentiation related to cells administered with DMSO vehicle control resulted from treating hPSC-CPCs with Wnt inhibitors (53AH and XAV939). The results show that the Wnt signaling pathway performs an essential role in cardiac differentiation (Patsch et al. 2015; Wang et al. 2011; Willems et al. 2011; Ao et al. 2012; Lian et al. 2013). When the cells were treated with 53AH (i.e., a distinct structural inhibitor of Wnt signaling),

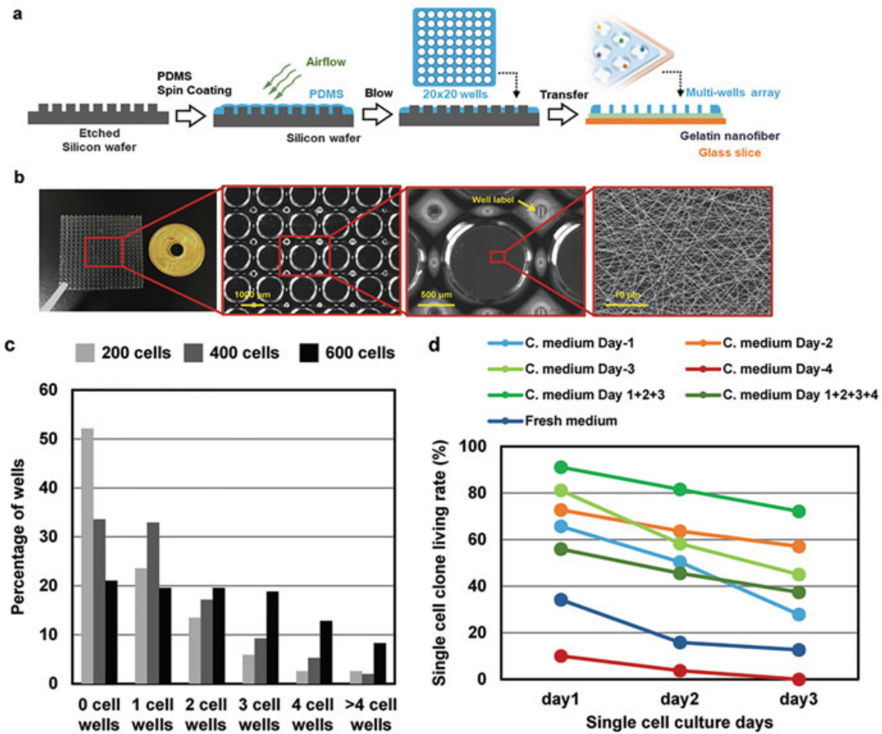

Fig. 4.8 Single-cell isolation system. (**a**) The process of making culture device for cell isolation. (**b**) Photograph of the culture device and single-cell isolation. The device is made of a polydimethylsiloxane multiwell array (400 wells) for single-cell isolation, and for the single-cell culture, it consists of gelatin nanofiber substrates. (**c**) The single-cell distribution rate of the multiwall array. (**d**) The highest viability was shown by the single cells with a mixture of days 1, 2, and 3 conditioned medium (C. medium) in the beginning 3 days of culture ($n \geq 20$). (Adapted from (Yu et al. 2019))

the expression of CM marker genes TNNT2 and MYH7 that are predominantly expressed in fetal ventricles was higher, and when the cells were treated with XAV939, the expression of MYH7 was also elevated (Fig. 4.9). These 3D-aligned nanofiber scaffolds mimic the structure of the native ECM.

Wanjare et al. (2019) fabricated microfibrous polycaprolactone by electrospinning to simulate the established physiological cellular organization of the entire myocardium to control the assemblage of induced-ESCs (iESCs) and induced-CMs (iCMs). By reprogramming peripheral blood mononuclear cells by transduction of cardiac differentiation-related genes (Sox2, Oct3/4, KLF4, and c-myc) mediated by Sendai virus, the hPSC (P356) cell line was developed. hESC (H9) was used along with hPSCs. After the cell seeding, visualization of the in vitro establishment of iCMs and iESCs within scaffolds was achieved according to phenotypic markers of troponin-T (TNNT) for CMs and CD31 for embryonic cells

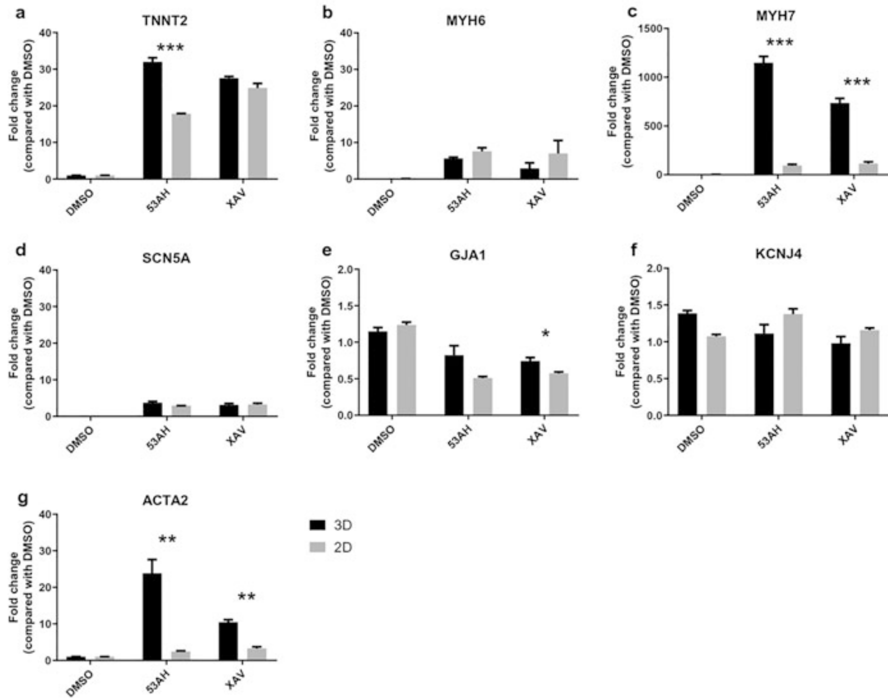

Fig. 4.9 Real-time RT-PCR analysis of CPC differentiation and proliferation in 3D vs. 2D culture. (**a**–**e**) Expression of different cardiac expression markers at day 7 of differentiation in 2D and 3D cultures. (Obtained with permission from (Ding et al. 2020))

(Fig. 4.10). NOD SCID mice (nonobese diabetic-severe combined immunodeficiency mutant mice) were utilized toward subcutaneous implantation studies. These scaffolds successfully induced and precisely directed the differentiation in the experimental cells.

With the use of an electrospun nanofiber as the substrate instead of tissue culture polystyrene plates (TCPs), CMs display more stable and long-lasting, spontaneous syncytium (Şenel Ayaz et al. 2014). Jingjia et al. (Han et al. 2016) designed the aligned and isotropic polycaprolactone fibers. The fibrous scaffolds were gold or palladium coated and were analyzed for characterization. After the culture of hiPSC-CMs was done on scaffolds coated with Matrigel, the alignment of cells on these substrates was confirmed using a polystyrene substrate as the control. Yan et al. (Chen et al. 2015) studied the impact of 3D polycaprolactone scaffolds on the CM differentiation of murine-iPSCs while performing in vitro examinations. A unique CM-inducing effect exists in the exchanges between the nanofibers of 3D polycaprolactone and the intracellular Wnt/β-catenin signaling of iPSCs. It was found that the gelatin-coated 3D scaffolds were suitable for iPSC cultivation and differentiation. Also, the conventional TCPs are less effective than 3D scaffolds for inducing the CM differentiation of iPSCs using the monolayer culture method.

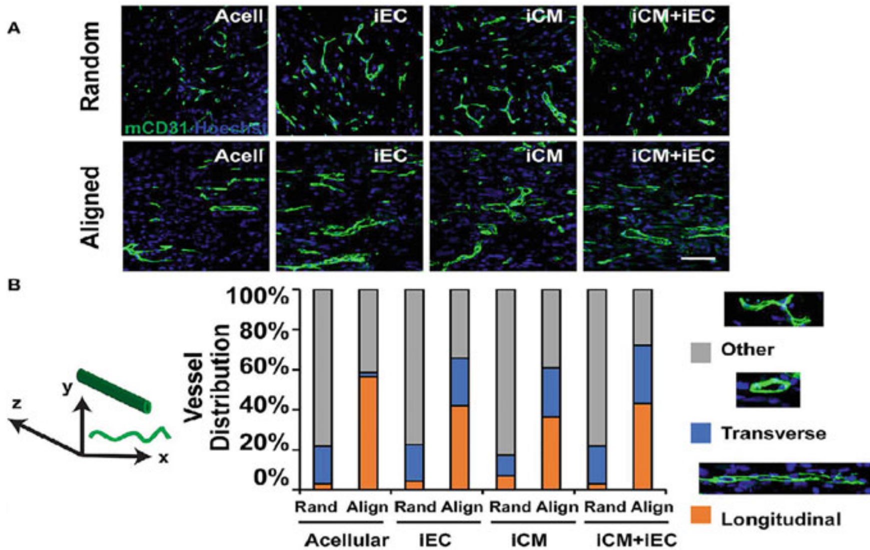

Fig. 4.10 The vascularization of cardiac tissue after successful implantation in mice model (**a**). Confocal microscopy images of CD31 staining (green) of engineered tissues obtained from scaffolds containing iCMs, iECs, or iCM+iECs after implantation. Acell means acellular scaffold (**b**). The orientation of vessels in engineered myocardial tissue relative to the axis of the aligned fibers. (Adapted with permission from (Wanjare et al. 2019))

To increase myogenic differentiation of hESCs, Leung et al. (2013) used chitosan-polycaprolactone fibers resembling the native muscle ECM microenvironment along with the Wnt3a protein. Polycaprolactone nanofibers were fabricated by electrospinning, and hESCs were seeded on this scaffold in media containing Wnt3a. An elongated morphology of hESCs was observed along fiber direction as compared to control substrates. Cells cultured on chitosan-polycaprolactone with Wnt3a expressed a high percentage of myogenic proteins over total hESCs after 2 days of cell culture.

4.5.3 Polydimethylglutarimide Nanofibers

Polydimethylglutarimide is a biocompatible polymer that can be easily electrospun into nanofibers (Orlova et al. 2011). Li et al. (2016) investigated hiPSC-CMs on fibers made of polydimethylglutarimide and pursued their cardiac tissue-like construction. Electrospinning was performed using a rotating drum to prepare aligned polydimethylglutarimide while increasing the rotation speed of the collector. The fibers of concentrations 19% and 16% demonstrated the best alignment than those from lower concentrations. The density of sheets was manipulated by changing the spinning time, and the 90-s electrospun fibers were chosen for CM culture. The as-spun polydimethylglutarimide threads on transferring to the surface of

microelectrode array let the extracellular recording of mimicking activities. Recording electrical activity in CMs enables the evaluation of their electrophysiological characteristics (Meiry et al. 2001).

4.5.4 Poly(lactic-co-glycolic) Acid Nanofibers

This polymer has ample versatility to some drugs, including hydrophobic, hydrophilic, micromolecular, and macromolecular. It also limits drug degeneration and the chance of easy surface modification to give more considerable interaction with biological surfaces (Pick 2009). Poly(lactic-co-glycolic) acid does not cause any inflammatory response and becomes absorbed well, not concentrating in the tissues or organs (Ali et al. 1993). Torabi et al. (2020), in their study, developed platelet-rich plasma-incorporated poly(lactic-co-glycolic) acid nanofibrous scaffold. The results demonstrated that the fabricated poly(lactic-co-glycolic) acid scaffold, in comparison to standard TCPs, exhibits enhanced biocompatibility. The platelet-rich plasma in poly(lactic-co-glycolic) acid is believed to increase biodegradability and hydrophilicity of scaffold and results in a suitable increase in the CM differentiation potential of hiPSCs. Its enhanced biocompatibility caused a rise in proliferation rate and PSCs survival due to the present growth factors. It may be noted here that there has been a positive impact of platelet-rich plasma on the growth and proliferation rate of other cells (Choi et al. 2005; Drengk et al. 2009). Platelet-rich plasma-incorporated scaffolds showed the maximum expression of cardiac genes, such as MLC2A, ANF, and MLC2V. Prabhakaran et al. (2014) analyzed the ability to differentiate ESCs into CMs on the poly(lactic-co-glycolic) acid and poly(lactic-co-glycolic) acid/collagen electrospun fibrous scaffolds as the cardiac patch. They made uniform bead-free fiber of poly(lactic-co-glycolic) acid and poly(lactic-co-glycolic) acid with collagen using the electrospinning technique. The ESC differentiation was induced by embryoid body formation, which proliferated and differentiated into CMs. Higher proliferation on poly(lactic-co-glycolic) acid with collagen scaffolds was observed by scanning electron microscope; the reason behind this could be the small diameter or high surface area of the fiber.

Most hiPSC-CMs morphologically and functionally look like naive CMs rather than adult ones, limiting their administration. Li et al. (2017a) produced high-quality constructs similar to cardiac tissue by culturing hiPSC-CMs on nanofibers of low girth made of biodegradable poly(lactic-co-glycolic) acid polymer. They described that multi-layered and elongated CMs could be arranged at high density with ordered fibers in a one-step seeding process, developing in upregulated cardiac biomarkers and enhanced cardiac functions. Constructs similar to cardiac tissue were used for assessing drugs, and they were more vigorous than the 2D control. They also highlighted the usability of cardiac constructs for in vitro designing of engraftments and in vivo treatment of MI.

4.5.5 Polyaniline and Polyethersulfone Nanofibers

Polyaniline is receiving much attention due to its excellent electrical conductivity. It has gained wide tissue engineering usage due to its electroactive qualities, high endurance, biocompatibility, easy synthesis, and being economical (Qazi et al. 2014). It scavenges reactive oxygen species and can mitigate oxidative stress in a myocardial injury (Gizdavic-Nikolaidis et al. 2004). It can be doped from the nonconductive form of meraldine base into the conductive state of emeraldine salt by protonic acids (e.g., camphor-10-sulfonic (β)) (Khuspe et al. 2014) and has been employed to assist in the transmission of electrical pulses (Balint et al. 2014). Furthermore, among different doped states of the poly(lactic-co-glycolic) acid, this polymer with camphor-10-sulfonic (β) is considerably aligned in the direction of the fiber. Moreover, the better interchain charge transfer occurs in poly(lactic-co-glycolic) acid with camphor-10-sulfonic (β) due to its dedicated polymeric chain packaging (Pouget et al. 1995). These semiconducting polymers concurrently support the adhesion and reproduction of CMs and induce cardiac differentiation (Bidez et al. 2006). Polyethersulfone nanofibers are other biocompatible materials that induce mesodermal differentiation (Ardeshirylajimi et al. 2013). Polyethersulfone is mechanically stable for differentiation but its electrical conductivity is low. Hence, blending polyethersulfone with polyaniline is practiced to create a scaffold with added mechanical stability for cardiac differentiation and electrical conductivity.

Mohammadi et al. (2017) practiced electrical stimulations onto aligned and random scaffolds to see how it affects the differentiation of PSCs to CMs. The effect of multidirectional electrical stimulation generated by random scaffolds on CM differentiation was negative, but the aligned unidirectional electrical stimulation of the aligned scaffold was positive. Recapitulation of the requirements and events leading to cardiac differentiation and maturation is a significant challenge (Courtney et al. 2006). The electrical impulses were applied to hiPSCs taken from patients with cardiovascular disease (referred to as CVD-iPS cells) seeded on aligned polyaniline/polyethersulfone scaffolds. This setup worked as an electrically effective cell culture system, including features similar to in vivo conditions of the heart that have been shown to generate cardiogenesis (Serena et al. 2009; Chi et al. 2010; Hernández et al. 2016). Through embryogenesis, the primary pacemaker cells originate from fetal CMs in the center of the sinoatrial node to create the first electrical impulses. Studies have been performed about exogenous electrical stimulation and cardiac differentiation of diverse kinds of stem cells (Serena et al. 2009; Hernández et al. 2016) by changing intracellular ion concentrations (Trollinger et al. 2002), yielding reactive oxygen species (Serena et al. 2009), or locating growth factor receptors and lipids in the cell membranes (Zhao et al. 2002). In this work, a bioreactor that applied exogenous electrical impulses was designed to apply electrical stimulation to cells via stainless steel electrodes. Analysis of the properties of this bioreactor showed that it is suitable for cardiac differentiation.

4.5.6 Polydimethylsiloxane Nanofibers

An important factor of the culture substrate that plays a vital role in determining the cell fate is the elasticity (Banerjee et al. 2009; Kshitiz et al. 2012; Sun et al. 2012; Li et al. 2017b). The self-renewal and differentiation of hPSCs are affected by the flexibility of cultures and morphology (Liu et al. 2014, 2017; Macrí-Pellizzeri et al. 2015). Engler et al. showed that the differentiation of mesenchymal stem cells toward a particular lineage is significantly based on substrate elasticity (Engler et al. 2006). The matrix stiffness and cell density assist hiPSCs in intercellular network formation, preserving phenotype and contractile function (Lee et al. 2017). A stencil method was revealed to investigate the outcome of substrate elasticity on the clustering and cardiac differentiation of hiPSCs. Dense elastomer pillars of altering stiffness were designed by adjusting the height of the pillars. To form uniform cell clusters, an elastomer stencil with a honeycomb pattern was created before cell seeding. It was demonstrated that both cell clustering and cardiac differentiation are dependent on the elasticity of substrate. They showed that the pillar of moderate elasticity (9 kPa) was better for both stiffer and softer ones.

4.5.7 Collagen Nanofibers

Joanne et al., in one of their works, have shown the feasibility of deriving collagen scaffolds mixed with biological solvents and crosslinking agents (Kitsara et al. 2015). In their other study (Joanne et al. 2016), they generated collagen scaffolds and cultured hiPSC-CMs and injected these scaffolds epicardially in a dilated cardiomyopathy (DCM) mouse model. Atelocollagen extracted from animal dermal tissue was electrospun by applying a proper voltage between the collector and the syringe needle. The suturing of collagen scaffolds on the ventricles of mice was done. The results showed the feasibility of the hiPSC-CM-seeded scaffold for the treatment of DCM. Hansen et al. (2018) developed and characterized hiPSC-CM-seeded fibrin suture that could be used for the delivery platform in repairing cardiac issues. hiPSC-CMs were seeded onto micro fibrin threads, and their contractile properties with time were characterized. These researchers fabricated a fibrin microthread suture for direct cell delivery to the myocardium (Guyette et al. 2013). In their study, various ECM and surface coatings were applied for improving cell attachment. Collagen IV and fibronectin were selected because of their occurrence in the cardiac basement membrane (Moyes et al. 2013; Rodriguez et al. 2014). Other hiPSC-CMs seeded on microthreads showed contraction within 7 days after seeding. The contraction of cells was in the direction of fiber for over 21 days. Also, this ordering was proved by immunohistochemical stains as over 21 days, the cells ordered further close to the thread, having the final alignment within 8° to the thread. Their findings suggest that hiPSC-CMs are able to attach to fibrin microthreads while the collagen IV protein-coating improves the potential of hiPSC-CMs attachment to fibrin microthreads. By the 14th day, the fibers contracted at a frequency that resembled the human heart and generated strains like those developed by

myocardium. Like the previous studies (Guyette et al. 2013; Hansen et al. 2016; Tao et al. 2017), this microthread scaffold could impact cardiac delivery approaches.

A blood vessel is composed of fibrillar ECMs and the other cell composition at the micro-/nanoscale (Stehbens and Martin 1993). Nakayama et al. (2015) designed a bi-layered vascular graft extracted from hiPSCs that reiterates the cell construction, alignment, and anti-inflammatory operation of blood vessels. In this graft, longitudinal-aligned nanofibrillar collagen-containing endothelial stem cells consisted of the luminal layer. Collagen with iPSC-derived smooth muscle cells consisted of the outer layer. Cells aligned on aligned fibers showed the association of F-actin within 8° from the direction of scaffolds. Endothelial stem cells seeded on ordered scaffolds had significantly lowered immune response due to attachment to monocytes. There is a significant influence of anisotropic scaffolds in directing cell construction and function.

4.5.8 Peptide Nanofiber

RADA-16-I (Ac-(RADA)4-CONH2) is a peptide nanofiber that causes the attachment, development, and differentiation of stem cells and mature somatic cells (Davis et al. 2005). RADA16-I can be modified with the addition of functional peptides to its C-terminal end. One useful peptide, e.g., PRG (Ac-(RADA) 4GPRGDSGYRGDS-CONH2), contains RGD (a natural cell adhesion motif to regulate the localization and proliferation of cells by ligation with integrin). Another operative peptide, i.e., KLT (Ac-(RADA)4G4KLTWQELYQLKYKGI-CONH2), augments angiogenesis by copying vascular endothelial growth factor (Liu et al. 2012). Functionalized self-assembling peptide nanofibers are of good biological histocompatibility. They have the capability to sustain the reproduction and differentiation of cells for repairing nervous tissues in vertebrate studies and are having applications for cardiovascular diseases as well. Peptide amphiphile is self-assembling peptides that join a hydrophobic and a hydrophilic peptide sequence exhibiting an excellent approach to gain this purpose (Hartgerink et al. 2001). Self-adhesive ligand Arg-Gly-Asp-Ser (RGDS), when incorporated with peptide amphiphile (PA-RGDS) and matrix metalloprotease-2 (MMP-2) degradable sequence, Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln (GTAGLIGQ), forms an ECM mimicking injectable nano-matrix. When interjected into PA, RGDS and fibronectin-derived ligand enhance cell adhesion and endurance and are verified by several cell types, including mesenchymal, umbilical, aortic, and pancreatic beta cells (Benton et al. 2009; Yu et al. 2009, 2010; Sapir et al. 2011; Gandaglia et al. 2012; Mihardja et al. 2013). MMP-2 degradable sequences on incorporation into peptide amphiphile cause a gradual degeneration of the fiber and are replaced by ECM of cells because the damaged tissue exhibits enhanced MMP production under ischemic conditions (Bendeck et al. 1994; Spinale et al. 1998; Cheung et al. 2000). This increases the migration of CMs into the myocardium (Jun et al. 2005). PA-RGDS nearly simulates the physical and biochemical complexity of ECM. Peptide amphiphile is amphiphilic and provides assembly into 3D networks of nanofibers similar to ECM

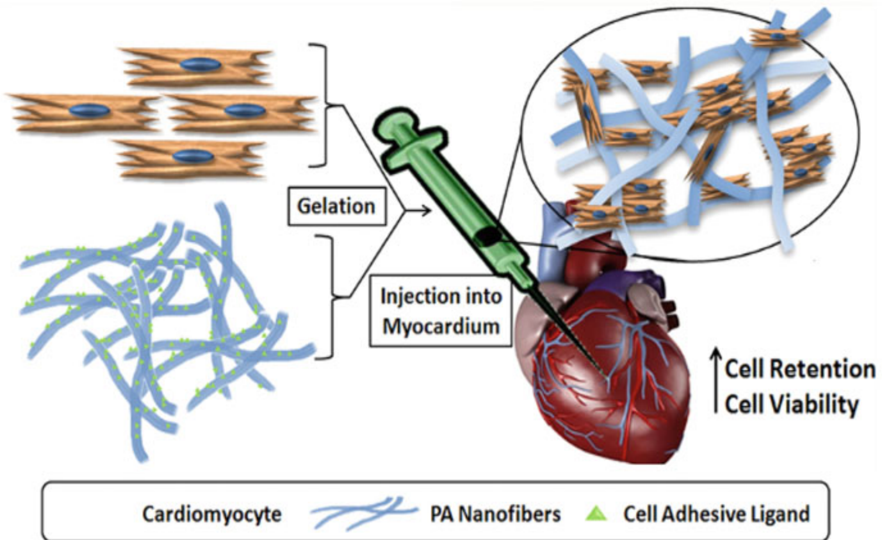

Fig. 4.11 PA-RGDS intensifies the endurance of mouse ESC-CMs and boosts heart function post-MI. (Adapted with permission from (Ban et al. 2014))

proteins under biological conditions. To know the underlying performance and machinery of transplanted stem cells in vivo, molecular imaging by dual-modal or multi-modal is more accepted to comprehend and review stem cell therapy with comprehensive information (Nguyen et al. 2014). To trace the transplanted stem cells and estimate their clinical effects, steadfast dual-modal imaging that utilizes bioluminescence and magnetic resonance imaging is set up (Cao et al. 2015). Li et al. (2017c) co-transplanted the bone marrow-derived mesenchymal stem cells using RAD/PRG or RAD/KLT, which promoted their localization and endurance in the infarcted myocardium, and their therapeutic effect was increased by co-transplantation with either RAD/PRG or RAD/KLT.

Ban et al. (2014) derived the CMS from mouse ESCs and encapsulated them in peptide amphiphile-RGDS to check their usage in MI therapy (Fig. 4.11). The incorporation of RGDS and GTAGLIGQ did the generation of peptide amphiphile-RGDS into peptide amphiphile. The CMs were taken from the rat model. They elucidated that most CMs survived in PA-RGDS for a week. The CMs were injected into the myocardium of mice model with PA-RGDS, and a threefold increase was seen in the incorporation in the models with CM+PA-RGDS compared to those with only CMs. A well-established cardiac function was seen in the group of mice with CM+PA-RGDS from 3 weeks and was sustained for 12 weeks.

4.5.9 Polyurethane Nanofibers

Tissue constructs with similar properties of structure and mechanics as that of myocardium were generated by Guan et al. (2011). In this regard, the electrospun polyurethane nanofibers were produced. The tissue construct mesenchymal differentiation was recorded by analyzing the expression of cardiac markers and the development of ion channels. The differentiation of cardiac cells was seen to be initiated by recording the mRNA expression. Tissue constructs were stretched statically to achieve cell alignment. The strain was increased from 25% to 75%, and it increased the degree of 3D alignment of cells. The RT-PCR determined that with a strain of 75%, the expression of GATA4, Nkx2.5, and MEF2C, which are the markers of differentiation of CMs, increased. Their work suggests that the pre-differentiation of mesenchymal stem cells into CMs before injection results in a higher cardiac regeneration rather than only injecting undifferentiated mesenchymal stem cells into the heart.

4.6 Conclusion and What Is Next

Cardiac tissue engineering is a field that repairs, reconstructs, and replaces cardiovascular structures, especially the heart, with engineered tissues. With the technology of deriving CMs from iPSCs, CVD phenotypes can be modeled, screening of the drugs can be done, and new ways of producing regenerative medicines can be achieved. It also offers ways to isolate iPSC-CMs from patients with genetic mutations or understand their pathophysiology. Direct reprogramming of the somatic cells into CMs without a transitionally pluripotent state is possible now. Genes are introduced at particular loci, and gene mutations are introduced to reverse the mutations that lead to diseases in iPSC-based CVD models in vitro. It is a novel and fast budding technology having thrilling applications. In the future, with more refinements, it will create means for the progress of personalized medicine for CVDs. Biomaterials can enhance stem cell function, and more knowledge of biomaterial engineering will help in molecularly designing biomaterials that resemble naturally occurring ECM of cardiac tissues. This way, biomaterials can guide the differentiation and function of progenitor cells. Biomaterials can be designed molecularly to control many of the factors that drive the differentiation of progenitor cells and function. Injectable nano-matrices could be designed to maintain biophysical and biochemical microenvironments of transplanted cells for better engineered cardiac tissues.

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5 How Physics Can Regulate Stem Cells' Fate: An Overview on Cellular Interactions with Their Substrate

Parinaz Abdollahiyan, Fatemeh Oroojalian, and Ahad Mokhtarzadeh

Abstract

Despite astounding developments in regenerative medicine, efficient cell differentiation has still met some struggles in conventional methods. Since mechanical ques are responsible for the communications between stem cells and their niche, advanced tissue engineering has introduced the novel branch of mechanobiology to manipulate organogenesis. Taking advantage of unique topographical microenvironment and physiochemical features, nanomaterials provide the opportunity to control cellular functions through multidimensional approaches. An overview of mechanobiology science reveals an interface of the disparate scientific disciplines from biology to mechanics. Accordingly, mathematical modeling is instrumental in the explosive progress of this area, assisting experimental studies on myriad levels. This chapter is inspired to generate discussions in mechanobiology from the fundamental conceptions to the cutting-edge developments. Moreover, the role of various biomaterials, magnetic nanoparticles, and conductive segments are examined, besides their relative physio-mechanical computations.

Keywords

Biomaterials · Cells' fate · Mechanotherapy · Stiffness · Stimulative cues · Tissue engineering

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5.1 Introduction

A glance at advanced studies on the microenvironments of the cells reveals the ability to control the biochemical and biophysical features through in vitro synthetic patterns of their niche (Dulak et al. 2015). In terms of regenerative therapies, artificial niches can be designed, thanks to mechanobiology techniques (Vining and Mooney 2017). The mechanobiology of cells refers to control the stem cells' behavior through the mechanical forces in vitro (Wang and Thampatty 2008). In a narrower sense, cells' fate is perfectly regulated by communications between cells and their niche, in which the stiffness of the ECM plays a decisive role (Lee et al. 2011). The differentiation behaviors of the cells are determined by mimicking the stiffness of the native substrate, which specifies the type of generated tissues. However, the variety of differentiation manners in multi-lineage types of cells leads to abnormalities in terms of the wrong place or undesired cell types (Huselstein et al. 2017). To eliminate the non-functional tissue or pathophysiological states, determined biological cues should be adjusted to regularize the differentiation responses of the stem cells (Wang and Thampatty 2008; Castillo and Jacobs 2010). The applied mechanical factors lead to fulfill the opportunity of selective differentiation for cells (Hao et al. 2015). Further, nano-topography, stiffness, colony sizes of the cells, and fluid flow can be considered as determinant external factors to adjust the intercellular signaling pathways (Mousavi and Doweidar 2015). The intercellular signaling pathways refer to the transmitted information within a cell through a series of molecular events. These events are affected by transmitting forces through intercellular generated forces. The mechanical interplays mediated by cell adhesion to the matrix and cell-cell junctions are considered as major internal forces (Vining and Mooney 2017; Huang et al. 2010). Cell junctions are intercellular bridges that provide contacts within the adjacent cells or their ECMs through particular proteins, namely, communicating junctions (Friedl and Mayor 2017; Mitchell et al. 2007). Biochemical cues (natural chemical process of body), epigenetic states (a series of mechanisms which can control gene activities during complex organisms), and physical environments of cells are main factors with the ability to regulate the intercellular mechanical forces. These intrinsic forces are generated by cells through cytoskeletal assembly and actomyosin contractility (fundamental cellular events based on cells' ability to apply the contractile force) (Murrell et al. 2015; Chen et al. 2014). The applied forces may be stored or dissipated within the microenvironment of the cells, according to its linear elastic or viscose types (Fig. 5.1) (MacQueen et al. 2013).

In detail, the applied energy, either stemmed from the internal or external origins, can be stored in the linear elastic microenvironments and pushes back to the cells (Reilly and Engler 2010). The cross-linked hydrogels are typical types of elastic materials, in which the density of crosslinking determines the elasticity and Young's modulus. Forces distribute in a viscoelastic environment, a combined state of viscose fluid and elastic solid. Accordingly, creep (gradual increase in the strain over time through sustained stress), stress relaxation (gradual decrease in the stress over time to maintain a sustained level of strain), or force dissipation may occur in the viscose

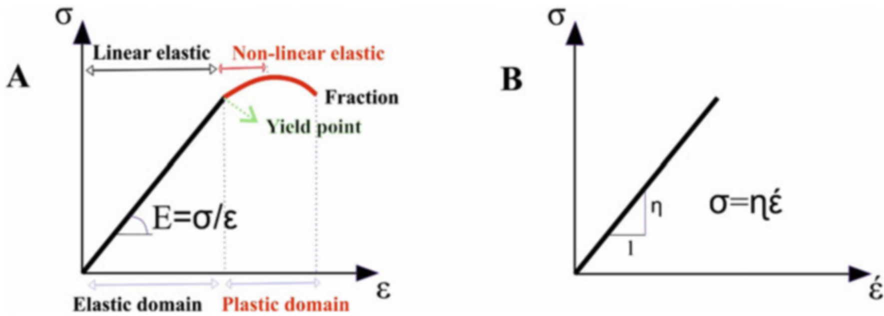

Fig. 5.1 The stress (δ)-strain (ε) curves of (**a**) linear elastic materials (E refers to the elasticity) and (**b**) viscose materials (η refers to the coefficient of viscosity)

environment. Various tension or compression tests are applied to measure the mentioned elastic parameters (Vining and Mooney 2017; Shao et al. 2015). In this regard, researchers used indentation test to evaluate the elastic mechanical characters of the tissue (Evans et al. 2013). In mechanobiology, the indentation test refers to a mechanical-based measurement. The rigidity of a solid determines through the distance passed by the indenter and the size of the created indentation (Tan et al. 2012). However, the variety of the obtained Young's modulus of the one-layer models is unreliable because of the inevitable role of the subcutaneous parts (Shin et al. 2013). Hence, a two-layer elastic model was assumed to overcome the issue by considering the mechanical features of the layers beneath the shallow parts (Kim et al. 2009).

A salient example of the association between cellular mechanical features and their metabolic states is illustrated in the patterning and organogenesis of embryonic development (Vining and Mooney 2017). Pertaining embryogenesis, embryonic development is considered the procedure by which the embryo generates and develops (Nowlan et al. 2010).

With regard to pluripotent stem cells (cells with the ability to differentiate to several types of tissues), the physical environment regulates their differentiation and self-repair ability. Beyond that, adult stem cells such as hematopoietic stem cells maintain their potency through physical interplays with the matrix, so the mechanical cues are considered as a pivotal point in their fate (Vining and Mooney 2017; Earls et al. 2013). Recently, induced pluripotent stem cells (iPSCs) have been known as an alternative for pluripotent stem cells with respect to their potency for clinical usage (Abagnale et al. 2017). This chapter discussed the role of several kinds of forces on and stem cells' fate, responses, organogenesis, and macrophage polarization as critical factors in tissue engineering to promote tissue regeneration or cancer therapy.

5.2 Mechanobiology Refers to the Intimacy Between Physics and Biology

The origins of mechanical applications in the biological fields are traced back to 20 years ago, when initial interests in cell proliferation and migration emerged. In relevance to the focal adhesion, it has been elucidated that the cell-matrix interactions are mechanosensitive, which, spurred by the mechanical forces, can be escalated in size under stiff microenvironments (Iskratsch et al. 2014; Brezulier et al. 2018). In the former times, “bioscience thought to have unique features quite unabated to the fields of physics and chemistry: ‘motion created from within,’ ‘chemistry of a very different type,’ ‘replication,’ ‘progress,’ ‘consciousness’ – each of which turned in to principles that become strange to the physicist to the gamut that several physicists look upon biology as an element outside their domain” (Iskratsch et al. 2014). However, some of the early researchers did perceive the necessity of mechanical stress and shape in the progression and operation of the organisms, which is categorized as a discipline called “physiology” or “physics in biology” (Li et al. 2017). The twentieth century witnessed the obsolescence of this disciple due to a lack of appropriate tools to measure physical parameters. Still, it primarily focuses on organ-level phenomena instead of molecular-level types (Shivashankar et al. 2015). Simultaneously, the knowledge about genome and proteome had a soaring trend in molecular biology, mass spectroscopy, and DNA sequencing (Palmieri et al. 2011). However, due to the lack of sufficient DNA-encoded information, the complex functions have still remained a mysterious debate (Georgiou 2019). A significant point of organ formation is dynamic interaction through cell and microenvironment (i.e., hormones, adjacent cells, ECM, and the loaded mechanical stresses to them) (Eweje and Ardona 2019). Despite the fact that the forms of the organisms are encrypted in their genome, the procedure that affects the ultimate form of vertebrates entails stable feedback within dynamic mechanical forces and cell proliferation and viability (Grashoff et al. 2010). Mechanobiology has transpired as an interdisciplinary field (among chemists, physicists, engineers, and material scientists) absorbed in studying the mechanical stress and geometry impacts on cell expansion and viability through mechanotransduction (Iskratsch et al. 2014).

Biochemical components illustrated disparate impacts on cells when mechanical features of their environment are changed. The gist of the debate is related to the primarily myosin motors that apply stresses on actin filaments in the forms of cell-matrix or cell-cell adhesions (Bouzid et al. 2019). Mechanosensors, moreover, are the other elements that are susceptible to the counter-forces from matrices. Providing cells to determine the tension and shape of an organ, actomyosin contractility mechanosensors are responsible for quasi-steady conditions in cellular tension, which can be construed by multidisciplinary approaches (Song et al. 2017).

From a physical viewpoint, the singularity of these detections is related to the strength of the supramolecular assemblies under force conditions rather than dissociating. Hence, merging potential mechanisms, this detection led to the expansion of a broad study in mathematical models (Bershadsky et al. 2006). So, in

parallel with the signs of progress in cell migration, the application of modeling and physics has become a major part of several works in this field. The quantitative methods are discussed in a triplet topic, adjusting numbers, integrating data, and perceiving biological phenomena (Feng et al. 2014). The first point is the gradual process to define beneficial mechanisms, which is called mechanotransduction, while the second refers to explaining the rare counterintuitive or contradictory data. Creating novel insights on biological phenomena is the third hypothesis for stimulating the next experiment (Liu et al. 2013). Concerning the importance of the numerical experiments in mechanobiology, Pelham and Wang (Pelham and Wang 1997) have found the more increased motility, more irregular focal adhesion, and less spread of the cultured cells on soft elastic polymeric scaffolds (about 5 kPa) in comparison with that of on the stiff plastic, elastic, and glass ones. According to their results, the defined elastic modulus was considered a threshold value for the cellular phenotypic switch, which was later corroborated by physics arguments. Further, they are already concerned that the dynamic of focal adhesion strongly impressed the stiffness reaction of cells (Balaban et al. 2001). The attention presents another confirmation of the threshold value for stiffness sensing. In fact, cells can calibrate their stiffness modulus against some inner reference value. A rudimentary approximation revealed a 5 kPa stiffness which concurs with prognostications from models that exert the principles of polymer physics about cytoskeleton. The developments in mechanobiology are considerably supported by the progress in novel technologies, particularly modern approaches to evaluate forces (Roca-Cusachs and Conte 2017). As one of the most favorable techniques in mechanobiology, traction force microscopy has been known as a tool to transform metamorphosis of the cellular environment into evaluations of the cellular traction forces (Mulligan et al. 2018). Lately, this method was integrated with modeling that inflicted particular hypotheses concerning the mechanics of cells, for instance, the biological features of the fibers at focal adhesion (Malandrino et al. 2018). Thus, as a correlative technique, traction force microscopy has recently found its way to complete various data types into one framework. In order to elucidate the importance of the mathematical models in mechanobiology, Schwarz considered the manner of the lamellipodium of a spreading cell in sensing the physical force (Schwarz 2017). Although several mechanosensitive molecules are concerned with the principles of lamellipodia expansion, there is the theoretical feasibility that the interaction within the growth, ramifying, and capping of actin filaments in a force-loaded condition caused a global shift in the cytoskeleton, which is managed by the details of s dynamical system, rather than involving with a particular molecular player (Ravasio et al. 2015). While it has been suggested before through various theoretical studies, the statics of electron tomography has recently corroborated the fact. Hence it is clear that the most salient interest of mathematical modeling is not the incipience of a unique universal model that completely elucidates the principles of mechanobiology, but the progressive extension of a toolkit for mechanobiological hypothesis and trains that supply an approach for new findings by investigating the subjects that go beyond biochemistry and genetics (Schorb et al. 2017). This accretion with physics was imperative for mechanobiology to raise beyond the studies of cell adhesion and

conceal the genome and the ECM and the physical perspectives of the cytoskeleton (Roca-Cusachs and Conte 2017). Chief among the major techniques in mechanobiology, quantification and mathematical models are significant elements to further enhance this extraordinary field (Sladitschek and Neveu 2017).

5.3 Quantifying Forces in Mechanobiology

Regarding the cells' response to the exerted physical forces, various tools are developed to quantify the forces created by cells. Nowadays, it has been known that cells have the potential to probe their surroundings through physical forces appropriate to differentiate stem cells (Xia et al. 2019) and primary transcriptional process (Wall et al. 2018), drive morphogenesis (Yan and Fei 2019), control malignancy (Miroshnikova et al. 2016), and direct cell migration (Sunyer et al. 2016). Whether the applied force is tensional, compressive or sheared stress, the principal of the matrix deformation, can be defined by elastic modulus and Young's modulus. According to the deformation mode, the elastic modulus represents the scaling between strain and stress of cells (Fig. 5.2a–c). The Young's modulus complies with the cell elasticity under extension. These properties of living tissues, as viscoelastic materials, are related to settling their three-dimensional (3D) shapes down under mechanical stress (Fig. 5.2d) (Mohammed et al. 2019). The applied mechanical forces lead to deforming the viscoelastic materials, which can be dissipated or stored within the deformable matrix (Tajik et al. 2016). The viscoelastic characters of tissues cause to enhance the deformation of the matrix and relax the mechanical stress over time (Shivashankar 2019) (Fig. 5.2e, f). The viscoelastic manner of tissues/cells can be described by various rheological models (Fig. 5.2d) (Mohammed et al. 2019).

Single-step mechanochemical switches at cell-cell (Yonemura et al. 2010) and cell-ECM (Sigaut and von Bilderling 2018) interactions are attributed to the procedures by which forces mediate the aforementioned reactions. Leaving no downstream function for mechanics, signaling cascades of pure biochemical types are triggered through the mechanochemical switch. Recent studies, in contrast, reveal a stable cross-talk between mechanics and biochemistry during mechanotransduction, which elucidate the importance of devices to estimate cellular forces (Elosegui-Artola et al. 2016). However, according to Newton's second law, it is impossible to measure force directly. Consequently, force sensors and measuring instruments are applied to quantify forces. As a physical device, the force sensor receives a force-type physical stimulus as the input and converts it to a measurable physical quantity. Newton's dynamometer is one of the most familiar force sensors by which the weight of a mass may transfer to a length deformation. Cell biology benefits of force sensors through force transduction into quantifiable physical quantities included light or mechanical deformations (Polacheck and Chen 2016; Sugimura et al. 2016). Thus, with regard to force sensors, materials with defined properties were considered, in which forces are measured from the distortion of materials of recognized mechanical properties. These approaches prepare an exact

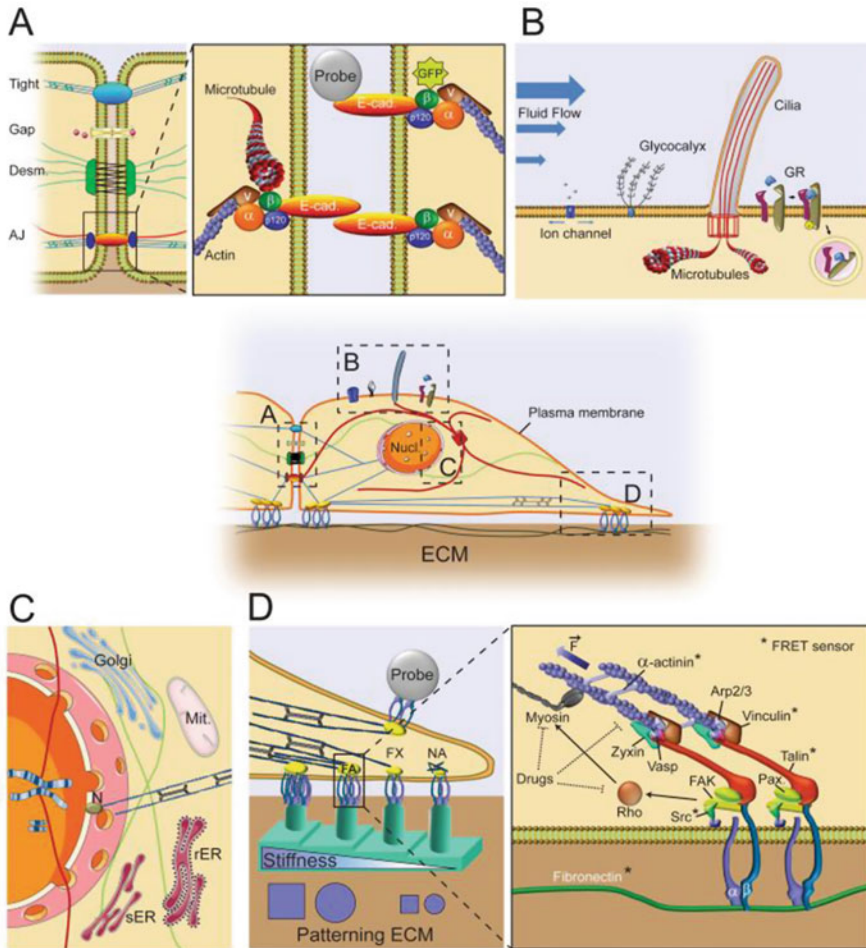

Fig. 5.2 An overview of ECM-cell and cell-cell connections. (**a**) Mechanotransduction at junctions. (**b**) Mechanoreceptor sites at the cell membrane. (**c**) Mechanotransduction at the nucleus. (**d**) Mechanotransduction at the focal adhesion. (Adapted from Eyckmans et al. (2011))

readout through evaluation noise triggered by various orders of magnitude in force, length, and time. Ranging from few piconewtons to hundreds of nanonewtons, the measuring noise spans from an immediate unfolding cryptic binding sites to gradual remodeling of epithelial tissues (Lynch et al. 2020). Based on the mapping stresses at the cell surface, traction microscopy evaluates cellular forces by estimating deformations of circumambient material. Adherent cells exert contractile stress on their underlying 2D ECM-coated substratum, which may be affected by the applied forces (Harrison et al. 2019).

These deformations are measured by traction microscopy by comparing two patterns of fiduciary markers inserted in the underlayer or bound to its facial parts. The former image is captured when the cell is exerting a force on the sublayer, and the latter when the film is fully relaxed. The latest performance of this method entails printing fluorescent markers with standard spacing on the gel to eschew the necessity of a reference image (Bergert et al. 2016). The loaded and unloaded figures can be compared by image-processing algorithms to give a displacement field, that is, a map that discerns the transformation of the peaks from the relaxed position. For this aim, as applied substrates for traction microscopy, silicon-based gels or polyacrylamide are mainly in demand (Bashirzadeh et al. 2018). These gels are optically transparent and linear elastic with tunable elasticity. The application of traction microscopy is not limited to the exerted force of the cells on a 2D substrate but to 2D scaffolds of arbitrary stiffness profiles and to multicellular clusters. However, cells normally exert 3D forces, and some sorts of cells generally present normal traction similar to the in-plane one. So the term of 2.5D tractions referred to as 3D forces on 2D undercoats can be estimated through the same aforementioned principle (Hur et al. 2020). The evaluation of 3D traction fields of the inserted cells in 3D ECMs is a far more complex issue, due to its inherent deformations and remodeling by the cells. In fact, at the cell-surrounded area, the chief cause of a large deformation is unclear, which may be on the base of the local ECM degradation or high traction. Further, physiological ECMs are fabricated of fibers with non-linear force-extension linkages, in which the fibers are in both forms of extend or buckle. To overcome the issue of non-affinity and non-linearity of the ECM, researchers have proposed the use of 3D traction fields by applying metalloprotease-cleavable polyethylene glycol (PEG) as an alternative gel matrix instead of the native ECM (Legant et al. 2010). With the same vein, a continuum approach had developed based on the non-affine features of the ECM. The force applied by breast carcinoma cells on the 3D matrix revealed an independent behavior of stiffness and ECM concentration which elucidated the distinct mechanical manner in a 3D scaffold in comparison with a 2D one (Okamoto 2019). However, the necessity of advanced software has restricted the popularity of this microscope. Despite the risk of experimental caveats such as precisely assigning the displacement field from the traction field, the limitations of traction microscopy have recently been mitigated through open-source codes (Rana et al. 2012). Moreover, regularization and filtering are applied to meet high-quality displacement fields to minimize possible errors.

As an additional method, the contractile forces of deformable substrates such as hydrogels can be measured through cantilevers and micropillars. Cantilevers are elongated frameworks with an elastic cross-sectional material that is free at one end, where force is exerted, and linked to a stiff substratum at the other end. So, cell forces can be measured from displacements through cantilever bending if the spring constant is known. On the other hand, micropillars are referred to microfabrication techniques in which the fabrication of arrays of micrometer cylindrical cantilevers is evaluated (Lachner-Piza et al. 2019). So cellular forces can be mapped through pillar displacements that are close enough to the ECM. Traction microscopy possesses some advantages based on continuous scaffolds, such as the calculation of

displacements from undeflected pillar sites, a simple calculation of the applied forces to the pillar, the generation of steep rigidity gradients through changes in pillar shapes, and additional mechanical stimuli by using magnetic actuators to the individual pillars (Yang et al. 2019).

5.4 Intercellular Interactions

A glance at the cell differentiation process elucidates the role of differential gene expression on cell determination, which depends on the expressed genes and the generated proteins. In fact, the inductive signaling pathways within the cells, called ligands, have been considered as a definitive factor in the process of cell differentiation (Benayahu et al. 2019). Direct mechanotransduction has occurred when cells translate adherent stimuli to a response that alters gene expression and guides cell differentiation. Indirect mechanotransduction talks about biochemical cascades resulting from cellular attachment by activation of mitogen-activated protein kinase (MAPK) and focal adhesion kinase (FAK) mediated by G-proteins among Cdc42, Rho, and Rac. Rho is a member of Ras family and is responsible for the direction of stress fiber (Changede and Sheetz 2017). Rac is also a G-protein in the cytoskeleton that is responsible for the regulation of lamellipodia and cdc42 to manage filopodia. Adipogenic and osteogenic phenotypes can be regulated by indirect mechanotransduction through MAPK/extracellular signal-regulated kinase (ERK) signaling. In fact, at both proteomic and genomic levels, the expression of ERK is related to the osteogenic topographies. Nuclear transcription factors such as RUNX2 (the osteogenic master gene for osteoblastic differentiation) can be controlled by ERK signaling. ERK signaling also adhered to PPARγ and stat1, which are important for adipogenesis and osteogenesis, respectively (Kennedy et al. 2017). Diffusion, direct contact, and gap junctions are chief among the major intercellular signaling pathways, which are based on ligand diffusion through specific receptors on the adjacent cell, gene expression altering through internal signal stimulation, and the signal transmission through the intercellular junctions, respectively (Ladoux et al. 2016). Hence, following the aforementioned intercellular signaling procedures, the differentiation behavior of the stem cells and the number of the various differentiated types can be determined, termed as a reflection of the potency (Adamopoulos et al. 2017). Moreover, the maintenance of the potency is significantly related to the physical interactions of the stem cells with their surroundings matrix. Thus, apart from chemical factors, physical cues can be considered as a variable factor to control the stem cells' fate and their reflection of the potency. So with regard to regulate the physical interactions between cells and their microenvironments, the features of the ECMs, such as their stiffness, can take into account as a variant factor, leading to the design of the artificial niches (Li et al. 2017). Stiffness, in detail, is referred to the ability of materials for elastic deformation, regularly determined by elastic modulus. On the base of the aforementioned definition, materials are divided into two major groups, as viscoelastic materials and linear elastic types, related to the line slope of the stress-strain curve which are

evaluated during the mechanical tests (i.e., optical tweezers, micro-aspiration probes, atomic force microscopy, nanoindentation, and force traction microscopy) (Vining and Mooney 2017). The sensing and reacting to the mechanical changes, termed as mechanical load effects, are dependent on the molecular half-life of the protein binds. Slip bonds, also called normal bonds, are the non-covalent interactions whose lifetime demolishes by applying the tensile forces. In controversy, although catch bonds are referred to the same type of interactions with that of in the slip bonds, their lifetime increases in advance to a critical point during application of the tensile forces, followed by decreasing with the higher forces (Xia et al. 2019). While at the same conditions the lifetime of the slip bonds is lower than that of in the catch bonds, the bases of mechanobiology field are relied on both two types of forces. So cell-ECM bonds and cell-cell interactions are promoted during the mechanical stimulus, thanks to intrinsic features of the catch bonds. The mechanical transduction can be mediated in mammalian cells through the specific ion channels (leakage channels and gated ones) (Ng et al. 2017). Governing stem cell differentiation, piezo proteins are stretch-activated ion channels, through which the mechanical forces are converted to the biological signals. Piezo functionality, which is defined by ectopic expression of the ion channels, is rescued via operating the level of cytosolic Ca^{2+} in the cells. In fact, acting as second messengers, the low concentrations of the calcium ions in the cytosol may be affected by embracing external stimulus, such as hormones or extra actions, through influencing the calcium channels, which simulate the over-expression phenotypes of piezo (Douguet et al. 2019). As calcium ions can stimulate several types of proteins through strong interactions, keeping a balance of Ca^{2+} in cell construction is considered a basic part of cell biology. Yet, the concentration of Ca^{2+} is not in an equivalent prevalence throughout the cellular structure, suggesting more concentration in the lumen in comparison with the cytoplasm (Lv et al. 2018). Thus, elucidating the role of Ca^{2+}ATPase as a pump, the level of Ca^{2+} has to be maintained in a low concentration to protect the cytoplasm from protein precipitation. In fact, the conformational changes of the proteins, as the result of their interactions with the calcium ions, cause proteins to be exposed to the aqueous media by their hydrophobic part, which leads to produce the calcium-protein complexes (Zhou et al. 2019). So, causing to damages, these complex structures of calcium-carbonyl groups of proteins precipitate in the cytoplasmic regions of the cells by a high concentration of Ca^{2+}. Piezo is essential for cytosolic Ca^{2+} adjustment in response to the guest mechanical stimulus (Gupta et al. 2018). Thus, studies on piezo have yielded pivotal insights into the biological fields; chief among them are associated to the mechano-related bioprocess such as entroendocrine functions or bladder distention and cellular activities such as secretion, fertilization, migration, proliferation, and neurotransmission. The mechanical stimulus at the membrane leads to affect not only the ion channel activities but also cytoskeletal filament dynamics and the yield of the molecular motors (Caluori et al. 2019; Takahashi et al. 2019).

Single-cell movement and deformation are complex operations that rely on cell contractility, actin polymerization, and dynamics of adhesive compounds at the interface of the ECM: traction forces are applied to the substrate by cells through

the generation of adhesive complexes and the communications of internal forces released within their cytoskeleton (Sarangi et al. 2017). There is the possibility to modulate the endogenous forces by applying external factors such as biochemical factors (Saxena et al. 2017) and matrix physical properties (Sarangi et al. 2017). In addition, neighboring cells are also mechanically linked by various junctions, leading to adjoining another layer of complexity and providing collective cells (Ladoux et al. 2016). How cells organize their functions within various biological processes, comprising tissue remodeling, cancer progression, and morphogenesis, is affected by the mechanical characters of the external environment. The behavior of collective cells, in contrast to single cells, depends on the cellular interactions with both the neighboring cells and surrounding ECM. Collective dynamics are coordinated by active interaction of cells with each other controlled by mechanosensitive adhesion complexes, in response to transmitting physical signals, at cell-cell junctions cell-substrate interface. The mechanotransduction and mechanosensitivity at adhesion complexes are pivotal for directing tissue cohesiveness and, in consequence, are pivotal for collective cell actions. The behavior of collective cells can be governed by mechanical and structural qualities of the cellular environment, such as matrix topography and geometry stiffness besides the application of external forces (Ladoux and Mege 2017).

5.5 Mechanotransduction; Developments and Functions

What is the secret of different shapes of organisms? This question remains a controversial debate that elucidates the importance of the relation between mechanotransduction processes and variations in the form of the cellular microenvironment. The cellular deformation in response to the applied stress can be interpreted by physical and mechanical equations (Steinwachs et al. 2016). D'Arcy Thompson (1917) has discerned the issue in an archival description, responding to the matter of shaping biological forms. With the same vein, by the early twentieth century, researchers began to prospect various methods to mechanically manipulate cells (i.e., through microneedles or adjusting osmotic pressure) concerning the growth of limited types of cancerous cells in agar gel (Sanford et al. 1954; Temin and Rubin 1958), which, later, turned to a crucial experimental tool in cancer studies, called mechanosensing. The approach relies on the coupling interactions between the outer and the inner plasma membrane layers. Evidence shows that the shape of the erythrocytes changes in the exposure of cationic and anionic drugs due to the differential changes in EC and intercellular membrane tension (Hou et al. 2011; Sheetz and Singer 1974). The importance of the issue is related to cellular mechanisms that can sense the changes of the membrane tension, known as the gist of cell migration and proliferation (Gauthier et al. 2011). Bearing this in mind, a significant number of research were assigned to perceive the communication mechanisms within cells, ECM, and their adjacent cells. This led to the discovery of focal adhesions, by which the multi-protein structures link to the matrix through the integrin sites on the core and allow the outside-in and inside-out

mechanosignaling (Iskratsch et al. 2014; Izzard and Lochner 1976). Pertaining the impact of the cellular forces in the procedures such as cell locomotion, it was deemed that the cell-matrix contacts may be destroyed by the actomyosin machinery (Kolega 1986). Concurrently, originated through the studies of Andrew Huxley (Huxley and Niedergerke 1954) and Hugh Huxley (Huxley and Hanson 1954), muscle biophysics was greatly widely enjoyed in the mechanobiology field in the 1950s, clarifying the main elements of actomyosin contractions. These studies were a milestone in the swinging cross-bridge pattern of myosin action on actin filaments and sliding filament opinion (Spudich 2001). The importance of the advanced techniques for the loaded mechanical stress evaluations at a subcellular level is discerned when the cytoskeleton-generated forces are transferred to the cellular microenvironment (Abercrombie et al. 1971). A salient example for the usage of this technique is an elastic silicon substrate, which was applied previously for cell culturing. The results of the study illustrated that the cellular forces were applied to the elastic substrate in the non-muscle cells, which was a prelude for the primary force-sensing tools (Harris et al. 1980). These devices are the base of traction force microscopy, where the motions of the embedded fluorescent beads are used to evaluate dynamic cellular forces on the elastic gels (Oliver et al. 1995). Simultaneously, at a micrometer scale, the impact of the matrix patterning on the growth and viability of the cells was evaluated. In this regard, as a major point for downstream signaling, the potential of cells to produce forces was tied to integrin-mediated mechanotransduction (Nguyen et al. 2016). In confirmation with the issue, researchers applied the magnetic forces to the cells which were cultured on the integrin ligand-modified surfaces and achieved an immediate strengthening of the cytoskeleton (Wang et al. 1993). Implementing passive and active forces to cells was previously introduced in the 1990s, explaining the mechanosensing process. Proposing the mechanism of muscle stabilization against overstretching, results of atomic force microscopy (AFM) concurred that titin was mechanically unfolded to untangle its immunoglobulin-like domains (Rief et al. 1997). AFM, moreover, is used to evaluate the features of intramolecular bonds among receptors and their ligands, leading to offer paramount assessments of the energy of molecular bonds (Merkel et al. 1999). The solid connections between the cytoskeleton and the ECM which were coerced by high amounts of passive rigidity of matrix molecules were illustrated via submicrometer-diameter beads (Choquet et al. 1997). Matrix rigidity was improved by altering the crosslinking features of acrylamide gels, and the following modifications in cell adhesions further augmented the concept that ECM rigidity influences stability and adhesion size. Later, the phosphatase inhibitor-treated cells revealed that dephosphorylation cycles and tyrosine phosphorylation were engaged in sensing rigidity, particularly the kinase FYN with respect to fibronectin-mediated rigidity sensing and tyrosine-protein phosphatase-α (von Wichert et al. 2003). Addressing the limitations of element analysis, a device which applied a hanging pad on movable cantilevers was used to track cellular forces in a limited gamut of subcellular area. Based on the localization of the force transmission and adhesion assembly fluorescent proteins, microcontact printing of elastic layers, moreover, presented a simpler solution to the restricted element analysis (Balaban et al. 2001). A needle-like 2D tool was another

device for evaluating the traction forces, which suffer from the concerns of changing traction force patterns, and cellular geometry by the gaps between pillars, though, the pillar arrangements provided similar traction force patterns to those developed by the former techniques (Tan et al. 2003). These patterns highlighted the intimacy of cells with their matrix substrate, which was contracting isometrically through balancing the forces exerted from the focal contacts of a cell at the narrow tail by several smaller adhesion forces. The concentration of forces and adhesion were much lower at the middle parts of the cells. Moreover, pertaining to the study of traction forces, further devices for evaluating the mechanical characteristics of individual cells (i.e., stress sensors on micromanipulators for estimating the contractile and elastic attributes of a particular cell over time, lonely and in regard to bioactive materials) were expanded. Sphingosylphosphorylcholine, for instance, provided interpretation for how metastatic cells shrink via membrane pores, leading to an increment in the elasticity of cancer cells (Beil et al. 2003; Micoulet et al. 2005). In relevant to the signaling and sensing facial rigidity, prior experiments discerned the importance of crosslinking of integrins at the molecular level (Coussen et al. 2002). To concur the force-dependent behavior of the binding between the cytoskeleton and the adhesion proteins entailing p130CAS (also known as BCAR1) (Sawada et al. 2006), paxillin, and focal adhesion kinase (FAK; also known as PTK2), stretching of exposed cytoskeletons accompanied with the Triton X-100 treatment was evaluated. In addition, technical advances modified the force-strength, spatial, and temporal resolution of biomechanical signs. These forces were perceived by fluorescence resonance energy transfer (FRET) at a light resolution. Further, to modify the resolution to nanometer levels, fluorescence quenching-based tension sensors have been prepared. Equally importantly moreover, relaxation cycles of talin molecules and dynamic stretch were evaluated by the single-molecule tracking method. Attached magnetic nickel particles accomplished local measurements of traction forces on pillars to the pillars, in which the resolution could be amplified by the gold veneer (Zhao et al. 2013). In fact, gold coating provided covalent tagging of the pillar points with a broad diversity of ligands to evaluate the contributions of α5β1- and αvβ3-integrins in cell traction forces (Rahmouni et al. 2013). Concerning the role of the pillar size on cell spreading, it has been dedicated that smaller pillars (submicrometer diameter) presented not only a better performance than the larger ones but also the greater spatial resolution of cellular stresses (Ghassemi et al. 2012). Hence, based on the appropriate tools, the field of integrin-dependent mechanosensing expanded rapidly. One particular study focused on the catalysts with the potential to realize the cell-ECM contacts which were prepared in a stepwise method with standard assemblies of cellular protein (such as podosomes or integrin adhesions) and functional modules. For each cell state, the behavior of the cells is reproducible, and hence it is feasible to analyze at a nanometer resolution. Striking alters in cell state appear abruptly; the deactivation of several functional modules was developed subsequently (Uto et al. 2017). A transition from primary swift isotropic spreading to following contractile spreading is a salient example in the case of cell spreading on a substrate, which entails a momentary growth in membrane tension. Hence, the substrate rigidity was tested through positional contraction

units when periodic contractions were activated. The resulted cellular morphology prepares through disparate levels which may vary slightly in various cell types (Huebsch 2019). The steps, however, share multiple functional modules that can evaluate the impact of different modules on cell behavior. Thanks to nanofabrication techniques and advanced microscopy, these days are witness of the potential to follow the provisional and spatial diffusion of functional complexes to specify whether the proteins are concerned with the initiation, disassembly of integrin adhesion, and quasi-steady-state behavior (Lerebours et al. 2016). The initial functional modules for stress manufacture in animal tissues and cells are myosin II motors which contract actin filaments followed by transferring force to actin-anchoring regions. Despite the corroborations about the existence of disparate sites of actin polymerization, the main part of actin polymerization was revealed at cell edges by integration of G-actin into filaments (Pollard and Borisy 2003). Actin assembly is an extremely ordered process entailing bundling, severing proteins, multiple assemblies, and crosslinking, which are specific for various kinds of actin networks (Campellone and Welch 2010). Latest experiments on the beginning steps of cell-ECM adhesions revealed that the formin protein FH1/FH2 domain-containing protein 1 (FHOD1) was employed in a force-independent behavior to animate actin assembly from early ligand-bound integrin clusters (Iskratsch et al. 2013). As formins, DIAPH1 (mammalian diaphanous homologue 1) and FHOD1 were crucial for the development of adhesion sites (Riveline et al. 2001). The role of FHOD1 and mDia1 in the formation process of actin structures, however, is distinct. While mDia1 was involved in the organization of dorsal stress fibers, FHOD1 was along with the actin-related protein 2/3 (ARP2/3) complex, which is concerned with transverse arcs and ventral stress fibers (Schulze et al. 2014). Hence, FHOD1 preferably assembles antiparallel actin constructions that contain contractile units occupied in early adhesion generation (Ghassemi et al. 2012; Iskratsch et al. 2013). A force-dependent alignment of actin assembly was evidenced through the queuing of stress fibers in the force orientation during periodic stretching (Hayakawa et al. 2001). According to a theoretical model, a pulling force would enhance formin-driven actin assembly in the piconewton range from the increasing barbed end. Flow forces, moreover, performed advanced actin assembly rate within a microfluidic tool from the immobilized formins Bni1p and mDia1 (Jegou et al. 2013). Adhesion-localized formins, hence, could straightly couple integrin-sensed forces to conversions in actin assembly. The major mechanical graft from the ECM-actin cytoskeleton is, though, presented by proteins such as α-actinin and talin, which bind to both actin filaments and integrin (Burridge and Connell 1983). Primitive discoveries performed that talin is engaged in force-dependent adhesion amplification, which, explained as a stick-slip mechanism, bonds actin to the integrin tail. During the process, the rearward-flowing actin links to talin (“stick”) and applied a stress that stretches it to the break point (“slip”) (Margadant et al. 2011). Vinculin found the possibility to binding through stretching to modify the linkage to the actin cytoskeleton. In addition, the actin crosslinkers filamin A and α-actinin challenge with talin for integrin linking (Roca-Cusachs et al. 2013). While α-actinin recruitment to generate adhesions provides the transfer of high forces throughout the

ripening of the adhesions, filamin appears to be an original mechanosensor (Lynch et al. 2013). As a dimer, filamin appeared to crosslink and stabilize the integrin clusters, which are attributed to its linking and protease calpain "buffering" (Xu et al. 2010).

5.6 The Role of Biomaterials in the Fate of Cells

In the last 30 years, biomaterials have been categorized into three types of generations. The first generation entailed biocompatible and mechanically stabile materials and highlighted their biodegradability and potential for cellular and in vivo studies (Ariga et al. 2016). Bioceramics such as hydroxyapatite and bio-glasses have been suggested for tissue engineering applications. The third generation of biomaterials is responsible for regenerating tissue by activating the genome of cells (Denisin and Pruitt 2016). These materials can manipulate biochemical control of cell functions. Studies for efficient cell culture approaches have focused on the modification of three pivotal material features: surface chemistry, topography, and stiffness. In fact, besides the physiochemical properties of scaffolds, their mechanical properties are determinant factors for programming cell differentiation (Klosterhoff et al. 2017). Thus, the ability to manipulate the microscale mechanical interplays between cells and their substrate is important for designing scaffolds. In this regard, micropatterned scaffolds with controllable elasticity and structure can induce cell behaviors in vitro. Multiscale computational modeling is studied to evaluate the mutual effects of microstructures on the microscale mechanical properties of the scaffolds (Giménez et al. 2017). A range of natural and synthetic biodegradable polymers have blended to fulfill both biocompatibility and mechanical properties. The following section discussed about the role of biopolymers and fillers on cells fate in relevance to the main aim of mechanobiology, by providing a microenvironment to regulate cell behaviors and examine their physiological functions (Argentati et al. 2019). The cellular functions can be adjusted by several external cues/stimuli. The salient examples of these factors are matrix structure and stiffness, soluble biochemical factors, and mechanical stress of the cell substrate. For instance, the proliferation behavior of neural stem cells and directional growth of axons were controlled by the fiber orientation of artificially designed nanofibers (Dado and Levenberg 2009). To interact with the matrix, cells use receptors that link to specific motifs of the ECM. In the former example of nerve tissue regeneration, neural cells were anchorage-depended and attached to the specific ECM proteins to proliferate (Najafi et al. 2013). Each anchorage site has its particular motifs in its sequence which is recognized by its individual cell receptor, called integrin. For example, the specific moieties of laminin are IKLLI, IKVAV, PDSGR, and YIGSR, of fibronectin are RGD and LDV, and of collagen is DGEA, which lead to mechanotransduction. The type of scaffold affects stems cell migration, proliferation, alignment, and differentiation. The directional movement of stem cells on a scaffold is triggered by single or combinations of external cues, such as substrate-bound chemoattractant, gradients in substrate stiffness, and the cells' potential to

proliferate and orient along the aligned patterned substrate (Rana et al. 2016). Polymers are the most favorable classes of scaffold in mechanobiology to direct stem cell function, with respect to their functional properties, which have the potency to mimic the microenvironment of natural tissues/cells. Among other types, the nanofibrous scaffolds are highly in demand due to their resemblance to the native ECM. The orientation of fibers can be tailored during construction to match the properties of the tissue to form this scaffold. Several techniques have been applied to manufacture nanofibers, such as electrospinning, self-assembly, and phase separation. Stem cells can respond to the structural properties of the microenvironment and make a dynamic relationship with it (Sridhar et al. 2015). In this regard, the high specific surface area of the nanofibers and their controlled nanoscale dimension make them capable of harmonizing with the cultured stem cells and regulating cellular functions. The alignment of nanofibers can manipulate the anisotropic mechanical properties of the cellular substrate and, hence, is a crucial factor for guiding stem cells' behavior (Subramanian and Sethuraman 2016). The alignment and organization of human MSCs and actin filament and their behavior could be controlled on anisotropic nanofibers. The migratory profile of MSCs showed accelerated speed on aligned and tiny diameter tussah silk fibroin nanofibers (TSF) (Xue et al. 2018). Likewise, MSCs presented topography-dependent differentiation to various lineages such as osteogenic, tenogenic, and chondrogenic on poly (L-lactic acid) (PLLA) nanofibers in both in vivo and in vitro. MSCs showed excellent tenocyte-like morphology and excellent tenogenic differentiation on the aligned fibers, while randomly oriented fibers guided MSC differentiation toward osteogenic lineage (Sisakhtnezhad et al. 2017).

Aligned and random carbon nanofibers (CNFs) were examined for the neural differentiation behavior of human endometrial stem cells (hEnSCs). More upregulation in neuronal markers, but less proliferation of hEnSCs, was revealed on the aligned CNFs under inductive neural conditions. Likewise, oligodendrocyte-specific markers showed slight upregulation on the random CNFs. Phenotypically, while cells differentiated along the main axis of the aligned CNFs, random fibers showed a multidirectional stretched profile. Apart from the topographical cues, electrical conductivity could affect the regenerative potential of neuronal cells (Mirzaei et al. 2016). Moreover, elasticity and stiffness of the polymer matrix are considered as other effective factors for stem cell's fate. The mechanical properties of polycaprolactone (PCL), a biocompatible polymer, were manipulated by gold nanoparticles. The mechanically tuned substrate could regulate MSCs' behavior to generate contractile proteins and gain to the typical cardiac phenotype. The high expression of cardiac markers was revealed on the gold-treated PCL scaffold with a Young's modulus of 2.56 Mpa (Sridhar et al. 2015). Similarly, the chondrogenic and osteogenic induction potentials of MSCs were compared on polyurethane (PU) and PCL nanofibers. The results indicated a more differentiation profile on PU with higher elasticity than PCL (Kuo et al. 2014). In order to examine the relations between MSC differentiation and the compressive elasticity of the scaffold, PEG dimethacrylate substrate was fabricated with various photocrosslinking degrees which regulated elastic modulus (2 to 15 kPa). Vascular-specific phenotypes were

expressed by varying matrix elasticity; thus, 3 kPa elasticity revealed the expression of Flk-1 endothelial markers from 95% of the cultured MSCs, while just 20% of MSCs showed Flk-1 marker on matrix with >8 kPa elasticity. In contrast, ~80% of MSC illustrated smooth muscle α-actin marker on the substrate with >8 kPa elasticity, whereas less than 10% of MSC revealed α-actin marker on scaffold with <5 kPa elasticity (Wingate et al. 2012).

5.6.1 Graphene Derivatives

Graphene is hexagonal sp2-hybridized carbon sheets that are randomly oriented. Graphene derivatives (graphene, graphene oxide (GO), and reduced graphene oxide (rGO)) have unique properties because of their specific honeycomb structure and strong covalent bonds within the carbons in the sheets (Niu et al. 2018). Besides proper mechanical properties, the biocompatibility of the graphene family is responsible for their broad applications in biology among drug delivery, biosensors, antibacterial activity, diagnosis, and cancer treatment (Liu et al. 2018). Tissue engineering has also found great developments with regard to the role of graphene derivatives in cellular functions, bone regeneration, and wound healing. Furthermore, graphene derivatives represented a determinative role for the fate of various stem cells such as neural stem cells (NSCs), MSCs, and induced pluripotent stem cells (iPSCs) (Chen et al. 2012).

Regeneration of the central nervous system has still remained an ongoing challenge because of the difficulties in directing stem cell differentiation toward a particular neural cell lineage (neurons and oligodendrocytes) (Kang et al. 2017). Recently, graphene-based nanomaterials have been used in favor of stem cells guiding toward oligodendrocytes, which solved the traditional limitations of neural regeneration (Kim et al. 2015). Further, the effect of graphene on the cardiomyogenic differentiation of MSCs was evaluated on the graphene monolayer. A single layer of graphene was coated on titanium (Ti) foils through CVD method; the Ti segments were etched at the next step, and mono-layers of graphene were provided. The cultured MSCs on graphene showed commitment toward cardiomyogenic lineage by upregulating the cardiomyogenic-related cell signaling molecules and expressions of the ECM protein gene, with no interference of exogenous chemical inducers. Thus, due to the potential of the cardiomyogenically differentiated MSCs for myocardial contractility treatments, graphene has been considered as a good substrate for cardiac therapies (Park et al. 2014).

Another study revealed the role of graphene in guiding cardiac differentiation of embryoid bodies (EBs). An adequate amount (0.2 mg per mL showed minimum cytotoxicity) of graphene was introduced to the mouse EB structure by the hanging drop method. The accelerated cell differentiation toward cardiac tissues was observed in the graphene-incorporated samples, which led to decrease proliferation of the stem cells, 5 days after culture (Ahadian et al. 2016). The 2D monolayer of coated graphene on a glass surface could adjust dynamic epigenetic variations to promote pluripotent epigenetic reprogramming of mouse somatic fibroblasts into

induced pluripotent stem cells (iPSCs) (Yoo et al. 2014). Furthermore, single-layer graphene was coated on the glass slides by the chemical vapor deposition (CVD) method. The epigenetic role of the synthesized graphene substrate was evaluated on bone marrow MSCs and adipose-derived stem cells (ADSCs) in terms of osteogenic differentiation. The upregulating methylation of H3K4 was revealed at the osteogenesis-associated gene regions (Runx2, OSX, OCN) after 14 days, which confirmed more osteogenic differentiation of MSCs on the graphene-coated substrate than the untreated sample (Fig. 5.3a) (Liu et al. 2016). Multilayer 3D structure graphene foam was constructed by coating graphene on the nickle (Ni) scaffold, which, at the next step, was removed through the $FeCl_3$ etching process. The osteogenic and neuronal differentiation of the cultured cells was traced by the fluorescent patterns of the cultured human MSCs (hMSCs) on graphene foam and tissue culture polystyrene (as control). The dramatic differences in the morphology of the cells and protein expressions proved the role of graphene foam in spontaneous osteogenic differentiation of hMSCs, without any external biological inputs (Fig. 5.3b, c) (Crowder et al. 2013; Zhang et al. 2016).

The interference of graphene derivatives in mechanobiology leads to direct the differential manner of the cells on polymeric substrates. The reason is attributed to the effect of the graphene moieties on the structural crystallinity of the scaffold, which raises its tensile strength, consequently (Qavamnia and Nasouri 2015). The Young's modulus of the polyaniline-grafted-polyacrylonitrile nanofibers were increased from 1.33 ± 0.56 to 2.51 ± 0.62 and to 2.23 ± 0.41 after incorporating with graphene and GO, respectively (Mahmoudifard et al. 2016). The comparison between the mechanical factors (i.e., tensile strength, tensile modulus, and tensile strain) of these two treatments showed a partial increment in the strength of the graphene-polymer scaffold than that of in the GO-polymer one. In fact, the presence of the hydrophilic moieties on the basal plane of GO caused to decrease the mobility of the polymeric chains, which has a direct impact on the crystallinity of the polymer. With regard to the less potency of cell spreading on the scaffolds with higher tensile strength, the SEM images of the satellite cell-seeded scaffolds showed more accumulated cell populations on graphene-treated polyaniline than GO-treated and bare polymer substrates (Mahmoudifard et al. 2016; Ammar et al. 2016). The results were reaffirmed by DAPI staining in which it revealed less cell proliferation on graphene-containing mats than the other samples (Mahmoudifard et al. 2016). In order to clarify the distinct effects of graphene and GO in cellular functions (attachment, proliferation, and differentiation), iPSCs were cultured on these surfaces and compared with a bare glass substrate as control. It was dedicated that cell attachment and proliferation were accelerated on GO substrates and iPSCs were differentiated to mesodermal and ectodermal lineage spontaneously. However, no evident changes were observed for the cultured iPSCs on the graphene mats, and the majority of iPSCs remained in their undifferentiated state or suppressed toward the endodermal lineage (Chen et al. 2012).

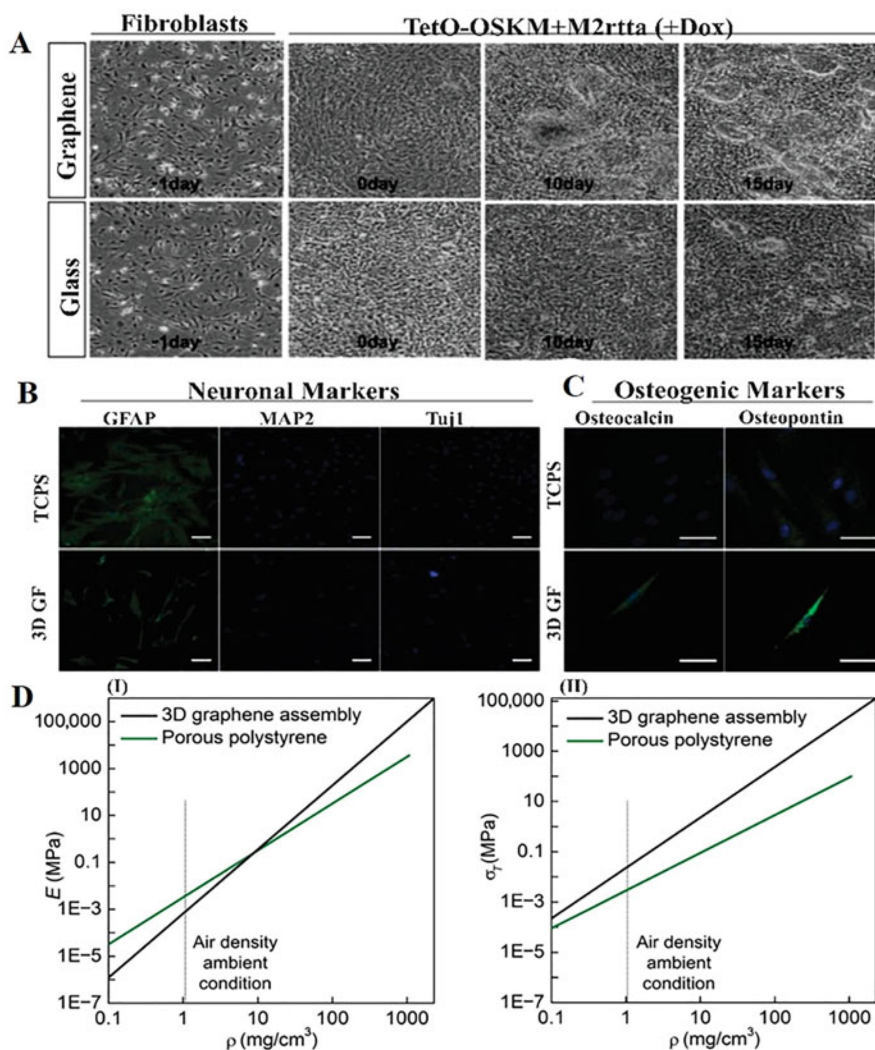

Fig. 5.3 (**a**) The differentiation behavior of the cultured mouse somatic cells on glass and graphene-coated glass substrates. The gradual formation and propagation of iPS cells are imaged on both surfaces within 15 days, which reaffirmed the suitability of graphene mechanical properties for reprogramming of the cultured cells. (Adapted from Yoo et al. (2014). Copyright 2014, Biomaterials). **b** and **c** represent spontaneous **(b)** neuronal and **(c)** osteogenic differentiation of hMSCs on the 3D graphene-based foam and polystyrene. Tracing neuronal differentiation by specific neuronal markers showed no significant changes for the cultured hMSCs on the graphene foam, unlike the control. However, the osteogenic patterns revealed the decisive role of graphene on the expression of osteogenic markers (osteopontin and osteocalcin) ((**b**) Scale bars = 100 μm, **(c)** Scale bars = 50 μm). (Adapted from Crowder et al. (2013). Copyright 2013, Journal of Nanoscale). (**d**) Comparison between the ***(I)*** Young's modulus and ***(II)*** tensile strength of 3D graphene foam and polystyrene. The stiffness of 3D graphene is higher than the woven polystyrene structures with $\rho > 10$ mg/cm^3, but not for lighter materials. (Adapted from Qin et al. (2017), Copyright 2017, Journal of Scientific Advances)

5.6.2 Titanium Dioxide (TiO_2)

Implantation is one of the most prevalent methods for hard tissue defects. However, when they play the role of artificial ECM, their therapeutic efficiency faces some drawbacks. The current metal implants possess poor ability to direct stem cell's fate; thus, the regeneration of hard tissue disturbs as the cultured MSCs differentiate toward soft tissues (Cao and Desai 2020). Controlling over cell differentiation toward an intended cell lineage is a pivotal factor for efficient implantation. The topographical and structural features determine the elongation, migration, growth, and differentiation of the stem cells (Raja et al. 2020). In this regard, the facial nanostructures of the implants, where the fate of the stem cells can be regulated, are challenging branches of study in hard regenerative therapies such as dental and bone implants. Titanium (Ti) has been considered as one of the most prevalent materials for hard tissue implantation, with respect to its admirable osteoconductivity, mechanical stability, and biocompatibility (Abdollahiyan et al. 2021). TiO_2 is the native oxide layer on the surface of Ti, which is suitable for orthopedic applications due to its capability to connect with the adjacent bones. Despite biological advantages of this metal, their application in nano-dimensions is disputable due to not only high surface activity of the nanoparticles but the dissemination of the small particles in the blood circulation system (Wang et al. 2016a). Thus, their applications as veneers, films, and nanoarrays are widely extended. Recently, it has been considered that the fate of the stem cells can be regulated by TiO_2 nanostructures. TiO_2 nanotube arrays with various dimensions that stabilized on Ti mats by anodization showed different effects on the MSCs functions. While tiny nanotubes (30 nm diameter) enhance MSC adhesion, larger ones (70–100 nm diameter) dramatically affect stem cell elongation. The induced cytoskeletal stress on large TiO_2 nanotubes regulated MSC differentiation toward osteoblast-like cells. It was reported that TiO_2 nanotubes with 70 nm diameter represented optimal osteogenic differentiation in vivo. Based on the reported results, researchers suggested TiO_2 nanorod array as the potential surface for osteoblast regeneration (Pang et al. 2019; Park et al. 2007). Based on the potency of TiO_2 nanorod for bone implantation, samarium (Sm)/strontium (Sr) was doped to these nanorods. Sr was introduced to improve bone formation, and Sm was added to raise the antibacterial activity of the scaffold. Thus, in comparison with the untreated sample, Sm/Sr-treated TiO_2 with enhanced hydrophilicity showed more osteogenic gene expression of the adhered MC3T3-E1 cells (Zhang et al. 2020). Other methods to fulfill the aim of regulating antibacterial properties and cells' fate were studied by Bhardwaj and Webster. They treated Ti by nanophase TiO_2 using electrophoretic deposition. They reported an 81.1% reduction in ampicillin-resistant *Escherichia coli*, 95.6% reduction in *Staphylococcus aureus*, and 90.2% reduction in *Pseudomonas aeruginosa*, compared to the untreated Ti as control. Moreover, in comparison with the bare Ti substrate, the osteoblast proliferation on the nanostructured TiO_2-treated Ti was increased after 3 days and 5 days, by 120.7% and 168.7%, respectively (Bhardwaj and Webster 2017). Likewise, the role of polyisoprene was evaluated in dental pulp stem cell differentiation. It was reported that the Young's moduli of the polyisoprene-coated TiO_2 could be adjusted

by varying the thickness of the hydrophobic elastomer. Dental pulp stem cells showed more growth and proliferation on the coated substrate than the uncoated sample, though the differentiated profile had to be regulated by the thickness of the polymer. In this regard, polyisoprene with various thicknesses of 15 nm, 120 nm, and 200 nm was coated on Ti-based substrate, in which the Young's moduli were of 2.76, 1.16, and 0.70 MPa, respectively. While, in comparison with the thin polymer segment, the doubling time of dental pulp stem cells was lower on the thicker veneer, the deposition of hydroxyapatite and the expression of OCN and COL1a1 were higher (Chuang et al. 2019). Because of the decisive role of surface morphology in cells' fate, plasma discharge treatment, under O_2, N_2, O_2/N_2 combinations, and He, was applied to improve surface roughness and hydrophilicity. Plasma surface modification of TiO_2 arrays led to enhance cell-matrix interactions and consequently promote the proliferation of the bone cells (Mahmood et al. 2011).

5.6.3 Magnetic Nanoparticles

According to advanced cell regeneration in terms of mechanobiology, cell differentiation can be programmed by regulating the operation of intercellular mechanosensors and mechanical loadings (Hughes and Kumar 2016). The living cells' internal architecture is organized through the heterogeneous intercellular spaces that are necessary for the generation of the decisive gradient enzymatic activities for cellular functions. Signaling proteins which can be localized on cellular membranes, cytoplasm, or organelles play roles in several cellular processes (Li et al. 2016). For instance, information transferring and cytoskeleton morphogenic features are controlled through the MAPK signaling cascade and GTPase proteins, respectively (Adebayo et al. 2019). Thus, the cellular functions can be adjusted by manipulating protein concentrations through the intracellular spaces via various methods such as surface organizing of biomolecules and microfluidics. Despite the restrictions related to the gradient of proteins, these methods evaluate the responses of the cells in the vicinity of perturbations of the artificial ECM (Lee et al. 2019). To regulate the protein activities, the optogenetic methods have been applied as alternative strategies. However, the need of labor-intensive activities for protein engineering has still led to made limitations in this area. In this regard, an interdisciplinary approach (based on chemical, optogenetic, and genetic) is magnetic stimulation of nanoparticles to control over cells (Tay et al. 2016). Magnetic nanoparticles are being able to target cell components. There are various possibilities for spatiotemporal manipulation and mechanical stimuli of the magnetized cells under external magnetic fields (Shen et al. 2017). Magnetic energy was received by magnetic nanoparticles and converted to mechanical forces that require piconewtons for mechanical sensitive biomolecules. In this regard, the importance of the role of magnetic nanoparticles in energy conversion has been highlighted. The magnetism property of these particles is related to electrons' motion, which contributes to the spin and magnetic moments in the atom. Some specific physical terms have been introduced to elucidate how magnetic moment (m) is a factor of volumetric

magnetization (m) and the volume of the particles (V); $m = MV$. Further, it has been proved that the strength of M is a coefficient of the magnetic susceptibility (χ) and the strength of the magnetic field (H); $M = \chi H$. An electromagnetic constant (μ_0) is defined to estimate the magnetic induction (B) through the equation of $B = \mu_0 (H + M)$. The magnetic moment can be aligned toward the field direction within uniform magnetic fields by applying a torque (τ) on the magnetic nanoparticles ($\tau = mB$). The magnetic forces are generated in a gradient condition that is a factor of magnetic field energy of the particles (U): $F = -\nabla U$. The applied force leads to rotate the particles in parallel to the magnetic field and attract the particles toward the stronger fields (Monzel et al. 2017). Considering the producing device, two kinds of magnetic fields (dynamic and static fields) can affect mechanotransduction. Ferromagnetic particles with great magnetic susceptibility χ are favorable types for mechanotransduction. The orientation of the magnetic particles toward the direction of the field and their intrinsic properties are critically dependent on oscillating frequencies, the strength of the field, and the main parameters of the magnetic field, such as configuration and geometry. Reorientation of the magnetic segments in the exposure of external stimuli such as thermal fluctuations is a factor of the particle size; the tiniest ones represent superparamagnetic behavior (Issa et al. 2013). Organized assembly of the magnetic moieties leads to improve the general magnetic moments. The shape of the particles also influences the magnetization properties. In this regard, because of the disordered positioning of the spins, the cubic magnets revealed better magnetization features than the spherical ones. Although these spins accumulate in the corners of the cubic shapes, the sphericals are their host on the facial parts of the particles. Iron oxide nanoparticles are a salient example for cellular mechanotransduction. Their magnetomechanical stimulation could be promoted by doping extra ions such as Mn^{2+}, Zn^{2+}, and CO^{2+} due to the increment of unpaired electrons and decrement of the off-site magnetic spins (Zhao et al. 2017). Researchers regulate the fate of MSCs toward smooth muscles by controlling mechanical incitement of platelet-derived growth factor receptor α and β (PDGFRα and β) through cycling magnetic fields. Time-varying magneto-mechanical incitement of PDGFR α led to smooth muscle α-actin in both mRNA and protein level, over a 3-h period. PDGFRα phosphorylation is found in response to stimulation, and the upregulation of mRNA is repealed by cell pretreatment with the neutralization antibody or receptor inhibitor. The fluorescence images of the magnetic particle-labeled cells confirmed the same size of the particles on the cell membrane with those that were applied for magnetic stimulation (Fig. 5.5b, (I) and (II)). The tagged magnetic nanoparticles were coated by a layer of dextran, which is dedicated in clung of cells by dextran staining (Fig. 5.5b, (III) and (IV)). The enhanced expression of SMA proteins was revealed after magnetic particle activation of hBMSCs (for 3 h), which was increased by the conjugation of anti-PDGFRα and anti-PDGFRβ antibodies (55% for PDGFRα, 22% for PDGFRβ) (Fig. 5.5b). An elevated trend was revealed in SMA expression, in particular with PDGFRα, after 24 h in the presence of cyclical magnetic fields. Moreover, SMA mRNA in PDGFRα targeted hBMSCs group raised by 1.5-fold by 3 h after culture. In contrast, no significant increase was reported for PDGFRβ-treated groups at the same condition. The

resulted SMA mRNA expressions were backed to their basal level (similar to control) in both of the targeted groups a further 24 h culture (Hu et al. 2014). Another study evaluated the effect of magnetic nanoparticles on Wnt pathway activation through mechano-stimulation of the Wnt/Frizzled receptor in MSCs. Anti-Frizzled functionalized MNPs were labeled to the Frizzled receptors, which were remotely stimulated by an oscillating magnetic bioreactor. It was reported that Wnt/β-catenin signaling was activated by Fz-MNP. Wnt signaling pathways have crucial effects on MSCs' fate which make it attractive in bone and cartilage therapy. To investigate the role of Fz-MNP on Wnt signaling activation, the nuclear localization and mobilization of the β-catenin were investigated. Partial β-catenin nuclear localization in the non-treated cells was assessed after 24 h (Fig. 5.4) (Fu et al. 2019).

In comparison with non-treated cells, considerable growth in nuclear localization was shown in anti-Fz MNP-treated cells without magnetic field. The β-catenin mobilization was the ascent to the peak point when Fz-MNP-labeled cells were stimulated by the magnetic field. It was reported that a negligible β-catenin localization was revealed in the untreated cells, a non-significant rise in nuclear localization appeared in cells treated with RGD-MNP or IgG-MNP, and no considerable effect was obvious in the exposure of magnetic field. The magnetic field-treated cells illustrated a moderate rise in β-catenin localization. Significant nuclear localization appeared in the presence of anti-frizzled magnetic nanoparticles in non-magnetic conditions in conjugation with the magnetic field. A significant nuclear β-catenin was also revealed for treated cells with Wnt-conditioned media after 24 h. Nuclear pixel intensity quantification showed the rates of nuclear (active) β-catenin. The similar progressing trends in levels of nuclear β-catenin were appeared for all of the magnetic field-treated RGD-MNP (in both magnetic field-treated and untreated mode), and IgG-MNP groups Wnt-CM and Fz-MNP promoted β-catenin mobilization, and further increment was revealed when the magnetic field-conjugated Fz-MNP was applied (Rotherham and El Haj 2015). Gene expression analysis was also performed to evaluate mechano-stimulations through stress response genes (NF-κB, c-Myc, and Cox2). The control particles showed a considerable upregulation in NF-κB expression at the initial periods, which gradually decreased over time. Compared with the control, though, the other groups were faced with small levels of elevations. c-Myc and Cox2 genes revealed low-varied response in expression, yet TREK-coated particles in the exposure of oscillating magnetic field have still the most extended peak. The expression of NF-κB gene was increased by treating with the magnetic field (for 3 h) and with Trek-MNP (for 1 h), in comparison with cells + magnet as control. A minor shift has appeared in the treated samples with Fz-MNP or Wnt 3A. Moreover, the expression of c-Myc was increased in the presence of a magnetic field (for 1 h) accompanied with a little raise in conjunction with Trek-MNP, in comparison with the control. A similar negligible shift has appeared again for c-Myc expression in treatment with Fz-MNP or Wnt 3A. Also, a significantly increased shift was shown in COX 2 gene expression in the presence of magnetic field for 1 h, followed by a decreasing trend after 3 h (Rotherham and El Haj 2015). The osteogenic differentiation of MSCs was evaluated in another study by means of the effect of iron oxide nanoparticles on the activation of MAPK signal

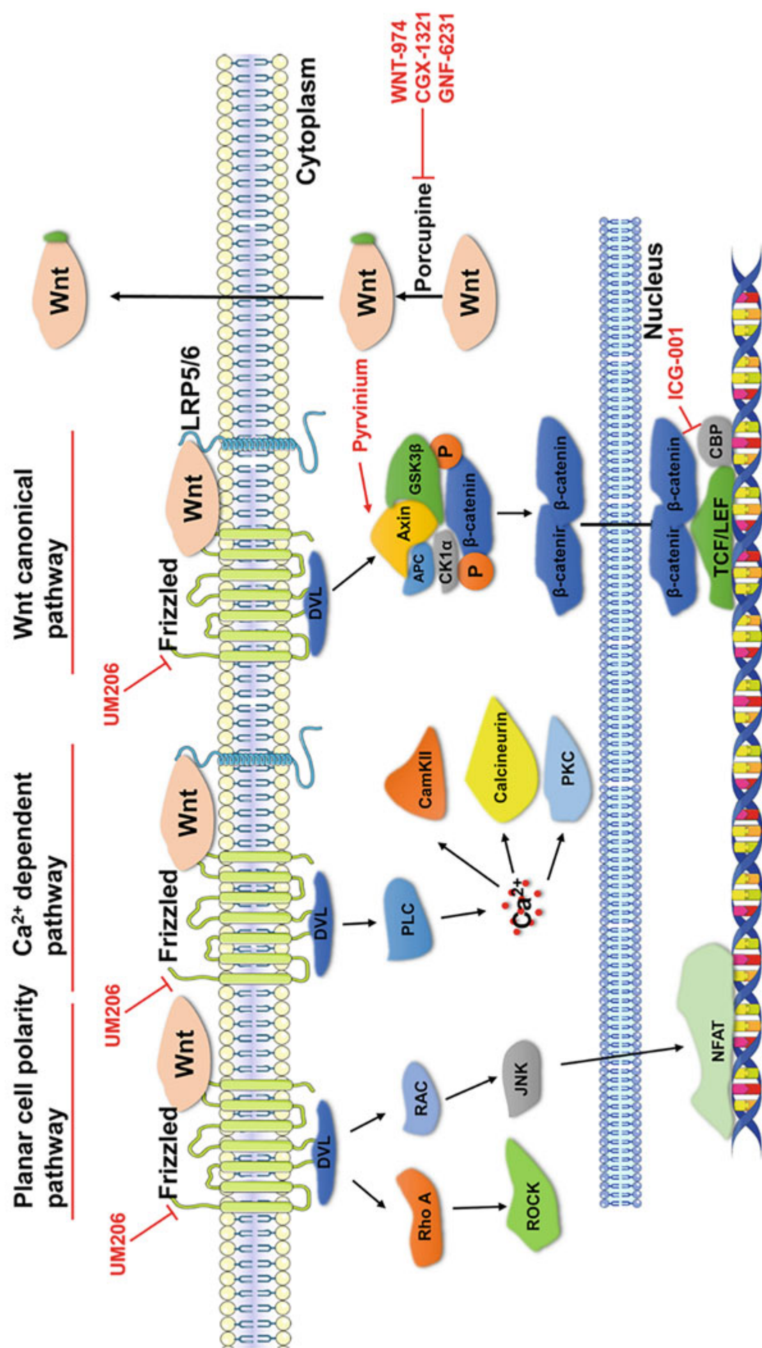

Fig. 5.4 Wnt signaling pathways and the Wnt pathway inhibitor intervention targets. (Copyright from Fu et al. (2019), Acta Pharmacologica Sinica, 2019)

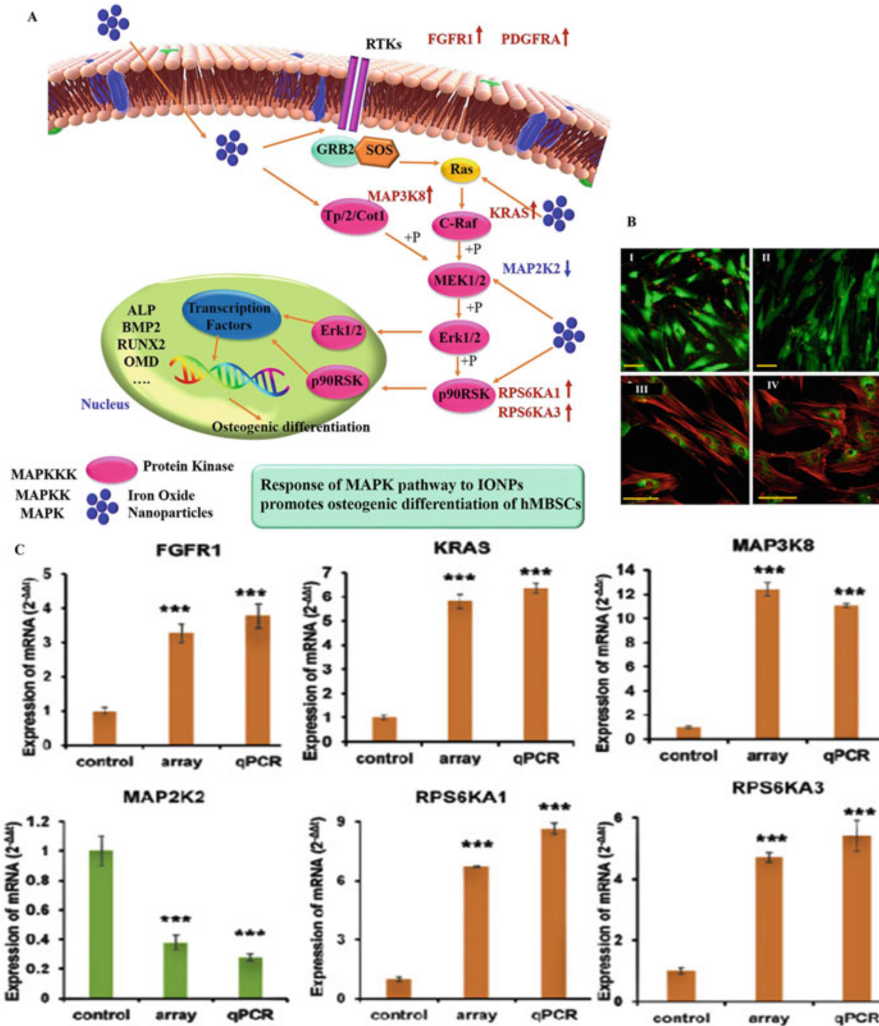

Fig. 5.5 The effect of magnetic nanoparticles in guiding the fate of the MSC-derived cells. (**a**) IONP-enhanced osteogenic differentiation. After modifying cells with IONPs, the classical MAPK signal pathway was stimulated, and the osteogenic genes were controlled by this activation. (Data were adapted from Wang et al. (2016b), Copyright 2015 Biomaterials). (**b**) The fluorescent image of Anti-PDGFRα ***(I)*** and PDGFRβ ***(II)*** antibody-conjugated magnetic nanoparticles. The cell membrane of hBMSCs embraced the Dextran (green color)-stained PDGFRα ***(III)*-** and PDGFRβ ***(IV)***-treated nanoparticles in Phallodin-Atto 565-labeled hBMSCs (red color). The scale bar is 100 μm. (Data were adapted from Hu et al. (2014), Copyright 2014 Biomaterials). (**c**) Identification of genes activated with classical MAPK signal pathway. Expression of mRNAs identified by Q-PCR in exposure to 100 μg/mL IONPs for 7 days, elucidated by $2^{-\Delta\Delta T}$ method. ($n = 3$, *** $p < 0.001$). (Data were adapted from Wang et al. (2016b), Copyright 2015 Biomaterials)

pathway. In this regard, a quantitative analysis was performed to assess the genes involved in MAPK signaling pathway, comprising FGFR1, KRAS, MAP3K8 (Tpl2/Cot), RPS6KA1, MAP2K2 (MEK2), and RPS6KA3 (Fig. 5.5c). The results revealed an upregulation about 4–11-folds in the mRNA levels of all of the studied genes except MAP2K2 (MEK2) which was fourfolds downregulated. Thus, it was suggested that the main members of MAPK pathway were expressed abnormally. The results of the detected phosphorylation events showed a significant increment in the phosphorylated form of p90RSK, MEK1/2, and ERK1/2, but down-expression of MEK1/2 and no significant change of ERK1/2 (Fig. 5.5c) (Wang et al. 2016b).

Following the magnetic fingerprint after cell magnetization revealed a destroying trend for magnetic nanoparticles caused to release iron within the cellular environment. The free iron was then held in ferritin in non-magnetic form, which could be a possible way for cell detoxification over time (Van de Walle et al. 2019). The magnetized nanoparticles act as not only reporters of biomolecules in this strategy but also operating biological responses upon interactions with external magnetic fields. Mechanosensitive proteins, proteins related to the signaling pathways, such as TRPV4 ion channel (Elliott et al. 2015) can also be actuated through magnetization, which leads to influence the clustering of the receptors by inducing the spatial positioning of the cytoplasmic intracellular pathways and endocytic internalization. Yet, their applications are limited due to the exclusive performance of clustering nanoparticles for mere cytoplasm and cellular membranes that are bound to the receptors and to the intracellular proteins, respectively. Researchers engineered signaling protein gradients in Xenopus egg extracts (as animal cytoplasm models) that were confined in the droplets by means of superparamagnetic nanoparticles with adjustable length (5 to 35 μm). In fact, with focusing on the Ran pathway, the protein gradients were synthesized through conjugating the signaling proteins to magnetic nanoparticles that were sensitive to magnetic forces and Brownian motions. Active Ran (RanGTP) with the spatial gradient of concentration encrypts Ran pathways around the chromosomes. Thus, two proteins among TPX2 and CDK11 are released by activating a signaling cascade, which plays a role as a stimulant for nucleation of microtubules and stabilizers for microtubules. The confined egg extracts in the droplets showed the perseverance of biological activity for RanGTP proteins after conjugating with the magnetic nanoparticle. Although a gradient of RanGTP proteins represented a destructive manner for self-assemblies of microtubules, the aster-like patterns were formed by the homogeneous concentrations of these proteins (Bonnemay et al. 2013). In order to stimulate integrin-ligand binding formation, Arg-Gly-Asp (RGD) peptide was conjugated with magnetic nanoparticles. The synthesized formulation was used for regulating stem cell's fate and adhesion by targeting the integrin $\alpha\nu\beta3$ under an oscillating magnetic field. It has resulted that the optimum functionality was regarded to the lower altering field about 0.1 Hz. However, this amount was reported at about 0.9–1 Hz in the presence of anti-Frizzled functionalized magnetic nanoparticles, where superparamagnetic iron oxide with 250 nm in size was used as the magnetic segment to stimulate Wnt signaling of the cells (Wong et al. 2017; Wu et al. 2018).

5.7 Conclusion and Future Prospects

In conclusion, numerous strategies have been progressed for the purpose of mechanobiology and the evaluation of the effects of mechanical incentives on cellular answers. Overall, the coupling of physicochemical, biological, and engineering has provided the opportunity to enhance technological scaffolds that hold countless potential for a comprehensive evaluation of cell mechanobiology. As a future outlook, we suppose technological developments to drive the progression of the abovementioned techniques, leading to a broad range of measurable and applicable forces and an amended spatiotemporal resolution. The next generation of devices to assess cellular forces is anticipated to form complicated cell microenvironments within combinatorial guidance signals in a single study. Therefore, individual cells can recognize a dynamically altering set of biochemical and mechanical situations more illustrative of in vivo settings. Hence, researchers will possibly support a stronger convergence of the machineries, as anticipated for high-speed optical tweezers (OTs) and AFM with regard to integration of nanoscale resolution pictures with manipulation skills at the molecular level (Ando 2018). The consideration of scientists in mechanobiology will undoubtedly be drawn to the amounts of cell and mechanical characteristics of tissue in vivo. Thus, micro-electro-mechanical systems are promising tools to detain a significant role in light of their design flexibility and broad force range. The challenge is translating the accumulating indication of mechanical regulation of cellular functions in disease and physiology into treatments and next-generation diagnoses.

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An Overview of the Use of Dental Stem Cells and Polycaprolactone Scaffolds in Tissue Engineering

6

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Abstract

Dental tissues have been discovered as a pool of stem cells with multiple differentiation potencies in recent decades. Stem cells with different differentiation potential, but particularly of mesenchymal origin, could easily be isolated from dental pulp, periodontal ligament, apical papilla, dental follicle, and gingiva, as well as the exfoliated deciduous teeth and third molar teeth germs. Polycaprolactone is a well-known biomaterial that has been used in the engineering of many tissues for more than 30 years because of its excellent tailorability and availability. This chapter will review the types of dental stem cells,

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techniques in producing polycaprolactone scaffolds, and different tissue engineering applications involving dental stem cells and polycaprolactone.

Keywords

Dental stem cells · Polycaprolactone · Scaffold manufacturing · Tissue engineering

6.1 Introduction

Tissue regeneration strategies require the use of a proper stem cell source and scaffolds that these cells could grow on and differentiate. Adult stem cells that are obtained from dental tissues are frequently being used in tissue engineering applications due to their ease of isolation and maintenance. Dental stem cells could be isolated from different parts of teeth like a germ, pulp, follicle, periodontal ligament, and apical papilla, along with alveolar bone, gingiva, and oral mucosa (Yamada et al. 2019). Tooth germ contains mesenchymal stem cells (MSCs) that can be used in osteogenic, adipogenic, neurogenic, and chondrogenic differentiation. Dental pulp stem cells (DPSCs) are of mesenchymal origin and can differentiate into odontoblasts, osteoblasts, adipocytes, and chondrocytes. Periodontal ligament (PDL) is a connective tissue that provides the connection of cementum to the alveolar bone, homing pluripotent dental stem cells. Like DPSCs, periodontal ligament stem cells (PDLSCs) are a source of progenitor cells that are capable of differentiation into derivatives of mesenchymal cells. At the tooth root tips, there are stem cells isolated from the apical papilla (SCAP), with the ability to differentiate into mesenchymal lineage cells such as odontoblasts and adipocytes. Dental stem cells could also be extracted from the teeth that are shed during childhood. These stem cells from human exfoliated deciduous teeth (SHEDs) could be used in regenerative applications. Dental follicle cells (DFC) are known to be more pluripotent than other dental stem cells because they are isolated from a developing tissue. Gingiva is a home for gingival mesenchymal stem cells (GMSCs) that are proven to be more potent than MSCs from other sources. These stem cells will be reviewed in terms of their properties and potencies for different applications. PCL is an FDA-approved synthetic scaffold biomaterial that is widely used in many tissue engineering applications because of its non-toxicity and biocompatibility. In addition, it is vastly soluble in organic solvents with a melting point of around 60 °C, and it can be blended with various natural and synthetic biomaterials. A wide variety of techniques, from particulate leaching to 3D printing, could be utilized to form PCL scaffolds of unique architectures. The techniques could be used alone or in combination, according to the desired application. Some of these scaffold production methods will be discussed in this chapter. In addition, different tissue engineering applications utilizing dental stem cells, PCL scaffolds, and their combination will be examined.

6.2 Tooth Development (Odontogenesis)

Odontogenesis is a unique complex order of events that occur by the interaction between the oral epithelium cells and mesenchyme from the neural crest (Volponi et al. 2010). Two sets of dentition are observed in humans: deciduous and permanent teeth, undergoing the same developmental stages at different times. Deciduous teeth are also called as primary or baby teeth and are replaced by permanent teeth later in childhood (Kwon and Jiang 2018). Development of tooth starts with the induction stage in which the oral epithelium thickens, sending signals to the underlying mesenchymal tissue (Fig. 6.1). As these mesenchymal cells gather signals from the epithelium, they condense around the bud which is formed by proliferating epithelial cells. Then, these epithelial cells start to differentiate into enamel-forming ameloblasts that lead to the formation of the enamel knot, which is responsible for the folding of the epithelium. Formation of the tooth crown is initiated in this so-called cap stage. By the bell stage, mesenchymal cells start to form dental papilla that brings about the dental pulp and dentine-forming odontoblasts. Simultaneously, ameloblasts begin to form the enamel organ around which the peripheral condensed mesenchymal cells expand, building the dental follicle. Terminal differentiation of odontoblasts and ameloblasts occurs at this stage. Tooth eruption is the last and postnatal stage of tooth development. Root formation takes place, and then the dental follicle gives rise to cementum generated by cementoblasts and the periodontal ligament that attaches the root to the alveolar bone (Volponi et al. 2010; Thesleff

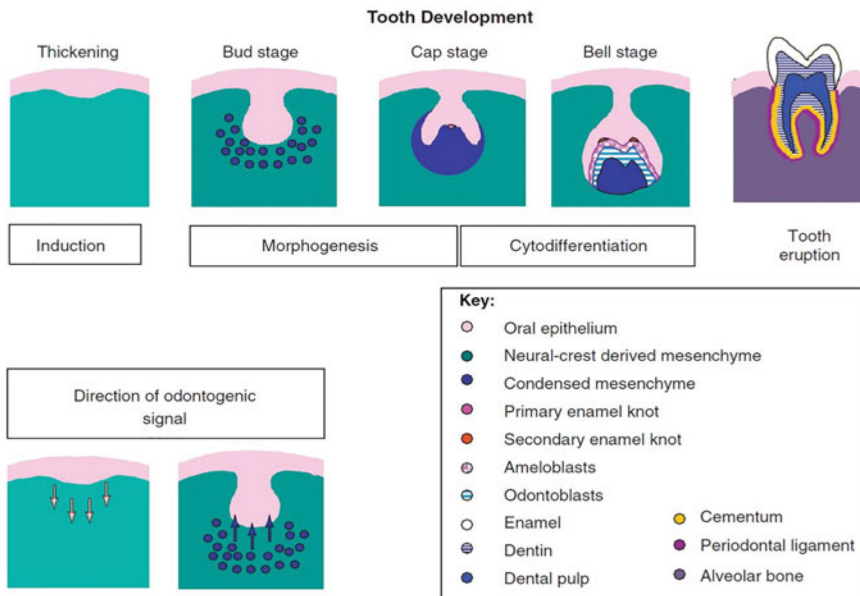

Fig. 6.1 Schematic illustration of tooth development (Volponi et al. 2010), reproduced under creative commons CC-BY license

and Sharpe 1997). Development of deciduous and permanent teeth occurs before birth, whereas the second and third molars develop postnatally, initiating at the bud stage. The third molars start to develop at the ectodermal dental lamina, which then interacts with jaw mesenchyme. The development of wisdom teeth begins at around the age of 5–6 (Zou et al. 2010) and finishes about the age of 18–25 (Volponi et al. 2010). Currently, although the third molar teeth are displeasing for many people because of causing some jaw and dental problems, the wisdom tooth germ is a pool of dental stem cells that have become an attractive adult stem cell niche within the last two decades.

Dental tissues are usually preferred as a collection of MSCs for research in regenerative medicine because dental stem cells can be extracted noninvasively compared to the other types of MSCs, such as bone marrow-derived ones (BM-MSCs). In addition, they have high proliferation and differentiation capacity, as well as being ethically permissible. In recent years, it is very common for wisdom teeth to be extracted because most modern people suffer from these third molars and are troubled about the problems these third molars will cause in the future. By the early extraction of the third molars, degeneration of the dental tissue and the diseases they cause can be prevented. Removing the third molars before they develop may be beneficial in terms of providing a potential stem cell pool in addition to being painless (Yalvac et al. 2010; Zou et al. 2010).

6.3 Dental Stem Cells

Up to date, there are many different sources of dental stem cells with diverse differentiation capacities. Due to their origin, the dental stem cells have an intrinsic tendency toward osteogenic differentiation. Identification of dental stem cells was first demonstrated by the pioneering work carried out by Gronthos et al. (2000) in which a pool of odontogenic progenitor cells isolated from postnatal dental pulp, **DPSCs**, were shown to be highly proliferative and clonogenic, with the ability to regenerate tissue. DPSCs could also be obtained from permanent third molar tooth pulp (Fig. 6.2). They have a mesenchymal origin with the potential of differentiation into odontoblasts, osteoblasts, adipocytes, and chondrocytes (Koyama et al. 2009; Yu et al. 2010; D'Aquino et al. 2007, 2008); however, the differentiation capacity decreases as the passage number increases. After the ninth passage, the differentiation of DPSCs is restricted to osteogenic lineage (Yu et al. 2010). In adult tissues, DPSCs remain dormant under healthy conditions but become active upon injury, regenerating odontoblastic progenitors. Because these cells are formed from the neural crest during embryogenesis, they have the potential to differentiate into neurons. Induction of neurosphere formation in human DPSCs was proven to be an efficient way to commit these cells to neurogenic lineage (Gervois et al. 2015). Expression of neurotrophic factors that promote regeneration and elongation of axons was shown to be higher in DPSCs than MSCs (Mead et al. 2014), verifying that these cells could be a suitable cell reservoir for nerve tissue engineering. In a scaffold-free nerve tissue engineering strategy, DPSC sheets were successfully used

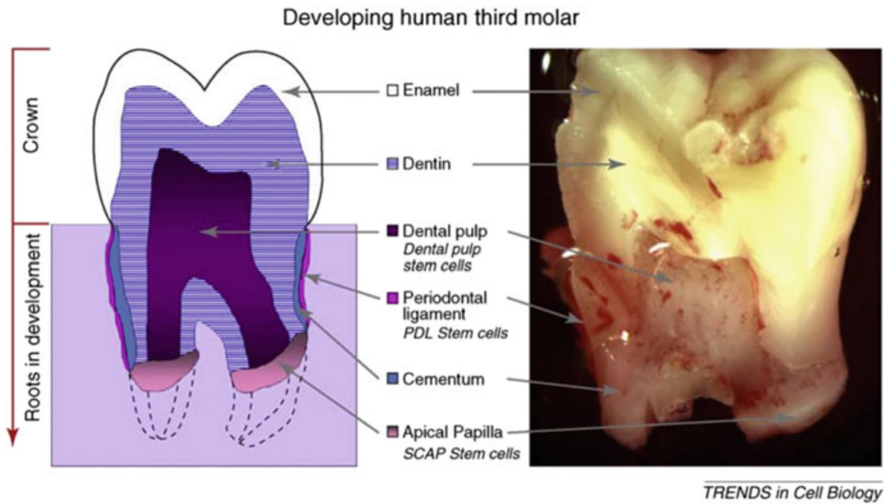

Fig. 6.2 Illustration and photograph of an extracted human third molar in development, showing the locations of dental stem cells (Volponi et al. 2010), reproduced under creative commons CC-BY license

for the repair of peripheral nerve injury on a rat disease model, which was attributed to the release of neurotrophic factors from the cell sheet (Ahmed et al. 2020). The regenerative potential of the cell sheets could be increased by the addition of fibroblast growth factor 2 into the culture medium.

The alveolar bone of the tooth and cementum are bound by the PDL, which contains dental stem cells that typically maintain homeostasis in the tissue (Volponi et al. 2010). These **PDLSCs** are responsible for the production of not only the extracellular matrix (ECM) fibers but also some pro-inflammatory cytokines to recruit leukocytes to repair PDL upon damage (Jönsson et al. 2011). Similar to DPSCs, PDLSCs are progenitors that could be differentiated into stem cells of mesenchymal origin in vitro (Seo et al. 2004; Sonoyama et al. 2006; Gronthos et al. 2006; Gault et al. 2010). Besides, the potency of PDLSCs in osteogenic, chondrogenic, and adipogenic differentiation decreases with aging (Li et al. 2020a, b).

SCAP, which are present only at the growing root tips (Fig. 6.2), were shown to be mesenchyme-originated and are capable of differentiating into osteoblasts and odontoblasts, as well as adipocytes (Nada and El Backly 2018; Sonoyama et al. 2006). It is somewhat difficult to gain access to stem cells from tissues in their early stages of development; however, extraction of SCAP is a relatively easy process, involving the use of tweezers to separate the tissue at the root tip and subsequent tissue culture protocols (Nada and El Backly 2018). Since the development of third molar tooth roots occurs after birth, pluripotent stem cells can easily be isolated from the apical papilla, which is one of the most important advantages of SCAP extraction from third molars (Volponi et al. 2010).

SHEDs were first recognized by Miura et al. (2003), suggesting that SHEDs were multipotent stem cells with a higher proliferation rate and better differentiation capacity compared to other dental stem cells. After the deciduous teeth are shed in childhood (around age 7), SHEDs could be extracted as a potential source of stem cells for tissue engineering. Having similar differentiation potential with MSCs, SHEDs are capable of differentiation into various cells, including bone, cartilage, dental, adipose, and nerve tissue cells (Saez et al. 2016).

The dental follicle is a connective tissue surrounding the un-erupted tooth enamel and papilla (Morsczeck et al. 2005), which contains stem cells that can differentiate into PDL cells, cementoblasts, and osteoblasts (Yao et al. 2008). The pluripotency of **DFCs** is higher than the other dental stem cell types because DFCs are isolated from tissue in development (Volponi et al. 2010). DFCs from human third molars were shown to have the capacity to form osseous tissues both in vitro and in vivo (Yagyuu et al. 2010). It was demonstrated that dental stem cells extracted from the human third molar germs had mesenchymal-like characteristics, being able to differentiate into osteogenic, adipogenic, and neurogenic cells (Yalvac et al. 2010). Because these human tooth germ stem cells (HTGSCs) are isolated from the un-erupted and developing tooth tissue, they represent a highly multipotent pool of dental stem cells. HTGSCs were further found to have the tendency to deposit cartilage-specific ECM when grown on macroporous polycaprolactone-poly(L-lactide) (PCL-PLLA) scaffolds (Calikoglu Koyuncu et al. 2017).

Gingiva is a type of soft and dense vascular connective tissue surrounding the neck of teeth and alveolar bone, holding the teeth in the proper position. Within the last two decades, it was evidenced by numerous studies that gingiva contains multipotent stem cells with mesenchymal origin. The **GMSCs** could be isolated from tissue explants after gingivectomy. When compared to bone marrow-derived MSCs, GMSCs were suggested to have a higher proliferation rate and differentiation capacity, maintaining a stable stem cell phenotype (Fawzy El-Sayed and Dörfer 2016). Furthermore, these characteristics do not significantly change when GMSCs are isolated from inflamed gingiva (Ge et al. 2012).

6.4 Production of Polycaprolactone Scaffolds

PCL is a semi-crystalline polyester that is among a few of the FDA-approved biomaterials. Because it is highly biocompatible and its hydrolytic degradation products are non-toxic, it is widely used as a biomaterial in tissue engineering applications (Ratner and Hoffman 2013). The first synthesis of PCL by ring-opening polymerization of the ε-caprolactone dates back to 1934 (Van Natta et al. 1934), but it was not used in biomedical applications until the 1980s when bioresorbable polymer research has gained popularity (Woodruff and Hutmacher 2010). It was in the mid-1980s when PCL was first commercialized after defining that synthetic polymers could be degraded by microorganisms (Huang 1985). The semi-crystalline nature of the polymer drives it hydrophobic with a degradation time over 2 years, allowing it to be suitable for long-term biomedical applications such as cartilage and

bone tissue engineering and sustainable drug delivery devices (Miller et al. 2011). Owing to its high solubility and blendability, the degradation rate of PCL can be adjusted by blending with other polymers according to the purpose of utilization. For instance, the addition of PLA to PCL enhances the mechanical properties of the composite scaffold, making it useful in bone tissue engineering. Another characteristic of PCL is its low melting point (59–64 °C), which makes it so ductile that it can be processed in different ways such as molding, particulate leaching, electrospinning, and three-dimensional (3D) printing (Arakawa and DeForest 2017).

6.4.1 Particulate Leaching/Solvent Casting/Freeze-Drying

Being one of the cheapest and easiest scaffold fabrication techniques, salt leaching involves creation of a porous scaffold by using a porogen, such as sodium chloride crystals. Depending on the tissue type to be engineered, pore size of the scaffold might be adjusted simply by using salts with specific particle sizes. Using a centrifuge technology during salt matrix preparation could provide homogenous distribution of the porogen, improving the uniformity of scaffold pore size (Yang et al. 2006). Particulate leaching is frequently used in combination with solvent casting method, in which polymer is dissolved in a solvent, cast into molds, and then fixed by allowing evaporation of the solvent. As PCL is an aliphatic polyester, the choice of solvent type is usually organic solvents. In this solvent casting/particulate leaching combinatory technique, PCL is dissolved in chloroform or dichloromethane, and then porogen, generally salt particles that are insoluble in organic solvents, is added to the solution. This mixture is poured into molds of desired shapes, and the organic solvent is evaporated. After dissolving the salt particles in water, known as dialysis, the polymer is dried, and a porous PCL scaffold is obtained. In order to further remove the organic solvent residues, the scaffold is subjected to lyophilization or freeze-drying. A study involving the use of PCL in bone tissue engineering was shown in Fig. 6.3. PCL/phospho-calcified cellulose

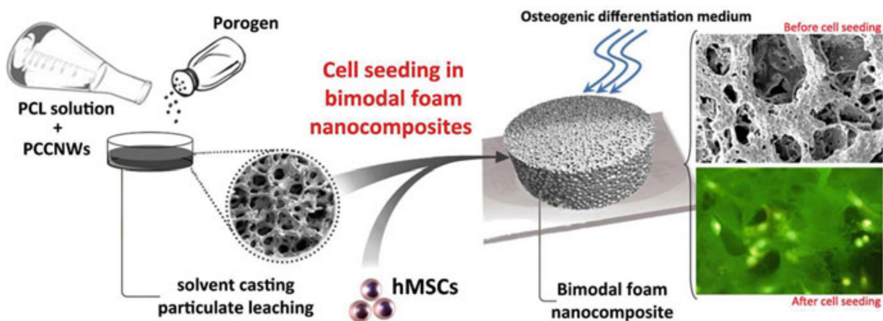

Fig. 6.3 Osteogenic differentiation of human MSCs on PCL scaffolds produced by solvent casting/particulate leaching method. *PCCNWs* phospho-calcified cellulose nanowhiskers, *hMSCs* human MSCs (Jafari et al. 2018). (Reproduced with copyright permission from the American Chemical Society)

nanowhiskers (PCCNW) were prepared by solvent casting/particulate leaching method, and then human MSCs were induced to differentiate into osteoblasts on these nanocomposites (Jafari et al. 2018). The addition of PCCNW was shown to increase hydrophilicity, and hence osteoconductivity. PCL/PCCNW scaffolds could mimic the native bone ECM, supporting the attachment and osteogenic differentiation of human MSCs. Scaffolds for tissue engineering can also be produced by only freeze-drying. After the polymer is dissolved in the organic solvent, it is subsequently frozen at -80 °C for a few days. Finally, the process of lyophilization results in a porous scaffold, frozen water crystals acting as the porogen. The pressure that is applied during lyophilization was suggested to determine the scaffold pore size and porosity (Autissier et al. 2010). Freeze-drying at low pressure offered scaffolds with higher porosity and mean pore diameter in comparison to those obtained by high freeze-drying pressure. The relationship between pore formation and freeze-drying was evidenced in a study in which a pullulan/dextran-based hydrogel scaffold was produced by lyophilization, suggesting that the pore shape and size were controlled by the nucleation of ice crystals (Grenier et al. 2019). As a result of solvent casting/particulate leaching and freeze-drying, one can obtain scaffolds with pore sizes ranging from roughly 20μm to 500μm. The pore size and porosity depend on the biomaterial, casting temperature, type and size of porogen, and lyophilization pressure, which should be carefully chosen according to the specific application since the optimal pore size changes from one tissue type to another.

6.4.2 Electrospinning

The electrical fiber drawing (electrospinning) method is one of the most preferred methods in nanofiber scaffolding due to its simple procedure and its advantages, such as controlling the shape and number of pore diameter. It is based on the principle of fiber pulling with the help of electrostatic forces. In this method, polymer solutions can be charged with thousands of volts of electric current and fibers with nano-diameter can be formed. An electrospinning unit consists of a high-voltage power supply, a syringe with pump, metal needle tip, and collector. For different applications, the unit can be modified, such as double syringes or rotating collector. In the electrospinning method, firstly, an electric current is created by charging the polymer solution with high voltage. Initially, the polymer solution stops like a drop due to the surface tension at the capillary end. However, as the voltage increases, electrical charges begin to act on the drop, and when these electrical forces reach a magnitude to overcome the surface tension, the charged polymer solution begins to move from the capillary end to the grounded plate. During the movement of the polymer jet toward the plate, the polymer jet solidifies as the solvent evaporates, and a web-like layer of nanofibers is obtained on the grounded plate (Tülü and Kaya 2020). PCL can be used in the engineering of many kinds of soft tissues by changing its properties and reducing its molecular weight due to its high tailorability. Production of PCL via electrospinning, including the technical and biological advantages, is summarized in Fig. 6.4. PCL has been reported in the

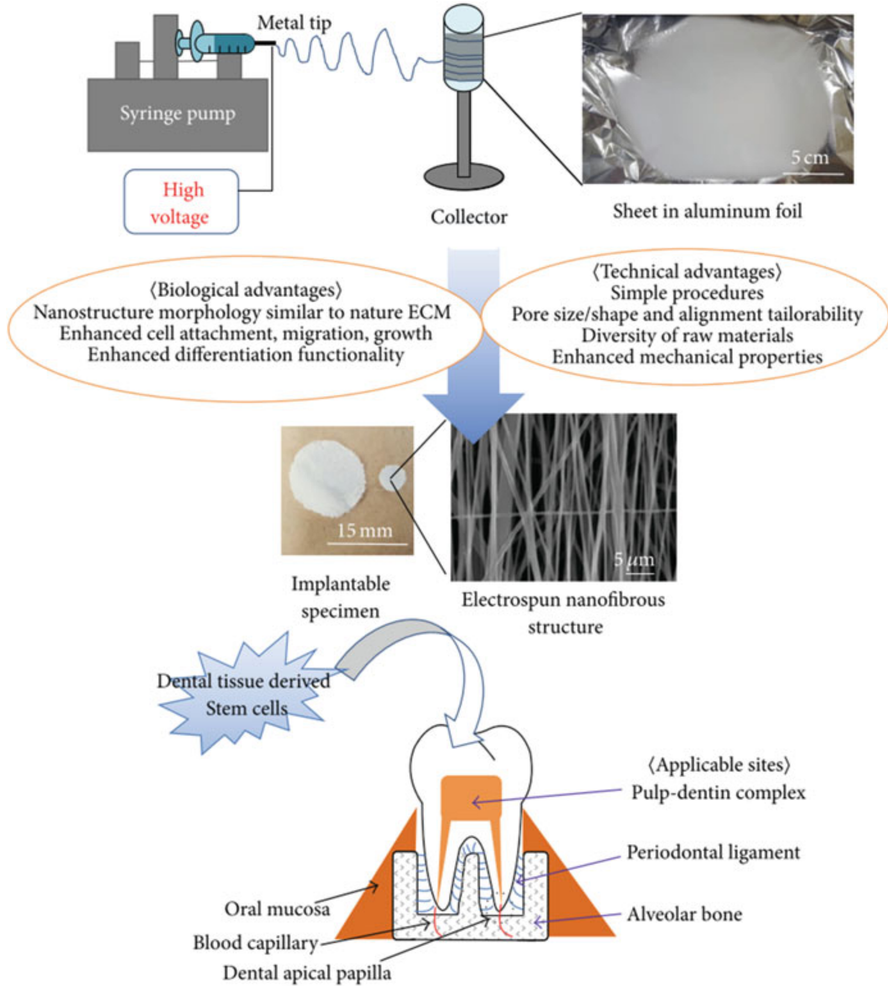

Fig. 6.4 Production of electrospun nanofibrous PCL scaffolds that were used for dental tissue engineering using dental stem cells. (Adapted from Seo et al. (2016) reproduced with copyright permission under the Creative Commons Attribution License)

construction of artificial periodontal ligament tissue as a scaffold material produced by electrospinning after dissolving PCL powder in glacial acetic acid (Safi et al. 2020). Yang et al. (2010) examined both in vitro and in vivo behavior of DPSCs in the absence and presence of nanohydroxyapatite (nHA) in PCL/gelatin scaffolds obtained by electrospinning method. They showed that scaffolds containing nHA combination increased the expression of specific odontogenic genes, and as a result, adding nHA to scaffolds increased the odontoblast-like phenotype differentiation of DPSCs both in vitro and in vivo (Yang et al. 2010). Guo et al. (2014) examined the behavior of dental pulp stem cells (DPSC) by adding or not adding fluorapatite

(FA) to PCL fibers obtained by electrospinning. After the first planting, the stem cells adhere well to both scaffolds. However, it was observed that there was multicellular aggregation in FA-added PCL scaffolds, and cell proliferation in FA-coated PCL scaffolds was slower than the scaffold consisting of PCL only after 14 days. It has been reported that although proliferation is fast in the PCL scaffold, multicellular aggregates were not detected, possibly due to differentiation hampering proliferation (Dreesmann et al. 2009). As a result, it has been reported that FA-modified PCL scaffold could be a favorable biomimetic material for bone and dentin/pulp regeneration in odontogenic-osteogenic tissue engineering (Guo et al. 2014). PCL nanofiber scaffolds produced by electrospinning were proven to enhance osteogenic differentiation of human MSCs from the bone marrow, umbilical cord, and adipose tissue, as evidenced by the increase in osteogenic markers upon the cultivation of MSCs on PCL nanofibers (Xue et al. 2017). Using an auxiliary electrode during the electrospinning of PCL nanofibers was shown to provide stability of the initial polymer jet ejected from the apex of the Taylor cone (Kim and Kim 2007). The addition of the chemical blowing agent azodicarbonamide to the PCL solution increased the porosity of the blown nanofibrous mats, serving as a favorable microenvironment for the attachment and growth of human dermal fibroblasts. The decomposition of the chemical blowing agent resulted in high porosity and increased pore size. Near-field electrospinning (NFES) was utilized in a research to obtain a proper 3D scaffold, claiming that the direct writing allows for fabrication of layered meshes with adjustable pore sizes (>100μm) and for controllable degradation of scaffolds (He et al. 2018). The 3D PCL/hydroxyapatite scaffold produced by NFES technique was shown to be well-suited for the attachment and proliferation of mouse pre-osteoblasts evidenced by in vitro analyses. Similarly, PCL nanofibrous mats produced by electrospinning promoted the formation of a cell sheet composed of periodontal ligament stem cells, which could potentially be used as a cover in dental implants (Safi et al. 2020). The control of pore size is also essential for the engineering of some tissues that require cell aggregation for regeneration. In cartilage tissue engineering, the ideal scaffold pore size is approximately 100–150μm (Nava et al. 2016), because the small pore size allows for cell aggregation and provides a hypoxic environment in which chondrogenic differentiation factors could be expressed in a higher amount than normally occurs in normoxic conditions. It is possible to manufacture scaffolds with pores in tens of nanometer scale by electrospinning techniques.

6.4.3 Three-Dimensional Printing (Additive Manufacturing)

3D printing technology, which first appeared in the field of material production only, has become very popular in recent years and started to be applied in a variety of areas over time (Değerli and El 2017). 3D printers do not require special tools and equipment during production, which reduces the workforce. The quality properties of the final 3D product, such as color, shape, texture, aroma, and raw material content, can be easily adjusted when necessary (Sun et al. 2015). These two concepts

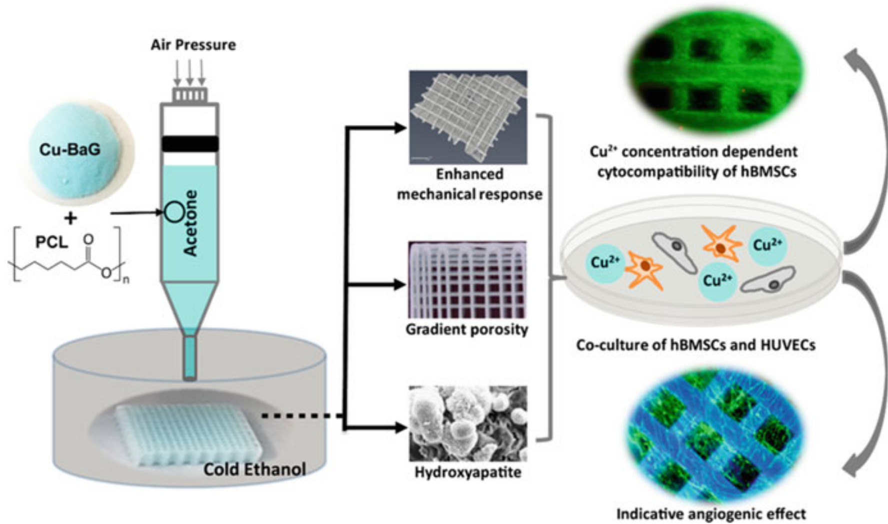

Fig. 6.5 The use of 3D-printed PCL copper-doped bioactive glass (Cu-BaG) scaffolds for bone tissue engineering. Cu was used for the purpose of angiogenesis. hBMSCs, human bone marrow-derived MSCs; HUVECs, human umbilical vein endothelial cells. (Adapted from Wang et al. (2019) with copyright permission)

can be summarized as forming physical structures in certain geometries by shaping various materials such as ceramics, metals, and polymers in layers (Godoi et al. 2016). A bone tissue engineering study using the 3D printing technique is summarized in Fig. 6.5. In order to blend immiscible biomaterials, a new method called indirect 3D (i3D) printing has been developed (Jung et al. 2015). In this technique, a temporary mold, which is made of a photopolymer resin in an alkaline solution, was printed using projection-based microstereolithography (pMSTL). PCL/gelatin blend solution was injected into the mold and let to solidify after removal of solvents by immersing the mold in isopropyl alcohol. After cross-linking gelatin with glutaraldehyde, the mold was dissolved in alkaline solution to obtain the final PCL/gelatin scaffold, which was suggested to be biochemically and mechanically more suitable for cartilage tissue engineering than pure PCL. Many objects can be created at once with 3D printing, increasing productivity, making 3D printing an attractive method in science and engineering (Chen et al. 2019). Jiao et al. (2019) reported that nano-HA/PCL and micro-HA/PCL tissue scaffolds could be produced by melt differential FDM 3D printer, which has the advantages of rapid processing, reduced cost, and toughness. The pores of these 3D scaffolds were interconnected and homogeneously distributed. Cell adhesion, proliferation, and nutrient transport can be easily performed by these pore structures (Jiao et al. 2019). Li et al. (2020a, b) reported that artificial bile ducts could be produced in vitro to repair and replace injured bile ducts. In this study, a composite PCL scaffold was printed via 3D method in the form of a tube. To improve the scaffold performance, hydrogel addition was done, and then gelatin methacryloyl (GelMA) with appropriate

thickness was applied. The application of the hydrogel layer protected and supported the scaffold, increasing the biocompatibility of the synthetic bile duct. The study implied that PCL/GelMA bile duct scaffolds enabled cells to proliferate, and these scaffolds could be used as artificial bile duct substitutes (Li et al. 2020a, b). Controlling the structure and porosity of PCL scaffolds could also be done by extrusion-based cryogenic 3D printing using PCL/GAC (glacial acetic acid) bioink. Combined extrusion-based cryogenic 3D printing (ECP) at 20 °C and subsequent freeze-drying processes were easily performed to fabricate the PCL scaffolds. It was stated that the PCL scaffold could be used in tissue engineering applications because of its advanced structural, physicochemical, and biological properties (Zhang et al. 2019). Ma et al. (2019) proposed a special material system containing poly-(-ε-caprolactone) (PCL), polyvinyl acetate (PVAc), and hydroxyapatite (HA) was used to produce porous scaffolds by 3D printing method for bone regeneration. PCL, PCL/PVAc, PCL/HA, and PCL/PVAc/HA groups were used in the study. Cell proliferation and bone formation rate in the PCL/PVAc/HA scaffold were found to be higher than the other groups, indicating that 3D-printed PCL/PVAc/HA scaffolds could be suitable for individual bone repair applications. Strontium-containing hydroxyapatite (SrHA) is a material that could be used for bone repair as it contains inorganic compounds similar to natural bone. It has been determined that Sr and Ca ions were continuously released from the PCL/SrHA scaffolds, which could be easily obtained with 3D printing technology and could be used in bone repair. Likewise, SrHA to the 3D printed system facilitated cell proliferation and supported bone regeneration (Ma et al. 2019). Using a new double-layered polymer scaffold combined with human BM-MSCs production of a bile duct for the treatment of biliary disease was hypothesized by Zong et al. (2017). The study proved that the PCL/PLGA scaffold promoted adhesion, proliferation, and matrix deposition of human MSCs, yet the cell-scaffold construct hMSC-PCL/PLGA (MPPC) provided improvement in bile duct injury repair. Patrício and Bártolo (2013) reported a new 3D scaffold production technique called BioCell Printing. PCL and PCL/PLA scaffolds that were produced using this system had regular and repeatable architectures, in addition to their biocompatible nature favoring cell attachment and proliferation.

6.4.4 Phase Separation Method

Production of 3D porous PCL scaffolds could be performed by a technique called thermally induced phase separation (TIPS). The phase separation technique depends on the separation of a thermodynamically unstable polymer solution into two phases as polymer-rich and polymer-poor (Kulkarni and Rao 2013). First, a polymer solution is prepared at high temperatures and mixed with a non-solvent that cannot dissolve the polymer below a certain temperature. When the temperature of the solution is suddenly reduced below the melting point, the polymer solution becomes thermodynamically unstable, ready to separate into two phases. The addition of water to the mixture removes the solvent, resulting in the precipitation of the

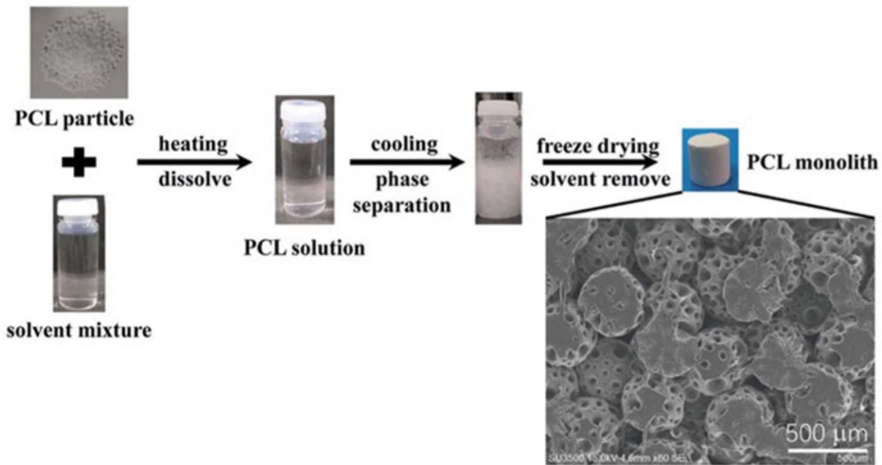

Fig. 6.6 Graphical summary of TIPS technique for the production of PCL scaffolds. Scanning electron micrograph of the scaffolds was shown at the bottom right corner of the figure. (Adapted from Cao et al. (2020) under Creative Commons Attribution-NonCommercial 3.0 Unported Licence)

polymer scaffold from the polymer-rich phase, while the polymer-poor part forms the pores of the scaffold (Katsogiannis et al. 2015). Finally, freeze-drying is performed to obtain the porous 3D scaffold. The technique is easy to perform, allowing for the manipulation of the porosity of the final scaffold through each level of production. In a study using the TIPS process, HA nanoparticles were added to PCL to make composite and osteoconductive scaffolds (Sultana and Khan 2013). It has been determined that the compressive mechanical properties of HA/PCL composite scaffold were increased, making the scaffolds suitable for regeneration of bone tissue. The addition of HA to PCL scaffolds during TIPS technique was thought to reduce the pore size of the scaffold (Hassan et al. 2012). TIPS is also used for increasing the hydrophilicity of PCL scaffolds (Fig. 6.6).

6.5 Use of DSCs in Combination with PCL Scaffolds in Tissue Engineering Applications

6.5.1 Cartilage Tissue Engineering

Since the cartilage tissue has limited ability to regenerate itself, various ways of administering therapeutic cells to the damaged tissue have been studied in chondral and osteochondral injuries. Cartilage tissue engineering has been introduced as a promising solution to these problems. The artificial cartilage obtained as a result of tissue engineering should be biomechanically identical to the native articular cartilage, allowing the joints to perform their normal functions in vivo in cases of

excessive strain. An ideal scaffold for cartilage tissue regeneration requires a highly porous, flexible, and elastic structure that is suitable for cell attachment (Siddiqui et al. 2018). Blending PCL with PLLA was suggested to enhance the biocompatibility of scaffolds for dental stem cells isolated from the third molar tooth germ (HTGSCs) which were successfully used in the engineering of cartilage (Calikoglu Koyuncu et al. 2017). It was demonstrated that these HTGSCs differentiated toward chondrogenic lineage, as evidenced by collagen type II and aggrecan deposition on PCL/PLLA scaffolds in vitro. Wan et al. (2014) produced four-layer porous scaffolds with chitosan/PCL/collagen type II in order to mimic knee joints. Photothermal heating and freeze-drying methods were used to improve these composite scaffolds while using sodium tripolyphosphate, a crosslinker. Chondrocytes isolated from rabbit knee joint cartilage were grown in the developed scaffolds and spread over the surface of each layer of the scaffold (Wan et al. 2014). Mesenchymal stem cells (MSCs) can differentiate into the bone, cartilage, or adipose tissue cells upon appropriate induction; therefore, they can be used for therapeutic procedures. Alves da Silva et al. (2010) isolated fibroblastic cells from human bone marrow (hBM) aspirates of knee arthroplasty patients, which were then cultured on electrospun PCL nanofibrous scaffolds inside a multichamber flow perfusion bioreactor to analyze the formation of cartilaginous ECM. It was suggested that the PCL nanofiber networks produced by electrospinning were suitable for chondrogenic differentiation of hBM-MSC in the perfusion bioreactor. Li et al. (2005) stated that adult BM-MSCs seeded on electrospun PCL fibers and induced with transforming growth factor-beta 1 (TGF-β1) could display chondrogenic phenotype at levels highly similar to typical pellet cultures. This structure possessed a zonal architecture with layers of cartilaginous ECM, mainly composed of collagen type II and aggrecan. Electrospun PCL/PLLA nanofibers were used for in vitro characterization and chondrogenic capacity of available human adult stem/progenitor cell sources (Shafiee et al. 2016). In vitro analyses of BM-MSCs, adipose tissue-derived MSCs (AD-MSCs), articular chondrocyte progenitors (ACP), and nasal septum-derived progenitors (NSPs) suggested that the cell source with the highest proliferation potential and chondrogenic capacity was NSPs in terms of articular cartilage tissue engineering. Thorvaldsson et al. (2008) used electrospinning method to cover single microfibers with nanofibers because in tissue engineering, nanofiber-coated microfibers could be produced in any size, shape, and porosity. Nanofiber-coated microfibers can be formed into special porous scaffolds for adequate cellular infiltration. PCL nanofibers were electrospun onto PLA microfibers to produce approximately 95% porous scaffolds. Seeding of human chondrocytes onto these electrospun fibers proved that high porosity increased the infiltration of cells inside the scaffold (Thorvaldsson et al. 2008). Shafiee et al. (2011) investigated the potential of a hybrid PVA/PCL nanofibrous scaffold seeded with rabbit BM-MSCs. PVA/PCL matrices positively contributed to the proliferation and chondrogenic differentiation of MSC in vitro and provided tissue healing in vivo, suggesting that PVA/PCL scaffolds combined with MSCs could be suitable grafts for articular cartilage reconstruction (Shafiee et al. 2011). In another research, Shafiee et al. (2014) produced PLLA/PCL hybrid scaffolds by the aligned and random directed electrospun method to

determine its effect on cell proliferation and chondrogenesis. Nasal septum-derived progenitors (NSPs) were found to behave differently in the two types of scaffolding. NSPs cultured on aligned fibers had higher expression of chondrogenic markers, suggesting that constructs produced by the combination of aligned nanofibrous scaffolds and stem cells derived from the nasal septum could be used in cartilage regeneration (Shafiee et al. 2014). In a BM-MSC-laden PCL-based nanofibrous scaffold system studied in the porcine model to repair full-thickness cartilage defect, hyaline cartilage-specific ECM could be deposited, repairing the cartilage surface (Li et al. 2009). Casper et al. (2010) implanted PCL nanofibrous scaffolds (with and without chitosan) into rabbits and interestingly observed that the presence of sulfated glycosaminoglycan (sGAG) and cartilage matrix formation were more typical in chitosan-free scaffolds. A study evaluating the effect of PCL/gelatin electrospun nanofibrous scaffolds on the chondrogenesis of iPSCs and repair of joint cartilage suggested that iPSCs on the scaffolds expressed high levels of chondrogenic markers (Liu et al. 2014). It was determined that cartilage defects showed remarkable histological development when implanted with the scaffold-iPSCs construct, evidenced by high cartilage-specific gene and protein expression, as well as a high level of tissue regeneration. Garrigues et al. (2014) stated that reinforcing multi-layered PCL scaffolds with native tissue components, like cartilage-derived ECM, could enhance infiltration and chondrogenic differentiation of human adipose-derived stem cells.

6.5.2 Bone Tissue Engineering

Although bone tissue can repair small-sized fractures upon damages, regeneration of the whole tissue is complicated when the damage results in significant bone loss. Bone tissue engineering has emerged as a feasible solution to this problem, in which fabrication of biocompatible materials mimicking the ECM of native bone is essential in order to serve as a tissue substitute. Microstructure, porosity, rate of degradation, and mechanical properties are important factors to consider in scaffold designing. Bioactive glass, bioactive ceramics, natural or synthetic polymers, and their composites could be used as bone materials. PCL is one of the synthetic biopolymers that is frequently used for engineering of bone tissue. Scaffolds produced from PLA/PCL/HA composites by 3D printing technique were proven to be suitable for bone tissue engineering when used in the ratio of 70/30 PLA/PCL containing 35% HA (Hassanajili et al. 2019). In all scaffolds produced with different PLA/PCL ratios containing 35% HA, osteoblasts could attach, proliferate, and maintain their morphology, suggesting that PLA/PCL/HA scaffolds could be used for bone tissue engineering. In another study, hybrid PCL scaffolds that were loaded with NaOH and Bio-Oss were produced by rapid prototyping (Wang et al. 2020). It was observed that NaOH-treated hybrid scaffolds promoted BM-MSC adhesion and increase in osteogenesis in vitro and in vivo. Interconnected porous bioactive materials could be a proper scaffold choice for bone tissue engineering. It was found that PCL/PEG/HA scaffolds were biocompatible and non-toxic, and the

addition of HA to the scaffolds enhanced alkaline phosphatase (ALP) activity (Koupaei et al. 2015). Heydari et al. (2017) developed a composite scaffold made up of octacalcium phosphate (OCP) particles produced by co-precipitation method and PCL produced by electrospinning technique. Being compatible with and non-toxic for human osteoblasts, the PCL/OCP composite scaffold had mechanical properties appropriate for bone tissue engineering. The addition of biphasic calcium phosphate (BCP) was found to accelerate osteogenesis, evidenced by a study in which PCL/PLGA-BCP scaffolds produced by solvent evaporation were used for bone tissue regeneration (Thi Hiep et al. 2017). The use of bone cement in bone implants is known to increase biocompatibility and osseointegration. A novel injectable calcium phosphate cement (CPC) containing the antibiotic ornidazole (ORZ) was developed recently (Chen et al. 2020). While encapsulating human periodontal ligament stem cells (hPDLSCs) in alginate microbeads, ORZ was added to the CPC-chitosan scaffold for offering an antibacterial scaffold. The construct was proven to support osteogenic differentiation of hPDLSCs and bone matrix mineralization, as well as being injectable, mechanically suitable, biocompatible, and antibacterial.

6.5.3 Nerve Tissue Engineering

Nerve tissue engineering is the most promising method of repairing defects in central and peripheral nervous systems as these tissues cannot recover spontaneously. The development of a proper nerve tissue substitute depends on the design of scaffolds with high flexibility, allowing cell alignment as in the native nerve tissue. PCL is frequently chosen as a synthetic biomaterial for nerve tissue engineering applications, not only because of its biocompatibility but also its flexibility. When combined with a cell source that is capable of neurogenic differentiation, PCL scaffolds could be used as support material for repairing nerve gaps. In order to fully restore the function of nerve tissue, either central or peripheral, related cell types in the tissue should be combined. For this purpose, stem cells with neurogenic potential could be chosen. AD-MSCs were shown to have neurogenic differentiation potential on porous PCL scaffolds (Barbarisi et al. 2015). Blending PCL with other synthetic or natural biocompatible materials could result in biomaterial scaffolds with remarkable therapeutic potential. Scaffold morphology is of high significance in nerve regeneration. The scaffold should positively affect neural cell function, supporting the attachment of cells and extension of neurites from the neurons. Micro- or nanofibers produced by electrospinning can facilitate neurite outgrowth (Lee and Arinzeh 2011). Randomly aligned PCL/gelatin composite scaffolds showed a positive effect in cell proliferation and neural differentiation evidenced by neurite outgrowth (Ghasemi-Mobarakeh et al. 2008). Among the varying ratios of PCL/gelatin, 70:30 was suggested to be the most promising one in terms of nerve tissue engineering. Similarly, aligned nanofibrous chitosan/PCL scaffolds promoted Schwann cell adhesion, proliferation, and neurite growth much better than the randomly oriented scaffolds (Cooper et al. 2011). Adhesion and controlled

orientation of pheochromocytoma (PC-12) cells were stimulated by these nanofibers, suggesting that aligned chitosan/PCL fibers could be used as scaffolds in advanced nerve tissue reconstruction. The positive effect of PCL/chitosan electrospun nanofibers on neurosupportive Schwann cells was also evidenced by Prabhakaran et al. (2008), who suggested that PCL/chitosan nanofibers ensured adhesion of Schwann cells and maintenance of their characteristic morphology and phenotype. Electrospun fibrous PCL/collagen/nanobioglass-based scaffolds were reported to have favorable hydrophilicity and toughness for peripheral nerve regeneration, promoting adhesion and proliferation of human endometrial stem cells (Mohamadi et al. 2017). The use of electrically conductive nanofibrous scaffolds in nerve tissue regeneration seems to be reasonable because action potential is generated during nerve cell signaling. Due to their unique physico-chemical properties, gold nanoparticles (AuNPs) can increase the conductivity of scaffolds, enhancing the transfer of electrical signals between nerve cells. In a recent peripheral nerve tissue engineering study, AuNPs doping onto electrospun nanofibrous PCL/chitosan scaffolds was demonstrated to increase Schwann cell attachment and proliferation compared to AuNP-free scaffolds (Saderi et al. 2018).

6.5.4 Periodontal/Dental Tissue Engineering

The purpose of periodontal tissue engineering, which involves the regeneration of PDL, gingiva, cementum, and alveolar bone, is to produce substitutes for the teeth that are lost due to infection in the periodontium. PCL is one of the most used biomaterials to build composite scaffolds for the engineering of periodontal tissues. Beta-tricalcium phosphate (β-TCP) has been used to manufacture PCL composites with high mechanical strength and porosity (Park et al. 2017). The 3D printed PCL/β-TCP scaffolds enhanced cell proliferation and alkaline phosphatase activity of mouse mesenchymal stem cells, suggesting that these scaffolds could be used in dental tissue regeneration. Mohandesnezhad et al. (2020) reported the proliferation of human DPSCs on nanofibrous PLA/PCL composite containing nHA and zeolite. Similar to TCP, zeolite is a silica-based biomaterial containing sodium, potassium, and calcium in its structure, enhancing osseointegration. Consequently, the addition of zeolite to the scaffolds increased DPSC viability and growth compared to the nHA-added scaffold, emphasizing the importance of mineral doping of scaffolds in the engineering of mineralized tissues like the bone and tooth. Design and production of composite hybrid polymeric scaffolds were studied for the formation of in vivo human dentin-bone complexes with the help of transplantation of gingival fibroblasts that were genetically modified to express bone morphogenetic protein-7 (BMP-7) (Park et al. 2010). It was suggested that PCL-PGA hybrid scaffolds could be used for the production of personalized periodontal tissue constructs, such as cementum, PDL, and alveolar bone. PCL scaffolds produced by jet spraying were proposed as 3D support in pulpodental tissue engineering, which was evidenced by the expression of collagen type I, osteocalcin, ALP, and dentin sialophosphoprotein

by DPSCs, indicating the odontoblastic differentiation of these cells (Louvrier et al. 2018).

6.5.5 Skin Tissue Engineering

Skin tissue engineering could be explained as the repair of damaged skin or skin renewal. Skin can be damaged by burns, surgeries, and diseases like diabetic ulcers. Due to the soft and moist environment of the tissue, porous nanofibrous scaffolds such as hydrogels, flexible polymers, and their blends are chosen as the scaffold for wound repair. The use of bilayer scaffolds made up of one layer of synthetic nanofibers and a second layer of natural hydrogel was reported by Franco et al. (2011). The first layer was made of PCL and PLGA composite electrospun nanofibers, while the hydrogel layer was composed of gelatin and chitosan. The resulting bilayered product had a porous and hydrophilic structure, ensuring cell viability, making it suitable for skin tissue engineering. In another similar study, corn protein Zein, PCL, and gum arabic were combined in the production of electrospun nanofibrous scaffolds (Pedram Rad et al. 2018). Zein and gum arabic resulted in a natural polysaccharide hydrogel layer with antibacterial properties, while PCL provided flexibility, strength, and controlled degradation of the scaffold. This porous and hydrophilic composite scaffold was reported to be resistant to shrinkage that is generally a problem with hydrogel usage, suggesting that the scaffold could be a potential wound patch to repair skin. Oxygen plasma modification could be an effective method to manipulate the hydrophilicity and biodegradation rate of PCL for wound healing studies. The technique involves the use of a plasma generator to generate oxygen plasma that will clean the scaffold surface of the unbound chemical residues prior to blending. Scaffolds modified with oxygen plasma contain highly hydrophilic functional groups, preventing the accumulation of fluids, referred to as exudate, in the wound area. The hydrophilicity and rate of degradation were shown to be increased when gelatin was infused into freeze-cast PCL scaffolds by plasma processing technique (Ghorbani et al. 2020). Gelatin-fused PCL scaffolds favored fibroblast attachment and proliferation more effectively than the bare PCL ones, which could be attributed to the RGD sequence in the gelatin, suggesting that these scaffolds could be suitable for skin tissue regeneration studies.

6.6 Conclusion

In recent years, stem cells isolated from dental tissues have gained popularity in regenerative medicine because of their ease of isolation, clonogenicity, and pluripotency. From different tissue layers of the tooth, dental stem cells with different potency can be obtained, which pulls the attention of tissue engineering professionals looking for an additional stem cell source toward these cells. Among the many materials that dental stem cells are compatible with, PCL stands out as an attractive source of scaffold biomaterial. PCL has been extensively used in various

biomedical applications for many decades due to its biocompatibility, flexibility, and blendability. Although its hydrophobic nature may not be appropriate for some applications, the desired properties can be achieved by blending PCL with other synthetic or natural biomaterials, as well as using different manufacturing techniques which allow for the manipulation of scaffold porosity. Due to the high tailorability and biocompatibility of PCL, engineering of both soft and hard tissues is possible by combining PCL-based scaffolds with dental stem cells.

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7 Injectable Biomaterials for Alveolar Bone Regeneration

Deborah Sybil, Shubham Datta, and Uqba Raza

Abstract

Substitutes for bone grafts have been extensively used since time immemorial for reconstructing bony defects, with the oral and maxillofacial region posing one of the greatest challenges in reconstruction owing to a wide variety of morphologically complex structures residing in a rather small area. Moreover, reconstruction of the defects in this region demands minimally invasive techniques on grounds of aesthetic considerations. Various shortcomings (including increased risk of infections due to bacterial contamination from the oral cavity as well as the sinuses) of the currently employed surgical methods have prompted to a dire need for tissue engineering. The injectable biomaterials have thus proved to be a great asset, since besides having great therapeutic value, they are minimally invasive, thereby reducing morbidity. This chapter outlines various injectable biomaterials (natural and synthetic polymers, inorganic and composite biomaterials, and some self-assembled peptides) that are available and are being used currently for regeneration of alveolar bone, in particular.

Keywords

Alveolar bone · Injectable biomaterials · Growth factors · Scaffolds

7.1 Introduction

A clear and thorough knowledge and understanding of the ideal alveolar bone anatomy and morphology are a pre-requisite for the restoration of proper form and function of alveolar bone and play a pivotal role in customization of various

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scaffolds for the regeneration of the same. Although alternatives to bone grafts have been used for reconstruction of bones, there has always been a search for synthetic biomaterials which can control the key biological properties like porosity, reduced cytotoxicity, etc. Injectable biomaterials are a boon when it comes to regeneration of damaged tissues as they provide various advantages over the conventional surgical techniques such as avoidance of repeated invasive procedures, hence minimizing the potential risk of trauma or secondary infections, easy incorporation and adaptation in irregular bone defects, better seeding and adhesion of cells, etc. A wide variety of materials have been developed and tested in this context which did yield excellent results.

7.2 Alveolar Bone: Anatomy, Morphology and Remodelling

The *alveolar process* is that part of the mandible and maxilla which forms and supports sockets for the teeth. Anatomically it is not distinctly demarcated from the body of both the jaws. In the anterior part of the maxilla, the palatine process fuses with the oral plate of the alveolar process. In the posterior mandibular region, the oblique line superimposes on the lateral aspect of alveolar bone.

The alveolar process has two parts: the alveolar bone proper and supporting alveolar bone. The roots of teeth are surrounded by the former (also called lamina dura). It is 0.1–0.4 mm thick and is composed of lamellated bone (which has the blood vessels surrounded by concentric lamellae to form the Haversian system) and bundle bone (which anchors the principal fibres of the PDL). It forms the interdental and the interradicular bone which has perforating canals of Zuckerkandl and Hirschfeld containing interdental arteries, veins, lymphatics and nerves. The supporting alveolar bone has two parts. The cortical plate is composed of compact bone and surrounds the alveolar processes. It is thicker in the mandible. It consists of longitudinal lamellae and Haversian systems. The second part is the spongy bone which is present between the alveolar bone proper and the cortical plates. It contains trabeculae of lamellar bone which are surrounded by marrow containing adipocytes and pluripotent mesenchymal cells. Radiologically, it is of two types: type 1, regularly arranged interdental and interradicular trabeculae in a ladder-like pattern and found mostly in the mandible, and type 2, irregularly arranged trabeculae found mostly in the maxilla.

In a study by Cho et al. (2019) on MDCT (multi-detector computed tomography) images, the average horizontal widths of maxillary and mandibular alveolar bone were measured at the crestal, mid-root and root apex level and reconstructed by medical imaging software which came out to be as mentioned below:

Maxillary: central incisor, 7.43 mm; canine, 8.91 mm; second premolar, 9.57 mm; and first molar, 12.38 mm.

Mandibular: central incisor, 6.21 mm; canine, 8.55 mm; second premolar, 8.45 mm; and first molar, 10.02 mm.

The mid-root level horizontal alveolar bone width was greater than that of apical and mandibular border region.

Alveolar bone proper remodels due to mesial drift of tooth and tooth eruption. On the distal surface, there is apposition of bone due to osteoclast-mediated resorption of bundle bone and deposition of lamellated bone. On the mesial surface, there are alternate periods of resorption and repair due to which islands of bundle bone are formed which are separated from lamellated bone by reversal lines. The presence of Howship's lacunae containing osteoclasts on the mesial side is a sign of active resorption. Ten Cate described the process of resorption as creation of an acidic environment on the side of mineralized bone by osteoclasts, through proton pumps which demineralize bone exposing organic matrix, which is then dissolved. Frost gave the concept of MES (minimal effective strain). MES is minimum effective signal of mechanical loads that convert their effect to bone architectural adaptation (Tyrovola and Odont 2015). A multicellular mediator mechanism, the "basic multicellular unit" (BMU), activated cells, osteoblasts, osteoclasts, turns lamellar bone over in compact packs. A BMU's osteoclastic action is biologically coupled to its osteoblastic action (Tyrovola and Odont 2015).

Another theory of bone remodelling involves the role of parathyroid hormone (PTH). Decrease in blood calcium levels leads to the release of PTH by the parathyroid glands which stimulates the release of interleukin-1,6 by the osteoblasts which mediate the migration of monocytes towards the bone area. These monocytes coalesce under the influence of leukaemia-inhibiting factor to form multinucleated osteoclasts that resorb bone, releasing calcium ions from hydroxyapatite, thus restoring blood calcium levels. The role of parathyroid hormone 1 receptor (PTH1R) has also been elicited. PTH1R signalling in $Prx1^+$ progenitors plays a part in alveolar bone regeneration. Prx1 is a transcription factor significantly expressed during the formation of limb buds and craniofacial development. $Prx1^+$ progenitors are present in mesenchymal stem cells in alveolar bone marrow around incisors, at the apex of molars and in the dental follicle and pulpal tissue of incisors (Cui et al. 2020).

7.3 Bone Tissue Engineering

Science Workshop foundation (1988) defined tissue engineering as the implementation of principles and methods of engineering and life sciences for the basic insight of structure-function association in physiologic and pathologic mammalian tissues and the creation of biological alternatives to re-establish, preserve or boost the functions of tissues (Chang et al. 2017). Three fundamental components are required for tissue engineering:

1. *Reparative cells* with considerable cell proliferative capacity, phenotype stability and immunogenicity which will form the functional matrix.
2. *Scaffolds* are the materials that simulate the extracellular matrix of the indigenous tissue. They permit cellular attachment and migration and diffusion of essential cellular nutrients and expressed products and modify the cellular phases.

3. *Growth factors* which are popular for bone tissue engineering are fibroblast growth factor 2 (FGF2), bone morphogenic protein 2,7 (BMP 2, BMP 7), transforming growth factor-β (TGF-β) and platelet-derived growth factor AB or BB (PDGF AB/BB).

Bone tissue engineering is the process of creating implantable skeletal substitutes for skeletal deficiencies due to trauma, tumours or developmental anomalies to restore normal tissue function (Langer and Vacanti 1993; O'Brien 2011). Bone tissue engineering includes direct placement of mesenchymal stem cells (MSC), placed in an appropriate scaffold, into the deficient region. To favour proliferation of the immature MSCs, they were expanded in hypoxic conditions and at a low seeding density (Jones et al. 2013). Recently, platelet lysates and platelet-rich plasma are being used for mesenchymal stem cell cultivation for bone tissue engineering.

Scaffolds are an integral part of tissue engineering. An ideal scaffold should be biocompatible and biodegradable, have mechanical characteristics mimicking that of the anatomical site where it is to be seeded, have an interconnected pore structure, maintain mechanical integrity and be cost effective. Additionally, for bone tissue engineering, scaffolds should be osteoconductive, osteoinductive and osteointegrating (Ghassemi et al. 2018; Matsuno et al. 2010). Based on the material, four types of scaffolds have been recognized for bone tissue engineering:

1. *Polymeric*: polylactic acid, polyglycolic acid and lacticoglycolic acid.
2. *Ceramic*: calcium phosphate ceramics, β-TCP scaffolds with 0.25% ZnO and 0.5% SiO_2 (0.5%).
3. *Composed structures as optimized scaffolds*: bioceramics which include calcium phosphate, hydroxyapatite, and tri-calcium phosphate with poly(L-lactic acid) (PLLA), collagen, gelatin, and chitosan, reinforced high-density polyethylene (HDPE) and poly(L-lactide-co-glycolide acid) (PLGA) with hydroxyapatite.
4. *Metallic*: Mg-RE (magnesium-rare earth alloys), Mg-Ca, pure Fe, Fe-Mn alloys and Fe foam.

Chang et al. classified scaffolds based on when they are shaped:

1. *Pre-formed scaffolds* have a definite shape prior to their application.
2. *Injectable scaffolds* form the shape in situ.

This chapter discusses injectable scaffolds in detail.

7.3.1 Need of Injectable Scaffolds for Bone Tissue Engineering

Since the preformed scaffolds have a regular conformation, they often fail to fit in the uneven defects in the body on incorporation at the site of defect (Migliaresi et al. n.d.). Presently used scaffolds have difficult modification, poor mechanical properties

Table 7.1 Differences between injectable and preformed scaffolds

Injectable scaffolds	Preformed scaffolds
Easily injectable using minimally invasive techniques and form a stable architecture in the body	Have predetermined shapes as per the nature of defect. Require surgical interventions for implantation
Provides homogenous distribution of bioactive materials	Uneven distribution of bioactive materials
Lower mechanical strength	Higher mechanical strength
Solidification of injectable scaffolds occurs post-injection	Cross-linked prior to implantation
Microparticles, pastes and gel forms of injectable scaffolds are popular	Synthetic polymers, metals and bioceramics are more popular
Degrade at a faster rate	Degradation process slower

and slow degradation, whereas injectable scaffolds have the following advantages (Hou et al. 2004):

1. Less invasive techniques, lowering infection and trauma risk.
2. Easy incorporation into irregular shaped defects.
3. Better cell seeding and adhesion.

Several factors including concentration of the macromonomer, pre-treatment ahead of injection, cellular adhesion peptide sequences, controlled growth factor release in the injectable scaffolds, etc. play a pivotal role in bone formation. Cell adhesive peptides added to injectable scaffolds improve cellular adhesion, proliferation as well as differentiation. A comparison of injectable and preformed scaffolds is given in Table 7.1.

7.3.2 Ideal Properties of Injectable Scaffolds

7.3.2.1 Injectability

The material should be flowable prior to injection and rapidly lose flowability after injection into the defect which can be brought about either by adding a crosslinking agent and/or enzyme or by altering the pH, light and temperature (Chang et al. 2017). For example, hydrogels can change from solution to gel. The concentration and viscosity of the solution before gelation, its gelation rate and process, extent of aggregation of particles and their size as well as the surface charge are a few components which may regulate injectability (Chang et al. 2017).

7.3.2.2 Cytotoxicity

All constituents of the hydrogel which is to be injected, any initiator(s), additives or crosslinking agents (Chang et al. 2017) and the released products should not induce an inflammatory response or cause toxicity. The scaffolds must degrade non-toxically. Natural polymers are less cytotoxic than synthetic ones.

7.3.2.3 Biocompatibility

Biocompatibility is assessed by the adhesion of cells on biomaterials. Hydrogels are usually biocompatible owing to the monomers of natural polymers which mimic the natural ECM (Chang et al. 2017). Synthetic scaffolds although are less biocompatible, but addition of RGD peptides, fibronectin segments and CD44 and CD168 surface receptors improves their biocompatibility.

7.3.2.4 Bioactivity and Biodegradability

Scaffold should be osteoinductive and osteoconductive. A controlled degradation of scaffolds by external-enzymatic/biological process is necessary which should correspond to the tissue formation rate. Rapidly degrading scaffolds lead to a loss of cell growth carrier function, while an indolent rate of degradation restricts the space available for the generation of tissues. Hydrogels degrade either by means of simple dissolution, enzymatic cleavage or hydrolysis (Chang et al. 2017). The rate of hydrolysis is influenced by the amount of remaining monomer, form, porosity, molecular weight, local pH, crosslinking density and filler.

7.3.2.5 Porosity

Cell diffusion and migration take place via a network of interconnected pores. Pores below 100 μm encourage adsorption of nutrients on their surface and are imperative for cell differentiation (Christy et al. 2020), while those in the range of 100 μm to 300 μm stimulate cell proliferation and migration (Li and Liu 2017). Pores help develop scaffolds that mimic the biomechanical properties of bone and impart bioactivity to the scaffolds for boosting bone growth (Christy et al. 2020).

7.3.2.6 Mechanical Properties

The mechanical properties of scaffold should be comparable to that of the ECM in order to support the cells besides bearing the biomechanical load. Because of being thermodynamically compatible with water, the injectable hydrogels are elastic and soft. A hydrogel should possess both considerable stiffness to grant a fairly substantial framework for cells and enough toughness for prevention of hydrogel from being friable. An elevated density of crosslinking betters the hydrogel stiffness as well as toughness and prolongs its time of degradation. The concentration and composition of the biomaterial, its process of fabrication, porosity, etc. may also alter the mechanical characteristics of injectable hydrogels (Chang et al. 2017).

7.3.2.7 Easy Manufacturing, Processing and Handling Along with Customization

Scaffolds should be easily fabricated, sterilized and clinically manipulated. They should have customizable properties according to the nature of application.

7.4 Classification of Injectable Scaffolds

7.4.1 According to Biomaterials

According to the source of origin, they can be classified as shown in Fig. 7.1.

7.4.1.1 Natural Polymers

They are derived naturally and are biodegradable, nonimmunogenic, biocompatible and biologically recognizable, thus promoting cell adhesion. However, they have the risk of pathogenic transmission. Some of the materials used as natural injectable scaffolds are discussed here. Several scientists consider chitosan, alginate and gelatin as a more suitable scaffold for bone tissue engineering among others due to their negligible immunologic response and increased biocompatibility. Cellulose is also being widely used.

- *Chitosan:* Obtained from crustacean exoskeleton, it is a deacetylated chitin derivative made up of *N*-acetyl glucosamine and glucosamine. It is degraded by lysozyme and is biocompatible, less toxic and osteoconductive. Only acidic solution can dissolve chitosan leading to a transition from sol to gel when glycerol-2-phosphate (b-GP) is added which increases the solution's pH towards neutral from acidic. Due to low osteoinductivity and poor mechanical properties of chitosan, several polymers are added to enhance its properties. Chitosan also has a commendable bactericidal action. Various modifications of chitosan have been studied and tested to yield some noteworthy results, some of which include *N*-(2-hydroxyl) propyl-3-trimethylammonium chitosan chloride (HTCC), a quaternized chitosan which displayed an improved bactericidal action towards *Porphyromonas gingivalis*, *Prevotella intermedia*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Actinobacillus actinomycetemcomitans* and methicillin-resistant *S. aureus* (MRSA) (Haugen et al. 2020); O-CMC-BMP2, a derivative of carboxymethyl chitosan, showed superior osseointegration. Moreover, chitosan

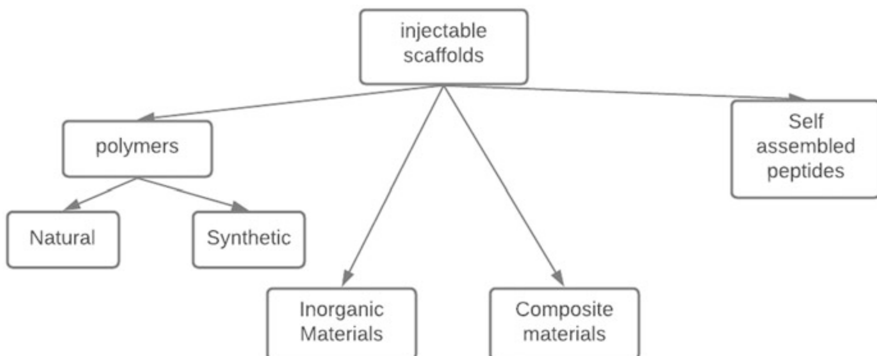

Fig. 7.1 Classification of injectable scaffolds according to biomaterials

with polycaprolactone-polyvinyl alcohol, polycaprolactone, collagen and poly (lactic-co-glycolic acid) revealed an improvement in the mechanical properties, and chitosan-nanohydroxyapatite-resol displayed a high metabolism, rate of adsorption of proteins and alkaline phosphatase action of the osteoblasts, i.e. MG-63. Furthermore, addition of calcium zirconate, hydroxyapatite and zirconium oxide boosted the rate of proliferation of OB-6 pre-osteoblasts and compressive strength.

- *Alginate:* It is an anionic, biocompatible, biodegradable and hydrophilic linear polysaccharide formed by repetitions of G-blocks, i.e. a-L-guluronic acid and M-blocks, i.e. (1,4)-linked b-D-mannuronic. Porosities in alginate aid in the seeding of cells and ensuring a smooth supply of nutrients necessary for tissue regeneration. Alginate has been modified to improvise the mechanical strength, cellular adhesion and degradability. In a study by Lin et al. conducted in the year 2018, on Sprague-Dawley female mice to evaluate magnesium ion release from poly(lactic-co-glycolic acid)-magnesium oxide-alginate core-shell microspheres, enhanced viability of MC3T3-E1 pre-osteoblasts and bone formation having around 75% bone volume with a sufficient modulus of elasticity were noted (Lin et al. 2018; Ghosh et al. 2019).
- *Gelatin:* A denatured protein and a collagen derivative, gelatin is constituted either by proline, hydroxyproline or units like RGD, i.e. arginine-glycine-aspartic acid. The cost-effectiveness, solubility in aqueous solution, immunogenic potential, biocompatibility and biodegradability, together with its ability to form porous three-dimensional structure make it a suitable injectable scaffold. Gelatin features a sol to gel transition with its lower critical solution temperature (LCST) close to human body temperature, i.e. around 37 °C due to which chemical crosslinking becomes mandatory for its structural stability.
- Fayyazbakhsh et al. (2017) used vitamin D3 ensheathed in gelatin and a double layered hydroxide-hydroxyapatite to study vitamin D3 release and cytotoxicity wherein they noted facilitated viability and proliferation of G-292 cells besides a high value of Young's modulus and density. Moreira et al. in the year 2019 concluded that chitosan-gelatin-bioactive glass was well suited for encapsulation of cells and drugs along with a good viability of osteosarcoma cell lineage (Moreira et al. 2019).
- *Cellulose:* A polysaccharide macromolecule having β-(1, 4) linked glycosidic bonds, cellulose is hydrophilic, cytocompatible, bioactive and optical transparent. Apt chemical, physical and mechanical properties make it useful for bone tissue engineering.

Other available natural polymeric scaffolds are collagen, hyaluronic acid, fibrinogen and elastin.

- *Collagen*: It is a natural biomaterial with one of the most extensive applications and is the most prolific mammalian protein. Collagen contains bases like Arg-Gly-Asp which establish cellular adhesion followed by the response. It is degraded in vivo by collagenases like matrix metalloproteinases. It has a low mechanical strength. Fu-Chen Kung blended type I collagen with calcium salt

form of poly(γ -glutamic acid) (γCaPGA) mixed with RGD-modified alginate which had a high cost/performance ratio and good structural stability and a high potential for bone regeneration (Kung 2018).

- *Hyaluronic acid:* Comprises of alternate beta- 1,3-*N*-acetyl-D-glucosamine and beta-1,4-D-glucuronic acid molecules. Hydrogel is formed by drawing in water due to the osmotic balance created by attraction of positive ions by the negatively charged polymer chain of hyaluronate. Hyaluronic acid can regulate cell differentiation and bone formation. *N*-cadherin-modified hyaluronic acid has been proven to have the ability to bring about osteodifferentiation of the mesenchymal stem cells. However, it is structurally complex and has low mechanical strength and immunogenicity. A new scaffold-type favouring osteoblast differentiation has been made of simvastatin-enriched graphene oxide-chitosan-hyaluronic acid which reported increased osteogenesis and scaffold mineralization. Another example is injectable click-crosslinking (Cx) hyaluronic acid (HA) hydrogel scaffold modified with a bone morphogenetic protein-2 (BMP-2) mimetic peptide (BP).
- *Fibrinogen:* A blood-borne glycoprotein made up of six non-identical polypeptide chains. It has an inferior value of mechanical strength besides a less governable biodegradability. But a graft comprising of an enriched platelet-rich plasma, mesenchymal stem cells of bone in a decellularized cartilage matrix developed by Feng et al. proved to have an ideal potential for bone remodelling with sufficient mechanical resistance (Feng et al. 2018).

7.4.1.2 Synthetic Polymers

Synthetic polymers can be mass produced with adequately controlled rate of degradation, microstructure and strength. In addition to this, synthetic biomaterials pose no risk of natural biomaterial-associated pathogen transmission. However, these are typically devoid of structural organic motifs which necessitate their complementation with distinct signal molecules for interaction between cells and material. They are less osteoconductive. Various synthetic polymers are PEG, α,β-poly-(*N*-hydroxyethyl)-DL-aspartamide, poly(L-glutamic acid), PEG-poly(*N*-isopropyl acrylamide) (PNIPAAm), poly(vinyl alcohol), methoxy polyethylene glycol, poly(propylene fumarate) and methoxy polyethylene glycol-poly(ε-caprolactone). Aliphatic polyesters, namely, poly(lactide-co-glycolide) (PLGA), poly(ε-caprolactone) (PCL), polylactide (PDLA, PLLA) and enamel matrix derivative are the most extensively used synthetic polymers (Lee et al. 2011).

- *Poly(ε-caprolactone) (PCL):* PCL is a semi-crystalline, biodegradable, non-toxic aliphatic polyester. It is hydrophobic, which hinders cellular adhesion and infiltration. PCL dissolves in organic solvents and is biocompatible with a comparatively slow rate of degradation. Abbasi et al. developed a melt electrowritten PCL scaffold with porosity gradient which enhanced bone formation (Abbasi et al. 2020). Qian et al. developed a new silver-modified/collagen-coated electrospun poly-lactic-co-glycolic acid/polycaprolactone (PLGA/PCL) scaffold (PP-pDA-Ag-COL) having heightened microbicidal and osteogenic potential and enhanced biocompatibility

with therapeutic value in alveolar/craniofacial regeneration of bones (Qian et al. 2019).

- *Polylactide (PDLA, PLLA):* It is cytocompatible and thermally stable and degrades non-toxically. Stereoisomers distributed in the polymer chains (L/D ratio) and their molecular weight affect its thermal stability and the degradation properties.
- *Poly(lactide-co-glycolide) (PLGA):* It is PLA and PGA copolymer whose GA/LA ratio controls the rate of degradation and its hydrophilicity.
- *Enamel matrix derivative:* Derived from foetal porcine tooth buds, it is primarily composed of about 90% amelogenin and traces of enamelin, tuftelin, ameloblastin and other nanomelogenin proteins. EMDs find extensive application in stimulation of formation of new periodontal attachment for acellular cementum, alveolar bone and periodontal ligament. Schwarz et al. studied the effect of EMD on the proliferation and differentiation of human osteosarcoma cell line (Saos-2) osteoblasts and also their viability on titanium implants wherein they noted that though EMD led to an enhanced Saos-2 osteoblast viability in addition to stimulated expression of fibroblast growth factor 2, it minimized the expression of alkaline phosphatase.

7.4.1.3 Inorganic Materials

Inorganic materials such as bioceramics, primarily made up of calcium and phosphate ions in varying amounts, are used as injectable scaffolds. They are biocompatible to a great extent, chemically bond with the host tissues, do not initiate any immune response and are osteoconductive. They also have some drawbacks like low biodegradability, challenging production and an inbuilt brittleness. Typical examples of bioceramics used as injectable scaffolds are:

- *Hydroxyapatite and beta-tricalcium phosphate (beta-TCP):* Hydroxyapatite and beta-TCP having calcium and phosphorus ratios in 1.50 to 1.67 range are well-established promoters of bone regeneration. While β-TCP has a faster rate of degradation, HA has a better osteogenic potential. Biphasic calcium phosphate (BCP) ceramics contain varying proportions of β-TCP and HA which are more and less soluble, respectively. Pores improve the biodegradability of bioceramics. Pan et al. developed injectable hydrogel-loaded nano-hydroxyapatite which could maintain dimensional alveolar ridge and facilitate healing of the soft tissues (Pan et al. 2020). Hydroxyapatite-containing hydrogel is also capable of promoting bone and soft tissue regeneration.
- *Calcium phosphate cements (CPC):* It is a commixture of anhydrous dicalcium and tetracalcium phosphate which may be shaped while setting in situ following the silhouettes of the defect. Pure CPC is not well injectable; therefore, chemicals like glycerol, polysaccharide xanthan and a few polymeric drugs were mixed which improved injectability, but at the same time, they also prolonged the cement's setting time quite significantly. A study conducted on human umbilical cord mesenchymal stem cells (hUCMSC) by Thein-Han et al. revealed that the hUCMSC on bioactivated CPCs resulted in enhanced expression of actin fibres,

cellular attachment and proliferation, differentiation of osteoblasts and mineral synthesis (Thein-Han and Xu 2011). Human-derived fibronectin (FN), engineered fibronectin-like protein, RGD (Arg-Gly-Asp) peptides, human platelet concentrate and extracellular geltrex matrix were used as bioactive materials.

7.4.1.4 Composite Materials

Composite materials used as injectable scaffolds can be classified into two types: ceramics with added polymers and ceramic particles encapsulated into a porous polymer carrier. Many different composite forms such as sponges, gels, films and blocks have been developed using different methods. In alveolar bone regeneration by guided bone regeneration, using sponges or gel composites is desirable, as they easily fit into alveolar bone defects. Sponge composites consisting of granule ceramics and natural polymers can simply be cut with scissors or a sharp knife and can therefore be easily moulded for use for various tissue disorders such as periodontal bone defects, cyst cavities and alveolar bone augmentation. The mechanical properties of the composites are relatively poor in comparison to the bone, although the graft site can be reinforced using membranes during GBR. Gradually, the collagen of sponge composites degrades, and the remaining β-TCP granules in the defect come into direct contact with the regenerated bone. Ultimately, the β-TCP granules replace the original bone during remodelling. Additionally, the composite scaffolds can be employed as drug delivery agents, for local release of growth factors from collagen and gelatin and for enhancing bone formation for correction of bone defects (Zhao et al. 2019).

7.4.1.5 Self-Assembled Peptides

Under favourable circumstances, some peptides have the ability to self-organize into biomimetic nanofibrous supramolecular conformation, thus becoming fairly good tissue regenerating biomaterials. The functional properties of the resulting peptide can be customized by modifying the peptide sequences which prove to be of a great advantage. No extra crosslinkers are required, thereby making the cell and protein incorporation into the gel without being subjected to harsh chemical agents, but the mechanical strength of these peptides is compromised due to absence of crosslinking. Also, they are less productive. Various examples of self-assembled peptides are the following:

- *Peptide-amphiphile (PA)*: These are made by means of standard solid-phase chemistry. When the pH is neutral, peptide-amphiphiles occur as amorphous aggregates owing to their negative charge which gets counterbalanced on adding polyvalent ions leading to formation of cylindrical micelles followed by their physical crosslinking eventually resulting in the formation of a gel.
- *PuraMatrix* (Filippi et al. 2020): It is a synthetic hydrogel comprising of recurring arginine-alanine-aspartic acid-alanine units in an aqueous solution. PuraMatrix has shown to have sufficient plasticity and absorption and is biocompatible. Moreover, it lacks animal-derived antigens and pathogens.
- *Fluorenylmethoxycarbonyl-diphenylalanine (FmocFF) peptide composite hydrogel*: Modifying sodium alginate by adding a self-assembling peptide FmocFF

results in a hydrogel which is rigid and injectable and does not require crosslinkers. Scanning electron microscopy studies show a nanofibrous structure similar to the extracellular matrix of natural bone. It has a thixotropic behaviour, high storage modulus and potential for induction of osteogenic differentiation, can facilitate mineralization of calcium and has high biocompatibility and excellent mechanical properties. This resultant hydrogel can provide a transitory 3D cellular microenvironment to promote bone regeneration.

7.4.2 According to Fabrication Technique

Accordingly, injectable scaffolds can be classified as hydrogels and microspheres which can be further classified as follows:

7.4.2.1 Hydrogels

Hydrogels are extensively used as injectable scaffolds. Hydrogels are made of 3D hydrophilic polymer chains; have superior mechanical strength; promote endogenous cell growth, similar to extracellular matrix; promote a controlled release of materials because of their network structure; are absorbable; and integrate well with surrounding tissues, reducing inflammation (Donnaloja et al. 2020). Due to ready availability of raw materials for hydrogel preparation, their geometrical shapes can be altered (Bai et al. 2018). They have controllable degradation rate and porosity. They may be classified either as physically crosslinked or chemically crosslinked hydrogels on the basis of their crosslinking manner (Fig. 7.2).

- *Physically crosslinked type*: Altered local conditions like pH, temperature and ionic concentration lead to the creation of these hydrogels by automounting of polymers. Their stability is less. Due to their moldability, physically crosslinked hydrogels are extensively used in bone tissue engineering. These may be temperature-induced crosslinked type of hydrogels, ionically crosslinked type or hydrogen-bonded crosslinked type.
- *Chemically crosslinked type*: These are formed by crosslinking covalent bonds of various polymer chains. Most of these linkages are irreversible which are mechanically stronger than a physically crosslinked hydrogel, while some possess reversible bonds with prolonged adaptability and stability. Hydrogels can be developed by radical polymerization, chemical reaction of complementary groups, ionic interaction and crystallization.

7.4.2.2 Microspheres

They are quintessentially small in size with a much larger surface area. They allow cell adhesion and proliferation prior to injection into the desired site, unlike hydrogels. They can be prepared by hot melt, solvent evaporation, solvent removal, spray drying technique and phase inversion microencapsulation. When used in combination with hydrogels, microspheres tend to improve the porosity as well as the mechanical properties of hydrogels.

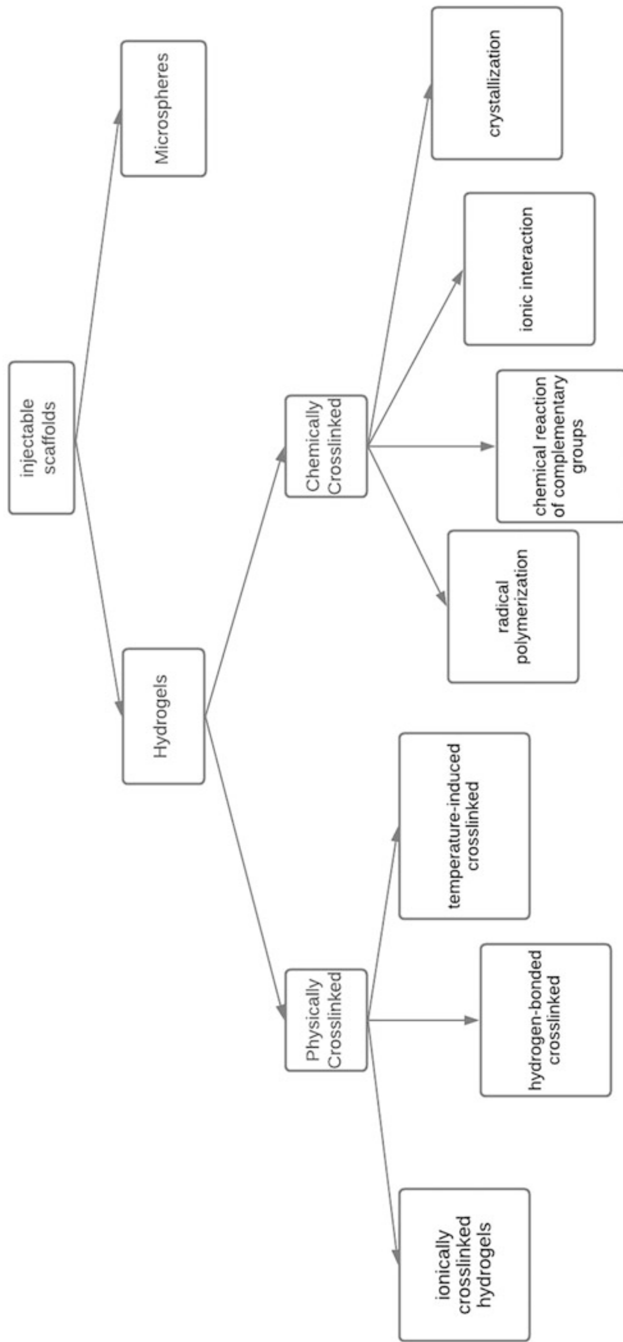

Fig. 7.2 Classification of injectable scaffolds according to fabrication techniques

7.4.3 Fabrication of Injectable Scaffolding Systems

7.4.3.1 Incorporation of Bioactive Substances into Injectable Scaffolding Systems

Physical or chemical encapsulation of bionductive factors such as cellular adhesion peptides (e.g., RGD sequences) and sequences like Ala-Pro-Gly-Leu l-Arg-Asn which can be degraded by enzymes into injectable scaffolds decreases their burst release profile. Covalent modification of alginate and RGD peptide, for instance, facilitates the adhesion as well as spread of the osteoblasts.

Various methods have been developed for growth factor delivery. De Witte et al. created a chitosan scaffold having a sustainable growth factor delivery. Using the "freeze-drying method", porous chitosan scaffold was developed which was biodegradable and osteoconductive and had storage modulus value of 8.5 kPa. Next, using carbondiamide crosslinker chemistry, poly(methyl methacrylate-co-methacrylic acid) nanoparticles were created and introduced into the scaffold. This system had a versatile controlled delivery of growth factors (De Witte et al. 2020).

7.4.3.2 Cell-Instructive Scaffolds

Scaffolds which instruct the cells for new tissue development are called cell-instructive scaffolds. One example is a synthetic metalloproteinase-sensitive hydrogel wherein the substrate used was PEG, the crosslinking agent was bifunctional peptide with an MMP cleavable site and a PEG-coupled integrin-binding RGDSP was used for cellular adhesion and recombinant human bone morphogenetic protein 2 (rhBMP2) as a growth factor. The structure and proteolytic sensitivity of the matrix together with rhBMP2 determined bone regeneration.

7.4.3.3 Self-Assembled Nanofibers

Many scaffolds such as RADA16-I simulate the nanofibrous framework of extracellular matrix. When combined with platelet-rich plasma and the mesenchymal stem cells, RADA 16-I acts as a three-dimensional scaffold for regeneration of bones.

7.4.3.4 Nanofibrous Microspheres

Liu et al. demonstrated that integration of polyethylene glycol (PEG)-co-functionalized mesoporous silica nanoparticles (MSN), poly(L-lactic acid) (PLLA) nanofibrous spongy microspheres (NF-SMS) and poly(lactic acid-co-glycolic acid) microspheres (PLGA MS) into a multi-biological delivery transporter led to manipulation of the regulatory T cells (Treg) (Liu et al. 2018).

7.5 Application of Injectable Scaffolds

7.5.1 Alveolar Ridge Preservation

Alveolar ridge needs to be preserved following tooth extraction for further dental procedures such as implants or dentures. Tao Yang et al. constructed an injectable woven bone-like hydrogel (IWBLH) in varying ratios using alginate integrated mineralized collagen and amorphous calcium phosphate (ACP) which was probed in rat's model of extraction post screening of optimal IWBLH by physiochemical categorization and biological assays in vitro. This could be conveniently manipulated and packed into the tooth socket and was found to prohibit bone resorption and complete remodelling within a time span of 4 weeks, thereby proving to yield encouraging outcome in the coming days for alveolar ridge preservation (ARP).

7.5.2 Maxillary Sinus Lift

Maxillary sinus floor augmentation or sinus lift as it is commonly called is a surgical procedure employed for bone regeneration in posterior maxilla, by means of lifting the Schneiderian membrane for the placement of bone graft. In the past, the osteogenic potential of sonication-induced silk hydrogels (Zhang et al. 2011) has also been investigated for their use as an injectable biomaterial for the regeneration of bones. Moreover, platelet-rich plasma was harnessed as an injectable biomaterial to carry out sinus lift procedure which yielded positive results in a clinical study.

7.5.3 Periodontal Regeneration

A tooth's anchorage is re-established by periodontal regeneration. Numerous injectable scaffolds have been put to test for regenerating the periodontium. For instance, periodontal ligament stem cells (PDLSCs) and drugs were delivered via an injectable carrier composed of glycerophosphate and quaternized chitosan (HTCC) which got converted to a gel at around 37 °C while remained in aqueous form at a temperature below 25 °C. Besides being non-toxic and an enhancer of the PDLSC's ALP activity, HTCC also slowed down the release of the drug and had a remarkable bactericidal action on the periodontal pathogens. Other than this, the addition of fibroblast growth factors to HTCC hydrogel was found to facilitate periodontal regeneration in the Canidae family. CPCs can also be used for this purpose.

7.6 Conclusion

Bones have innate reparative, regenerative and remodelling properties, and bone tissue engineering is involved in the harnessing of this intrinsic potential of the bones. Scaffolds are an integral part of tissue engineering. Besides possessing the fundamental properties like biocompatibility, biodegradability, mechanical integrity and cost-effectiveness, an ideal bone tissue engineering scaffold should be osteoconductive, osteoinductive and osteointegrating. Since the preformed scaffolds have a regular conformation, they often fail to fit in the uneven defects in the body on incorporation at the site of defect. Presently used scaffolds have difficult modification, poor mechanical properties and slow degradation, whereas injectable scaffolds require less invasive technique, can be easily incorporated even in irregular defects and have a better cell seeding and adhesion. These have yielded very encouraging results when employed for alveolar bone regeneration, thus helping to improve the health and quality of life of a large number of patients.

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8 Different Biomaterials for Dental Tissue Regeneration from Clinical Point of View

Deborah Sybil, Apoorv Rana, and Shradha Singh

Abstract

The tooth is a structurally complex tissue consisting of both hard and soft components. Dental enamel is one of the toughest substances known, and the dental pulp is the living connective tissue with blood vessels and nerves. In between the two is the mineralized tubular structure dentine. The supporting structures for teeth are also complex, varying from keratinized epithelium in the gingiva to highly mineralized cementum. Such complexities are not challenging to regenerate in physical form but also a challenge to restore functionally. Tissue engineering of the entire tooth and/or its components requires specialized biomaterials that provide the biological characteristics of tissue regeneration, physical strength to support and promote mineralization, and structural adaptability to conform to the desired shape. This chapter discusses the various biomaterials that have been tested as scaffolds for dental tissue engineering. It also describes the biomaterials, their properties, advantages, disadvantages, modifications, and future challenge. The chapter explains in detail both natural and synthetic biomaterials with a note on newer materials that could be researched for future use. Furthermore, stem cell source and the tooth tissue supported with context to tissue engineering approaches are also discussed in this chapter.

Keywords

Bioactive glass · Composite scaffold · Ceramic scaffold · Polycaprolactone · Silk · Collagen

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8.1 Introduction

One of the most widely bacterially induced oral diseases is dental caries. Dental caries first lead to the destruction of enamel (outer part) and dentin (inner part) of the tooth. However, if no necessary treatment steps are taken, it may further lead to irreversible pulpal damage and eventually tooth loss (Setzer and Kim 2014). Tooth loss can occur due to microbial diseases, traumatic or iatrogenic causes, poor oral hygiene, and failure of dental prostheses or restoration (Cooper 2009; Young et al. 2002). There are various treatment options available, e.g., conservative treatments, root canal therapy (RCT), dental implants, etc., for restoring missing tooth structure and tooth loss. But still, tooth loss is considered as an important health issue that needs to be addressed (Cooper 2009). It should be noted that in RCT, the tooth is denervated and therefore becomes non-vital. Dental implants are a good solution to tooth loss. These are generally made of titanium and other metals/alloys, function through osseointegration but cannot restore aesthetics or function as compared to their natural counterparts.

For regenerating a tooth or missing structure of the tooth, it is necessary to have a guided tissue formation along with revascularization of the tissue and surrounding tissues (Chou et al. 2006; Eap et al. 2014; Ravindran et al. 2014). Tooth regeneration is an emerging area in the field of medicine. It involves epithelial-mesenchymal interactions that promote development, revascularization, and reinnervation of the neo-bioengineered tooth and also periodontal regeneration (Nakahara 2011). Among these processes, revascularization is one of the significant challenges. A versatile range of biomaterials is used to fabricate ideal scaffolds for the tooth regeneration process. This chapter will discuss various biomaterials and their role in dental tissue engineering to develop a bioengineered tooth that can mimic natural teeth with the same functional and morphological characteristics.

8.2 Biological Structure and Functions of Tooth

There are mainly two generations of human dentition: primary, i.e., deciduous, and secondary, i.e., permanent dentition. The 20 deciduous teeth erupt between the ages of 6 months and 2.5 years in the oral cavity and are ultimately lost physiologically (exfoliation) (Nanci and Cate 2003). The permanent teeth are 32 in number and erupt in the oral cavity near 6 years of age (Nanci and Cate 2003). On a morphological basis, a tooth mainly has two parts: (1) crown and (2) one or more roots. The inner part of the tooth is made of soft connective tissue that is highly innervated and vascularized in nature, known as the dental pulp, which is surrounded by three calcified structures—the enamel, dentin, and cementum (Fig. 8.1 and Table 8.1).

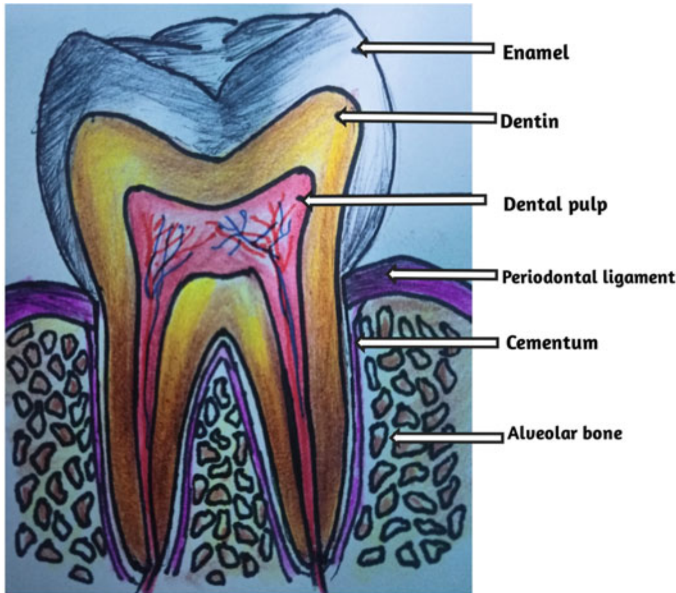

Fig. 8.1 A detailed structure of the teeth

8.2.1 Enamel

The most mineralized tissue of the body is enamel. It is very hard in nature and is produced by ameloblasts through amelogenesis. At the time of tooth eruption, a breakage of ameloblasts happens that disables the ability of the enamel to regenerate (Lacruz et al. 2017). On a morphological basis, the enamel is variable depending on various aspects like (1) color, i.e., ranges from yellow to yellowish to gray-white, (2) thickness, (3) hardness, (4) stains present on the enamel surface, and (5) mineralization. Enamel is acellular and lacks any blood and nerve supply, but it can bear heavy loads of masticatory forces. The hardness of the enamel emerges from its inorganic build-up that comes from hydroxyapatite, i.e., it is made up of 95%–98% of calcium and phosphate ions. However, 1–2% of it is composed of organic materials. The organic part majorly comes from the enamelins or extracellular matrix proteins. Water content is around 4% that makes up the remaining part of enamel (Deakins and Volker 1941; LeFevre and Manly 1938). Enamel protects against various bacteria and different types of acids. It also bears the daily occlusal stress and pressure. It also provides insulation against various physiological forces that may otherwise be harmful to the vital tissue of the dental pulp (Lacruz et al. 2017).

Table 8.1 Biological structure of tooth tissues and their properties and functions

S. no.	Tooth tissue	Composition	Characteristics	Functions	Reference
1	Dental enamel	Inorganic (hydroxyapatite) 96%, organic 1%, and water 3%	Varies in color (yellow to white/gray), acellular, and translucent in appearance	Bears heavy loads and pressure and protects from wear and tear	Uchida et al. (1997)
2	Dentin	Inorganic (hydroxyapatite) 70%, organic 20%, and water 10%	Calcified living tissue, light yellow in color, contains cells and minerals	Transmission of painful stimuli and protection of dental pulp	Butler et al. (2002)
3	Dental pulp	Comprises of one coronal pulp and one/few radicular pulp	Highly vascularized and innervated part of the tooth within the dentin	Formation of reparative dentin, response to various stimuli, provision of nutrients to dentin, and sensation to the whole tooth	Walton and Torabinejad (2002)
4	Cementum	They are composed of inorganic (hydroxyapatite) 45% and organic 55% parts	Light yellow in color, covers the root part of the tooth, mineralized and cellular	Protects the root of the tooth, provides a surface for attachment of periodontal ligament and alveolar bone	Cho and Garant (1989)
5	Periodontal ligament	–	With the help of Sharpey's fibers, it attaches the alveolar bone on one side to the root and cemental surface on the other side	Provides cushion effect at the time of mastication provides nutrients and sensory perception to the tooth	Cate (1994)

8.2.2 Dentin

Dentin is the most significant structural and most voluminous component of the human tooth. The process of formation of dentin is known as dentinogenesis. Dentin comes from the dental papilla of the tooth germ. Dentin formation starts before the secretion of enamel and is initiated by the odontoblast cells present inside the pulp

tissue (Tjäderhane 2019). In terms of mineralization, dentin is mineralized to a greater extent in comparison to both the bone and cementum but less mineralized than enamel. The composition of dentin is made up of mineral phase by 40–45% in volume and organic matrix by 30% in volume, and rest 20–25% in volume is water (Goldberg et al. 2011).

There are majorly three types of dentin, i.e., primary dentin, secondary dentin, and tertiary or reparative dentin (Leslie and Christopher 2002). Primary dentin is the first formed dentin and lies in between the enamel and the pulp chamber. Secondary dentin is a layer of dentin produced when the root of the tooth is fully formed. However, tertiary dentin, which is also known as reparative dentin, is formed in response to various stimuli, trauma, or wear (Goldberg et al. 2011). Dentin supports the enamel and resists fracture of enamel at the time of occlusal loading. It also provides protection to pulp against various harmful microbes and noxious stimuli. Dentin plays a dual function by protecting the continuity of the hard tissue (dentin) and soft tissue (pulp) and, on the other hand, by making a mechanical barrier in between pulp and oral cavity environment (Tjäderhane et al. 2012). It is the first vital tissue to meet external irritation, and instead of being merely a passive mechanical barrier, dentin also participates in the dentin-pulp complex defensive reactions (Tjäderhane and Haapasalo 2012). Though dentin is similar to the bone as it is also made up of type 1 collagen, its matrix never undergoes remodelling as that of the bone (Goldberg et al. 2011).

8.2.3 Dental Pulp

Pulp is the vascular part of the tooth that lies in the center of the tooth. It is made up of cells like odontoblasts and living connective tissue (Ghannam et al. 2020). The signalling processes and the pulpal cell activity regulate the vitality of the pulp-dentin complex (Bath-Balogh 2011; Smith 2003). The process of development of the pulp organ depends on the location of the pulpal portion as the portion of the crown of the tooth develops differently from the pulpal portion in the roots (Goldberg 2014). Histologically, the pulp has mainly four regions from the periphery to the center. Those are the following: (1) Odontoblastic layer is the outermost layer near the dentin region. (2) Cell-free zone lies next to the odontoblastic layer. This zone is narrow in width, and it is transversed by nerve fibers, various cytoplasmic processes and blood capillaries. (3) Cell-rich zone lies next to the cell-free zone, in which the fibroblasts cells are higher in number. It has cells like mesenchymal cells (undifferentiated), macrophages, and dendritic cells. (4) Core of the pulp is the main zone that is made up of blood vessels, nerve fibers, and loose connective tissues (Ghannam et al. 2020; Green 1955). The essential roles played by the pulp of the tooth are as follows: (1) It provides nutrition that makes the tooth vital and prevents it from being brittle in nature. (2) It has a major role in dentin secretion with the help of odontoblastic cells. (3) It plays a role in the stimulation of pressure and temperature in the form of pain. (4) It helps in the secretion of reparative dentin under certain specific stimuli (Ghannam et al. 2020).

8.2.4 Cementum

Cementum lines the dentin along the root of the tooth. It is considered as the part of periodontium connecting the tooth and alveolar bone together. Cementoblasts are responsible for the formation of cementum (Schroeder 1986; Hammarström et al. 1996). These cementoblasts are derived from mesenchymal cells of the dental sac that are undifferentiated in nature. The cementum is slightly lighter in color and softer in comparison to dentin. It has the maximum amount of fluoride content of all mineralized tissues. It is highly permeable to various materials. Cementum forms throughout life layer by layer in the form of deposition of a new layer. It consists of about 45% by weight of inorganic material and nearby 55% by weight of organic matter, and the rest is water. Since cementum is avascular, it derives its nutritional supply from the nearby periodontal ligament (Nanci and Cate 2003). Cementum can be broadly classified on the basis of the presence of cementocytes into cellular and acellular cementum (Yamamoto et al. 2016). Cellular cementum surrounds the cervical root and is thin, whereas acellular surrounds the root apex and is thicker. However, there are various other types of cementum: (a) acellular extrinsic fiber cementum, (b) cellular intrinsic fiber cementum, (c) cellular mixed stratified cementum, (d) acellular afibrillar cementum, and (e) intermediate cementum. The main function of cementum is to support the tooth and keep it all together with the adjacent structures like the bone (Nanci and Cate 2003). Thus, the acellular extrinsic fiber cementum is the major one providing support to the tooth. However, the cellular intrinsic fiber cementum works as a type of reparative cementum that does the filling of the resorbed root surfaces. Cementum also provides attachment to the collagen fibers present in the periodontal ligament (PDL). This helps in maintaining the uprightness and position of roots in the gums and bone. Also, it helps in the repair and regeneration of teeth.

8.2.5 Periodontal Ligament (PDL)

The periodontal ligament is a combination of connective tissues that maintain the integrity of the tooth by attaching to the tooth on one side to the nearby alveolar bone on the other side (Jiang et al. 2016). PDL cells come from the dental follicle at the time of root formation (Cate et al. 1971). The width of the PDL range is between 0.15 mm and 0.38 mm (Jiang et al. 2016). However, this width depends on age (Nanci and Bosshardt 2000). PDL consists of fibers, nerves, blood capillaries, extracellular matrix, and loose connective tissue (Jiang et al. 2016; Beertsen et al. 1997). There are different PDL fibers depending on location and orientation along with the tooth, i.e., there are six major types, i.e., (1) horizontal fibers, (2) oblique fibers, (3) interdental fibers, (4) alveolar crest fibers, (5) apical fibers, and (6) transseptal fibers (Sloan and Carter 1995). Sharpey's fibers are the ends of these principal fibers that insert on one side into the root(cementum) and the other side into the alveolar bone. The architectural structure of PDL is maintained by the different collagen fibers in the extra- and intracellular compartments such as type I,

III, V, V, VI, and XII collagen (Beertsen et al. 1997; Bartold 1998). PDL has a deterministic effect on a tooth's ability to withstand various stresses. It plays a major role in tooth eruption, tooth vitality, mechanical support, nutritive functions, remodelling, and physiological maintenance of the surrounding alveolar bone (Jiang et al. 2016; Beertsen et al. 1997).

8.3 Tooth Tissue Engineering and Its Triad

Regenerative medicine and tissue engineering is the field that is made up of the combination of principles and knowledge of engineering, biological sciences, physics, and chemistry, resulting in the formation of fully functional engineered products that can restore lost or injured tissues (Griffith and Naughton 2002). The triad constitutes biomaterial-based scaffolds, cells, and growth factors in this strategy.

Three main strategies have been used for tooth tissue engineering: (1) conductive, (2) inductive, and (3) cell-based approach.

1. Conductive: This approach is based on the utilization of biomaterials for promoting the desired tissue regeneration. Here, cells are able to regulate repair and can penetrate the defect site. Scaffolds made of biomaterials act as a barrier controlling unit where the barrier either is removed surgically or is resorbed over time.
2. Inductive: Inductive approach depends on the activation of cells in proximity to the injured site with specific biological signals. This involves the release of bioactive molecules which bind to receptors of certain specific hosts. The new extracellular matrix gets deposited when the desired cells travel to the defect.
3. Cell-based approach: This is based on the direct seeding of scaffolds made up of biomaterials along with bioactive factors and cells. Cells are seeded onto a scaffold in vitro, and the construct is then implanted into the defect site. As the host cells, along with the transplanted cells, travel into the defect, regeneration of tissue with formation of new tissue takes place. This approach is considered as the most promising strategy in tooth tissue engineering.

There are various cell sources that play an important role in dental tissue engineering. Cell sources can be (1) postnatal and embryonic dental cells and (2) stem cells from dental and nondental tissues—(a) adult stem cells and (b) embryonic stem cells. The stem cells that are embryonic are immature and undergo continuous differentiation. On the other hand, adult stem cells are differentiated into various tissues that are of certain specific types (Lv et al. 2014).

1. Embryonic stem cells: These cells are mainly derivative of blastocysts (inner cell mass of early mammalian embryos). This cell source is a renewable type of cell source as it can divide and renew itself without much differentiation for longer time periods in comparison to somatic/adult stem cells.

Table 8.2 Stem cell source and the tooth tissue supported

S. no.	Stem cell	Tooth structure supported	Proliferative capacity
1	SHED (stem cells from the human exfoliated deciduous tooth)	Dentine pulp complex, odontoblast-like cells	Excellent
2	DPSCs (dental pulp stem cells)	Bone, dentine pulp complex, odontoblast-like cells	Very good
3	DFPCs (dental follicle precursor cells)	Cementum matrix and Pdl-like cells	Not defined
4	PDLSCs (periodontal ligament stem cells)	Pdl-like and cementum-like cells	Not defined
5	SCAP (stem cells from apical papilla)	Bone, dentine pulp complex, odontoblast-like cells	Very good
6	Embryonic stem cells	Representatives of all three germ layers	Excellent

2. Adult/somatic stem cells: These cells can be derived at different stages of organogenesis. Stem cells like SHED (stem cells from the human exfoliated deciduous tooth), DPSC (dental pulp stem cells), and PDLS (periodontal ligament stem cells) are well-differentiated and do not continue their developmental process (Ghannam et al. 2020), while stem cells like DFPC (dental follicle precursor cells) and SCAP (Stem cells from apical papilla) continue their developmental process (Table 8.2).

The second essential component of the triad of tooth tissue engineering is a scaffold. Scaffolds are essential of three kinds: (1) synthetic biomaterials, (2) natural biomaterials, and (3) composite materials.

Biomaterials that fabricate scaffolds are broadly classified as natural or synthetic. These scaffolds can be used as tools to support cell sources so that cells can be cultured upon them. Also, they can be used as drug and growth factor delivery tools in dental tissue engineering (Ripamonti 2004). The composition of these scaffolds should be similar to the natural extracellular matrix of the desired tissue to be regenerated (Goldberg and Smith 2004; Du and Moradian-Oldak 2006). Each of these biomaterials is discussed in detail later in the chapter.

Growth factors—(1) Platelet-derived growth factor, (2) Bone morphogenetic proteins, (3) Transforming growth factors, and (4) Insulin-like growth factors

The third essential part of this triad of the tissue regeneration process is growth factors. Growth factors are directly attached to the receptors present on cell surfaces. These are normally released through the adjacent ECM. The growth factors activate several essential mechanisms and pathways. They control cellular adhesion, their proliferation, migration, differentiation, and survival as well (Oshima and Tsuji 2014; Kitamura et al. 2012; Goldman 2004; Grazul-Bilska et al. 2003; Atanasova and Whitty 2012). They mediate cell processes that are important for angiogenesis, growth, and wound healing (Grazul-Bilska et al. 2003). For example, TGF-β, i.e., the tissue forming growth factor, is important for differentiating odontoblastic cells, thus

providing further stimulation to pulpal stem cells (Oshima and Tsuji 2014). Likewise, BMP-2, i.e., bone morphogenetic protein, also does similar functions of differentiating pulpal stems cells into odontoblastic cells.

8.4 Strategies for Regeneration of Tooth

Successful tooth tissue engineering requires (1) regeneration of functionally and structurally sound tooth and (2) regeneration of each and every part of the tooth. The bioengineered tooth is expected to regenerate as similar to natural ones as possible. For regeneration of a bioengineered tooth with desired morphological and histological characteristics, two things are important, i.e., (1) epithelial and mesenchymal reactions on biomaterial scaffolds and (2) interaction with the extracellular matrix.

On a broad basis, two approaches are mainly used for regenerating bioengineered tooth:

1. Scaffold involved tooth tissue engineering: This technique involves tooth tissue engineering using a scaffold. In this approach, cells are planted on scaffolds by either cell homing or in vitro implantation (Fig. 8.2).
2. Scaffold-free tooth tissue engineering: This approach uses appropriate signals which guide embryonic tooth formation (Fig. 8.3).

8.4.1 Approach Using Scaffold

A three-dimensional scaffold is most suitable for tooth tissue engineering. This approach involves the seeding of cells in a planned manner on scaffolds in laboratory conditions. The seeded scaffolds are pre-cultured in vitro and then transplanted in vivo into host tissues for oxygen, nutrients, and blood supply. At the recipient site, the regenerated tissue can later be grafted to obtain the desired organ, which can be implanted into the jaws. This approach is highly dependent on the physical and chemical characteristics of biomaterials and their individual effects on extracellular matrix and cell survival. Tables 8.3 and 8.4 present the various scaffold fabrication techniques with their merits and demerits.

Using the approach, Young et al. (2002) made the first-ever bioengineered tooth crown that was histologically and morphologically similar to the natural tooth in terms of similarity in enamel organ, odontoblasts, pre-dentin, etc. They seeded disassociated cells on poly (L-lactide-co-glycolide) (PLGA) polymer scaffolds. Another study demonstrated that dental tissue regeneration could be performed in species like rats and pigs by seeding the embryonic tooth cells of adult rats on scaffolds made up of various rigid biomaterials (Duailibi et al. 2004). One major drawback of these teeth was that although they were histologically similar to the natural tooth with morphologic features of dentin, enamel, dental pulp, etc., their shape and size varied greatly from a natural tooth. Cell homing at the site of injury supports tissue regeneration with the help of bimolecular signals by the process of

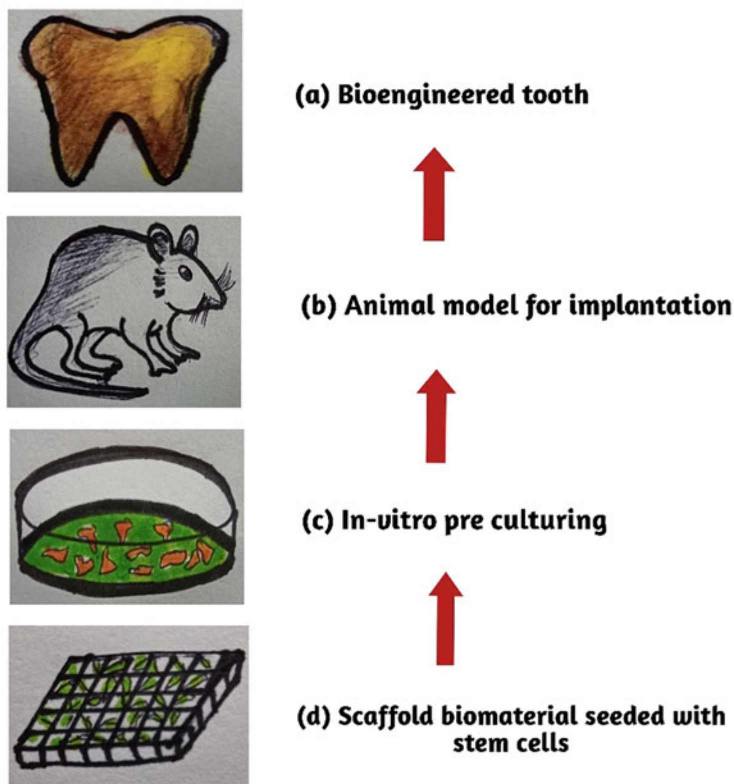

Fig. 8.2 (**a**) Bioengineered tooth. (**b**) Animal model for implantation. (**c**) In vitro preculturing. (**d**) Scaffold biomaterial seeded with stem cells. This schematic illustrates the bottom-up approach to culture the stem cells in presence of scaffolds and then fully shaping these scaffolds into the complete tooth

chemotaxis. The method of chemotaxis-based cell homing is most commonly used for periodontal ligament and pulp regeneration. It comprises inhabiting the implanted scaffold matrix and in situ inducing of nearby host site stem cells (Kim et al. 2010a). However, this technique does not involve isolation and manipulation of cells in laboratories. Thus, it reduces cost and improves clinical success.

8.4.2 Approach Without Using Scaffold

Scaffold-free approach is based on the production of desired tissue that mimics the natural tooth through inducing the embryonic developmental processes that are mainly guided by certain specific signalling molecules. In this approach, the aggregation of cells leads to the development of desired tissue without the use of any foreign carrier for cellular adjoining through the epithelial and mesenchymal cellular

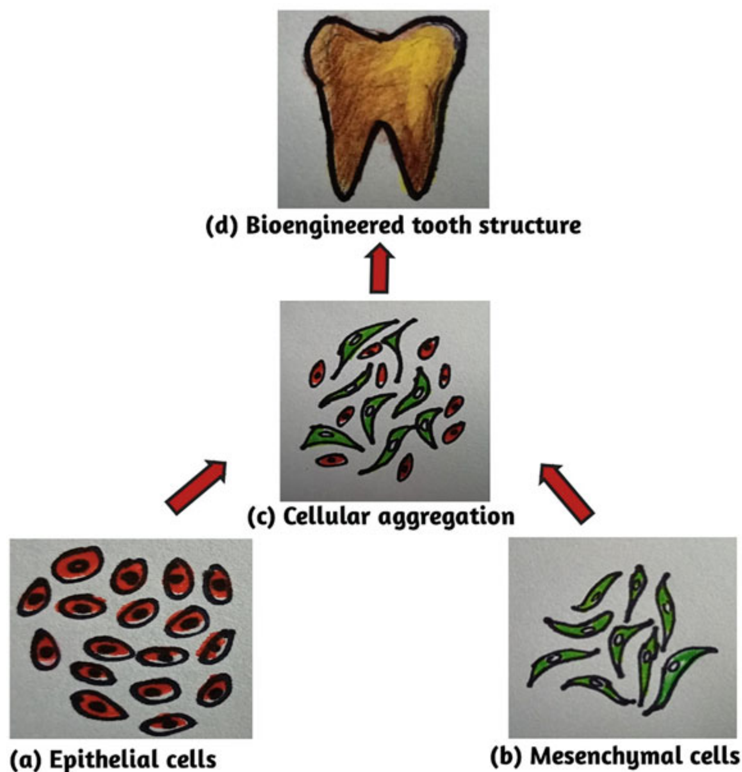

Fig. 8.3 (**a**) Epithelial cells. (**b**) Mesenchymal cells. (**c**) Cellular aggregation. (**d**) Bioengineered tooth structure. This schematic illustrates the formation of the tooth without the use of scaffolds using appropriate signals leading to embryonic tooth formation

Table 8.3 Conventional techniques for fabrication of scaffolds

S. no.	Technique	Merits	Demerits
1	Gas foaming	Produces scaffolds with high porosity (approx. 85%)	Rigidity of scaffold Closed pore structure
2	Freeze-drying	Mid-range porosity	Irregular pore size High-energy-consuming technique Use of cytotoxic solvents
3	Thermal induced phase separation	Highly porous scaffolds (>98%)	Can only construct thermoplastics
4	Solvent casting and practical leaching	Good porosity (50–90%) Thin-walled 3D structures	Tedious process with the usage of toxic solvents
5	Electrospinning	Scaffolds with high tensile strength	Difficulty in obtaining desired pore size Use of toxic solvents

Table 8.4 Rapid prototyping (RP) fabrication techniques with merits and demerits

S. no.	Technique	Merits	Demerits
1	Selective laser sintering (SLS)	Used to create a scaffold of desired properties as it provides a command over the microstructures	The operating temperature is high
2	Fused deposition modeling (FDM)	The deposition temperature is low	Limited use for biodegradable polymers
3	Bioprinting	Low cost High accuracy and speed Increased complexity in shape	Mainly dependent on the presence of cells
4	Stereolithography (SLA)	Less wastage, increased resolution with uniform interconnectivity in pores	Requires large quantities of monomers

interaction (Arakaki et al. 2012; Lee et al. 2011; Nakao et al. 2007). Signalling molecules play a major role in the epithelial-mesenchymal interactions, which play a crucial role in the tooth regeneration process.

1. Signalling molecules supporting amelogenesis: These include sonic hedgehog (SHH) signalling, which is able to preserve the niche of stem cell, bone morphogenic protein (BMP) signalling essential in the differentiation of ameloblasts, fibroblast growth factor (Fgf) signalling for deposition of enamel, wingless (Wnt) signals, etc.
2. Signalling molecules supporting dentinogenesis: BMP-2 helps in guiding the differentiation of odontoblasts and also helps DPSC-mediated mineralization. On the other hand, G-CSF signalling is involved in the regeneration of dentin. Also, it supports proliferating nature of stem cells.

Various studies had been conducted on this approach of tooth tissue engineering. Ohazama and his co-workers prepared a synthetic primordial of tooth embryo with the use of (1) oral epithelium and (2) mesenchymal cells that were of non-dental origin (Ohazama et al. 2004). They were also successful in making a histological copy of the natural tooth. In this study, they implanted tooth primordial of E14.5 rat into adult mice. Researchers have also been able to develop bioengineered tooth that is fully functional in nature with the use of three-dimensional organ culture through regenerative engineering (Ikeda et al. 2009). This approach is limited due to the need for an optimal quantity of mesenchymal cells.

8.5 Ideal Properties of Biomaterials for Tooth Regeneration

The biomaterial scaffolds that act as a physical matrix in tooth regeneration should meet certain ideal requirements such as the following: (1) It should be biocompatible and biodegradable. (2) It should be non-toxic. (3) It should have easy handling. (4) It

must be mechanically and physically stable. (5) It should have adequate pore size, shape, and volume for the diffusion of cells or growth factors. (6) The immunogenicity should be less, and it should support vascularity. (7) It should be ready to sterilize and stored in clinics with a reasonable shelf life. (8) It should be exhibiting cellular encapsulation or cellular adhesion. (9) It should maintain the functionality of tooth forming cells like odontoblasts, cementoblasts, ameloblasts, fibroblasts, etc.

8.6 Biomaterials in Tooth Tissue Engineering and Its Classification

Biomaterials that form the basis for cellular proliferation and differentiation are involved in odontogenesis and osteogenesis (Albrektsson and Johansson 2001; Bhui et al. 2018). These are the naturally or synthetically derived materials that construct the various types of scaffolds utilized in tooth regeneration. The interaction of the extracellular matrix with the systemic arrangement of epithelial cell layers is essential to form bioengineered teeth of determined morphology and characteristics. Biomaterials used in tooth tissue engineering can be classified into natural and synthetic. The basis of the chemical composition of these biomaterials can be polymeric, metallic, composites, or ceramics. These biomaterials offer certain advantages and disadvantages (Table 8.2). Biomaterials can also be used in various forms like gels, fibers, foams, etc.

A versatile range of natural biomaterials is obtained from animals and plants, providing excellent properties of bioactivity, biodegradability, and biocompatibility. Naturally derived biomaterials used in tooth regeneration are (1) proteins, collagen, fibrin, and silk, and (2) polysaccharide, alginate, chitosan, hyaluronic acid, and agarose. The naturally derived biomaterials have good cellular compatibility. However, they are less mechanically stable and also lack control over the size of pores. On the other hand, synthetic biomaterials are mainly polymers like poly glycolic acid (PGA), poly lactide-co- glycolic acid (PLGA), poly lactic acid (PLA), and polycaprolactone (PCL) that are generally used for forming highly porous scaffolds. Hydroxyapatite, beta-tricalcium phosphate, and constructs of silicate and phosphate glasses are also synthetic in nature. These biomaterials are cost-effective and can be made under controlled environments in abundant quantities. Synthetic biomaterials can be tailored into desired forms, and at the same time, they are flexible in the overall processing (Zhanga et al. 2013). The cell differentiation and chemical and mechanical properties of these manufactured biomaterials can also be altered as per need. The major drawbacks of synthetic biomaterials are reduced cell adhesion properties and lack of inherent bioactivity and biological site recognition. There is another category of biomaterials, composite materials, combinations of two or more polymers of desired characteristics, and advantages that are best suited to view the variations required in the bioengineered tooth (Table 8.5).

Table 8.5 Merits and demerits of biomaterial scaffolds used in tooth regeneration

Merits and demerits of biomaterials	Natural biomaterials	Synthetic biomaterials	Composite materials
Merits	Less manufacturing cost, less renewability, and less disposal cost Biodegradable and biocompatible Excellent property of cell adhesion, biological signalling, and biocompatibility	Flexibility in processing—Physiochemical and mechanical properties can be tailored easily as per required size and shapes	Permit combination of desirable properties of each polymer component Properties can be tailored as per need
Demerits	Biological properties may be lost during processing Rapid disintegration Risk of immune rejection	Risk of adverse tissue reactions Lack of cellular interaction and adhesion	Lack of biological activity

8.6.1 Natural Biomaterials for Tooth Regeneration

8.6.1.1 Natural Polysaccharides

Cellulose, alginate, hyaluronic acid, chitosan, dextran, and agar are the most widely used polysaccharides in dental tissue engineering.

Cellulose

Cellulose, a polysaccharide, mainly occurs in green plants. This biomaterial exhibits good biocompatibility and has average mechanical strength. Cellulose molecules, due to the presence of various hydroxyl groups, can be used to produce multiple hydrogel scaffolds for tissue engineering. The scaffolds obtained from cellulose have an angiogenic effect and are capable of building capillary tube-like structures, thus helping in increased revascularization and regeneration of dental pulp.

Alginate

Alginate, a natural polysaccharide, has been extensively used in both tooth and bone regeneration. The most widely used forms of alginate are (1) injectable hydrogels, (2) porous scaffolds, and (3) nanofibrous scaffolds. It is considered a viable alternative to proteins and polymers. Alginates can be used for cellular encapsulation and growth factor delivery. However, uncontrolled degradation rate and poor mechanical stiffness make it slightly unsuitable for hard tissue regeneration in its pure form.

Hydrogels made from alginate are excellent candidates for providing a balanced matrix that can support the regeneration of pulp, dentin, and periodontal ligaments. It is also utilized to deliver various growth factors such as TGF-β that can promote dental pulp regeneration. Moreover, it was studied that both the alginate hydrogels that were acid-treated and TGF- containing hydrogels could (1) help in upregulation

of the tubular dentin matrix secretion, (2) promote differentiation of odontoblastic cells, and (3) regulate the secretion of regular dentin (Dobie et al. 2002). Arginine-glycine-aspartic acid (RGD)-modified hydrogels of alginate have proven success in cell differentiation, cell adhesion, and cell proliferation. Alginate microspheres were able to enhance mineralization activity and maintain the viability of DPSCs immobilized within them (Kanafi et al. 2014). It should also be mentioned here that composite scaffolds made up of alginate and bioglass ceramics have shown greater ability to enhance the adhering capacity, proliferating rate, and alkaline phosphatase activity of human periodontal ligament fibroblasts cells (hPDLF) more in comparison to pure alginate scaffolds (Srinivasan et al. 2012).

Hyaluronic Acid

Hyaluronic acid (Hyl A) or hyaluronate, a naturally derived polysaccharide, is found majorly in all body fluids and the extracellular matrix (ECM) of several connective tissues like synovial fluid tooth pulp matrix, etc. (Fakhari and Berkland 2013; Guo et al. 2019; Zhai et al. 2020). The enzymes active in the regulation of Hyl A are hyaluronan synthase in the cell membrane, which produces Hyl A (Huang and Huang 2018; Xing et al. 2020), and hyaluronidase, which disintegrates it in the tissues (Xing et al. 2020; Sharma et al. 2014). Hyl A plays a crucial role in the regulation of various important processes like cellular adhesion, survival, differentiation, and proliferation (Xing et al. 2020; David-Raoudi et al. 2008). It is a promising biomaterial that has been utilized in many biomedical fields, drug delivery systems, etc. (Zhai et al. 2020; Hemshekhar et al. 2016). However, apart from its biocompatibility, its viscoelasticity makes it a potential candidate in tooth tissue engineering (Cowman et al. 2015). The other features that make Hyl A more appealing for fabricating scaffolds are non-thrombogenicity, biodegradability, and non-immunogenicity (Xing et al. 2020).

There were several studies that proved Hyl A as a contributor to the formation of dentin (Ferroni et al. 2015; Zhang et al. 2013). Through ligation of Hyl A and CD44 on the cell surfaces of the odontoblastic cells, these cells have shown a significant amount of differentiation and proliferation (Chen et al. 2016). In another study by Inuyama et al., in context to pulp regeneration, it was demonstrated that Hyl A-based spongy three-dimensional scaffolds could promote cell growth both in vivo and vitro (Inuyama et al. 2010). They also reported that the Hyl A sponge scaffold showed a lesser inflammatory reaction in comparison to the collagen sponge. In addition to this, Hyl A also promotes the regeneration of bone with the help of regulation of biological factors and induction of several cellular signalling pathways (Zhai et al. 2020; Xing et al. 2020). Hyl A scaffolds can be used resourcefully in dentin-pulp complex regeneration as the tooth pulp expresses Hyl A differently during regeneration and helps in the dentine matrix formation (Ferroni et al. 2015; Itano 2008). Hybrid Hyl A scaffolds with cross-linking and several chemical modifications have also been used in prolonging the growth factor release period (Xing et al. 2020; Ferroni et al. 2015; Chrepa et al. 2017).

Limitations of Hyl A scaffolds are instability in changing pH and temperature (Xing et al. 2020; Martínez-Sanz et al. 2011). Also, it has a rapid degradation rate.

Hyl A can be modified to overcome these limitations and improve physical and mechanical properties while maintaining its functional integrity (Hoffman 2012). Some of these modifications are cross-linking with hydrazide (Farzin et al. 2020) and tyramine (Chen et al. 2017) and making composite scaffolds with improved cellular and mechanical properties (Xing et al. 2020).

Chitosan

Chitosan is a linear and semi-crystalline naturally derived biopolymer (Lee et al. 2008; Husain et al. 2017). It has been widely used in various tissue regeneration processes due to its properties like biocompatibility, biodegradability, hydrophilicity, adhesive nature with biological molecules, etc. (Lee et al. 2008). It has been manufactured in various scaffold forms like microparticles, hydrogel, nanoparticles, and sponge (Aguilar et al. 2019). This biomaterial shows ionic bond formation with several anionic agents like lipids, DNA, and synthetically derived polymers (Aguilar et al. 2019; Croisier and Jérôme 2013). Also, this biomaterial is cost-effective (Husain et al. 2017; Kim et al. 2008). The biocompatibility and degradability of chitosan are dependent on its molecular weight (Husain et al. 2017; Aguilar et al. 2019; Croisier and Jérôme 2013). There are various methods for making of chitosan scaffolds (Kim et al. 2008). However, among those, lyophilizing and freezing of chitosan solution are the most commonly used method (Husain et al. 2017). The salt leaching method makes it more porous, and the electrospinning method with polymers produces fibrous scaffolds (Islam et al. 2020; Lim et al. 2011). Chitosan scaffolds have the ability to regenerate pulp and dentine (Husain et al. 2017; Pezeshki-Modaress et al. 2014). It supports the formation of dentin by differentiation of odontoblastic cells of pulp along with their migration and proliferation (Husain et al. 2017; Lauritano et al. 2020; Albuquerque et al. 2014). Moreover, it also promotes cellular adhesion, osteogenesis and proliferation, leading to the formation of new tissue formation (Kim et al. 2008; Ducret et al. 2019). Hydroxyapatite combined with chitosan has been shown to improve various biologic properties in dental tissue engineering (Li et al. 2019). It is challenging to produce chitosan of exact molecular weight as biocompatibility and degradability depend on its weight. There is variation in this property (Lee et al. 2008; Kim et al. 2008).

Agar and Dextran

Agar is jelly in consistency, a linear polysaccharide of agarose, while dextran is a polysaccharide of sucrose. Both have different uses as agar promotes the formation of odontoblasts, thus helping in the tooth regeneration process, and dextran supports bone formation (Shahnavazi et al. 2016). Agar has been used as a substrate for the growth of micro-organisms (Alshehadat et al. 2016). Agar aids in the differentiation of dental mesenchymal cells into functional odontoblastic cells (Mac Faddin 1985). Very similar observations were noticed with agar-based culture media after treating it with growth factors like TGF-β, fibronectin, or heparin (Kikuchi et al. 1996; Bègue-Kirn et al. 1994). Agar-based hydrogels are also utilized for cell encapsulation. Dextran-based heparan-like polymers alone or in association with other growth factors are used in promoting osteoblastic differentiation and thus stimulate

regeneration of the bone (Kikuchi et al. 2001). Dextran hydrogels laden with growth factors like BMP have enhanced the regeneration of periodontal ligament in a dog (Shahnavazi et al. 2016).

8.6.1.2 Natural Bioceramics

Natural ceramics are more biocompatible than synthetic ceramics. Bioceramics are obtained from marine and coral sponges. There is a huge similarity between the vertebral column in hard tissues and the sponge. They have a special interconnection that can sustain a good amount of water. The sponge also supports fluid flow that can be utilized in the fabrication of appropriate bone and dentin scaffolds suitable for tooth regeneration. There is also a mineral component known as biosilica. They have less frictional properties, excellent resistance, high compressive strength, and good mechanical properties. Also, they have the ability to proliferate and mineralize cells along with the formation of bone (Blanquaert et al. 1999). Due to their porous structure, they help in increased vascularization, cellular infiltration, and cellular adhesion.

8.6.1.3 Natural Proteins

Fibrin, collagen, silk, and gelatin are the main naturally occurring proteins utilized in tooth tissue engineering.

Fibrin

Fibrin is a naturally derived fibrous polymer that plays a role in the blood clotting process. It is produced naturally by fibrinogen polymerization (Lee et al. 2008; Ahmed et al. 2008). Its products are used clinically as sealants and bio-adhesives for hemostasis as in various surgeries (Bolhari et al. 2019). Fibrin offers several advantages of cost, biocompatibility, immune response, and cell adhesion as compared to synthetic scaffolds (Lee et al. 2008). It provides greater control over degradation rate with end products that are non-toxic (Bolhari et al. 2019). Moreover, it supports the growth and differentiation of human dental pulp stem cells (hDPSCc) (Galler et al. 2011) and promotes the formation of new blood vessels (Horst et al. 2012). Fibrin produces no immunogenic reactions and can thus be used as an autologous biomaterial having high biocompatibility with simple and easy preparation methods (Lee et al. 2008; Whitmore 1999).

Fibrin as a biomaterial is majorly used in three forms: fibrin hydrogels, fibrin glue, and fibrin microbeads (FMBs) (Ahmed et al. 2008). (1) Fibrin hydrogels are manufactured from purified forms of allogeneic fibrinogen and thrombin (Ahmed et al. 2008). These hydrogels are usually used in injectable forms (Li et al. 2015). For enhancing the osteoinductive properties of these gels, they are combined with BMPs (bone morphogenetic proteins) (Karfeld-Sulzer et al. 2015). Fibrin hydrogels can promote the growth and differentiation of cells derived from dental papilla in the tooth regeneration process, namely, odontoblasts and osteoblasts (Horst et al. 2012; Ohara et al. 2010). These hydrogels also provide better seeding capacity and high cellular adhesion (Ahmed et al. 2008). As these scaffolds have a high rate of degradation and shrinkage, stability needs to be enhanced. This is done by using

polyethyglycolated fibrin scaffolds which support dentin matrix and pulpal tissue regeneration (Dikovsky et al. 2006).

(2) Fibrin glue, also known as fibrin sealant, is quite different from the hydrogels of fibrin. Combination of fibrin glue with gelatin, hydroxyapatite, hyaluronic acid, etc. has been extensively used as a delivery vehicle and also facilitates an adequate scaffolding matrix material for various tissue engineering processes like regeneration of periodontium, blood vessel, and bone (Ahmed et al. 2008). (3) Fibrin microbeads (FMBs) are shorter spherical beads that consist of fibrin in cross-linked and condensed forms. Fibrin microbeads enhance the stability of fibrin. However, these are generated for growing mesenchymal stem cells from the blood and bone marrow (Ahmed et al. 2008). Galler et al. found that in comparison to other naturally derived scaffolds, fibrin-based hydrogels were better for stem cell differentiation into odontoblastic cells and also for regenerating pulpal tissue (Galler et al. 2018). Ruangsawasdi et al. in 2014 showed that fibrin-based scaffolds in the form of hydrogel could support the cellular infiltration better and also generate a highly vascular pulp-like tissue that had all significant zones of dental pulp (Ruangsawasdi et al. 2014). In his study, immature premolars with fibrin scaffolds were implanted in rats, and it was found that fibrin not only affected the growth and differentiation of the adjacent cells but also influenced the morphology of the whole tooth tissue. They also showed angiogenesis in the canals where fibrin was used. Fibrin glue enriched by platelet-rich fibrin (PRF) was used as a scaffold in a porcine alveolar socket. Dental bud cells, when seeded into the scaffold, showed complete tooth tissue formation and eruption into the oral cavity. The only drawback was that the erupted tooth had irregular morphology (Yang et al. 2012). Fibrin has a tendency to show shrinkage, has a rapid degradation rate, and also suffers from poor mechanical properties that need adequate modifications (Bolhari et al. 2019). These modifications include (1) combining fibrinogen with various synthetically or naturally derived materials, (2) adjusting the parameter of polymerization of fibrin, and (3) using some chemical fixing agents (Lee and Kurisawa 2013). All these modifications have been shown to improve the mechanical properties of this polymer and to prevent it from shrinking.

Collagen

Collagen is the most important fibrous protein found in various connective tissues in the body. Due to structural and chemical similarities with major structural proteins found in the extracellular matrix of dental tissues, it has been studied extensively for tooth tissue regeneration. Collagen promotes cellular adhesion, cellular growth, and cellular migration. It can also be woven into desired forms and shapes due to its great tensile strength and therefore is suitable for use in pulp regeneration. To improve its mechanical properties, cross-linkage is done with glutaraldehyde (Wu et al. 2007), and hybrid scaffolds are made with hydroxyapatite and polyethylene (Wahl et al. 2007). However, cross-linking with glutaraldehyde could compromise its biocompatibility and cell survival (Wu et al. 2007).

Several studies have used collagen scaffolds for regenerating tooth structures (Table 8.3). A triad of DMP1, dental pulp stem cells, and collagen scaffold has been

Table 8.6 Studies of whole tooth regeneration with collagen scaffolds

S. no.	Type of cell	Biomaterial used	Site of implantation	Result	Reference
1	Embryonic tooth bud cells	Collagen	In vivo implantation in the jaw of mouse	Bioengineered tooth bearing good mechanical stress and appropriate mastication hardness	Ikeda et al. (2009)
2	Incisor tooth germ obtained from mouse	Collagen gel	In vivo implantation in the subrenal capsule of mouse	Complete tooth formation	Nakao et al. (2007)
3	hDPSCs, mesenchymal cells, and gingival epithelial cells obtained from porcine teeth	Collagen	In vivo implantation in the subcutaneous tissue of rat	Tooth crown along with mineralized dental tissues	Zhang et al. (2017)
4	Tooth bud cells of porcine third molar	Collagen and PGA	In vivo implantation in the rat omentum	Collagen proved to be superior scaffold	Sumita et al. (2006)

used to produce a pulp-like tissue matrix (Prescott et al. 2008). The dental pulp stem cells effectively adhered with the collagen scaffolds. Collagen can offer a three-dimensional environment for several types of cell sources and support cell proliferation and differentiation in tooth tissue engineering (Ravindran et al. 2010). Kim et al. (Kim et al. 2010a) studied the regeneration potential of collagen scaffolds with cells and growth factors like PDGF, basic FGF, and VEGF in endodontically treated root canals. These real-sized native human teeth were then implanted into the dorsum of the mouse. They found dental pulp tissue revascularization and re-cellularization and also new dentin formation in the endodontically treated teeth. A preclinical study that implanted collagen sponges in omentum of immunocompromised rats resulted in the development of a complete tooth with morphological features (Sumita et al. 2006). Nakashima M. suggested that specific surface characteristics were necessary for odontoblast differentiation, and he demonstrated the formation of osteodentin in the presence of type I collagen-based scaffolds (Nakashima 1994) (Table 8.6).

Silk

The natural fibrous protein silk is very popular in dental tissue engineering. It is non-toxic in nature and biocompatible and also offers cellular attachment, proliferation, and 3D soft tissue augmentation. Silk scaffolds from electrospun silkworms promote gingival tissue regeneration as it supports the attachment of gingival fibroblasts and cellular proliferation on electrospun fibers. On the other hand,

hexafluoroisopropanol-based silk fibers support tooth pulp regeneration. It has a slow degradation rate and is better than aqueous-based silk, thus considered for the formation of the soft dental pulp. Moreover, in a study, it was found that these scaffolds can also induce osteodentin formation (Zhang et al. 2011). In this study hexafluoroisopropanol silk scaffolds were used on which tooth buds cells were seeded. Silk scaffolds can also be utilized to serve as load-bearing bone grafts. In a study, to produce high strength of this biomaterial, micro-sized silk fibers were incorporated into fiber composite as a reinforcement which favored bone formation and stem cell differentiation (Mandal et al. 2012).

8.6.2 Synthetic Biomaterials for Tooth Regeneration

8.6.2.1 Synthetic Polyesters (PLA, PGA, PLGA, PLLA, and PCL)

The most widely used synthetic polyester polymers for tooth regeneration are poly lactide-co-glycolide(PLGA), poly-glycolic acid (PGA), PLLA, and polylactic acid (PLA) (Table 8.4). These polymers are also known as rigid biomaterials that provide structural substitutes. Polycaprolactone (PCL) is also used infrequently. These biomaterials are mild inflammatory, biodegradable, and biocompatible in nature. By changing their molecular weight, polymer ratio, and crystallinity, desired modification of various properties like their viscosity, porosity, structure, and degradation rate can be done (Table 8.7).

PGA, PLA, and PLLA

PGA is a biodegradable biomaterial and is extensively used in tooth tissue engineering due to better cellular proliferation and high similarity to the normal dental pupal tissue. PGA alone or combined with other natural or synthetically derived biomaterials is mainly used in dental pulp tissue regeneration (Mooney et al. 1996; Buurma et al. 1999). It has also been demonstrated that the construct made from PGA fibers and human dental pulp-derived cells could form new pulp-like tissue in a 60-day period in vitro (Bohl et al. 1998). PGA supports fibroblast differentiation and increases angiogenesis. In a study conducted by Ohara et al., the PGA scaffold showed a high capacity to form dentin-like materials on subcutaneous implantation with fibrin and collagen gels in rats (Ohara et al. 2010). PGA and PLA with DPSCs and SHED have been used to obtain pulp and dentin-like tissues, and PGA and PCL composite scaffolds have been used for periodontal regeneration. In inflammatory conditions, the PLLA scaffold combined with simvastatin supports the odontogenic differentiation of pulp cells. PLLA/PGA has already shown that these scaffolds are able to regenerate the whole tooth crown (enamel + dentin + pulp) on transplanted rat omentum with the use of tooth bud cells, whereas, when a similar combination was used in the jaws of the rats, the periodontal tissues were formed.

PLGA

PLGA is a copolymer that has dual assets of both the PGA and PLA. PLGA can be made into a scaffold having optimum physiochemical and mechanical properties

Table 8.7 Studies of whole tooth regeneration that used rigid polyester polymers as scaffolds

S. no.	Type of cell	Biomaterial used	Site of implantation	Result	References
1	Tooth bud cells of porcine third molar	PLGA and PGA-PLLA	In vivo implantation in omentum of rat	In 35 weeks, identifiable tooth structure having enamel organ, HERS, and dentin	Young et al. (2002)
2	Embryonic tooth bud cells of mouse	PGA	In vivo implantation in kidney capsule of mouse	Teeth having the normal shape of the crown but were small in size	Iwtsuki et al. (2006)
3	Tooth bud cells of porcine third molar	PGA-PLLA	In vivo implantation in omentum of rat	Identifiable tooth structure with enamel, pulp, dentin, and odontoblasts	Young et al. (2005)
4	Tooth bud cells of canine first molar	PGA	In vivo implantation of canine tooth socket in jaw	Dentin and bone formation took place but no enamel and root was formed	Honda et al. (2006a)
5	Tooth bud cells of porcine third molar	PGA	In vivo implantation in omentum of rat	Bioengineered tooth with dentin, cementum, and enamel	Honda et al. (2005)
6	Tooth bud cells of 3–7 dpn rat	PLGA and PGA	In vivo implantation in omentum of rat	In 12 weeks, tooth structure with enamel, dentin, and pulp	Duailibi et al. (2004)
7	Tooth bud cells of porcine third molar	PGA	In vitro studies and in vivo implantation in omentum of a rat	In vitro—Increased expression level of vimentin, bone sialoprotein, and amelogenin. In vivo—Enamel and dentin formation	Honda et al. (2006b)

with the desired degradation rate. Scaffolds made from PLGA have shown promising results in the bioengineering of a tooth crown (Young et al. 2002). Moreover, PLGA fiber orientation also targets the morphology of stem cells (van Manen et al. 2014). The incorporation of various inorganic materials, e.g., hyaluronic acid (Hyl A) into PLGA biomaterials, creates a favorable environment for dental tissue engineering. The PLGA-tricalcium phosphate composite is found to be more suitable for differentiation and proliferation of DPSCs in comparison to the other types (Zhang et al. 2011).

Polycaprolactone (PCL)

PCL is a semi-crystalline, aliphatic synthetic polyester polymer. It has low tensile and good mechanical strength. Having a lower rate of biodegradation, PCL is a preferred candidate for a prolonged drug delivery system (Dwivedi et al. 2020). Also, being chemically inert, poly-ε-caprolactone (PCL) has been widely researched for medical and dental applications in tissue engineering processing (Dash and Konkimalla 2012). Furthermore, PCL is biocompatible and economical and also promotes cellular adhesion and proliferation at the same time (Siddiqui et al. 2018).

The study conducted by Rotbaum et al. demonstrated the dependency of the mechanical properties of PCL on the pore size and on the pore geometry of the scaffold (Rotbaum et al. 2019). PCL is generally used for the regeneration of hard tissues like alveolar bone. PCL scaffolds promote bioactivity and improve mechanical properties, cellular differentiation, and cell proliferation (Dwivedi et al. 2020). By the process of "cell homing," the PCL-HA-based scaffold also showed good results regarding PDL tissue regeneration in a rat model (Kim et al. 2010b). Also, this biomaterial has fewer limitations, hydrophobicity being one of them. Hydrophobicity can decrease cellular adhesion capacity, but this can be significantly reduced by active screen plasma nitriding (Gaona et al. 2012). Coating PCL with hyaluronic acid, gelatin, TCP, calcium phosphate, etc. (Huang et al. 2004) can enhance cell proliferation, attachment, and migration capacity (Erisken et al. 2008). Polydopamine coating on PCL-based scaffolds can also improve various other properties like osteogenic ability and surface wettability (Dwivedi et al. 2020; Lee et al. 2016).

8.6.2.2 Synthetic Polyanhydrides

Polyanhydrides are widely used for oral inflammatory pathologies (Uhrich 2010; Conte et al. 2018). Polymers (such as poly 1,3- bisp-carboxyphenoxypropane, polysebacic acid, etc.) containing two carbonyl groups linked with the ether group were explored for repair and regeneration of bone. In a study, Hasturk et al. (2014) demonstrated the use of polyanhydrides with light or chemically set polyhydroxyethylmethacrylate, polymethylmethacrylate, and calcium salt graft materials around extraction sockets and dental implants. The results showed high implant stability with the use of these synthetic grafts.

8.6.2.3 Synthetic Ceramic Scaffold

Calcium-phosphate biomaterials (like beta-tricalcium phosphate (β-TCP) and hydroxyapatite), glass ceramics, and bioactive glasses comprise a group of ceramic scaffolds. Ceramic-based scaffolds can be tailored to obtain the desired rate of dissolution, desired permeability, and desired surface characteristics that can be controlled.

Calcium Phosphate-Based Scaffolds

Calcium phosphate scaffolds include β-TCP and HA that have been successfully used for bone regeneration. These biomaterials offer several advantages like osteoconductivity, biocompatibility, bone regeneration, decreased immunogenicity,

and enhanced bone defect healing. Calcium phosphate scaffolds also increase the osteoblastic activity of implanted cells. β-TCP scaffold is generally used for the regeneration of pulp and dentin. Both β-TCP and HA are biodegradable. Porous 3D Ca-P granules provide excellent substrate conditions for the growth and maintenance of dental pulp stem cells – odontogenic cell growth and differentiation. However, due to its brittle nature, high density, and low mechanical strength, the material needs certain modifications. These drawbacks can be overcome by the addition of zinc oxide and silicon dioxide that increase the mechanical strength of the scaffold (Fielding et al. 2012).

Glass Ceramics

Glass ceramics are used in tooth regeneration as they provide a better crystallization environment and also support differentiation of dental pulp stem cells. Niobium-based fluorapatite glass ceramics are the potential candidates for proliferation, differentiation, and adhesion of human dental pulp stem cells. Mg-based glass ceramics enhance bioactivity and also increase mechanical integrity (Goudouri et al. 2012). Nano-active glass ceramic composite scaffolds are excellent for bone regeneration as they help in the maximum amount of mineral deposition (Srinivasan et al. 2012).

Bioactive Glass

For binding to the soft tissues and as well as bone, bioactive glass plays the same role as that of hydroxyapatite (HA). It also supports increasing hydroxyapatite formation when coated on dental ceramics.

8.6.3 Composite Scaffold

A variety of biomaterials can be made by combining two/more non-similar scaffold materials that reduce the disadvantages and enhance the advantages of each of the materials to get optimized results. These materials could be a combination of inorganic polymers and synthetic polymers (Sharma et al. 2014; Lim et al. 2010). Ceramic and glasses can be added to flexible polymers to increase their stiffness. Composite products are able to provide better cellular spreading, adhesion, and viability for cell growth. Bioglass-polymer composites are highly bioactive and biocompatible and indicated for hard tissue repair (Blaker et al. 2003). Other examples of composite scaffolds are zirconia hydroxyapatite, a porous scaffold and an excellent candidate for both bone regeneration and reconstruction (An et al. 2012), and a combination of PGA and PLGA with HA or β-TCP and PLA with enhanced mechanical properties. PLGA was studied along with three different combinations of PLGA composites with HA, CDHA (calcium-deficient hydroxyapatite), and TCP (tricalcium phosphate) (Zheng et al. 2011). It was found that the PLGA-TCP composite was the most suitable for dentin regeneration. All these four scaffolds were studied for dental pulp stem cell differentiation and proliferation. It was found that calcium phosphate-based composite scaffolds could promote tooth

regeneration. Also, calcium phosphate scaffolds were able to neutralize the acidic pH of polymers and thus could provide greater control of mild inflammations associated with the degradation of polymers. Table 8.8 presents a comprehensive update of the properties of the various scaffolds discussed in this chapter.

8.7 Challenges Associated with Regeneration of Tooth and Concluding Remarks

With the current available regenerative biotechnologies, tooth tissue engineering has not matured to the extent to which regeneration of the whole tooth is possible as a functional unit in humans. There are still various hurdles to overcome in the process of regeneration of a fully functional tooth.

8.7.1 Challenges Associated with Cell Source

There are various problems in the accessibility of the source of cells, in inducing odontogenic process in these cell sources and speeding up the whole regeneration process with the help of gene control. It should be noted that it is often difficult to obtain cell sources like SHED and DPSCs in abundant quantities. These stem cells can be extracted from allogenic or autogenic cell sources. Allogenic cells have risks of rejection by the immune system and ethical concerns for use. Embryonic cells derived from xenogenic sources also show immune rejection. Moreover, it is also technique-sensitive to store, isolate, and culture these stem cells, and even after in vitro expansion, these cell sources lose odontogenic capacity.

8.7.2 Challenges Associated with In Vivo Implantations

These tissue-engineered constructs are made with the help of very potent molecular signals that can cause stem cell growth modification, e.g., induced pluripotent stem cells (iPSc) eliminated the restricted pluripotency of adult stem cells. But cancer-like uncontrollable growth was found to be associated while using iPSc. Moreover, these cells have to be kept alive and functional with suitable nutrient, blood, and oxygen supply from the starting stage of fabrication till the implantation stage, which is a complicated task.

8.7.3 Challenges Associated with the Storage, Cost, and Aging

The products associated with tissue engineering need specific storage like hypothermic storage or cryopreservation. Also, at the same time, it should be cost-effective to the patient. The most common organ failure still remains tooth loss which is being currently replaced by the available prosthetic clinical procedures like dental

Table 8.8 Properties of scaffolds for tooth tissue engineering

Biomaterial	Mechanical strength	Porosity	Adhesion property	Antibacterial activity	Biocompatibility	Structural integrity	Bio-conductivity	Biodegradation	Mineralization
Natural polysaccharides									
Cellulose	High Young's modulus >15 GPa	High	Cellular adhesion present	Not in bacterial cellulose	Very good	Physical integrity maintained	Excellent Osteoconductivity	Not degradable in humans	Support
Alginate	Weak	High	Limited (possible with the help of ligand)	Present	Good	Possible with RGD alginate gel	Good Osteoconductivity	Present	Support
Hyaluronic acid	Weak	Medium	Weak cell adhesion	Present in developed hydrogels	Very good	Poor physical integrity	Osteoconductive	Rapid degradation	Supports (with modification)
Chitosan	Weak	High	Muco- and bio-adhesive	Present	High	Weak structural integrity	Osteoconductive	Present	Biomineralization possible
Agar and dextran	Moderate	High	Weak	Present	Present	Poor structural integrity	Osteoconductive	Low degradation	Mineralization possible
Natural bioceramics	High Young's modulus 73–113 GPa	High	Promotes cell adhesion	Present	Very good	Maintains structural integrity	Highly osteoconductive	Slow degradation	Promotes mineralization
Natural proteins									
Fibrin	Low	Moderate	Promotes cell adhesion	Present	Excellent	Poor	Supports osteoconductivity	Rapid degradation	Supports

(continued)

Table 8.8 (continued)

Biomaterial	Mechanical strength	Porosity	Adhesion property	Antibacterial activity	Biocompatibility	Structural integrity	Bio-conductivity	Biodegradation	Mineralization
Collagen	Low 0.0018–0.046 Gpa	High	Promotes cellular adhesion	Present	Excellent	Weak physical integrity	Supports osteoconductivity	Rapid degradation	Supports
Silk	High (Young's modulus 15–17 Gpa)	High	Promotes cellular adhesion	Present	Present	Maintain structural integrity	Supports osteoconductivity	Slow degradation	Supports
Synthetic polyesters									
PGA,PLA, PLLA	High tensile strength (PGA 7000–8400 Mpa, PLA 3000 Mpa)	High	Less cellular adhesion	Present	Present	Weak structural integrity (PLA > PGA)	Support osteoconductivity	Slow degradation with PLA and PLLA compared to PGA	Supports mineralization
PGLA	Tensile modulus of 2000 Mpa	High	Less cellular adhesion	Present	Present	High molecular weight enables better structural integrity	Pure PGLA shows poor osteoconductivity	Fast degradation in comparison to PLA	Pure PGLA does not induce adequate mineralization
PCL	Tensile modulus of 700 Mpa	Moderate	Less cellular adhesion	Antibacterial activity is increased with the addition of ZnO	Present	PCL 3D scaffolds maintain structural integrity	Supports osteoconductivity	Slowest degradation among polyesters	Supports mineralization

Synthetic ceramic scaffold									
Calcium phosphate scaffold	Weak mechanical strength	Low	Support cellular adhesion	Ag-TCP-coated Ti showed a significant decrease in bacterial colonies	Present	Maintains structural integrity	Promotes osteoconductivity	Low degradation	Promotes mineralization
Bioactive glass	High mechanical strength	High	Promotes cell adhesion	Present	Present	Maintains structural integrity	Promotes osteoconductivity	Controllable biodegradation	Supports mineralization

implants, but they present various disadvantages. Biomaterials used in dental tissue engineering are indispensable. They are the frameworks for tooth regeneration. Biomaterial research has focused on the creation of scaffolds with optimum properties ideal for tooth tissue regeneration. However, tremendous efforts are still needed with an aim to produce fully functional dental tissue structures to get clinical success.

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3D-Printed Lanolin-Based Sodium Alginate Wound Dressings

9

Muhammet Uzun, Ani Diana Kuyumcu, and Oguzhan Gunduz

Abstract

This chapter aims to develop a realistic view of creating novel wound dressing materials by making use of 3D printing technology. Wool wax, which is known as textile waste, is used as an agent material. Various concentrations of wool wax (lanolin) and sodium alginate-based scaffolds were developed by additive manufacturing technology. The use of lanolin as wound dressing material and 3D production have been successfully achieved in the study. As lanolin concentration is amplified, the pore size of the scaffolds also increases serially. The results showed that the optimum concentration of wound dressing was developed using 2% of lanolin and 4.5% of sodium alginate solution. The antibacterial activity of the developed structures was performed against *Escherichia coli*. The result of the antibacterial activity demonstrates that the addition of lanolin in the scaffolds provides the antibacterial activity. The mechanical properties, such as swelling and degradation, were also studied. The discharge mechanism of

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the scaffold was investigated by using agar gel chromatography and chromatography. All the results suggest that the 3D-printed lanolin-based sodium alginate scaffolds have an excellent potential for wound dressing applications.

Keywords

3D printing · Wool wax (lanolin) · Sodium alginate · Wound dressing · Industry · Additive manufacturing

9.1 Introduction

Burn wounds are caused by chemical, electrical, fire, and sun contact with human skin, irritations, and traumas. As mentioned within the literature, burn wounds are characterized by the severity of injury to the skin. The Wallace rule of nines is applied to assess the depth of the burn. As given in Fig. 9.1, there are mainly three significant sorts of burn wounds (What Are the Types and Degrees of Burns? n.d.).

A first-degree burn, predominantly epithelial burns, is erythematic without vesicating. A second-degree burn involves the epidermis with a variable thickness of the dermis. This is often categorized into different types like second degree, i.e., superficial, where vesication and inflammation are seen in the skin as only the papillary dermis is involved. The second-degree deep-eschar formation is seen because it involves deep reticular dermis. In interrogation burn, also referred to as full-thickness burn, eschar formation is present (Tiwari 2012) (Fig. 9.2).

Wound healing ends with reepithelialization and restoration of the epidermal barrier. It takes years for scar recovery, even after complete epithelialization. As shown by the current situation globally, over one million people are moderately and/or severely burned per annum in India. In Bangladesh, Colombia, Egypt, and Pakistan, 17% of youngsters with burns have a short-lived disability, and 18% result in permanent disability. In 2008, over 410,000 burn injuries occurred within America, with approximately 40,000 requiring hospitalization (Burns n.d.).

In this year, the wound dressing market is 7.0 billion USD. Wound care is vital since prehistory. Although wounds can heal naturally, hunter-gatherers empirically discovered many factors like herbs for accelerating the process of wound healing. There are many milestones. For example, the wound dressing is developed in ancient

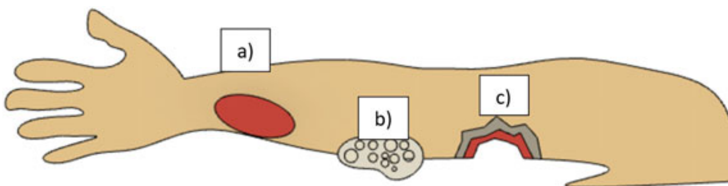

Fig. 9.1 Characterization of the burn wound: (**a**) first-degree burn wound, (**b**) second-degree burn wound, and (**c**) third-degree burn wound

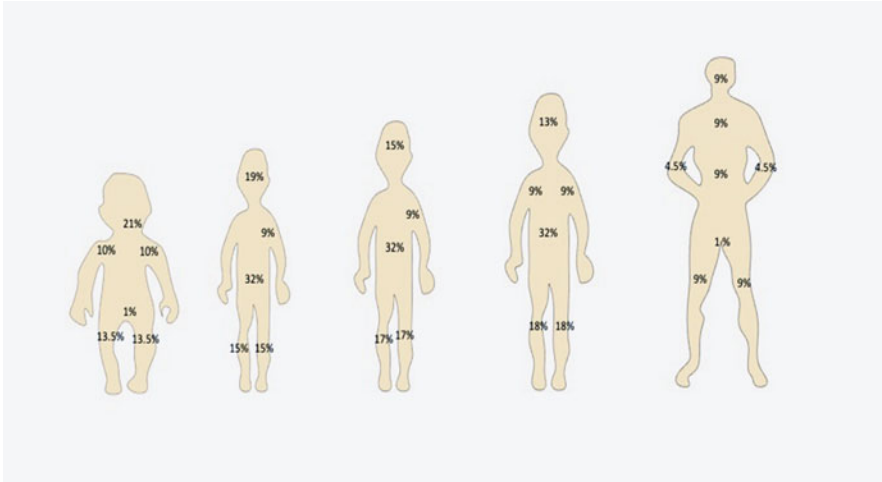

Fig. 9.2 The Wallace rule of nines

Greek, ancient Egypt, the nineteenth century, twentieth century, and twenty-first century. History of the natural wound dressing materials: First of all, in ancient Greece, the herbal treatment industry was so huge that traders skipped doctors and sold herbal treatment. This is the foundation of the primitive pharmaceutical sector. The earliest list of herbal medicine was written on the papyrus, which includes 850 plant medicines by Diocles of Carystus, the Greek philosopher Aristotle. This chapter is the initial of herbal scientific research. Furthermore, ancient Greeks were aware of wound closure; they tried to close a wound by using herbs. Consequently, ancient Greece was crucial in wound dressing. Secondly, ancient Egypt believed that closing wounds prevents from external evil spirits. They used animal grease and honey as topical treatments for wounds. Obviously, their wound dressings are animal grease and honey. The use of honey and animal grease is written in the Ebers Papyrus circa 1500 BC. Briefly, ancient Egyptians used herbs and animal grease as a wound dressing. Thirdly, in the twentieth century, polymer synthetic wound dressings began with the development of polymers.

Fourthly, in the 1950s, nylon, polyethylene, polypropylene, and polyvinyl, which are fibrous synthetics used for wound dressing, were explored for the protection of wounds and accelerated the healing process. Afterward, in the 1960s, George Winter and Howard Maibach realized that moist wound dressing enhances reepithelialization and healing of the wound. Fifthly, in the 1990s, composite and hybrid polymers were used as a wound dressing. Also, a new class of wound dressing, which is called living skin equivalent, has risen with the development of biotechnology and tissue engineering. The main property of living skin equivalent is the release of growth factor for wounds, serving as a cellular matrix. Also, biomembranes, skin substitutes developed, and the concern of burn wound pain were reported. In brief, the nineteenth and twentieth centuries bring a new dimension

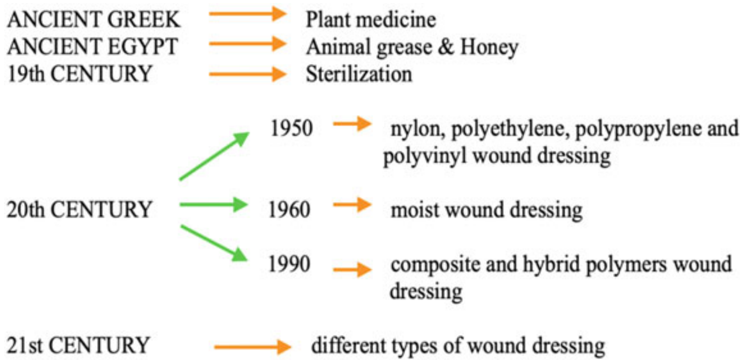

Fig. 9.3 The history of wound dressing

to wound dressing. Sixthly, in the twenty-first century, different materials and different types of wound dressing were produced. In conclusion, there are sharp indications in ancient Greece, ancient Egypt and the nineteenth century for the progression of wound dressing (History of Wound Care—Wikipedia n.d.) (Fig. 9.3).

There is another issue that burns are dynamic and alter in appearance, particularly within the first 48 h ($1728*10^2$ s). Therefore, the burn service practice is to review burns after 48 h ($1728*10^2$ s) before decisions regarding definitive dressings or surgery are made. The initial burn dressing should be one that will remain intact for 48 h ($1728*10^2$ s) and stop infection. It is a protocol within the burn service to cover all burns with dressing for 48 h ($1728*10^2$ s) (Douglas and Wood 2017). Additive manufacturing may be a technology that builds three-dimensional solid objects from their digital models; this enables to pick a material layer by layer. Due to the manufacturing complex, shape additive manufacturing may be a part of modern manufacturing. Using additive manufacturing can reduce the environmental effect and make sustainable yield. To accomplish the design problem of additive manufacturing, inverse problem-solving methods are often used. During this study, to unreveal the difficulties when fabricating wound dressing, the inverse problem-solving method is employed (Williams and Seepersad 2012) (Fig. 9.4).

The primary objective of this study is to develop novel structures which may be applied to second-degree wounds, which entail innovative wound dressings to move at the preliminary stage and, through the healing process, protect burn wounds. The scaffold acts as a barrier between air and skin. It prevents the connection of bacteria, germs, dust, and wound. It is aimed that the wound dressings provide a suitable environment for healing. Commercially, the utilization of lanolin in medical cream is common for burn wound treatment. It is well-established that lanolin has a reepithelialization rate, the thickness of the dermis, and cell counting impact (Chvapil et al. 1988). There are some lanolin-based skin creams; however, there are no lanolin-based 3D-printed structures, which are one of the important novelties of this work. In today's world, sustainability, recycling, and renewability are significantly important and challenging. Nowadays, the priority has been growing over the disposal of wastes produced by textile industries. The utilization of wool is so

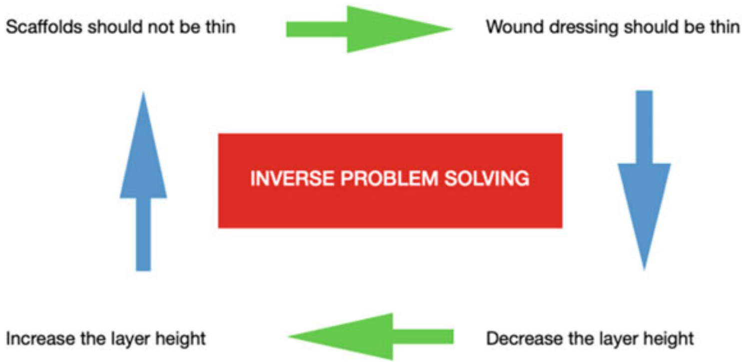

Fig. 9.4 Problem-solving method

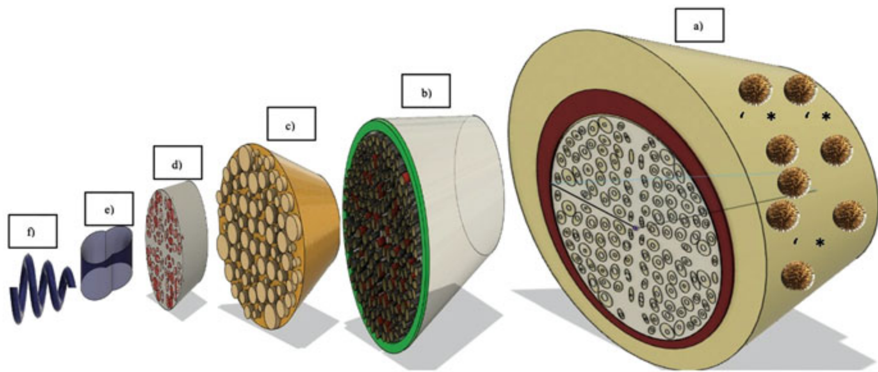

Fig. 9.5 The structure of wool: (**a**) cuticula, (**b**) cortex cell, (**c**) microfibril, (**d**) microfibril, (**e**) molecular chain, (**f**) alpha helix

prevalent that the precise parts of the wool are used in textile manufacturing. Textile industry-based wastes are not used as a by-product for intelligent and technical textiles. Lanolin, also referred to as wool oil/wax/grease, has prominent molecular alcohol and grand molecular acid esters. Wool wax may be a yellowish substance, whitish in color, and soluble in organic solvents. The removal operation of wool wax from wool is completed during the washing process with emulsification. If requested, it's recoverable from the washing bath. When wool wax first leaves from washing bath, it is dirty yellowish in color, with the smell of sheep. After the purification period, wool wax attains odorless, light yellowish color with 38–44 °C freezing point commercial material. Lanolin is acquired after diluting purified wool wax. Soxhlet device is used to remove foreign materials in animal grease (Fig. 9.5).

9.2 Materials Used in the Experiments

9.2.1 Consumables

Sodium alginate (SA) with a mean relative molecular mass of 216.121 g/mol was obtained from Sigma-Aldrich (Istanbul, Turkey). Calcium chloride dihydrate was obtained from Merck (Darmstadt, Germany) to be used as a crosslinker. Mueller Hinton agar (CM0337B, Oxoid), Mueller Hinton broth media (CM0405B, Oxoid), and ampicillin containing disks (CT0002B and CT0003B, Oxoid) were obtained from Thermo Fisher, Basingstoke, UK. Dichloromethane and *N*,*N*-dimethylformamide were purchased from Sigma-Aldrich, USA. Wool oil was kindly gifted by Pozitif Wool Ltd., Uşak, Turkey.

9.2.2 Preparation and Characterization of Scaffolds

In the present study, the animal oil-modified alginate scaffolds were developed and characterized. For this objective, sample 1 solution was prepared. Sample 1 solution was found to be optimum for the 3D printing method, and animal oil was added to the solutions at different ratios of 0.5%, 1%, 2%, 5%, and 10%. The sum of the answer contents is given in Table 9.1. Firstly, sodium alginate was dissolved in distilled water; twenty milliliters ($2*10^{-5}$ m^3) of sample 1 was stirred at 800 rpm for 3 ($108*10^2$ s) h at room temperature. Secondly, different animal oil solutions were taken and added to 10 mL (10^{-5} m^3) sample 1 solution. Then, animal oil sodium alginate solution was stirred in a vortex machine for 5 min (300 s) at room (20–22 °C) temperature. The solutions were named as sample 1 (4.5% sodium alginate), sample 2 (0.5% animal oil, 4.5% sodium alginate), sample 3 (1% animal oil, 4.5% sodium alginate), sample 4 (2% animal oil, 4.5% sodium alginate), sample 5 (5% animal oil, 4.5% sodium alginate), and sample 6 (10% animal oil, 4.5% sodium alginate). Thirdly, to take out the scaffolds from lamella, 1% $CaCl_2$ dehydrate was used as a crosslinker.

Table 9.1 Table of concentrations

Samples	Alginate (%)	Wool oil (%)
Sample 1	4.5	0
Sample 2	4.5	0.5
Sample 3	4.5	1
Sample 4	4.5	2
Sample 5	4.5	5
Sample 6	4.5	10

9.2.3 Optimization of Wool Oil

Hydrous lanolin or lanolin contains 25% water. It is imperative to mention that during this study, thanks to the properties of lanolin for medicinal usage purposes, 2% of lanolin is kept at the maximum ratio. A study by Chvapil M, Gaines JA, and Gilman T (1988) showed that that lanolin with a concentration of up to 2 was not effective in the healing process, mainly within the valuation (Mariani Neto et al. 2018). The wool oil dichloromethane/dimethylformamide (dcm/dmf) with ratio 4/1 solution's density synchronized with lanolin. For this aim 0.148 g ($148*10^{-6}$ kg) animal oil was dissolved in dichloromethane/dimethylformamide (dcm/dmf) with ratio 4/1. Afterward, 4.5% sodium alginate solution was prepared and mixed with discrete animal oil solution (0.5%, 1%, 2%, 5%, and 10%). After mixing animal oil solution with 4.5% sodium alginate, the color change was observed, which was transparent to white. Since the animal oil concentration was increased, white color visibility was increased. Furthermore, after mixing animal oil solution with 4.5% sodium alginate, odor change was observed. Then, to get rid of the odor, it was ventilated. A three-day experiment was conducted. Consistent with the experimental results, the animal oil prevented sodium alginate degradation (Fig. 9.6).

9.2.4 3D Design of Scaffolds

In this study, twill woven fabric structures with a pattern of diagonal parallel ribs were designed and analyzed by the 3D printing technique. 3D scaffolds' design was inspired by twill woven structure due to its drape properties; it is aimed to cover the wound area well as the wound is not within the specific shape (Twill—Wikipedia n.d.). Twill is employed in many areas of textiles and has wound dressing properties.

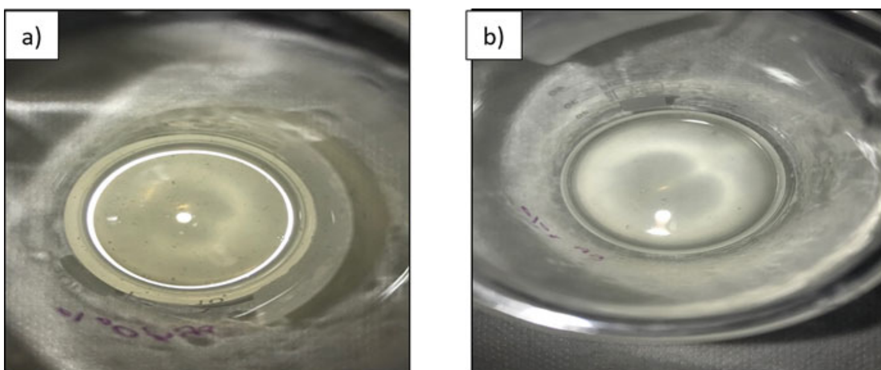

Fig. 9.6 As seen in the figure, wool oil was pretended corruption of sodium alginate solution. (**a**) 3 days after preparation of a solution of sample 2 (0.5% wool oil, 4.5% sodium alginate solution). (**b**) 3 days after preparation of a solution of sample 3 (1% wool oil, 4.5% sodium alginate solution)

The size of scaffolds was calculated and designed consistent with Wallace's rule of nines. The scaffold was produced as 20 mm × 20 mm ($2*10^{-2}$ m × $2*10^{-2}$ m); the size can change depending on the appliance area on the body. The scaffold 3D design was made in Adobe Fusion 360. The designed wound dressings were manufactured employing a modified extrusion method by 3D printer (Ultimaker 2 + Netherlands) (Fig. 9.7).

9.2.5 Characterizations

9.2.5.1 Fourier Transform-Infrared Spectroscopy (FTIR)

The wound dressing was characterized for the functional groups by Fourier Transform-Infrared Spectroscope (Jasco, FT-IR-4700). The spectrum was recorded at 400 and 4000 cm^{-1} scanning range and 4 cm^{-1}. This experiment was done to manage the modification of lanolin to SA.

9.2.5.2 Antibacterial Assay

All scaffolds were cut and sterilized using UV light (254 nm). The antibacterial assay was done according to the Kirby-Bauer procedure. The medium was Mueller Hinton agar 4 mm ($4*10^{-3}$ m) deep and poured into 150-mm ($15*10^{-2}$ m) Petri dishes. *Escherichia coli* bacteria were used. The pH level of the agar was 7.2, which was

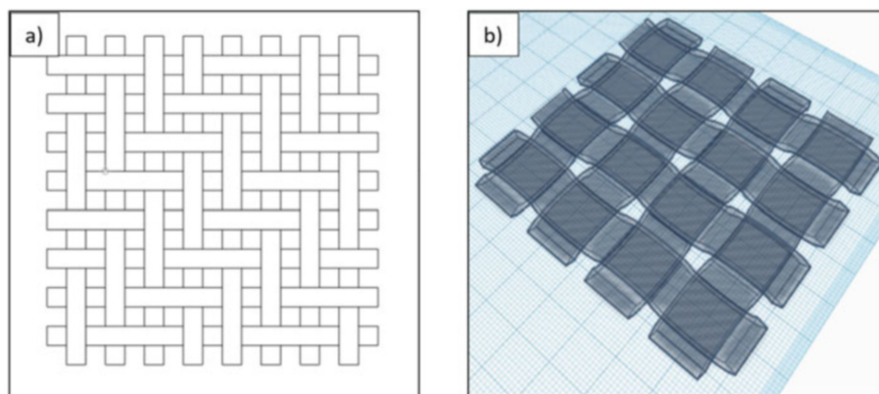

Fig. 9.7 Designing of scaffolds. (**a**) Top view of the scaffolds. (**b**) Side view of the scaffolds

sterile and was placed into the broth culture using the aseptic method. The plate was rotated 90° and was streaked again therein direction three times. It was dried for 6 min (360 s). Finally, the ampicillin antibiotic disk was used. The plate was incubated at a temperature of 37 °C.

9.2.5.3 Swelling Analysis of Scaffolds

Water-holding capacities of the scaffolds are crucial for burn wound treatment. Swelling capability may be a significant index for a scaffold that helps wound healing. Swelling tests were administered to specify the water-holding capability of scaffolds. The dry weights of the scaffolds were measured (W_0). Then the scaffolds were inserted into 1 mL (10^{-6} m^3) of PBS at 250 rpm. They were incubated in the thermal shaker. Samples were far from the buffer after appropriate intervals of 10 s using Whatman paper, and their weights were determined (W_s). The water-holding rate of the samples was calculated using the following equation.

$$\text{Swelling ratio} = \frac{W_s - W_0}{W_0}$$

9.2.5.4 Degradation Analysis of Scaffolds

The degradation of scaffolds was decided using the gravimetric method. The weights (W_0) of the scaffolds that do not contain liquid were determined. They were inserted into 1 mL (10^{-6} m^3) of PBS, pH 7.4, at 37 °C at 250 rpm. They were incubated in the thermal shaker. At the top of the acceptable interval, the samples were dried for 2.5 h ($9*10^3$ s) using Whatman paper at room temperature. Then weight (W) and, therefore, the deformation values of the scaffolds were calculated using the following formula:

$$\text{Weight loss (\%)} = \left[\frac{W_0 - W}{W_0}\right] * 100$$

9.2.5.5 pH Analysis of Scaffolds

In the present study, experiments are administered to work out the pH of the scaffold. Firstly, all the samples were taken in the middle of Petri dishes. Then, two different solutions were prepared in 0.5 mL ($5*10^{-7}$ m^3) distilled water using 0.184 g ($184*10^{-6}$ kg) of salt dihydrate ($CaCl_2$) and 1.148 g ($1.148*10^{-3}$ kg) of common salt (NaCl). The pH measurement was appropriated for a trial period of 7 days. The pH meter was immersed in each of the solutions. In each measurement, pH meter calibrated with 4.0 pH solution and 7.0 pH solution at temperature. Results were controlled using a pH paper.

9.2.5.6 Paper Chromatography

Whatman paper was used. The paper was cut in the dimensions of Petri dishes. The paper was wet using 0.73 mL ($73*10^{-8}$ m^3) Fe_3Cl solution and waited for a quarter-

hour. They were then transferred to petri dishes. Then using pressure, sample 3 solution was put at the center of the paper. Then diffusion distances were measured. Diffusion distance was calculated using the formula. Colorful area (x), scaffold area (y) calculating average.

$$\text{Diffusion distance} = (x - y)/n$$

Results found were determined with mathematical formulas and graphs. Diffusion distance versus time graph decided $y = ax^{\mathrm{b}}$ parabolic graph.

9.2.5.7 Microscopy

To determine the morphological character of scaffolds, a microscope was used. Consistent with lanolin concentration, notable changes in scaffold were seen.

9.2.5.8 Statistical Analysis

Using RStudio software, the mean and variance were calculated. All measurements were done three times. Statistical analysis of experimental results was done using RStudio software.

9.3 Results and Discussion

9.3.1 Synthesis Result

Many different commercial sorts of sodium alginate scaffold material, also as medical creams containing lanolin, are out there on the present market. This study aimed to organize sodium alginate incorporated with animal oil wound dressing. For this purpose, alginate hydrogels with and without animal oil different samples were prepared to be used as a wound dressing. The scaffolds are successfully designed and printed by making use of the 3D printer. The optimum printing was observed in sample 3. $CaCl_2.2H_2O$ was used as a crosslinker. Animal oil revenged degradation of sodium alginate solution. The previous studies showed that less than 2% lanolin does not have a medical effect. In this study, optimum printing is additionally in sample 3. Swelling tests were performed, and oil concentration increased by the swelling ratio. There are not any worthy changes in the degradation percentage on tested samples. The very best degradation percentage was observed in sample 6. Due to the very fact that animal oil softened alginate, sample 6's degradation percentage is found to be the very best value. The pH of the wound dressing is substantial for burn wound healing. It has been observed that animal oil concentration decreased the pH values.

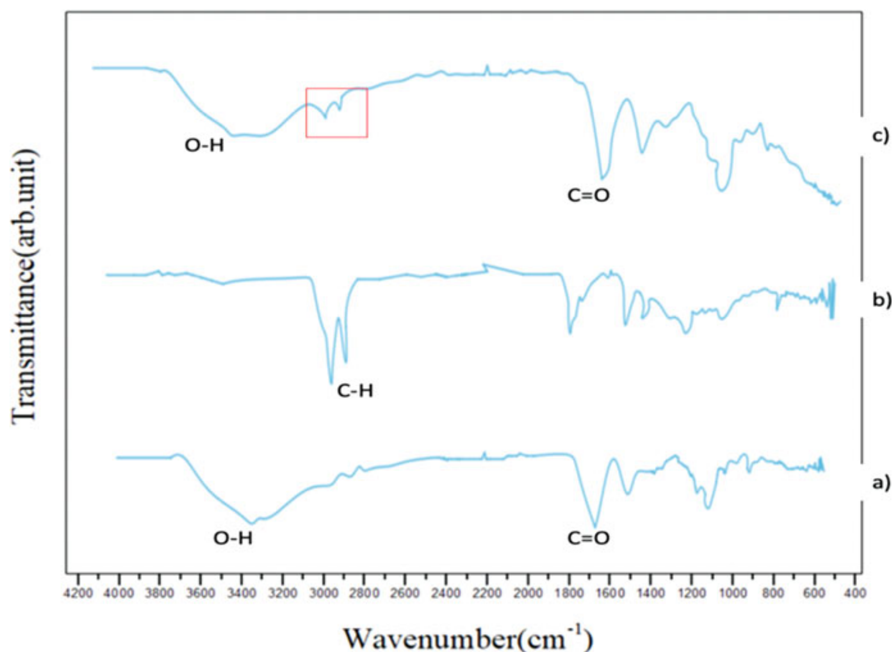

Fig. 9.8 FTIR graph of sample 3. (**a**) Pure SA, (**b**) sample 3, (**c**) the red area shows that lanolin is integrated with alginate

9.3.2 Characterization Result

9.3.2.1 Fourier Transform-Infrared Spectroscopy (FTIR)

FTIR uses the infrared region of the spectrum. It measures what proportion of infrared was absorbed by the bonds. It provides a unique fingerprint to molecules. Different functional groups absorb unique heat. During this study, FTIR was used only to manage the incorporation of lanolin into SA. As seen in Fig. 9.8, the blending of lanolin is 2900 cm^{-1} wavenumber.

9.3.2.2 Antibacterial Assay Result

Determining the growth inhibition zone of wound dressing is proper. Lanolin has antifungal and antibacterial activity. Lanolin has the same chemical composition as human skin (Lanolin, Wool and Hand Cream—OpenLearn—Open University n.d.). This study showed that lanolin has antibacterial activity. It was compared with the ampicillin zone, and *Escherichia coli* was used. The disk diffusion method was used (Fig. 9.9).

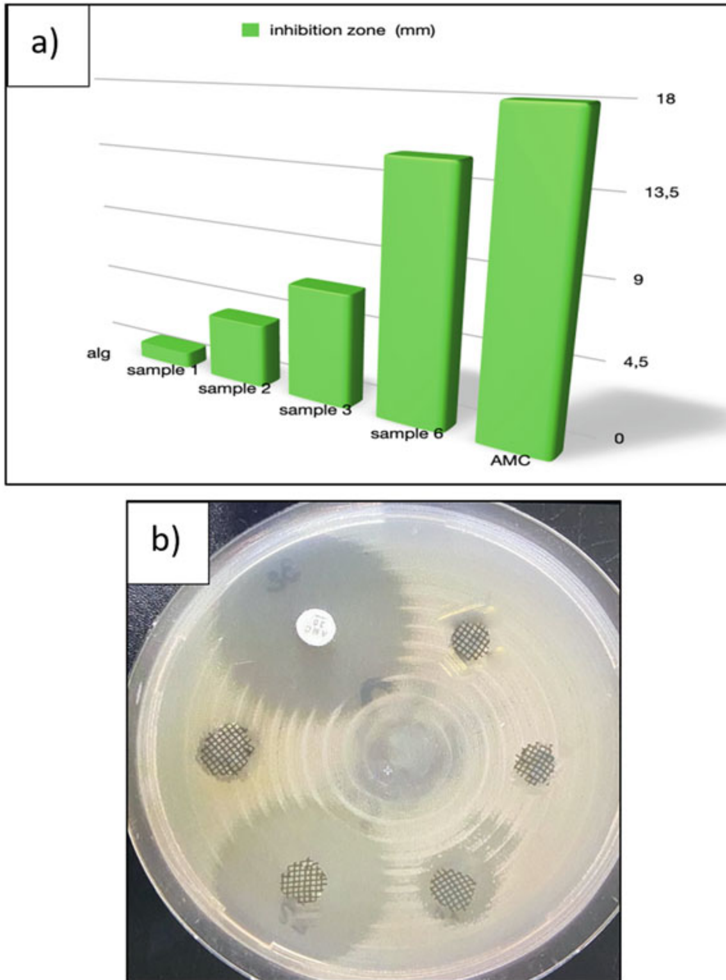

Fig. 9.9 İnhibition zone graph

9.3.2.3 Swelling Analysis of Scaffolds

The swelling capacity of scaffolds is decided by the quantity of liquid material absorbed by them. The swelling and elastic forces refer to the swelling capacity of hydrogels. Therefore, the swelling parameters allow hydrogel scaffolds to regulate the diffusion of bioactive molecules and cell migration through the complex network structure (Zhu and Marchant 2011). Second-degree burns affect the skin, called the dermis. Infection and dehydration are the main vital problems for the burned patients. When an individual is burned and depending on the severity of the burn, the blood vessels, including the capillaries, could also be affected. Combined with the discharge of chemicals into the blood, this may increase

capillary permeability to fluids, resulting in leaking fluids from the blood vessels into the tissues. The upper the share of burned skin, the more severe the loss of fluid is going to be and therefore, the more significant dehydration will directly affect the skin (Third Degree Burns and Dehydration—Burn Injury Resource Center—September 13, 2011 n.d.). Moist-providing wound dressings are suitable for second-degree burn wounds. The sort controls the total absorbency and swelling capacity and degree of cross-linkers want to make a gel (Advanced Textiles for Wound Care—Google Kitaplar n.d.). During this study, a 1% salt dehydrate solution was used as a crosslinker.

Alginates are one of the leading materials for developing wound dressings due to their high absorbency values, which help to provide an optimal moist environment. The swelling and diffusion properties allow the wound exudates to be absorbed and stop causing secondary trauma upon removal. The alginate dressing is often applied either pre-wetted to provide a desiccated wound with moisture or dry to assist in the absorption of exudates (Advanced Textiles for Wound Care—Google Kitaplar n.d.). During this study, the swelling behavior of samples 1, 2, 3, 4, 5, and 6 was analyzed. The lanolin percentage in sodium alginate solution increases the swelling ratio of scaffolds increases. The minimum ratio of swelling was found in sample 2, and the maximum swelling ratio was found in sample 6. This shows that when animal oil solution enters in alginate solution, swelling changes toward the ratio of animal oil. It is surprising that as a result, animal oil addition in the structure increases the water capacity of the alginate wound dressing (Fig. 9.10).

9.3.2.4 Degradation Analysis of Scaffolds

Degradation percentage is critical for the preservation of burns. The wound dressing may be a physical barrier to prevent physical damage to the wound. Burn wounds are dynamic and alter in appearance, particularly within the first 48 h ($1728*10^2$ s). Therefore, it is the practice of the burn services to review burns after 48 h ($1728*10^2$ s) before decisions regarding definitive dressings or surgery are made. The initial burn dressing should be one that will remain intact for 48 h ($1728*10^2$ s) and stop infection. It is a protocol in the burn service to cover all burns with dressing for 48 h ($1728*10^2$ s) (Douglas and Wood 2017). Therefore, degradation percentage is vital in 48 h ($1728*10^2$ s). During this study, degradation tests were administered in appropriate time intervals; degradation was not visible and memorable. At 72 h ($2592*10^2$ s), degradation was visible. The very best degradation percentage was in sample 6 at the appropriate interval. The minimum degradation percentage was observed in sample 1 at the appropriate interval. Therefore, animal oil solution affects degradation percentage; consequently, as animal oil solution concentration increases, degradation percentage increases. In sample 1, degradation was also observed. This might be described by the presence of alginate as an elementary material in both hydrogels, which covered Ca^{2+} ions. When alginate-based hydrogels were dipped in PBS media containing monovalent ions like Na^+, these ions could compete with original Ca^{2+} ions and begin to degrade the hydrogels over time, thanks to a natural process reaction between Ca^{2+} ions and Na^+ ions (Droge and Goss 2012) (Fig. 9.11).

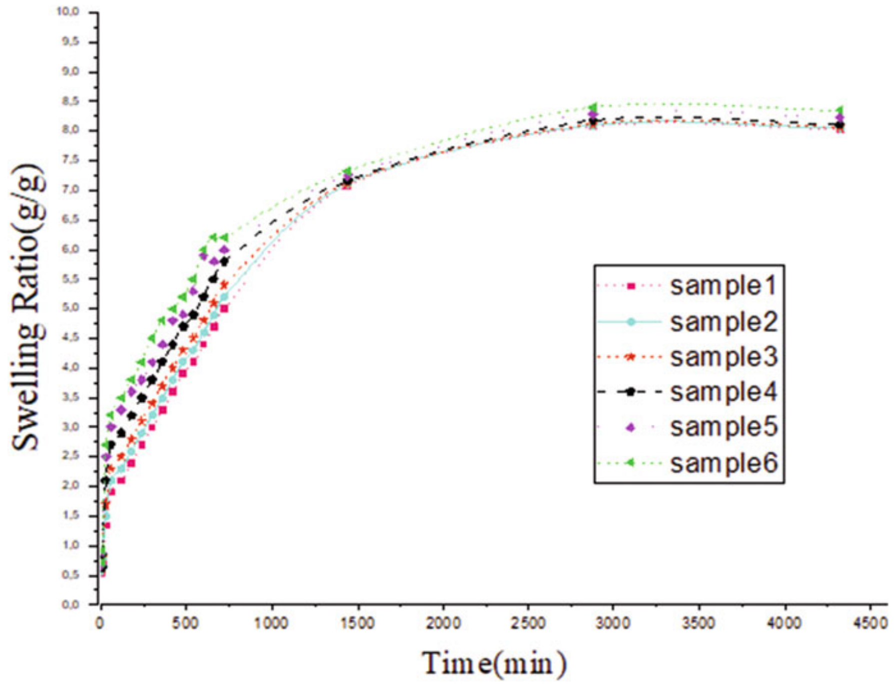

Fig. 9.10 The swelling graph of samples

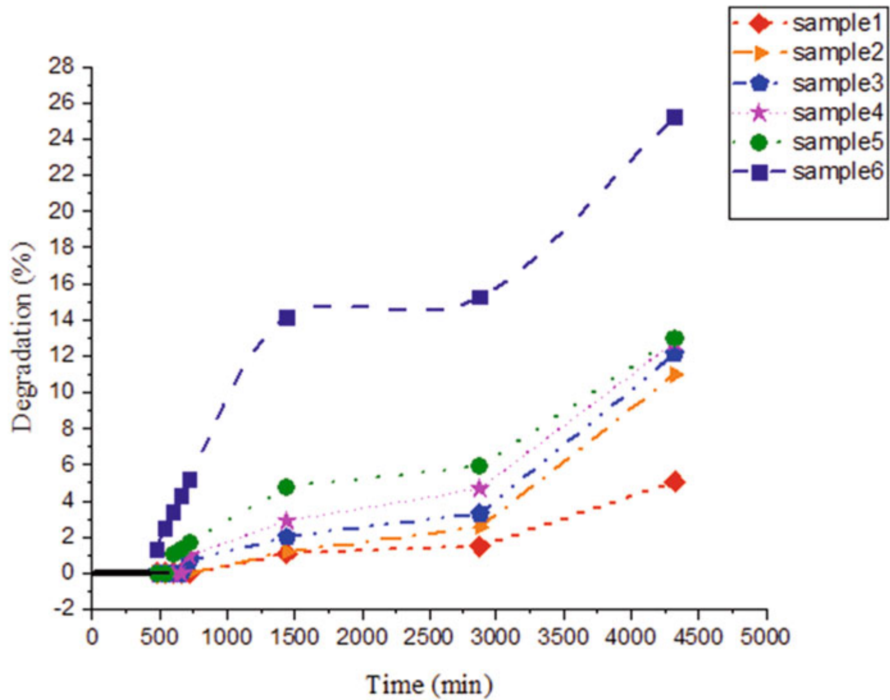

Fig. 9.11 Degradation pattern of samples

9.3.2.5 pH Analysis of Scaffolds

Wound healing may be a difficult period. It has been proven that the surface pH of a wound plays a crucial role in wound healing because it helps control infection and increases antimicrobial activity, oxygen release, and bacterial toxicity. The previous study showed that an acidic environment created by the use of acids, algin, ethanoic acid, boric acid, vitamin C, and mucopolysaccharide helps in wound healing (Nagoba et al. 2015).

In pure water, the concentrations of H_3O^+ and OH^- are equal. However, when acid and base are added to water, the H_3O^+ and OH^- ions are not any longer present in equal amounts. By comparing the values of H_3O^+ and OH^-, pH is often determined (General Chemistry: Principles and Modern Applications—Ralph H. Petrucci, F. Geoffrey Herring, Jeffry D. Madura, Carey Bissonnette - Google Kitaplar n.d.). In this study, pH values were primarily stable. This shows that samples decreased the pH of the control solution. The pH changes of all samples were observed on the fourth, fifth, and sixth days. The sample degradation was not marked at a temperature in two of pH measurement solutions. Thus, the dressing was not dissolved in the solution, and ion change did not occur and these changes are not remarkable. The pH range of solutions was 5.38 to 5.08. The solutions are acidic and suitable for burn wound healing (Fig. 9.12).

9.3.2.6 Paper Chromatography

Paper chromatography is employed to separate mixtures into individual substances. It is utilized in the process to separate substances and determine the quality of drugs. Vitamins, preservatives, amino acids, and proteins are analyzed by chromatography. Also, detection of alcohol in the blood is often done by using chromatography (How Is Chromatography Used in Industry? n.d.). During this study, diffusion distances were measured and calculated. As seen in Fig. 9.13, diffusion distances are increasing in 4 h ($144*10^2$ s). After 4 h ($144*10^2$ s), oscillation has proceeded and distance is stabilized.

9.3.2.7 Microscopy

Microscopy is employed to magnify small objects. During this study, the microscope is employed to define how lanolin concentrations affect the structure of wound dressings. As seen in Fig. 9.14, we can observe as oil concentrations increase, the visibility of the cell membrane increases. As oil concentration increases, the pore size increases (Fig. 9.15).

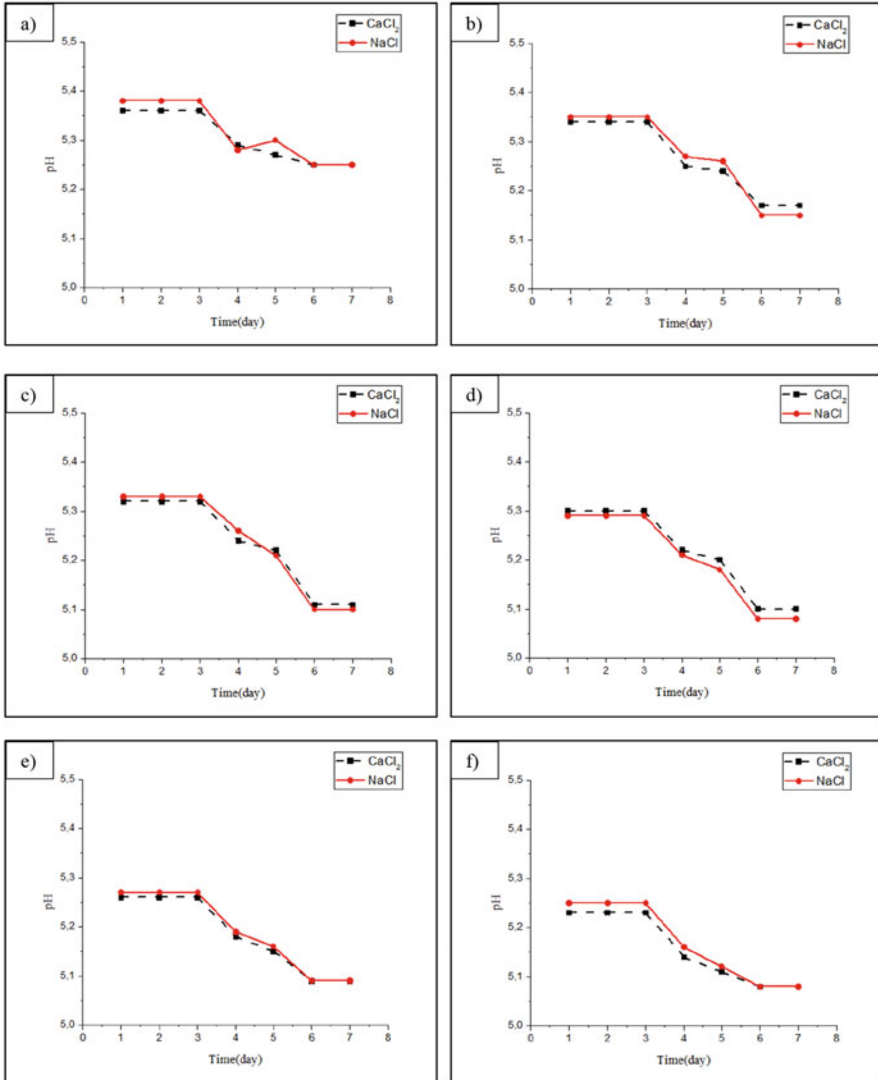

Fig. 9.12 The pH graph of samples. (**a**) Sample 1, (**b**) sample 2, (**c**) sample 3, (**d**) sample 4, (**e**) sample 5, and (**f**) sample 6

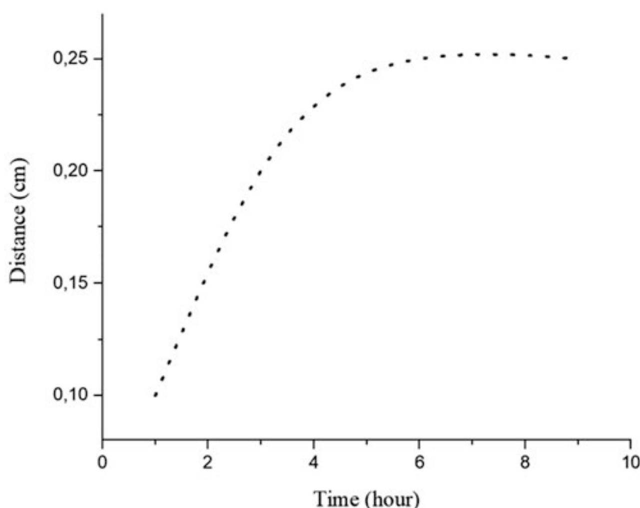

Fig. 9.13 Distance vs. time graph of oscillation

9.4 Conclusion

In conclusion, lanolin is a widely used substance in pharmacy. Also, animal grease is used in the past to protect the wound from the environment. Wool oil concentration is crucial for wound dressing due to 3D printability and healing criteria. In this study, the scaffold was designed to be inspired by a twill textile structure using Adobe Fusion 360 3D drawing program, and many tests have been done. These tests are FTIR, antibacterial assay, swelling, degradation, pH, paper chromatography, and microscopy analysis. Antibacterial assay results showed that lanolin has an antibacterial property. Swelling tests and degradation tests showed that as wool oil concentration increased, swelling and degradation increased. pH analysis showed that there is no notable change as oil concentration is increased. Paper chromatography results showed that wound dressing has oscillation. Microscopy analysis showed that as wool oil concentration is increased, the visibility of the cell wall is increased. All experiments were repeated three times; all statistical analyses were done using RStudio. Due to 3D printing difficulty, more than 5% concentration of wool oil is not appropriate for manufacturing.

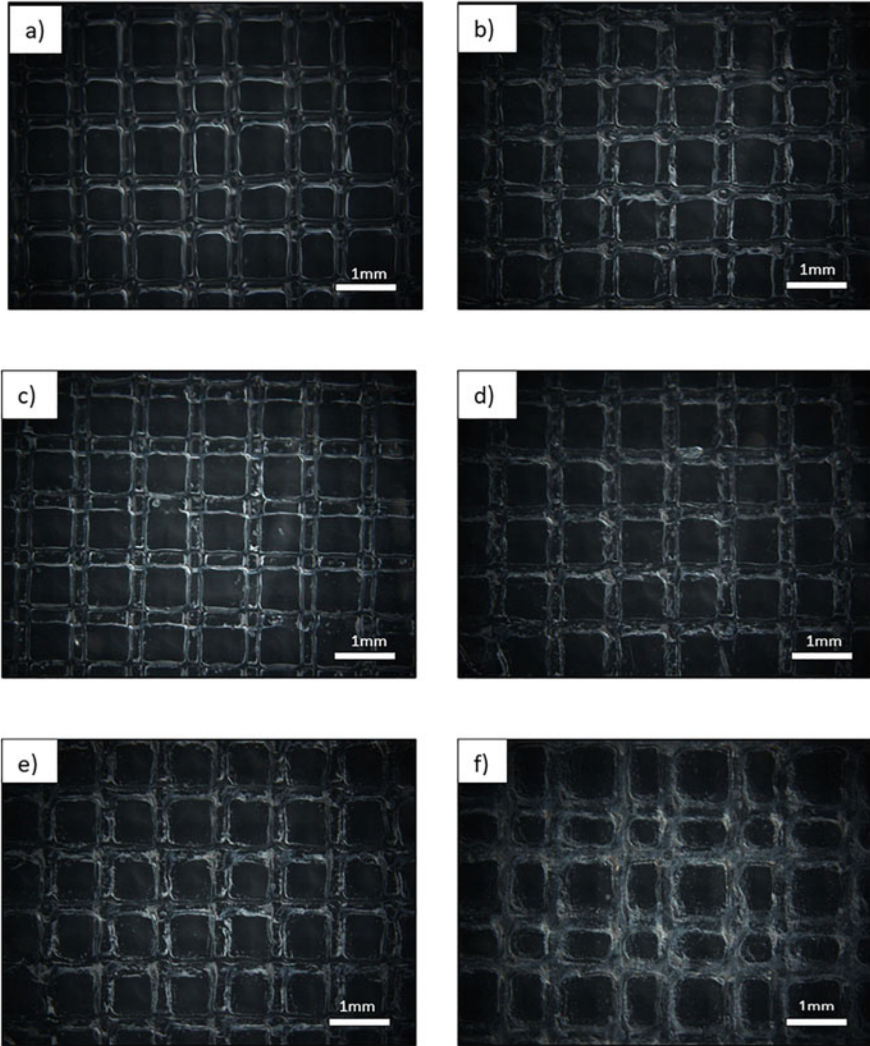

Fig. 9.14 Microscope pictures of samples. (**a**) Sample 1, (**b**) sample 2, (**c**) sample 3, (**d**) sample 4, (**e**) sample 5, and (**f**) sample 6. As seen in the figure, as oil concentrations increase, the visibility of the cell wall increases

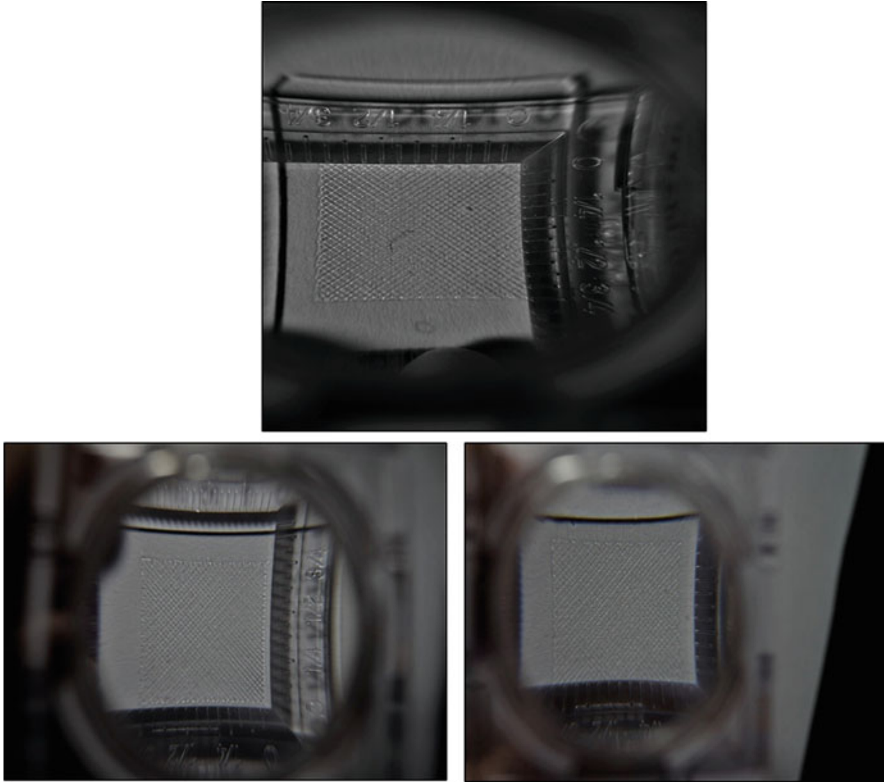

Fig. 9.15 Photographs of scaffolds

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Engineering Biomaterials for Testicular Tissue Engineering and In Vitro Spermatogenesis

10

Rakesh Bhaskar, Balaram Mishra, and Mukesh Kumar Gupta

Abstract

Male infertility is a severe medical and social problem worldwide, with one in every 15 males being diagnosed as infertile. In any case, the loss of fertility is due to pre- or post-meiotic barriers to spermatogenesis and is challenging to treat. The regular treatments include supplementation or substitution of androgen and testicular prosthetic graft but have been found to cause medical complications. To date, no appropriate methods mimicking the physiological conditions have been developed in the reconstruction of testis functions. Testicular tissue engineering is emerging as a potential approach to solve this problem. It requires developing a scaffold with a unique structure and morphology and mimics the extracellular matrix (ECM) structure to imitate various functions performed by the ECM. This chapter discusses different biomaterials that might provide structural support for the spatial distribution of cells to create a niche for in vitro spermatogenesis.

Keywords

Infertility · Scaffold · Microtubular architecture · Testicular tissue engineering · In vitro spermatogenesis

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10.1 Introduction

Infertility has significant demographic and health impacts on the human population. It is progressively increasing in the urban males worldwide and is a cause of societal stigma to the affected individuals (Kumar and Singh 2015). Various medical interventions and strategies are available to treat male infertility, but it remains a thrilling medical challenge. Among various interventions, hormone augmentation and testicular tissue grafting have helped restore fertility (Caires et al. 2012). More recently, testicular transplantation of male germline stem (GS) cells has been viewed as a novel approach for the preservation and restoration of male fertility. However, GS cell transplantation cannot restore fertility in cases wherein infertility is associated with pre- or post-meiotic barriers to spermatogenesis, which accounts for 20–25% of spermatogenic disorders in humans (Poongothai et al. 2009). In such cases, it is imperative that the GS cells be induced to initiate and complete spermatogenesis in vitro (Jung et al. 2010; Kanatsu-Shinohara et al. 2005; Ning et al. 2012). However, appropriate methods of mimicking the physiological condition of in vitro spermatogenesis are suboptimal. Moreover, donor-derived GS cells are needed to be injected into a recipient testis to achieve in vitro spermatogenesis under the air-liquid interface of organ culture, which is not feasible in humans (Sato et al. 2013).

Testicular tissue engineering offers an attractive opportunity to prevent the need for donor-derived testis for in vitro spermatogenesis by the development of artificial tissue constructs. The tissue-engineered constructs can be fabricated to resemble testicular tissue's three-dimensional structures and provide the appropriate niche for GS cell proliferation and differentiation. Studies have shown that in vitro spermatogenesis can occur in the microenvironment of engineered testicular tissue that provided three-dimensional niches to GS cells, mediated by hormones and paracrine/autocrine factors (Discher et al. 2010). Further, the tissue-engineered constructs can also be fabricated to reconstitute the testicular tissue, using GS cells, for in vitro spermatogenesis in an organ culture system. Tissue engineering with the patient's autologous cells or immunologically inactivated xenogeneic or allogeneic cells can also overcome the pitfalls in the restoration of missing tissue function and may offer novel remedial techniques for testicular diseases that cannot be cured with the currently available clinical methods (Discher et al. 2009).

Classical tissue engineering involves the seeding of three-dimensional scaffolds to develop an appropriate microenvironment for attachment and proliferation of cells and reconstruct injured tissues or organs. These specifically designed scaffolds should imitate the innate in vivo microenvironment and allow cell interaction and response to the mechanical signal acquired from the molecules present in the neighboring 3D environment (Bi et al. 2014). Therefore, the material characteristics of the scaffolds used for tissue engineering are essential in regulating cellular response and fate.

Various natural or synthetic polymers and their copolymer have been used previously to prepare engineered tissue constructs from tissues of multiple origins. Thus, different biopolymers may be explored to design the matrices and scaffold that

can mimic the extracellular matrix (ECM) and eventually assemble into complex functional tissue of the testes (Loh and Choong 2013). Polymers having desirable properties such as biocompatibility, nontoxicity, relatively inexpensive, and gelation by supplement of divalent cations like Ca^{2+} ions may be explored. Highly porous biodegradable and biocompatible scaffolds with interconnected macropores can be fabricated from biopolymers and copolymers of natural materials such as silk and chitosan and synthetic materials such as poly(D,L-lactic acid-co-glycolic acid) and polycaprolactone. The scaffolds can be prepared by different molding methods, such as particulate leaching, freeze gelation, and/or freeze-drying process, and can be impregnated with various bioactive molecules (e.g., GDNF, LIF, bFGF, and ATRA) for a time-controlled release. Besides, the electrospinning technique can also be used to produce nanostructured fibers that can be aligned to mimic the micro-architecture of the testes by layer-by-layer (LBL) and/or solid free-form (SFF) fabrication technique to fabricate the 3D scaffolds.

10.2 Scaffolds for Testicular Tissue Engineering

Material characteristics of the scaffolds used for tissue engineering are essential in regulating cellular response and fate. However, only a few biomaterials, derived from natural or synthetic sources, have been employed in testicular tissue engineering to restore the functionality of spermatogenesis (Baert et al. 2017). Further, demonstrations of developing haploid spermatids or spermatozoa from the GS cells have been limited. To be useful in testicular tissue engineering, the scaffold should provide a suitable niche for spermatogonial stem cells (SSCs) to self-renew and complete spermatogenesis in vitro, which requires optimization of scaffolds' physicochemical characteristics and organ culture systems at the air-liquid interface (Fig. 10.1). The diameter of seminiferous tubule ranges from 80 μm to 300 μm in mammals, and thus, the scaffold for testicular tissue engineering should be tailored to provide desired porosity for cells to proliferate and populate. However, unlike natural seminiferous tubules, SSCs do not seem to require tubular morphology of the scaffolds. Studies have shown that SSCs can self-renew in scaffolds that were nontubular, provided suitable factors (e.g., GDNF) and nutrients (e.g., lipid-rich albumin) were available in the culture medium (Azizi et al. 2017). The scaffold to be used for testicular tissue engineering should also have some fundamental properties such as biocompatibility and biodegradability. The scaffold must not evoke an immune response to preclude it from generating a dreadful inflammatory reaction by the body. It should also be biodegradable to permit the cells to secrete their native ECM (Drury et al. 2004). The by-products of the scaffold biodegradation should be nontoxic and should be able to clear from the body without causing any intervention to other organs of the body. Preferably, the scaffold must also possess mechanical characteristics compatible with the anatomical locale into which it has to be incorporated. In addition to these facts, scaffolds should also have high porosity with interconnected pore structure to promote cellular dissemination and sufficient

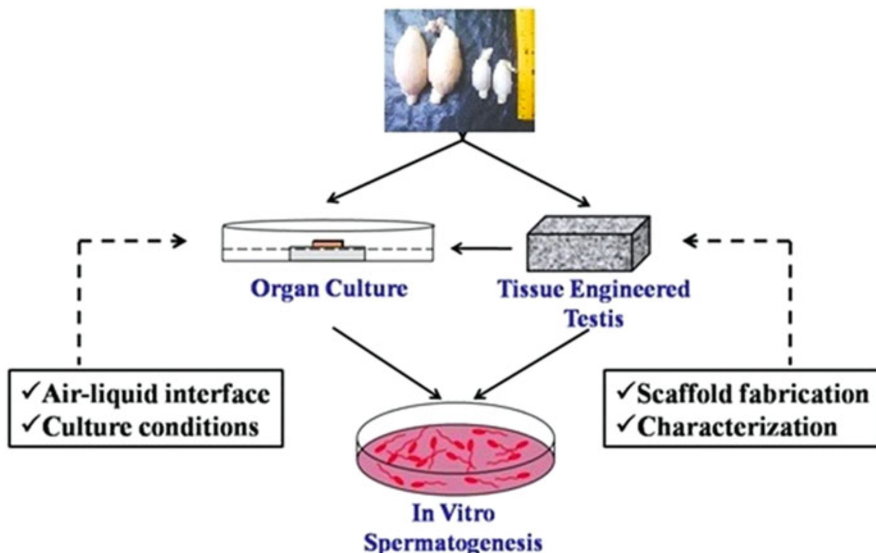

Fig. 10.1 In vitro spermatogenesis by testicular tissue engineering. The biomaterial-derived scaffold may replace the need for donor-derived testicular tissue for in vitro spermatogenesis from SSCs under air-liquid interface culture

nutrient distribution to cells present within the model and the extracellular matrix produced by these cells.

When generating artificial tissues by testicular tissue engineering, the scaffold has to carry out the essential functions performed by the ECM, at least for the time required for the tissue construct to fully integrate and remodeled by the native tissue following implantation (Loh and Choong 2013). However, it remains a challenging task to construct scaffolds that convene all the necessities depicted above. Hence, several researchers have also attempted to utilize the native ECM with their native chemical composition and physical properties such as structure at micro and nanometer scale, or unique properties such as anisotropy that is an essential characteristic of most tissues (Wang et al. 2020; Baert et al. 2015; Peng et al. 2017; Rezaei Topraggaleh et al. 2019). Biomaterials, derived from natural sources, are generally used to restore or replace the structure and function of impaired tissue or organs because of their advantages over synthetic biomaterials (Chawla et al. 2020). They can effectively bolster cell attachment, proliferation, movement, and differentiation of testicular stem cells (Bi et al. 2014; Baert et al. 2017; Rezaei Topraggaleh et al. 2019; Richer et al. 2020). In particular, when introduced into a damaged area, nature-derived biomaterials can improve the adhesion and movement of cells in the local environment, impel the formation of extracellular matrix, and enhance tissue repair (Campiglio et al. 2020).

Biological materials such as collagen, alginate-based substrates, chitosan, and various proteoglycans are frequently utilized to create scaffolds for testicular tissue

engineering (Atala 2012; Baert et al. 2017; Shams et al. 2017; Jahanbani et al. 2020). Unlike scaffolds produced from a synthetic polymer, natural polymers tend to be biologically active and mainly stimulate excellent growth and cell adhesion. However, creating scaffolds from biologically derived materials, having uniform and consistent structures, poses an engineering challenge. Scaffolds having regular, aligned microtubular structures would be perfect for testicular tissues (Baert et al. 2015; Peng et al. 2017). However, they are challenging to fabricate. Various biomaterial-derived scaffolds with the 3D structure have been shown to regulate cellular proliferation and regeneration in both testis and other tissues (Oğuzkurt et al. 2007; Baert et al. 2015) and need to be further fine-tuned to develop tissue-like constructs. However, in most cases, the development of 3D scaffolds for in vitro spermatogenesis supported their differentiation up to the pachytene stage (Gohbara et al. 2010), and very few have been successful in the generation of fertilizing spermatozoa (Sato et al. 2011, 2013).

10.2.1 Porous Scaffolds

Biomaterial porous scaffolds are the best choices for testicular tissue engineering owing to the large natural diameter of seminiferous tubules (Peng et al. 2017). Mainly three types of scaffolds are being used in this field, such as tissue-derived scaffold through decellularization, scaffold consisting of natural polymers, and scaffold made up of synthetic materials (Atala 2012; Jahanbani et al. 2020). The porous scaffolds mimicking the ECM and subjected to slow degradation are ideal for the generation of testicular tissue matrices (Del Vento et al. 2018). Such porous scaffold can provide an environment for sustained cell growth and form the tissue construct using growth factors and biologically active molecules. However, so far, testicular tissue engineering using a porous scaffold failed to create a transplantable artificial testis, although they could re-establish or maintain the cells within them under in vitro conditions.

10.2.2 Bio-Printed 3D Scaffolds

Bioprinting is an advanced technique to construct a 3D scaffold with precise geometry and clear pore structures that can enhance scaffold fabrication for personalized architecture and biomimicry. The 3D printing of biological scaffold and printing the cells in required places together is called bioprinting. A testicular scaffold has been reported to be fabricated using 3D printing of alginate and was shown to promote testicular organoid formation (Baert et al. 2019). However, the biomimicry to testis tissue was not observed (Baert et al. 2019). The development of bioprinting in testicular tissue engineering is still a challenge because of bioink's shape fidelity.

10.3 Biomaterials for Testicular Tissue Engineering

10.3.1 Decellularized Testicular Scaffolds

10.3.1.1 Extracellular Matrix of Testis

The basement membrane is the ECM found between the connective tissue and epithelial structures in mammals. In the testis, the basement membrane that separates the seminiferous tubules from the connective tissue is denominated a tubular wall and constitutes collagens, laminins, and fibronectin (Baert et al. 2015). The interstitial compartment of the testis is also rich in ECM proteins that support the connective tissue and the vasculature (Oğuzkurt et al. 2007; Baert et al. 2015). In addition to their contribution to tissue integrity, the components of the ECM are also biologically active. More specifically, the ECM components of the tubular wall are involved in the transit regulation of growth factors, such as transforming growth factor beta 3 (TGF-β3), between the interstitium and the seminiferous tubules. Moreover, collagens and laminins and peptides resulting from their degradation contribute to the regulation of blood testis barrier (BTB) cellular permeability during spermatogenesis (Siu and Cheng 2004). The decellularized testicular matrix (DTM) may thus provide a microenvironment of the testis and could be helpful for structural support for cell attachment, proliferation, and property of native testis as a scaffold.

10.3.1.2 Decellularized Testicular Matrix

Decellularization of a testicular tissue can be done by physical, enzymatic, or chemical treatments to remove cells and cellular materials while the construct around the cells remains intact. The ECM is highly conserved between species, and therefore, it is also being investigated as a potential biomaterial for testicular tissue engineering. Decellularization protocol has been investigated by several researchers and in our laboratory to obtain optimal testis-derived scaffold, which could retain significant ECM components such as collagen, fibronectin, and glycosaminoglycan (Rezaei Topraggaleh et al. 2019). The DTM has also been obtained from human testicular tissue by applying sodium dodecyl sulfate (SDS) and Triton X-100 to decellularize and develop 3D scaffolds with important ECM contents (Baert et al. 2015). Testicular tissue-derived scaffold can also be utilized to understand the role of tissue matrix interaction in seminiferous tubule formation and spermatogenesis (Vermeulen et al. 2018). Testis organoids, produced from human testis tissue constructs, were shown to support spermatogonial cell proliferation and secrete testosterone (Gargus et al. 2020). Human Sertoli cells have also been cultured on decellularized porcine testis ECM and were suggested to be suitable for human testicular tissue engineering.

The desired testicular stem cells may be cultured on the DTM scaffolds, which contain the ECM, growth factors, nutrients, proteins, etc., required by the cells to proliferate and differentiate into a tissue (Fig. 10.2). The recellularization of the desired testicular stem cells on DTM aims to mimic the native testicular environment to give rise to new testicular cells that can produce the sperms. The DTM scaffolds may be a good model for solving the problem of infertility in males.

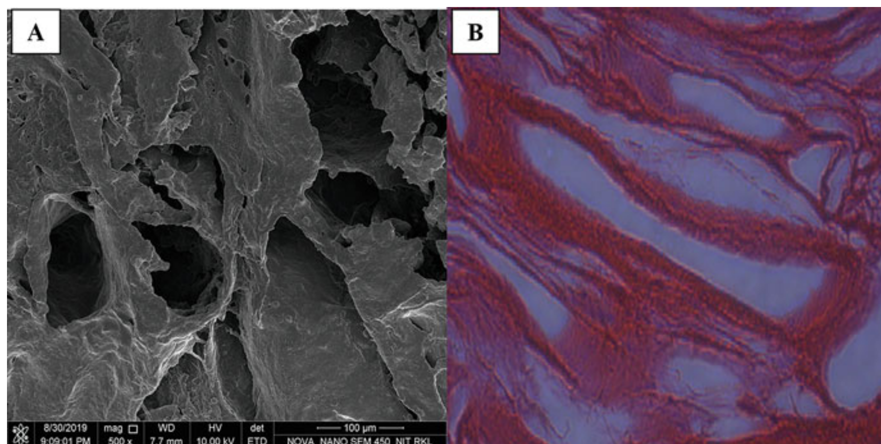

Fig. 10.2 Decellularized testicular matrix (DTM) as a scaffold for testicular tissue engineering. (**a**) Scanning electron micrograph of DTM; (**b**) Hematoxylin–eosin-stained DTM

10.3.2 Natural Biomaterials

Natural biomaterials for testicular tissue engineering have the biocompatibility, biodegradability, and physicochemical properties of several native tissues. Natural polymers such as chitosan, alginate, silk, collagen, and hyaluronan can maintain the native organ's biochemical activities and microenvironment. A number of natural polymers have thus been investigated for their use in testicular tissue engineering and are elaborated below.

10.3.2.1 Collagen

Collagen is the most abundant protein found in the ECM, and it contributes to cells' dynamic behavior and intracellular communication. The porous scaffold made up of collagen mimics the tissue integrity and is highly biocompatible. Three-dimensional collagen fiber scaffolds provide microstructures, good mechanical behavior, and quick integration with new tissue matrix with proper adhesion (Jahanbani et al. 2020). Additionally, collagen is having higher flexibility and tensile strength, which helps maintain the scaffold's flexibility. Collagen's tripeptide arginine-glycine-aspartic acid (RGD) domain helps to adhere to the cells through adhesion molecules present in the cell surfaces. There is evidence to culture spermatogonial stem cells on a collagen-coated surface for enhanced self-renewal (Shams et al. 2017).

10.3.2.2 Alginate

Alginate is an α-L-guluronate and β-D-mannuronate containing polysaccharide, mostly recovered from the marine algae. Alginate is biocompatible and biodegradable and gives better adhesion to the cells. An alginate scaffold was found suitable for neovascularization, spermatogonial cell survival, and seminiferous tubule integrity (Poels et al. 2016). Sodium alginate cross-linked with calcium (Fig. 10.3) and

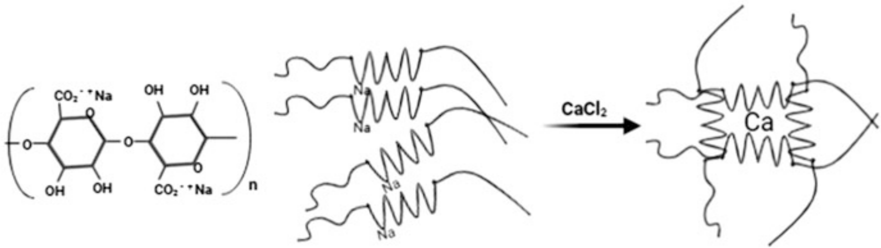

Fig. 10.3 Cross-linking behavior of the sodium alginate and calcium ions

derivatives are primarily used in tissue engineering for biological applications. Alginate is suitable for fabricating scaffolds of porous structures having a pore diameter of 200 μm, while others are also subtle to find good porosity. Alginate hydrogel scaffold has low toxicity, allowing differentiation of male germ cells to haploid cell stage (Del Vento et al. 2018). A different type of cross-linking techniques can produce hydrogels from alginate, and their structural resemblance to ECM of natural tissues permits a variety of uses in wound repair, the release of bioactive molecules such as minute chemical therapeutics and proteins, and cell transplantation (Baert et al. 2019; Chawla et al. 2020).

Alginate possesses the exceptional property of creating tubular channels via polyelectrolyte complexation with positively charged polymer chains (Fan et al. 2014). When alginate makes contact with electrolytes, coating through a membrane or covering develops a gel with aligned channels of microtubular structure within the gel (Zhao et al. 2016). The concept behind these structural arrangements is the complexation of alginate polymer. The electrolyte results in excluding water molecules from the gel in the reverse direction of the developing gel layer. Later, it was revealed that the basis of this channel formation is diffusion as well as convection. Thus, various fabrication techniques have been employed for the development of a microtubular scaffold with calcium alginate and copper-capillary alginate. Such alginate scaffolds have a great potential in mimicking the seminiferous tubules of vertebrates because of their prominent tubular nature. Biological characterization of these gels can be done to verify their compatibility with animal cells, especially spermatogonial stem cells (SSCs). A regular, microtubular structure is drawn onto the newly forming alginate gel due to the convective-like process that the system goes through to release energy (Chawla et al. 2020). The porosity of scaffolds can be tuned by controlling the preparatory alginate and which methods are employed. In one study, macroporous scaffolds were produced by ionotropic gelation of sodium alginate and copper sulfate (Kubota et al. 2004). Channels of diameter ranging from 100 μm to 250 μm were obtained in the anisotropic gel (Fig. 10.4). The tubular nature of the scaffold mimicked seminiferous tubules in dimension (Reda et al. 2016).

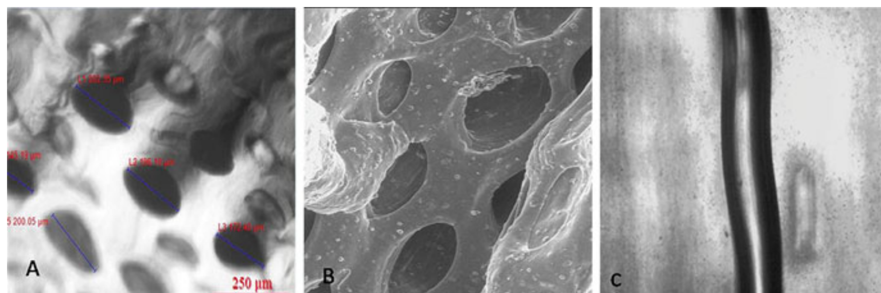

Fig. 10.4 Macroporous aligned scaffolds fabricated from alginate. (**a**) Cross-section of scaffolds under optical microscopy. (**b**) Cross-section of scaffolds under electron scanning microscopy. (**c**) Longitudinal section showing pore channels with a pore diameter of 100–250μm

10.3.2.3 Silk

Silk is a natural protein produced by spiders and silkworms. The coating-free (sericin) silk is highly biocompatible and enhances adhesion, movement, proliferation, and differentiation of cells. This has excellent mechanical properties like elasticity, elongation, toughness, and ductility. Additionally, the silk scaffold can be made in different forms like sponge, fibrous, powdered, and hydrogel (Peng et al. 2017; Atala 2012; Narimanpour et al. 2020). Silk electrospun nanofibrous scaffold is suitable for SSC culture because it provides a microenvironment for the SSC attachment and proliferation (Narimanpour et al. 2020). The limitations of silk scaffold are fast degradation and restricted length for revascularization.

10.3.2.4 Fibrin

The use of fibrin in the tissue engineering field is widespread as an alternative to collagen. Fibrin is derived from blood and used to construct scaffolds for tissue engineering and regenerative medicine. The most important properties of fibrin are controlled degradation and high porosity level up to 1-μm-diameter pores. Other blood-derived bioactive substances are also being useful to the cells for growth and proliferation. Collagen and fibrin matrix gel has also been used as a basis of cryoprotective medium for testicular tissue (Volkova et al. 2018). The fibrin-based scaffold also triggers angiogenesis and spermatogonial cell survival (Del Vento et al. 2018) and can be used as an injectable system.

10.3.2.5 Hyaluronic Acid

Hyaluronic acid (HA) or hyaluronan is a linear polysaccharide found in the extracellular matrix of most animal tissues. It has some distinctive biological functions, such as maintenance of homeostasis in tissues and permeability regulations. HA is biodegradable, biocompatible, and non-immunogenic, which allows its use as a promising biomaterial for testicular tissue engineering (Wasupalli and Verma 2018). Hyaluronans' unique hygroscopic property and viscoelasticity allow the creation of a suitable microenvironment for cell attachment and migration. HA coating on biomaterial scaffold also allows binding with sperm receptors present

in mature sperm cell surfaces and increases sperm motility, which is critical for fertilization (Morra 2005).

10.3.2.6 Chitosan

Chitosan is a natural polymer, deacetylate version of chitin, and used in different tissue engineering applications. It has unique physicochemical properties for the designing and fabrication of desired scaffolds. Chitosan has excellent potential for combining with other biomaterials, nanoparticles, cells, and other substances for testicular tissue engineering. It has particular biological and mechanical properties, which mimic the natural ECM. Chitosan also has high biocompatibility, biodegradability, and strong antimicrobial properties against Gram-positive and Gram-negative bacteria. These properties make chitosan a common choice for testicular tissue engineering (Ahsan et al. 2018). The microfibrous/tubular architecture scaffolds made up of chitosan provide adequate tensile strength, swelling behavior, and biodegradability. Bearing such property of chitosan may be a success in obtaining functional tubular structures with the capability of promoting the regeneration, repair, and replacement of damaged, injured, or lost tissues (Silva et al. 2018). The microporous scaffold promotes cell adhesion, differentiation, proliferation, tissue integration, and vascularization. Chitosan provides functionality to biologically active molecules such as laminin peptides, RGD containing peptides, and γ-polyglutamic acid, thereby supporting cell growth, differentiation, and proliferation (Ahsan et al. 2018). Moreover, chitosan also promotes encapsulation and sustained release of cytokines, growth modulators, chemokines, etc., and adds to the application of chitosan as a microenvironment molecule for testis tissue engineering.

10.4 Synthetic Biomaterials for Testicular Tissue Engineering

Synthetic polymers are the new prospects for testicular tissue engineering due to their ability to be tailored for mechanical properties such as elasticity, mechanical stability, and controllable degradation ability (Shams et al. 2017; Del Vento et al. 2018). Synthetic materials are readily available than natural materials for tissue engineering applications. However, they may cause inflammatory reactions and immunogenicity upon their transplantation and have not been used extensively. Synthetic polymers like polylactic acid (PLA), polyglycolic acid (PGA), polylactic-co-glycolic acid (PLGA), and polycaprolactone (PCL) have been tried with limited success in the reproductive tissue engineering field (Peng et al. 2017). An electrospun scaffold consisting of PCL and collagen having high porosity and higher surface-area-to-volume ratio was found to limit application in testicular tissue engineering (Atala 2012). PCL scaffolds were also suitable to maintain the structural characteristics; therefore, it was used for 3D printing scaffold. The 3D-printed scaffold was able to keep the porcine follicles' architecture (Gargus et al. 2020). A highly porous (400–500 μm), biodegradable scaffold made from PLGA by salt leaching method was also found to differentiate spermatocyte into spermatid (Lee et al. 2011). Polyglycolic acid and high-density polyethylene-based testicular

prosthesis supported the formation of fiber-like tissue within the scaffold for testicular tissue engineering. However, studies on the use of synthetic biomaterial for testicular tissue engineering are very limited.

10.5 Conclusion

Mammalian spermatogenesis is a complex and the most extended biological process of sequential cell division and differentiation of putative GS cells that are found in the basal compartment of the seminiferous tubule and produce unlimited numbers of spermatozoa throughout life. The development of a scaffold with microtubular structure and desirable pore size and porosity can provide a 3D environment for the growth and differentiation of germ cells and can develop artificial testicular tissue construct. It may also provide a platform for understanding the mechanism of spermatogenesis and may have future clinical applications. The obtained spermatids and sperm might help produce reproductively competent offspring through assisted reproductive technologies (ART).

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Cardiac Tissue Engineering: Stem Cell Sources, Synthetic Biomaterials, and Scaffold Fabrication Methods

11

Pallavi Pushp and Mukesh Kumar Gupta

Abstract

Cardiovascular diseases (CVDs) are one of the leading causes of death worldwide. Despite the remarkable progress made in the biomedical field, their treatment remains a significant challenge for clinicians. Advancements in cardiac tissue engineering (CTE) may offer unique opportunities to replace diseased myocardium using stem cell-based tissue constructs. Different types of scaffold-free beating cardiac tissue constructs have been developed from stem cell-derived cardiomyocytes (CMs) that could compensate for the lost beating cells and recover cardiac function in the damaged heart. The stem cells could also be seeded into biomaterial-derived scaffolds to develop functional tissue construct for transplantation into the diseased heart. This book chapter discusses various types of stem cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), and germline pluripotent stem cells (GPSCs) for CTE. This chapter also discusses various synthetic polymers and scaffold fabrication methods useful for CTE.

Keywords

Cardiac tissue engineering · Cardiomyocytes · Scaffolds · Polymers · Stem cells

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11.1 Introduction

Various cardiovascular diseases (CVDs) such as cardiac ischemia, infarction, and dilated cardiomyopathies are the leading cause of death in the industrialized world and are estimated to account for 31% of all deaths (Wang and Christman 2016; Cardiovascular Diseases 2020). Several therapeutic approaches, including bypass graft surgery, heart transplantation, angioplasty, etc., are available to treat CVDs. However, the heart is a complicated organ system with extremely limited self-renewal or regenerative potential of adult cardiac cells to compensate for the lost cardiomyocytes (CMs) in CVDs. Further, the death of CMs affects cell-to-cell communications with cardiac fibroblasts to cause irregular electrical signal propagation and formation of scar tissue (Van den Borne et al. 2010). Thus, despite significant improvement in the medical field, the treatment of CVDs is challenging and results in high mortality. Earlier attempts have also used cardiomyoplasty wherein different types of patient-derived cardiac cells were injected into the damaged heart that improves the function of cardiac cells. However, cardiomyoplasty suffers from demerits such as poor cell survival and retention, inflammation, injury, and blockage of the vessels after injections of cells (Chang et al. 2013).

Cardiac tissue engineering (CTE) may offer a desirable therapeutic approach for treating CVDs using patient-derived stem cells. The autologous stem cells such as induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) can be isolated from adults and differentiated into CMs for fabrication of cardiac construct, which can be used for in vivo transplantation in the diseased heart (Chong et al. 2014). Various other sources of stem cells such as embryonic stem cells (ESC), hematopoietic stem cells (HSC), male germline stem cells (GSC), and somatic stem cells (tissue-specific stem cells) such as cardiac stem cells (CSC) have also been reported to differentiate into functional CMs for CTE applications. Further, in cardiac tissue, the CMs are surrounded by different muscles and non-muscles supporting cells such as endothelial cells, fibroblast cells, and smooth muscle cells. Thus, regeneration of cardiac tissue requires not just repopulation of functional CMs but also the development of angiogenesis to support, nourish, and stabilize newly formed beating cells. Biomaterials are used to form a scaffold that provides a three-dimensional physiological environment that enhances the support for the proper cell attachment, growth, and proliferation to direct the cells in a specific type of differentiated cells. Additionally, cells on the scaffold can prevent anoikis, i.e., programmed cell death of cells detaching from the extracellular matrix (ECM) (Sarig and Machluf 2011), and replace damaged cells with new cells; hence, they improve cardiac function. Thus, the choice of cell source and biomaterials are significant factors that must be considered for the fabrication of construct for the CTE application. This book chapter discusses cell sources and biomaterials for fabrication of cardiac tissue constructs. Various scaffold fabrication techniques are also described.

11.2 Scaffold for Cardiac Tissue Engineering

All types of cells require macro- and microenvironments related to their native tissue structure, such as ECM and cell signaling molecules. Thus, a cardiac scaffold can be fabricated, which may mimic the native ECM of the target tissue and should be able to provide an environment that resembles as closely as possible to in vivo conditions. An ideal scaffold for CTE should also be biodegradable, biocompatible, biomimetic, and cell-friendly to provide a functional and living construct for transplantation in the host body. Different biological substances, polymer (natural and synthetic) and non-polymeric materials such as ceramic and metals, have been explored for the fabrication of biomaterials. This construct can be further improved by incorporation of various growth factors, small molecules, angiogenic factors, and culture with differentiated CMs and other cell types of the heart (Jackman et al. 2018).

Natural polymers have attractive biological properties but are difficult for their chemical or physical modification as compared to synthetic materials. Other challenges are the availability of the material source, risk of disease transmission, and immunogenicity as they are derived from mammals. Several studies have suggested that decellularized matrix from the ECM of cardiac tissue or from other types of the organ can be used and incorporated in the scaffolds to develop a composite type of biomaterials that may improve and enhance the application of CTE (DeQuach et al. 2010; Kochupura et al. 2005). Consequently, decellularized matrix, derived from the ECM of different types of tissues such as cardiac tissue, skeletal muscles, nerves, liver, ligaments, and tendons, has been tested as a biological scaffold for CTE. The decellularized matrix-derived scaffolds are biodegradable in nature but lack tensile strength. They are composed of polysaccharides such as glycosaminoglycans; collagen types I, II, III, IV, V, and VI; laminin; fibrin; and various growth factors such as vascular endothelial growth factor, bone morphogenetic proteins (BMP), transforming growth factor, and basic fibroblast growth factor.

11.2.1 Synthetic Biomaterials

Several synthetic biomaterials such as polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(glycerol sebacate) (PGS), polyurethane (PU), poly (lactic acid) (PLA), poly(glycolic acid) (PGA), poly(1,3-trimethylene carbonate-co-lactide) (PTMCLA), poly(ethylene glycol) (PEG), poly-vinyl alcohol (PVA), and poly(*N*-isopropylacrylamide) (PNIPAAm) have been tested for CTE (Table 11.1). The use of synthetic polymers for CTE is advantageous because they can be synthesized in the laboratory in pure and consistent composition to avoid batch-to-batch variations. They also offer opportunities to be synthesized on a large scale and tailored to develop scaffolds with desired architecture pore size, orientation, mechanical strength, and controllable degradation patterns with respect to time. Furthermore, they can be surface modified to control their interaction with cells and/or provide mechanical and electrical cues for differentiation (Guo and Ma 2018;

Table 11.1 Overview of synthetic polymers used for cardiac tissue engineering

Polymer	Polymer detail	Copolymer/ crosslinking	Fabrication method	Cell type seeded	References
PCL	A semi-crystalline homopolymer and degradation product form is caproic acid, which is nontoxic in nature	Gelatin, chitosan	Hydrogel, fibers, sandwich	CMs	Pok et al. (2013) and Yeong et al. (2010)
PU	Synthetic elastomer, first used in biomedical engineering as cardiac pace makers and vascular grafts	Aniline pentamer	Multilayered aligned matrix	CMs	Herrmann et al. (2013) and Baheiraei et al. (2014)
PVA	A synthetic semi-crystalline homopolymer of vinyl alcohol	Cellulose, PVP, alginate	Film, fiber, hydrogel	CMs	Saleemi et al. (2013) and Gnanaprakasam Thankam et al. (2013)
PLA	Chiral molecule that exists in various forms such as PLLA PDLA and nontoxic product is lactic acid	MNPs	Nano-film, nanofibrous	ESCs, H9C2	Liu et al. (2015) and Ventrelli et al. (2013)
PLGA	FDA-approved polymer created with lactic and glycolic acid	Chitosan, gelatin-elastin	Porous, fibrous	CMs	Stout et al. (2012) and Hsiao et al. (2013)
PGS	Elastomeric biodegradable polyester synthesized from glycerol and sebacic acid	Fibronectin, fibrin, collagen	Honeycomb scaffold	CMs, ECs, fibroblasts	Radisic et al. (2008) and Engelmayr et al. (2008)

Rosellini et al. 2015). However, it has been reported that synthetic biomaterials used to have poor biocompatibility when compared with natural polymers due to the release of acidic (e.g., PGA, PLA) or toxic (e.g., PU) degradation products that results in allergy or inflammation and causes fibrous encapsulation (Thomson et al.

1995) and other problems (Yang et al. 2001). Most synthetic polymers are hydrophobic in nature that results in poor cell attachment on the scaffolds, and therefore, various treatments or fictionalization of the surfaces is required before cell culture on them (Moorthi et al. 2017).

Scaffolds used for CTE must also have properties such as good elasticity, high modulus, flexibility, and conductivity to mimic the contractility of native cardiac tissues (Ahadian et al. 2016). Various polymers used as biomaterials for CTE lack the major properties such as appropriate mechanical integrity and conductivity (Shin et al. 2016) that may be overcome by the use of composite biomaterials where we use to combine two or more natural and synthetic biomaterials to overcome the demerits of one or another. For example, composite nanofiber made of poly(lactide-co-caprolactone) and poly(ethyl oxazoline) better mimicked the cardiac ECM and showed enhanced tensile strength and expression of cardiac marker proteins (Lakshmanan et al. 2015). Another major concern with CTE is the anisotropic nature of the scaffold to imitate the native cardiac tissue.

11.2.1.1 PVA-PVP as a Composite Biomaterial for Cardiac Tissue Engineering

PVA, one of the synthetic semi-crystalline homopolymers of vinyl alcohol, shows high tensile strength, flexibility, tear strength, and biodegradability and is a promising biomaterial for CTE applications. It is an atactic material whose tensile strength increases with molecular weight. A significant drawback of PVA is its high solubility in water that causes rapid disintegration of scaffolds in cell culture media, due to which cell seeding is a limiting factor. Thus, PVA is usually used as a combination with various natural and synthetic biomaterials such as chitosan (Alhosseini et al. 2012; Charernsriwilaiwat et al. 2010), cellulose (Medeiros et al. 2008), hydroxypropyl methylcellulose (Uslu et al. 2010), alginate (Zain et al. 2011), poly(vinyl pyrrolidone) (PVP) (Saleemi et al. 2013), PEG, and poly(acrylic acid). However, some researchers have used PVA alone for the fabrication of scaffolds (Htike et al. 2012). Venugopal et al. fabricated composite polymer of xylan and PVA and reported a nanofiber diameter of 427 nm with 2.43 MPa and 3.74 MPa of mechanical strength and Young's modulus, respectively, required for the better cell culture and proliferation of CMs (Venugopal et al. 2013). PVA and alginate hydrogel were also reported to have good mechanical strength and promoted cell adhesion, proliferation, and 3D growth of CMs (Gnanaprakasam Thankam et al. 2013).

On the other hand, PVP is a vinyl polymer derivative and contains a pyrrolidone group that signifies its amorphous nature. It may quickly form bonds with other polymers due to the group called "pyrrolidone" with a robust proton-accepting carbonyl group. It has wide application in CTE and in biomedical and pharmaceutical sectors (Bühler 1992; Kurakula and Rao 2020) due to various properties such as solubility in water, biocompatibility, biodegradability, stability at pH, and absorption of ions. Risbudet al. reported the growth and culture of endothelial cells (ECs) on chitosan and PVP hydrogel for vascular applications (Risbud et al. 2001). The bioactive glass has also been enforced into the PVP to fabricate the scaffold and

evaluated with the help of MSCs under in vitro condition that provides enhanced mechanical strength and biocompatibility to the scaffold (Hatcher et al. 2003).

In one of our works, we prepared PVA-PVP blended film and nanofiber using solvent casting and electrospinning, respectively. Physicochemical properties such as swelling and degradation of PVA-PVP films were found to be improved after incorporating the glutaraldehyde in the composite. The PVA-PVP blends increase the viscosity of the composite polymers due to the hydrogen bonding and get reduced after the addition of glutaraldehyde. In addition, glutaraldehyde decreases the degree of solubility by decreasing the availability of the free hydroxyl group. The inclusion of glutaraldehyde in the PVA-PVP blend produced nanofibers with fewer beads than standard PVA-PVP fibers (Pallavi and Kumar 2017). Our existing composite scaffold of PVA-PVP for the use of tissue engineering can be modified further based on process parameters. Additionally, by keeping in mind the flexibility of the PVA-PVP composites for CTE application, the plasticizers have been added with the PVA-PVP. In this work, the PVA-PVP blends were plasticized with various concentrations of glycerol and propylene glycol to form a transparent film. The films fabricated were colorless with a folding endurance value of more than 300 and were homogeneous in nature. In addition, it showed good mechanical properties in terms of elongation of the film, which is pretty much good from the CTE application point of view. The swelling behavior of obtained films was decreased after adding the plasticizer that satisfied the desirable property of matrices for CTE application during in vivo and in vitro conditions. The plasticized films were also hemocompatible and cytocompatible in nature after we cultured the cardiac cells on them. All the plasticized samples showed noncorrosive and nonirritant properties as the obtained cell viability was more than 50% after 60 min of culture. Few physiochemical tests and in vitro drug release studies were also performed and evaluated in our laboratory. The results of experiments showed that the matrices with plasticizers showed better drug release patterns than non-plasticized ones, which may be due to properties of plasticized matrices such as the absence of aggregation, homogenous nature, and reduction in crystalline size due to the incorporation of the plasticizers into the matrices.

11.2.1.2 PCL as a Biomaterial for Cardiac Tissue Engineering

PCL is a semicrystalline homopolymer with a low melting temperature and a slow degradation rate of 2–3 years, whereas the copolymerization with various natural and synthetic polymers may reduce the degradation time. In addition, the product after degradation (caproic acid) is nontoxic in nature, and hence, PCL has better tissue compatibility as compared to other polymers. The cardiac contractile grafts were reported by Shin et al. using nanofibrous PCL cultured with CMs. The CMs started beating after 3 days with the expression of cardiac proteins such as α-myosin, connexin 43, and troponin (Shin et al. 2004). The increase in the PCL nanofiber diameter increased the cell penetration proportionally and showed homogenous tissue formation with sufficient matrix deposition (Balguid et al. 2009). PCL has also been combined with natural polymers such as gelatin and chitosan to provide mechanical strength. Sandwiching of PCL between the gelatin and chitosan

hydrogel provided high tensile strength, ease of handling the scaffold during the surgical reconstruction of heart defects, suitability, and increased cell attachment and pore size suitable for the CM migration and function (Pok et al. 2013). Yeong et al. used selective laser sintering to fabricate a porous PCL scaffold, which resulted in cardiac construct with better cell proliferation density and tensile strength similar to native myocardium in the range of kPa (Yeong et al. 2010).

In our study, we have fabricated collagen-coated PCL-aligned and random nanofibers and investigated their potential application in the field of CTE (Pushp et al. 2017). The coating of collagen was required due to the hydrophobic nature of the PCL. The aligned nanofiber coated with collagen enhances cell proliferation, adhesion, and cell growth of H9C2 compared to the cells cultured on random nanofibers. In addition, the aligned nanofiber controlled the orientation of H9C2 cells along the parallel direction of fibers and therefore mimicked the anisotropic nature of cardiac tissue. Thus, coated aligned PCL nanofibers hold wide applications and act as a promising material for CTE application as they increase cell proliferation, attachments, and growth.

11.2.1.3 Other Biomaterials for Cardiac Tissue Engineering

PLGA is composed of lactic and glycolic acid and is one of the most studied synthetic biopolymers for CTE. It is approved by the US Food and Drug Administration (FDA) for several biomedical applications due to good biocompatibility, non-immunogenicity, and controlled biodegradability with channeled microstructure and morphology. The scaffold of PLGA can be fabricated by various techniques such as lyophilization (Park et al. 2005), extrusion (Carrier et al. 1999), salt leaching (Caspi et al. 2007), and electrospinning (Xie et al. 2010). It may be used as a hybrid biomaterial with several natural and synthetic polymers. The composite of PLGA and chitosan showed increased hydrophilic behavior to provide good cell adhesion and proliferation (Xie et al. 2010). PLGA should be combined with carbon nanofiber to promote CM function as it enhances the mechanical strength/conductivity and protein expression that is comparable to the native cardiac tissue (Stout et al. 2012). Hsiao et al. reported aligned composite nanofibers of polyaniline (PANI) and PLGA scaffold for synchronously beating CMs. After incubation, the adherent CMs formed a cell cluster and expressed gap junction protein (connexin 43), which improved impaired heart function (Hsiao et al. 2013). Composite of PLGA with gelatin and elastin showed homogeneity and a fiber diameter of 380 nm less than the diameter of the individual polymer (Li et al. 2006). Zong et al. used the post-processing for PLLA and PLGA composite, such as mechanical stretching of electrospun fibers, which helped in the cell growth in parallel orientation (Zong et al. 2005). Other composites of PLGA that improved the expression of cardiac proteins and cardiac markers, CM proliferation, and contractile properties are PLGA with PCL and collagen I (Park et al. 2005) and PLGA/gelatin nanofibers (Prabhakaran et al. 2011).

PGS, synthesized from glycerol and sebacic acid, is an elastomeric polyester and biodegradable in nature. The degradation of PGA is prolonged, which maintains its tensile strength for a longer time period (Wang et al. 2003). It also shows good biocompatibility, mechanical strength, and intrinsic elasticity and, hence, is well

suited for the mechanically active CMs (Chen et al. 2008). A porous PGS scaffold with a parallel array of channels was fabricated by Radisic et al., which mimicked the capillary network in native myocardium upon tri-culture of CMs with ECs and fibroblast (Radisic et al. 2006, 2008). Engelmayr et al. reported a novel PGS-based accordion-like honeycomb scaffold whose stiffness and mechanical properties closely matched those of the native cardiac tissue, thereby helping in the formation of vascular grafts around beating cells aligned in nature (Engelmayr et al. 2008). Coating of PGS by proteins such as laminin, fibronectin, fibrin, collagen, and elastin increased cellularity, enhanced ECM production, and modulated the differentiation of endothelial progenitor cells (Sales et al. 2007).

PU is a synthetic elastomer that was first used in biomedical engineering as cardiac pacemakers and vascular grafts owing to its excellent mechanical strength and elastomeric properties. PU film has been used as a scaffold material for the formation of multilayered, aligned CMs as tissue construct for myocardial repair (McDevitt et al. 2003). The coating of PU film with laminin or collagen promoted cell attachment and increased contractile CMs (Alperin et al. 2005). The PU patch promoted the formation of contractile smooth muscle cells, cardiac remodeling, and improvement of contractile function (Fujimoto et al. 2007; Herrmann et al. 2013). Formation of toxic aromatic diamines such as diisocyanate and oxidation of PU surface after implantation are the major drawbacks in the use of PU in CTE. Therefore, newer approaches are required for controlled degradation of PU and to avoid the unwanted effects (Santerre et al. 2005). Baheiraei et al. synthesized a biodegradable electroactive scaffold of PU by incorporating aniline pentamer (Baheiraei et al. 2014).

PLA is a chiral molecule and exists in various forms such as poly(L-lactic acid) (PLLA), poly(D-lactic acid) (PDLA), and meso-poly(lactic acid), where PLLA and PDLA are primarily used in the field of CTE applications. They have high modulus and strength, low degradation rate, better processability, and easy metabolization inside the body. Lactic acid is the major degradation product formed from the PLA that is naturally present in our body system and enters in TCA cycle. Nanofibrous PLLA scaffolds with a highly interconnected porous structure supported cardiac tissue formation from ESC-derived cardiovascular progenitor cells (Liu et al. 2015). Ultrathin nano-films have also been developed by combining PLA with magnetic nanoparticles (MNPs), which enhanced proliferation and adhesion of H9C2 cardiac cells and increased the surface area of myotubes for cellular morphogenesis (Ventrelli et al. 2013).

11.3 Fabrication Techniques for the Development of Scaffolds

Various types of matrices can be fabricated from biomaterials using different techniques such as solvent casting, gas foaming, electrospinning, freeze drying, and rapid prototyping. The cells and tissues of our body are organized in a particular fashion, and to get the same architecture, one or more of the above techniques can be used to facilitate the desired porosity, strength, high surface-to-volume ratio, and 3D

structure for proper distribution, proliferation, and growth of the cells. Each of the fabrication methods has its unique principle and working conditions that depend on the application and different structural requirements to regenerate the whole tissue. The different methods are discussed below.

11.3.1 Solvent Casting/Particulate Leaching Method

Solvent casting or particulate leaching method has been the most commonly used method for the fabrication of scaffold and matrices in the area of CTE and biomedicine. The method involves mixing polymers in their respective solvents, casting in a desired mold, and drying by solvent evaporation. If desired, porogens such as NaCl can be added to the solution and, after solvent evaporation step, it can be leached out by using suitable solvent (e.g. water for NaCl) to obtain a porous structured matrix. The later method is called particulate leaching method. Both methods are straightforward and cost-effective and hence can be applied to almost all polymers that are soluble in nature. This method was developed by Mikos et al. (Mikos and Temenoff 2000) in the year 1994. The major limitations are varying pore sizes, toxicity due to residual toxic solvents, and thermal degradation of polymer solution (Weigel et al. 2006). However, by controlling the size and amount of the salt particle and the concentration of the polymer, it is possible to control the pore size of the scaffold to some extent (Gokmen and Du Prez 2012). Ghaziof et al. fabricated composite scaffolds of PCL and carbon nanotube via solvent casting method followed by vacuum drying. The addition of carbon nanotube enhances its mechanical strength and electrical conductivity of the scaffold for CTE (Ghaziof and Mehdikhani-Nahrkhalaji 2017). In 2018, Bagdadi 2018 et al. reported a novel promising biodegradable material such as poly(3-hydroxyoctanoate) for CTE with a particular size of pores. The scaffold showed similar mechanical properties to that of cardiac muscle with coordinated beating that supports the cells during the regeneration process (Bagdadi et al. 2018). Recently, Behr et al. reported a tubular graft of poly(lactide-co-caprolactone) with PVA and polyethylene glycol as a porogen. The scaffold was subsequently submerged in water to dissolve the porogen and formed the pore sizes ranging from 2 to 9 μm. The graft fabricated through this method is compliant as in vivo implantation experiment results suggested its potential for clinical applications (Behr et al. 2020). Our study observed that PVA-PVP films fabricated by solvent casting methods had shown suitable cell attachment and proliferation of H9C2 cardiac cells that are suited for CTE applications (Pallavi and Kumar 2017).

11.3.2 Gas Foaming

Gas foaming method mainly uses gas in place of organic solvent to form a porous structure in the scaffold. Generally, these gases used in the foaming process are termed blowing agents. A blowing agent can be either chemical or physical such as plastics, natural and synthetic polymers, and metals that undergo hardening or phase

transition steps and therefore are capable of forming a porous, cellular structure inside the scaffold via the foaming process. The chemical blowing agents experience a chemical reaction and then produce some gases, whereas the physical blowing agents are gases inert in nature and do not react chemically to the polymeric matrixes of the scaffold. The most widely used gas in the gas foaming process is carbon dioxide, which creates pores of the desired size. In addition, combining particle leaching with the gas foaming method helps to get opened pores on the surface of a scaffold. It gives a highly porous scaffold up to 200–500 μm. It is free of toxic solvents but has limited mechanical property and inadequate interconnectivity. Dattola et al. developed a 3D PVA-based matrix for CTE using the gas foaming method with a pore size of 10 to 370 μm within the range of CMs that support the stem cell growth and differentiation into CMs (Dattola et al. 2019).

11.3.3 Freeze Drying

Freeze drying, in contrast to the conventional method of drying, is based on the low-temperature vacuum drying process where the water present in the polymeric solution gets converted to the frozen solvent with lowering of pressure. Further, the sublimation and removal of the bound frozen or water molecules take place under low-temperature vacuum conditions, which aid in the formation of pores inside the scaffolds that help to maintain the native biological activity of the seeded cells. The basic protocol is to dissolve the polymer material in a solvent and stir well until a homogenous solution of the polymer has been attained. Furthermore, the polymeric solution must be poured into predefined molds and kept for freezing for at least overnight. The frozen samples are kept in a lyophilizer until all the solvent evaporates. The prepared scaffolds used to be washed using phosphate buffered saline (PBS) and stored for further use. The process parameters that should be kept in mind for the freeze-drying process are the concentration of the polymer, the concentration of solvent, freezing temperature, freezing time, the concentration of gelation medium, and gelation time. It gives a pore size of the range 13–35 μm with the 3D scaffold. The advantage is that it is simple to process with complete solvent removal and highly interconnected pores, whereas the disadvantage is that it is a time- and energy-consuming process.

11.3.4 Electrospinning

Electrospinning is a simple technique to fabricate nanofiber by applying a strong electric field with the help of high voltage that creates a charge in the polymeric solution. Further, the applied high voltage allows the polymeric solution to overcome its surface tension and form an ultrafine jet solution that results in fiber formation, collected on the grounded collector. The nanofibers achieved are very thin in structure, usually less than 1 μm (1000 nm) in diameter with controlled fiber diameter and pore size. The critical parameters responsible for the fabrication of

Table 11.2 Different parameters and their effects on nanofiber morphology

Parameter	Change of parameter	Effect on	
		Fiber diameter	Bead formation
Solution concentration	↑	↑	↑
Molecular weight	↑	↑	↑
Viscosity	↑	↑	↑
Applied voltage	↑	↑	↑
Flow rate	↑	↑	↑
Tip to collector distance	↑	↑	↑
Surface tension	↑	↑	↑
Conductivity	↑	↑	↑
Humidity	↑	↑	↑
Temperature	↑	↑	↑

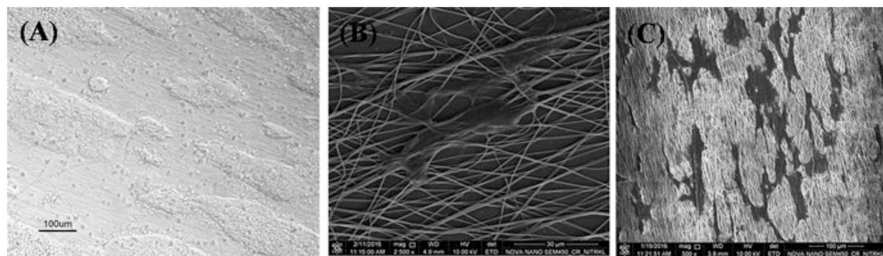

Fig. 11.1 Human iPSCs (**a**), MSCs (**b**), and H9C2 cardiac cells (**c**) grown in PCL nanofibers

robust nanofiber are viscosity, surface tension, the conductivity of the polymeric solution, applied voltage, flow rate per ml, distance between the tip and collector, and molecular weight of the polymer and nature of collector surface (Table 11.2). Environmental conditions such as temperature and humidity (30–50%) must be maintained during the spinning process. The unique properties such as the high surface-area-to-volume ratio, easily mimicking ECM due to nano-meter size, small pore size, free from toxic solvents, and flexibility in surface functionalities, which provides better cell attachment and proliferation, provided a broad application of nanofiber in the field of CTE. Advantages of this technique include the nano range diameter of the fiber that mimics the in vivo ECM structure and its time-efficient processing system with complete removal of toxic solvents. In contrast, the disadvantages are that it is an energy-consuming process, high voltage equipment may be risky for usage, and preparation of 3D scaffolds with internal pore network involves the proper design of collector. The culture of iPSCs, MSCs, and cardiac cells on nanofiber is shown in Fig. 11.1.

11.3.4.1 Random Versus Aligned Nanofibers

Two types of nanofibers are commoly fabricated using the electrospinning technique: (a) random nanofibers and (b) aligned nanofibers. Several researchers have

worked on random nanofibers (Prabhakaran et al. 2011; Kai et al. 2015; Feng et al. 2014; Borriello et al. 2011). However, in the last few decades, aligned nanofibers have been reported to have wider application in CTE due to the patterned and anisotropic nature of fibers that provide topographical similarity to the ECM of the native cardiac tissue (Baker et al. 2008). Aligned nanofibers can also induce guided orientation to cardica cells as compared to random nanofibers. One of the critical characteristics of CMs is their unidirectional nature (Laflamme and Murry 2005), which can be favored by nanoscale fibers to provide a native microenvironment for synchronized beating (Hsiao et al. 2013).

11.3.5 Rapid Prototyping

Rapid prototyping consists of a step-by-step process that can generate a model of prototype and functional components directly from computer-aided data (CAD). The lasers have been used as a power source to sinter powdered material, followed by binding them together to fabricate a solid model or structure. Basic data is obtained from computed tomography (CT) or magnetic resonance imaging (MRI), and the complex shapes are designed using computer-aided design software. Scaffolds are fabricated using a 3D printer according to the requirement with desired geometry and features. With the help of CAD, an STL file can be created, which can then be processed by the 3D printers to form the physical models in a layer by layer deposition of the polymer. It has excellent control over geometry and pore size with a 3D scaffold to fabricate complex type scaffolds with low budget energy and within a limited time frame in an efficient manner. The disadvantages are the following: the limited application to use the different polymeric materials, it is a sophisticated method, and heterogeneous pore morphology is not possible.

11.4 Stem Cell Sources for Cardiac Tissue Engineering

Stem cells are undifferentiated cells that have the capacity to self-renew and differentiate into a diverse range of specialized cell types and hence can be used to treat some diseases that currently have no cure. They are responsible for the development, maturation, regeneration, and repair of all tissue types. Stem cells are present in the embryo as well as in adult tissue. Ideally stem cells and their sources must be available in sufficient amounts and can differentiate into beating CMs upon transplantation. They must be immune-compatible, non-allergic, and electrophysiologically compatible with the myocardium of the host body, which can grow on electrospun fibers (Ehler and Jayasinghe 2014) or macroporous scaffold (Sapir et al. 2014) or microtemplated scaffold (Thomson et al. 2013) either alone or as tri-culture with other cells types such as ECs, cardiac cells, and fibroblasts to mimic the native heart tissue cellular organization. Various types of stem cells include ESCs, iPSCs, MSCs, HSCs, GSCs, and somatic stem cells (tissue-specific stem cells) such as CSCs, adipose-derived stem cells, and neural stem cells, which

can be differentiated into beating cells (~10^5 cells/cm^3) and therefore recommended for tissue engineering applications (Table 11.3). Numerous studies have shown that a fraction of these cells is capable of differentiating into beating CMs and replacing damaged myocardium tissue to improve heart function. Additionally, cells such as MSCs were shown to release various paracrine factors such as cytokines and other growth factors for cardiac cell repair, protection, and regeneration (Zhou et al. 2019).

11.4.1 Embryonic Stem Cells (ESCs)

ESCs are highly proliferating pluripotent cells that can be isolated from an undifferentiated inner cell mass of embryos of animals and humans and available as commercial cell lines. They have the ability to quickly differentiate into all three germ layers and therefore have wide application in CTE and the biomedical field of research (Parrag et al. 2012). Recently ESC-CMs served as pharmacological assays to separate and isolate the mature CMs from undifferentiated and undesired cell type (Veevers et al. 2018), whereas DNA methylated in ESCs has been reported to find the novel regulators of cardiogenesis (Fu et al. 2018). They have reported the isolation of cell surface markers such as cardiac troponin I and myosin light chain 2 from ESC-CMs with the help of bacterial artificial chromosome carrying GFP that can be used for better understanding of CM subtype specification for therapeutic and regenerative applications. Shen et al. isolated pure CMs and cultured them in an advanced bioreactor system by using physical signals such as pulsatile flow, cyclic strain, and extended culture time that mimics in vivo conditions important for heart development (Shen et al. 2017). They compared the static and dynamic bioreactor systems where dynamic systems showed the enhanced expression of cardiac-associated proteins such as cTnT and cardiac ion channel genes and increased sarcoplasmic reticulum activity similar to the primary CMs.

The ESCs can also differentiate into all other cell types known to exist in the heart, including CMs, ECs, fibroblasts, pericytes, and neurons and, therefore, are the most attractive cell source for CTE. Generation of CMs from ESCs/iPSCs by introducing stage-specific genes is shown in Fig. 11.2. Several researchers have been successful in developing cardiac patches from ESCs, which may be directly applied to the diseased area of the heart (Liau et al. 2011; Moon et al. 2013). However, the clinical use of ESC-derived CMs poses ethical issues and potential risks of immunogenicity, teratoma formation, and uncontrolled differentiation (Hynes 2008). Therefore, clinical application of ESC-derived CMs is prohibited almost all over the world in view of the above issues.

11.4.2 Induced Pluripotent Stem Cells (iPSCs)

iPSCs are pluripotent stem cells directly derived from the somatic cells under in vitro conditions and offer an alternative to ESCs for CTE application (Lim and Gong 2013). They can be derived by direct reprogramming of autologous patient somatic

Table 11.3 Different cell sources for cardiac tissue engineering

S. no.	Sources	Type of cell	Differentiation potential	Biomaterial used	References
1	Bone marrow	Mesenchymal stem cell	Cardiac progenitor cells Cardiomyocytes	Hyaluronic acid	Boopathy et al. (2013)
2	Adipose tissue	Adipose-derived stem cell	Cardiomyogenic cells	Chitosan	Liu et al. (2013)
3	Cord blood cells	Mesenchymal stem cell	Cardiac vascular cells	Fibrin	Roura et al. (2012)
4	Cord blood cells/cord tissue	Mononuclear cell	Cardiogenic cells	–	Khattab et al. (2013)
5	Blastocysts	Non-embryonic parthenogenetic stem cells	Cardiac lineage	–	Didié et al. (2013)
6	Adult or fetal heart	Cardiomyocytes/cardiac fibroblast cell	–	PGS/gelatin	Kharaziha et al. (2013)
7	Adult or fetal heart	Cardiac progenitor cells	–	Silk fibroin	Di Felice et al. (2013)
8	Blastocyst stage embryo	Embryonic stem cells	Cardiomyocytes	PLGA/collagen	Prabhakaran et al. (2013)
9	Spermatogonial stem cell	Germline stem cell	Mesodermal cells	–	Iwasa et al. (2010)
10	Umbilical cord	Mesenchymal stem cell	Cardiomyogenic lineage	Polyurethane	Herrmann et al. (2013)
11	Somatic cell	Induced pluripotent stem cells	Cardiomyocytes	–	Mandel et al. (2012) and Miki et al. (2012)
12	Amniotic fluid	Amniotic fluid-derived stem cells	Cardiogenic lineage	–	Maioli et al. (2013)
13	Adult skeletal muscles	Skeletal muscle-derived stem cell	Cardiomyocytes phenotype	Collagen	Clause et al. (2012)
14	Peripheral blood	Endothelial progenitor cell/precursor cell	Cardiomyocytes	–	Badorff et al. (2003)

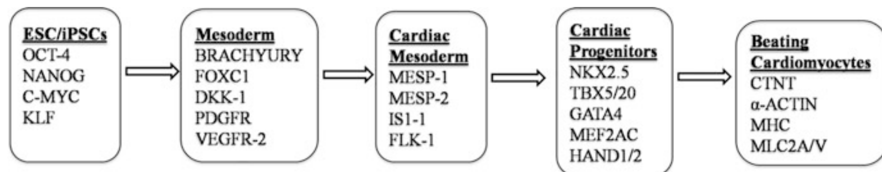

Fig. 11.2 Stage-specific markers of CMs generated from ESCs and iPSCs

cells by using various reprogramming factors such as Oct4, Nanog, Sox2, and Lin28 (ONSL) or Oct4, Sox2, Klf4, and c-Myc (OSKM or Yamanaka factor) (Takahashi et al. 2007; Takahashi and Yamanaka 2006). Later, various viral or non-viral delivery methods have been modified and explored to transfer the reprogramming factors into somatic cells that reported promising results (Table 11.4). Fully reprogrammed iPSCs resemble the structure, morphology, gene expression, phenotype, and self-renewal ability of ESCs and are able to differentiate into all three germ layers such as endoderm, ectoderm, and mesoderm, including beating cardiac cells (Narazaki et al. 2008; Zhang et al. 2009; Mauritz et al. 2008). Treatment of embryoid bodies with histone deacetylase inhibitor, activin A, BMP4, and trichostatin A (TSA) has induced the differentiation of human iPSCs into cardiac cells (Lim and Gong 2013). It has been reported that ESC- and iPSC-derived CMs generally do not differ significantly in their gene expression pattern, power-law behavior, beating rate, myosin and sarcomeric organization, and response to the adrenergic receptor (Zhang et al. 2009; Mauritz et al. 2008; Mandel et al. 2012). Therefore, on the basis of reported results, various researches and studies have started using iPSCs as an alternative to ESCs for cardiac-related applications (Miki et al. 2012). Gelmi et al. developed a PLGA fibrous scaffold coated with polypyrrole, which helped in electrical, mechanical, and topographical cues for differentiation of human iPSC into beating cells by following stimulated and unstimulated procedures (Gelmi et al. 2016).

The use of iPSCs offers the benefit of using autologous cells that minimizes the chances of immune rejection as well as avoids ethical, legal, and social issues related to ESCs. However, the exact mechanism of iPSC reprogramming and its functionality after getting reprogrammed and forming differentiated cells is still difficult to track under in vitro conditions. In some cases, incomplete reprogramming is likely to occur, resulting in poor results. The use of fully reprogrammed iPSCs does not guarantee to repudiate the formation of teratoma, as with the case of ESCs. In our study, we cultured iPSCs on a Matrigel-coated electrospun PCL-aligned nanofiber that enhanced the cell growth, attachment, and alignment on the nanofiber matrix (Fig. 11.1a). The iPSC 3D culture has been established by Shadrin et al. to develop cardiopathy for differentiation of CMs, which resulted in enhanced electromechanical coupling with the evidence of markers such as H-zones, I-bands, T-tubules, and M-bands comparable to the adult myocardium (Shadrin et al. 2017). Recently, Hatani et al. demonstrated an efficient protocol for iPSC differentiated CMs using

Table 11.4 Derivation and differentiation of induced pluripotent stem cells (iPSCs) into cardiomyocytes

S. no.	Sources of cell	Reprogramming factors[a]	Differentiation
Human			
1	Cardiac progenitor cells	OSKM	Cardiomyocytes
2	$CD34^+$ cells	OSKM	Hematopoietic progenitors
3	Cord blood	OSNL	Cardiomyocytes
4	Fibroblast	OSKM	Germ layers
5	Fibroblast	OSNL	Cardiomyocytes
6	Fibroblast	OSKML mRNA	Myogenic cell
7	Fibroblast	OSKML with Valproic acid	Cardiomyocytes, cardiac cell
8	Fibroblast-like synoviocytes	OSMN	Cardiomyocytes
9	Keratinocytes	OSKM	Cardiac cell
10	Neural stem cells	OK	Germ layers
11	Peripheral blood mononuclear cells	OSKM	Cardiomyocytes
12	Renal epithelial cells from urine	OSKM	Germ layers
13	Urinary progenitor cell	OSKM	Cardiac cell
Mouse			
14	Fibroblast	OS	Germ layers
15	Fibroblast	OK with GSK-3 inhibitor	Embryoid bodies
16	Fibroblast	miR302/367 cluster	Germ layers
17	Fibroblast	Nr5a2 shRNA with SKM	Germ layers
18	Fibroblast	OSK in mutant p^{53} vector	Germ layers
19	Fibroblast	OSKM	Neuronal cell
20	Fibroblast	OSKM	Germ layers
21	Fibroblast	Sox2-TAT protein	Germ layers
22	Pancreatic β cells	OSKM	Germ layers

[a]*O* Oct4, *S* Sox2, *K* Klf4, *M* c-Myc, *N* Nanog, *L* Lin28, *TAT* transactivator of transcription

embryoid body formation supplemented with dorsomorphin and SB431542 (Hatani et al. 1816). Another concern for CTE is related to cell sheet engineering, where the thickness of the sheet is a major concern. However, large cardiac tissue sheets have been generated using 10-cm-sized temperature-responsive culture dishes (Ishigami et al. 2018), which upon transplantation in myocardial infarcted pigs could restore the cardiac dysfunction of the infarcted heart.

11.4.3 Mesenchymal Stem Cells (MSCs)

MSCs are multipotent stem cells initially identified in the bone marrow but later isolated from the umbilical cord (Kadner et al. 2004), amniotic fluid (Petsche Connell et al. 2013), placenta, adipose tissue, Wharton's jelly, menstrual blood (Patel et al. 2008), amniotic membrane or fluid, trabecular bone, chorionic villi, dental pulp, deciduous teeth, synovial fluid (Jones and Crawford 2014), etc., for clinical applications (Shaer et al. 2014) as they do not pose immunogenic or ethical problems. MSCs have the ability to differentiate into mesoderm, ectoderm, and endoderm lineages. They secreted soluble molecules with anti-inflammatory and immunomodulatory properties that help during cell-based therapy (Hoogduijn et al. 2010).

Various preclinical and clinical trials have reported successful differentiation of CMs from MSCs (Abd Emami et al. 2018; Cai et al. 2016; Gerace et al. 2019; Li et al. 2017), where in their controlled delivery into cardiac patients promoted the blood pumping capacity and volume of left ventricle (Hare et al. 2009). In addition, Rahimi et al. compared differentiation potential of menstrual blood-derived MSCs and bone marrow-derived MSCs into cardiac cells in *Bombyx mori*-derived silk scaffold, and reported cardiac tissue construct with suitable mechanical and physical qualities for CTE applications (Rahimi et al. 2014). Various methods have been reported for the differentiation of MSCs into CMs by using pharmacological agents such as 5-azacytidine (Hou et al. 2013) and DMSO and growth factors like BMP, angiotensin-II (Xing et al. 2012), platelet-derived growth factor (Xaymardan et al. 2004), platelet-rich plasma (PRP) (Homayouni Moghadam et al. 2016), and TSA (Rouhi et al. 2013) with variable efficiency. In our study, the MSCs were isolated from the umbilical cord matrix using two methods, the explant method and enzymatic digestion method, and further treated with 5-azacytidine to differentiate them into cardiac type cells. The isolated MSCs from UCM were also differentiated into chondrocytes, osteoblasts, and adipocytes for multilineage characterization, and the cells were further characterized by flow cytometry analysis and successfully cryopreserved for a cell bank. The cells were further proliferated on a nanofiber, as shown in Fig. 11.1b. Ravichandran et al. reported cardiac differentiation of MSCs grown on hemoglobin/gelatin/fibrinogen nanofibers with the treatment of 5-azacytidine (Ravichandran et al. 2013). The improvement in the cardiac function derived from MSCs is due to the secretion of exosomes and paracrine factors, as well as the modulation of the immune response (Ju et al. 2018; Lukomska et al. 2019). Further, the number of differentiated beating cells and the expression of various cardiac cell markers increased after treating the MSCs with 5-azadeoxycytidine (Planat-Benard et al. 2004; van Dijk et al. 2008; Smith et al. 2012).

11.4.4 Hematopoietic Stem Cells (HSCs)

HSC improves cardiac cell function by secretion of various soluble growth factors with the activation of Wnt signaling pathways (Mirotsou et al. 2007) rather than

transdifferentiation (Jawad et al. 2007) and therefore can be used as an alternative source of ESC for CTE applications. HSC used for clinical applications can be isolated from the bone marrow by using Good Manufacturing Practices (GMPs). It has been routinely used for transplantation in many countries to alleviate blood disorders. However, various studies have reported the variance in the reported data and results regarding the differentiation capability of HSC into CMs.

11.4.5 Germline Stem Cells (GSCs)

GSCs, also known as spermatogonial stem cells, are testis-derived stem cells that have been regarded as a potential cell source of heart cells (Guan et al. 2006). Recently, Mahapatra et al. (2018) isolated GSCs from the human testis and differentiated them into cardiac cells that expressed cardiac-promoting paracrine factors and were capable of producing cells of all three germ layers. The absence of teratoma formation is the significant advantage of GSC due to the downregulation of pluripotency genes after differentiation with no ethical issues as compared with ESCs. Several studies have reported that when GSCs are cultured in the correct medium, they can even differentiate into beating CMs (Guan et al. 2006; Kanatsu-Shinohara et al. 2005; Ko et al. 2009). Kim et al. (2017) reported that mouse multipotent GSC-derived alpha cells helped in cardiomyogenesis under in vitro conditions and reduced fibrosis associated with MI injury. Similarly, Luan et al. also reported the differentiation of beating CMs from chicken spermatogonial stem cells similar to ESCs, which provides the basic idea for research and various regenerative applications for degenerative cardiac diseases of animals (Luan et al. 2014). In 2012, Kim and his group reported that mouse GSC supplemented with protein resulted in the increased potential of cardiac cells with an expression of primitive marker and transcription factors related to cardiac tissue. In addition, GSC transplantation enhances ischemic heart wall thickness, blood vessel formation, and systolic velocity of beating cells in ischemic mice (Iwasa et al. 2010). The adult mouse GSC-derived CMs showed similar gene expression patterns, such as cardiac markers like alpha-actinin, connexin 43, and troponin T, and electrical properties similar to the CMs derived from ESCs (Baba et al. 2007). Therefore, in the case of GSCs, the results pointed toward a new source of CMs for basic research of CTE and its therapeutic application in various regenerative fields. However, the exact mechanism behind the cardiac differentiation of GSC is still unknown.

Cardiac endothelial and smooth muscle cells have also been successfully derived from GSCs without the formation of any tumor (Guan et al. 2007). Therefore, they have been regarded as a new alternative to another type of stem cells for CTE (Nguyen et al. 2016). In one of our studies, mouse multipotent adult GSC was successfully differentiated into beating CMs (Jung et al. 2010), and therefore, all together, these results suggest that GSC may provide a unique source of differentiated cardiac cells and hence can be utilized for future CTE of a damaged heart tissue (Table 11.5).

Table 11.5 Differentiation potential of germline stem cell

S. no.	Type of cell	Cell source	Species	Differentiation	References
1	Multipotent germline stem cell	Testis from newborn mice	Mouse	Cardiomyocytes/ endothelial cell	Baba et al. (2007)
2	Spermatogonial stem cell	Testis	Mouse	Functional cardiomyocytes	Guan et al. (2007)
3	Multipotent germline stem cell	Testis	Mouse	Cardiomyocytes derived from Flk1^{+} cells	Iwasa et al. (2010)
4	Testicular stem cell	Testis	Mouse/ mice	Cardiomyocytes	Izadyar et al. (2008)
5	Sertoli cells	Testis	Rat	Osteoblast cells	Hu et al. (2007)
6	Sperm/germ cells	Testis	Mice	Mammary epithelial cells	Boulanger et al. (2007)

11.4.6 Cardiac Stem Cells (CSCs)

The mammalian heart is known to contain undifferentiated CSCs. The CSC from the human and the murine heart has been firstly reported by Messina et al. with the expression of markers such as CD117, Sca-1, and MDR-1 (Messina et al. 2004). They can be further characterized by the expression of marker genes such as c-kit, Sca-1, Abcg2, Isl-1, Mdr-1, Bry, and Flk-1 and capability to form a blast colony-forming unit (BL-CFC) and a cardiovascular colony-forming unit (CV-CFC). CSC's multipotent and self-renewing characteristics made it capable of forming beating cells, cardiac endothelial cells, fibroblasts, and cardiac vascular lineages when treated with various stem cell growth factors and cytokines (Bollini et al. 2011; Kattman et al. 2006). However, the cell density of CSC is very low (one cell per 10,000 myocytes), which is generally found in the atrium and ventricular apex (Ge et al. 2015; Beltrami et al. 2003). During fetal development, they are mainly located in the pharyngeal mesoderm called secondary heart field and in the neural crest (Engleka et al. 2012) and help in the formation of atria, right ventricle, and outflow tract by regulating fibroblast growth factor (Golzio et al. 2012). Unfortunately, CSCs are difficult to isolate as a pure population owing to their extremely low number in the heart and lack of authentic CSC markers. Some believe that the beneficial effect of CSCs is due to the presence of differentiating cardiac progenitor cells (CPCs) or other types of uncharacterized cardiac cells. In one of our studies, we proliferated cardiac cells on a nanofiber (Fig. 11.1c).

The CSCs have also been isolated from cardiac adipose tissue with inherent cardiac and angiogenic potential (Soler-Botija et al. 2012). Smith et al. differentiated endogenous c-kit^{+} CSCs, isolated from the adult mouse and rat hearts, into functional beating CMs, smooth muscle and endothelial cells (Smith et al. 2014). The combined delivery of pericytes with CSCs repaired the vascular and muscular mouse infarcted hearts by secreting various growth factors, angiopoietins, and microRNA

(Avolio et al. 2015; Santini et al. 2016). Di Siena found that activated c-kit receptor is required in the heart to promote CM differentiation and regeneration after injury, with a significant improvement of survival of animal models (Di Siena et al. 2016). Recently, Aquila et al. showed that c-kit-expressing CSCs impair adult myocardium and restore CM replacement in the heart (Aquila et al. 2019). CSCs have still been a major controversial topic in the field of CTE.

11.4.7 Commercially Available Cardiac Cell Lines

Under laboratory research settings, cardiac cell lines such as AC16, HL-1, H9C2, and C2C12 are the most commonly used CM source for CTE. H9C2 is a cardiomyoblast cell line derived from a rat heart tissue. They are mononucleated cells in their earlier stage, but upon reaching confluency, they begin to form multinucleated, large, flat, and spindle-shaped cells. Although these cells no longer have beating and nonproliferating properties like CMs, they share many similarities with primary CMs in their protein expression, hormonal signal pathway, and electrophysiological properties (Hescheler et al. 1991). On the other hand, C2C12 is a mouse myoblast cell line isolated from the thigh muscle (Davidenko et al. 2016). It is adherent in nature and does not show spontaneous beating. Similarly, Tallawi et al. seeded C2C12 on poly(glycerol sebacate)/PCL (PGS/PCL) composite patterned nanofibers fabricated by a combination of electrospinning and soft lithography techniques and reported that the alignment of cells starts as per the topographical feature of fibers (Tallawi et al. 2016). These cells are, however, recommended to be co-cultured with EC and fibroblast as tri-culture. Tri-culture of CMs with ECs and fibroblasts promotes the formation and stabilization of vascular beds in the tissue construct for better oxygenation and nutrient supply (Iyer et al. 2012). The presence of EC-derived endothelial capillaries also augments the proliferation of CMs without hampering their orientation and alignment (Caspi et al. 2007).

11.4.7.1 Tri-Culture of Cells

Tri-culture of CMs with other cell types such as ECs and fibroblasts was reported to increase the proliferation and viability of CMs by augmenting the endothelial capillary formation for delivery of oxygen and nutrients (Thomson et al. 2013). Caspiet al. successfully developed synchronously contracting human cardiac tissue constructs from human ESCs, which are known to possess the potential to differentiate into all cell types, including CMs, ECs, and fibroblasts (Caspi et al. 2007). Indeed, ESC-derived 3D cardiac construct contained CMs, ECs, and embryonic fibroblasts that formed vessels, which were stabilized by the presence of mural cells originating from embryonic fibroblasts. A modular CTE approach has also been proposed for generating a vascularized cardiac tissue, wherein ECs were seeded onto type I bovine collagen supplemented with Matrigel™ having a macroporous and sheet-like scaffold embedded with CMs rich in contractile function (Leung and Sefton 2010). The formation of capillary-like cords was facilitated by seeding a low number of ECs (8% of the total cell number) on Matrigel™-coated

microchannels for 24 h followed by seeding of fibroblasts (32% of the total cell number) for another 24 h to stabilize the endothelial network (sequential preculture) before seeding CMs (60% of the total cell number) to develop beating cylindrical organoids (Iyer et al. 2012). Several studies have shown that tri-cultured tissue constructs have a better ability to form a vascular bed, generate contractile force, and anastomose with the host vasculature (Stevens et al. 2009; Morrissette-McAlmon et al. 2018). Tri-culture also promoted the integration of the engineered cardiac tissue with native myocardium and thereby enhanced survival of engineered graft (Lesman et al. 2014; Valarmathi et al. 2018).

11.5 Prospects and Challenges

In this book chapter, we have discussed the aspects of biomaterials required for CTE and their fabrication techniques and cell sources. The properties of biomaterials such as biodegradability, biocompatibility, porosity, cell adhesion property, hemocompatibility, mechanical integrity, electrical coupling, etc., must be tuned up to fabricate the scaffold, which must be compatible with cells and mitigate and organize themselves similar to native cardiac tissue.

As we know, the heart is a complex organ, and its regeneration depends on various significant factors. It develops a complex relationship between native cells and matrixes under in vivo and in vitro conditions, which requires high-throughput testing of therapeutics. On this basis, we need to focus on the appropriate selection of the scaffold material and its fabrication with cellular selection and proliferation on them. However, various clinical challenges such as isolation and proliferation of cells from their respective cell sources and biomaterial method to generate relatively thick constructs and advancement in the new techniques to fabricate 3D construct with vascularization must exist. An automatic system and a standard protocol must be established to translate the transition phase of technology from in vivo methods to clinical trials on humans. Numerous studies have been reported in this review, including in vitro and in vivo conditions, and many are in progress, which include the regeneration of the whole heart or bioartificial heart. However, many more advanced works remain to be done to overcome the challenges of experimental techniques and to improve existing methods and protocols for CTE applications.

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Recent Advances in Stem Cells for Dental Tissue Engineering

12

Mohammed Zahedul Islam Nizami and Yuta Nishina

Abstract

Stem cell therapy has been signified as an important field of dentistry, regenerative medicine, and tissue engineering research since the uncovering and design of mesenchymal stem cells (MSCs). MSCs, which can be obtained from various sources, may accomplish a vital role in tissue/organ growth and repair and have immense potentials in bone and dental tissue regeneration. General dental therapies involve the use of specialized tissue-adapted materials that are still uncertain in terms of their effectiveness and durability. Efforts of the past few decades have focused on novel therapeutic approaches for tooth regeneration wholly or partially, which has employed sophisticated biomaterials and implants. Current dental treatments have focused on multidisciplinary approaches by combining advanced tissue engineering, biomaterials, scaffolds, digital technology, and stem cells, which have exhibited great potentials. Therapeutic approaches based on stem cells would be alternative treatments in dentistry since they are highly promising in physiologically advanced structural and functional outcomes. To take a look at future dentistry in terms of stem cell-based treatment, the objective of this chapter is to introduce the history of stem cells, various types of stem cells related to dentistry, their characteristics, differentiation, isolation, collection, and preservation. This chapter outlines strategies of stem cell therapy applied in various dental/tooth/orofacial organ and tissue regeneration to provide

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a theoretical basis for clinical workflow. Further, the current status of dental and medical applications is within the scope of this chapter.

Keywords

Dental stem cells · Tissue engineering · Regenerative dentistry · Oral and dental tissue regeneration · Stem cell therapy

12.1 Introduction

Stem cells are primitive cells with self-replicating and multidirectional differentiation potentials. Stem cells can be differentiated into various tissues, functional cells, and organs under certain conditions; thus, they are regarded as universal cells. These cells can reproduce themselves randomly by self-renewal processes (Slack 2008), and they differ in terms of their circumstantial position in the body and the type of cells they can produce. Stem cells were first reported in the scientific literature in early 1868 to describe a single-celled progenitor organism, which was assumed as the precursor of all multi-celled organisms (Haeckel 1874). However, stem cell research was highly perceived in the mid- and late twentieth century. Martin J. Evans, Mario R. Capecchi, and Oliver Smithies were awarded the 2007 Nobel Prize in Physiology or Medicine for their discoveries of principles for introducing specific gene alterations in mice using embryonic stem cells (Mummery et al. 2014). The therapeutic potential of stem cell research currently attracts the researchers' interest. Updated research in visualizing the hope of these cells in severe progressive degenerative diseases was well demonstrated (Bottai et al. 2003). After discovering their comprehensive sources, these cells have attracted particular attention from researchers. The types of stem cells depend on the cells isolated and located in the body. In recent years, several studies have reported that orofacial tissues are a great source of stem cells, easily approachable for dentists. Counting their exclusive capacities, stem cells are considered for developing advanced technologies. Stem cell therapy utilizes the multidirectional differentiation property of stem cells for repairing damaged cells or re-forming the normal operation of cells and tissues (Caton et al. 2011). These applications are introduced in tissue engineering strategies for regenerating or replacing injured, diseased, or lost tissues and organs by cell manipulation in vivo and design of the extracellular environment (Langer and Vacanti 1993).

The tooth consists of highly mineralized enamel, dentin, cementum, and soft connective tissues of dental pulp and periodontium. The enamel is developed from epithelium-derived ameloblasts and other tooth components from ectomesenchymal cells. Dental pulp cells are differentiated into odontoblasts and form the matrix of the dentin, and cells of the periodontium are responsible for forming cementum of the tooth and alveolar jawbone. The periodontal ligaments (PDLs), which stabilize teeth by connecting the cementum of root to the alveolar bone, are created by various types of cells (i.e., epithelial rests of Malassez, fibroblasts, neuronal cells, and

endothelial cells) (Mariotti 1993; Sonoyama et al. 2007). Periodontal infections, dental caries, and traumatic injuries usually lead to pathologies of teeth and adjacent tissues (Caton et al. 2011). To date, these are the main clinical challenges due to the lack of self-healing ability of dental tissues. Prophylactic treatments following dental or periodontal lesions involve particularly preserved gene programs active during embryonic tooth growth (Aberg et al. 1997; Giannobile and Somerman 2003; Jin et al. 2004; Magloire et al. 2001; Ripamonti 2007). Generally, in pulpitis (pulp inflammation), stem cells and/or progenitor cells initiate new odontoblast generations and replace the disintegrated odontoblasts. It was thought that stem and progenitor cells are attracted by signaling molecules released at the pathologic sites and initiate the healing process, which ultimately repairs dentin (Nakashima and Iohara 2014). Nevertheless, the reparative immune system of the dental pulp and periodontal tissues is often inadequate in the complete recovery of injured and diseased tissues. If such issues are not treated nor noticed, the lesions compromise tooth integrity, leading to tooth loss and more severe pathology.

Reputable researches on the reparative dentin formation by dental tissues have contributed to apply alternative treatments of dental diseases. At this stage, dentistry is standing to the previously recognized treatment protocols; subsequently, maximum numbers of the claimed therapeutic outlines are still not a practical stage. Biomaterials related to dentistry are repeatedly used to amalgamate growth factors and molecules to enhance orthodontal and periodontal tissues (Pilipchuk et al. 2015). In partial dental tissue repair, specialized dental materials with ambiguous efficiency and durability and advanced dental implants in tooth replacement are good examples (Esposito et al. 2013; Fron Chabouis et al. 2013). However, advances in regenerative tissue engineering likely lead to a long-term result employing biological restoration or artificial replacement of injured teeth. The structuring of dental tissue showed possible results in the regeneration of dental tissues or organs. Multiple factors are involved in alveolar bone resorption (i.e., different degrees of bone resorption after tooth extraction/loss due to periodontal disease, advanced caries, fractures of the root, or traumatic injuries) (Kirkwood 2008). Besides, the bone resorption in the residual ridge, mostly in the mandible, continues throughout life in many edentulous patients (Atwood 1971). The excessive bone resorption makes it tough to replace the missing teeth with implants or dentures (Darby et al. 2008; Egusa et al. 2010; Egusa 2012). Thus, stem cell and tissue engineering rehabilitations are anticipated to provide an innovative capability for significant regeneration in periodontal tissue defects (Izumi et al. 2011) and defects in the alveolar bone (Yamada et al. 2006a, 2008; Ueda et al. 2008) and to ultimately replace the lost tooth itself (Ikeda et al. 2009; Oshima et al. 2011). The area for such regenerative techniques in dentistry involves the tongue (Luxameechanporn et al. 2006), craniofacial skeletal muscles (Shah et al. 2005), the condylar cartilage of the temporomandibular joint (Dormer et al. 2011; Yu et al. 2011), as well as salivary gland (Lombaert et al. 2011). Various studies were conducted in animal models using stem cells and critical components of tissue engineering such as extracellular matrix scaffolds and bioactive factors for developing the idea of oral and dental tissue and organ regeneration for clinical dental applications (Akagawa et al. 2009; Kubo et al. 2011). However, it has already

been used in clinical trials for oral bone tissue regeneration (Yamada et al. 2006b, 2008; Ueda et al. 2008).

The clinical applications of jawbone regeneration using stem cells and tissue engineering strategies were reported in implant dentistry. Despite these auspicious achievements, a wide variety of stem cells from the oral and maxillofacial regions can be conquered, which may lead to confusion about the role of stem cells and regenerative biology in dentistry, usually about the optimal regeneration of stem cells for oral tissue. Thus, the details of the types of stem cells, clinical availability, sources in dentistry, differentiation capacity, accessibility, and potential immunomodulatory properties, as well as the current state of stem cell research and clinical trials in dental practice, are discussed in this chapter.

12.2 The Tooth Development and Strategy of Biological Tooth Replacement

The development of a tooth is generally considered a sequence of well-defined phases (Fig. 12.1) that involve the interface of the oral epithelium (originated from the ectoderm) and mesenchyme (originated from the neural crest) (Mina and Kollar 1987). Dental enamel derives from oral epithelium, while mesenchymal cells

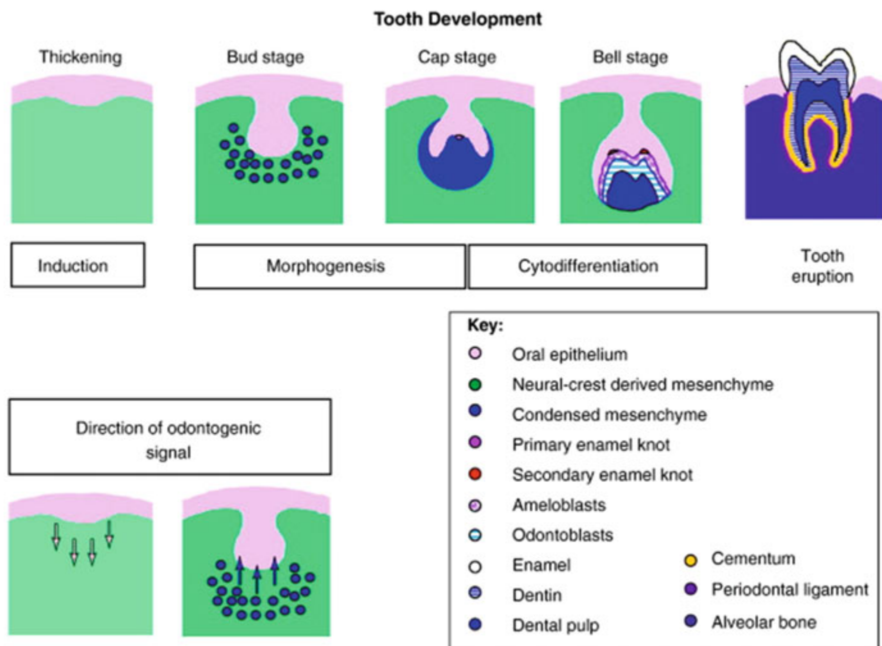

Fig. 12.1 Schematic representation of various stages of tooth development. (Reproduced with courtesy—Trends Cell Biol. 20:715)

produce cementum, dental pulp, and PDLs. Besides, progenitor cells (originated from mesodermal cells) and neural crest cells produce craniofacial bones (i.e., skull bone and face) (Huang et al. 2010a).

In tooth development, induction is the first step, where signaling from the epithelium to the mesenchyme initiates the tooth formation. After the mesenchymal cells receive signals from the epithelium, they transmit signals to the epithelium. With the limited proliferation of dental epithelial cells, the cells form a bud around which the mesenchymal cells are organized. Oral epithelium condensing leads to dental lamina formation on both the lingual side and the vestibule. While the dental lamina forms the teeth, the vestibular lamina generates a deep notch or groove within the teeth and cheek. Later, placodes are formed by the proliferation of dental lamina. They invade the mesenchyme and form the tooth bud. Local proliferation and differentiation of the epithelial cells in the bud initiate the cap stage in which crown formation is commenced by the epithelial signaling center, where an enamel knot controls the epithelium folding. The mesenchymal cells produce the dental papilla at the bell stage, where the predecessor of the specific tooth cells (i.e., ameloblasts and odontoblasts) differentiates to form tooth enamel and dentin. Progressively, the growth and expansion of the teeth continue postnatally; the outer enamel covering becomes hard and the teeth root develops next, and the tooth erupts in the oral cavity subsequently (Mina and Kollar 1987; Lumsden 1988; Ruch et al. 1984). Epithelial and mesenchymal cells synchronize each process by exchanging signals throughout the entire period of tooth formation. The biological replacement strategies may be utilizing these signal exchanges and identifying either epithelial cells or mesenchymal cells that can induce ingenuous mesenchyme or epithelium for stimulating tooth development (Caton and Tucker 2009). Restoration, repair, and replacement of teeth are unique among clinical treatments. The teeth are integral but nonvital organs for life and are therefore not considered a significant emphasis of regenerative research compared with neural or cardiac diseases. Mostly, the patients do not typically have severe complaints or life-threatening conditions and do not necessitate significant surgeries. However, the presence of overgrown stem cells in teeth, which can be easily found from naturally lost or surgically extracted teeth (i.e., orthodontic treatment purpose, impacted canine or third molar), can be employed for tooth restoration, repair, and regeneration in addition to non-dental uses like emerging stem cell-based treatments for serious life-threatening lesions. An essential but often overlooked advantage of teeth as a source of stem cells is the formation of postnatal roots, which are more embryonic than other sources of dental stem cells. They could play an essential role in future regenerative medicine and dental research (Caton and Tucker 2009).

12.3 Types of Dental Stem Cells (DSCs)

DSCs can be distinguished from different tissues in the craniofacial and oral regions. These start from the individual developmental stages of the teeth. To date, other human tooth stem cells have been segregated and classified based on their location, as typified in Fig. 12.2.

12.3.1 Dental Pulp Stem Cells

The dental pulp is a fibrous tissue comprising mesenchymal stem cells (MSCs) that originate from the embryonic cranial neural crest (Chai et al. 2000). Dental pulp stem cells (DPSCs) have been known since the discovery from human teeth in 2000 (Gronthos et al. 2000). They are found mainly in the pulp-rich cell area in perivascular and perineural areas of the pulp and are enormously proliferative, clonogenic, and multipotent and exhibit a high degree of plasticity (Perry et al. 2008). It was reported that the pulp contains cells, containing osteogenic markers that correspond to osteogenic differentiation inducers (Graziano et al. 2008a). DPSCs show a strong aptitude for proliferation and self-renewal and eventually differentiate to odontoblast and osteoblasts to produce dentin and bone. The most significant source of DPSCs is the third molar, which can be extended and stored for future use (Gronthos et al. 2000). DPSCs exhibit both MSC-like (i.e., CD29, CD90, CD105, CD146, CD166, and CD271) and neural stem cell-like (i.e., nestin and glial

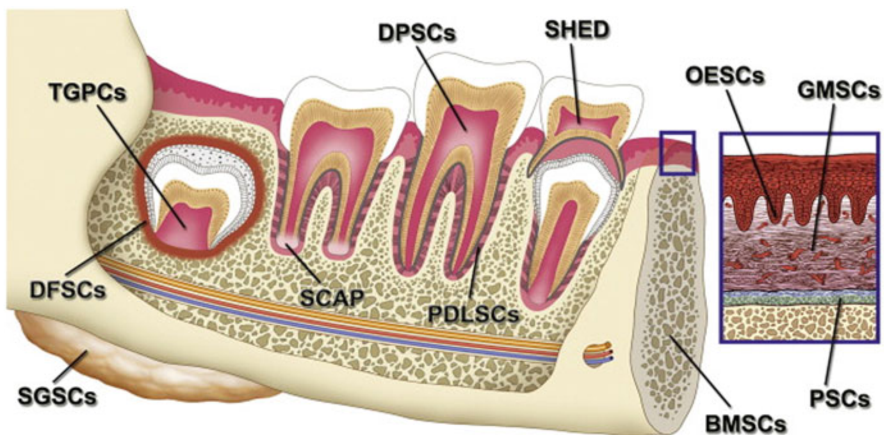

Fig. 12.2 Schematic illustration of different dental stem cells in the oral region, such as tooth germ progenitor cells (TGPCs), dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), dental follicle stem cells (DFSCs), stem cells from the apical papilla (SCAPs), periodontal ligament stem cells (PDLSCs), salivary gland-derived stem cells (SGSCs), bone derived mesenchymal stem cells (ABMSCs), oral epithelial progenitor/stem cells (OESCs), gingival mesenchymal stem cells (GMSCs), periosteum-derived progenitor/stem cells (PSCs). (Reproduced with permission from Egusa et al.)

fibrillary acidic protein (GFAP)) phenotypic stem cell markers but negative for hematopoietic markers (i.e., CD45) (Gronthos et al. 2000; Gronthos et al. 2002; Huang et al. 2009). DPSCs can be differentiated into odontogenic (Gronthos et al. 2000; Hayashi et al. 2015; Miura et al. 2003), chondrogenic (Waddington et al. 2009), osteogenic (d'Aquino et al. 2009; de Mendonca et al. 2008), neurogenic (Martens et al. 2014; Nosrat et al. 2001), adipogenic (Gronthos et al. 2002; Waddington et al. 2009), and myogenic (Kerkis et al. 2008; Pisciotta et al. 2015) cells in vitro and in vivo. Stem cells of dental pulp show an elevated immunosuppressive activity compared to adult mesenchymal stem cells of the bone marrow (d'Aquino et al. 2007). DPSC compounds with hydroxyapatite/tricalcium phosphate form dentin-like structures that contain pulp tissue and form an odontoblastic cell lining after transplantation into immunocompromised mice (Gronthos et al. 2000). DPSCs are important for postnatal tooth homeostasis and repair because of their capability to replenish odontoblasts during dentin restoration/regeneration (Laino et al. 2005; Shi and Gronthos 2003; Gronthos et al. 2002). In addition, DPSCs are of neural origin; they can be differentiated into functionally active neurons and glial cells after proper environmental stimulation. Similarly, DPSCs contribute to neuroprotection and neurite proliferation as they demonstrate a unique ability to conceal neurotrophic factors (Arthur et al. 2008; Ratajczak et al. 2016). They have diverse bone markers, including bone alkaline phosphatase, osteocalcin, osteonectin, sialoprotein, and collagen types I and III. During their breeding in substrates in conjugating with hydroxyapatite, bone and cement tissue are formed (Yamada et al. 2006a). DPSCs can be differentiated into functional osteoblasts in vitro and produce an extracellular mineralized matrix (Laino et al. 2005). They have all the properties needed for successful therapeutic usage; for instance, they are easily available for insulation, they are multipotential and exhibit interaction with biomaterials for cell proliferation, and they are enduring (Chang et al. 2005).

12.3.2 Stem Cells Isolated from the Pulp of Human Exfoliated Deciduous Teeth (SHEDs)

Primary teeth are the most important sources of stem cells. These are heterogeneous cell populations that have a clonogenic capability. In 2003, MSCs were isolated from the pulp tissue of exfoliated deciduous tooth crown, which vary from DPSCs and are termed SHEDs (Miura et al. 2003). SHEDs express the cell surface molecules such as STRO-1 and CD146 and different neural and glial markers (i.e., β-III tubulin and nestin) (Miura et al. 2003). SHEDs have pleasantly advanced proliferation rates associated with DPSCs and bone marrow-derived MSCs (Nakamura 2009). The gene expression profiles exposed 4386 genes differentially between DPSCs and SHEDs when twofold or more have been used. However, a broader expression was identified in the SHEDs for genes involved in alleyways related to cell explosion and extracellular matrix formation, with reasons for a lot of growth factors such as fibroblast growth factor and transforming growth factor (TGF)-β (Nakamura 2009). TGF-β is essential because it is released after damage to the dentin, which can

assemble pulp stem cells to differentiate into odontoblasts (Sloan 1999). The SHED-regenerated tissues express odontoblastic differentiation markers (i.e., DMP-1 and DSPP) (Sakai 2010). They can be differentiated into in vitro odontogenic, chondrogenic, osteogenic, myogenic, adipogenic, and neurogenic cells and can be carried in vivo to form bone and dentin (Miura et al. 2003; Kerkis et al. 2008). SHEDs illustrated functional odontoblasts that produced tubular dentin and angiogenic endothelial cells when they were seeded onto tooth slices/scaffolds and subcutaneously implanted into immunosuppressive mice (Sakai 2010). In in vivo dental pulp tissue engineering, the infected pulp was removed and replaced with stem cells. It was exposed that the newly formed tissue has analogous tissue architecture and cellularity to that of the dental pulp (Cordeiro 2008). The introduction of SHEDs through blood vessels and nerves in both animal models and traumatized patients also regenerated the three-dimensional whole dental pulp, and that pointed to SHEDs as an attractive cell source for bone and tooth regeneration (Xuan et al. 2018). In addition, SHEDs can appropriately induce bone-like matrix formation with lamellar structures using host cells (Miura et al. 2003; Seo et al. 2008). This specific property of SHEDs in bone formation can be explained by the nature of the thin teeth, which have combined root resorption with new bone formation around the roots.

The ability of SHEDs in the regeneration of dental pulp by depositing a mineralized and organized matrix using clinically operable techniques is of great interest in tissue engineering. Transplanted SHEDs in full-length root canals, including injectable scaffolds, were able to extend inside the root canal and reveal putative markers of odontoblasts (DSPP, DMP-1, and matrix extracellular phosphoglycoprotein). SHEDs were differentiated into functional odontoblasts that can generate new dentin. However, more investigations are suggested for the evaluation of the ability of SHEDs for the development of functional dental pulp tissue in an oral condition (Rosa et al. 2013). In addition, these cells can be differentiated into vascular endothelial cells. In vitro, vascular endothelial growth factor (VEGF) induces SHEDs to release the endothelial markers (i.e., vascular endothelial growth factor receptor 2 (VEGR2), platelet endothelial cell adhesion molecule (CD31), and VE-cadherin adhering molecules) and organize capillary-like sprouts (Ferrara et al. 2003). Besides, the therapeutic efficacy of SHEDs in alleviating Parkinson’s disease is also evident (Wang 2010). SHEDs can be used as a generic allogeneic source of MSCs. However, their use as autologous cells is currently limited to children who have not yet lost all their deciduous teeth. These are being expanded to enable commercial banking of these cells to be used by children as they become adults. Only a few studies have shown that frozen SHEDs retain their properties after cryopreservation for 2 years (Papaccio 2006), but long-term storage ($\geq$10 years) was not yet assessed.

12.3.3 Periodontal Ligament Stem Cells (PDLSCs)

PDL is the fibrous connective tissue situated between the inner wall of the alveolar fossa and cementum, which plays an important role in supporting the tooth by fortifying the alveolar bone.

(Chen et al. 2012). The presence of various types of cells indicates that PDLs comprise stem cells, which are accountable for physical conservation and the regeneration of periodontal tissue structure and function. PDLSCs were first discovered in 2004 using MSC-related markers (Seo et al. 2004). PDLSCs are usually found from extracted orthodontic teeth or impacted third molars (Zhai et al. 2018), extending the source of preserved deciduous teeth (Silvério et al. 2010) or cryopreserved human PDLs (Seo et al. 2005). The roots of human deciduous teeth are slowly becoming a new source of PDLSCs that are highly refined by magnetic cell extraction (Silvério et al. 2010). Different well-distinguished clonal human PDLSC cell lines were well utilized for the advancement of the reformative periodontal treatment protocol (Tomokiyo et al. 2008; Singhatanadgit et al. 2009). PDLSCs exhibit positive expression of the cell-surface markers STRO-1, CD146, and CD44 that facilitate the mesenchymal cell lineage differentiation to form osteoblast-like cells, adipocytes, and collagen-forming cells in vitro. Furthermore, PDLSCs have seen an improved level of tender-specific dictation factor expression, which makes it suitable for the postnatal MSC population (Seo et al. 2004). These cells exhibit a clonogenic nature, reveal various cementoblastic/osteoblastic markers, and can form mineralized nodules. In vivo replacement of PDLSCs can produce cementum and PDL-like structures (Seo et al. 2004). Transplantation of these cells into periodontal defects facilitates the attachment of the PDLs to the tooth surface and regenerates the lost alveolar bone tissue in vitro (Li et al. 2009) and may form the cementum/PDL tissue, which comprises thick collagen I positive tissue when transplanted into immunocompromised mice (Seo et al. 2008). This positive collagen tissue mimics the physiological attachment of Sharpey's fibers. Thus, it was expected that PDLSCs could form osteoblast cells, cementum/PDL tissue, and Sharpey's fibers. In addition, human PDLSCs were seen to recover defects and transfer into the PDL compartment when replaced into surgically created defects in the periodontal region of lower molars, suggesting that PDLSCs could be used to regenerate a periodontal tissue. The reproducible potential of bone marrow-derived stem cells, PDLSCs, DPSCs, and SHEDs for the treatment of periodontitis was well proved in miniature swine and dog models (Ding et al. 2010; Du et al. 2014; Fu et al. 2014; Khorsand et al. 2013). PDLSCs exist on both the root and the alveolar bone surface with a strong ability to differentiate and expand (Wang et al. 2011a, b).

Regeneration of alveolar bone defects is a significant challenge for clinicians. Some years ago, the first successful clinical trial of alveolar bone reconstruction was performed using autologous human DPSCs, combined with collagen scaffolds (d'Aquino et al. 2009). Over a 3-year follow-up, conventional and in-line holotomography assessment observations showed that the regenerated bone at the grafted sites was completely compact and entirely different from the common spongy alveolar bone of the mandibles (Giuliani et al. 2013). In another study, a

clinical trial was conducted on three patients to assess the recurrence of PDLSCs in severe periodontal disease and two of those patients were reported to restore healthy periodontal tissue with significant regeneration of periodontal tissue. Moreover, the probing depth and looseness were significantly reduced, and the periodontal attachment was stabilized in another patient. Thus, autologous PDLSCs were claimed to have a significant effect on severe periodontal diseases (Feng et al. 2010). However, the shortage of PDLSC collected from the same donor restricts the use of PDLSC for treating periodontal disease. On the other hand, PDLSCs were shown to have low immunity as BMMSCs and important immunoregulatory functions (Kim et al. 2010). It was shown that when it was transplanted into the periodontal bone defect pig model, allogeneic PDLSCs reversed periodontitis without immune rejection (Ding et al. 2010). Therefore, PDLSCs were considered as the first choice for periodontal regeneration therapy (Iwata et al. 2010), and clinical studies also had shown the use of autologous PDLSCs for repairing periodontal defects and they are proven to be safe and efficient in periodontal regeneration and the treatment of the defects (Chen et al. 2016). On the other hand, the microenvironment around stem cells has been shown to have a reflective effect on their functions, such as PDLSCs differentiated to the cementoblast lineage for the formation of cementum/PDL tissue, while apical tooth germ cells are cultured and supplied to produce a highly cementogenic microenvironment (Yang et al. 2009). However, in inflamed microenvironments, epigenetic modification and endoplasmic reticulum stress may regulate PDLSC osteogenesis (Li et al. 2016; Xue et al. 2016). Further study of molecular mechanisms may enhance researchers' knowledge of chronic inflammatory conditions and acclimate the theoretic basis for PDLSC-based stem cell treatment for inflammatory periodontal disease. Periodontal regeneration was promoted by platelet-derived growth factors (PDGFs) and bone morphogenetic protein (BMP) was reported (Howell et al. 1997; Lynch et al. 1989); BMPs increased alveolar bone and cementum formation (Selvig et al. 2002). Commercial amelogenin extracts have also been reported to be used clinically for periodontal tissue regeneration, but their activity is still unclear (Veis et al. 2000).

12.3.4 Stem Cells from the Apical Papilla (SCAPs)

SCAPs are isolated from the human immature permanent apical papilla, a unique variety of dental MSCs which express the characteristics of high proliferation, self-renewal capability, and low immunity (Sonoyama et al. 2006). It was reported that SCAPs could be differentiated to various cell lineages such as odontogenic, chondrogenic, osteogenic, adipogenic, neurogenic, and hepatogenic cells, which leads to a potential source for stem cell-based therapies (Sonoyama et al. 2006, 2008; Dong et al. 2013; Patil et al. 2014). They have shown positive response to STRO-1 and CD146 and negative response to CD34 and CD45, which are like DPSCs and SHEDs, except for CD24, which only exists in SCAPs. The degraded expression level of CD24 of SHEDs exhibits in vitro odontogenic differentiation. Furthermore, clonal cell populations may be obtained using type I collagenase and

neutral proteases to digest apical papilla tissue and formulate single-cell suspensions. SCAPs appear to be the source of primary odontoblasts when forming root dentin, where DPSCs are a potential source of additional odontoblasts to produce secondary reparative/replacement dentin. In addition, SCAPs show a higher expansion than PDLSCs in their third passages (Han et al. 2010). To create bio-root, SCAPs and PDLSCs were co-implanted in the alveoli of minipigs, and it was reported that the combined use of these stem cells could create a root/periodontal complex for root function replacement (Sonoyama et al. 2006, 2008). It has recently been reported that stem cells derived from the apical edge of developmental roots exhibit unique "embryonic" properties (Jo et al. 2007) and form a common in vivo cementum/PDL-like complex.

12.3.5 Dental Follicle Stem Cells (DFSCs)

The dental follicle is derived from ectomesenchyme. It is a loose connective tissue capsule that surrounds the enamel organs and tooth tissues during development before emergence. Human DFSCs are mainly extracted from the sac of the third molar. Basically, these cells are progenitor cells of the PDLs, alveolar bone, and cementum and exhibit the STRO-1 and CD44 markers (Morsczeck et al. 2005). In vitro orientation, DFSCs was reported to exhibit odontogenic, cementogenic, osteogenic, adipogenic, and neurogenic differentiation (Huang et al. 2010a; Morsczeck et al., 2005; Rai et al. 2013). In vivo implantation of DFSCs in an immunodeficient mouse shows the formation of fibrous and rigid tissues rich in osteocalcin, bone sialoprotein, and collagen type I (Luan et al. 2006). They are reported to form both in vivo and in vitro cementum and bone tissues (Kémoun et al. 2007; Yokoi et al. 2007). Additionally, the DFSCs comprise putative MSC markers (nestin and Notch-1) and other markers of STRO-1 (CD105 and CD90). They also display multipotencies like bone marrow-derived MSCs (BMMSCs) and PDLSCs. The ability to adhere to plastic surfaces makes it easier to separate DFSCs from human dental follicles during culture (Morsczeck et al. 2005). They were reported to differentiate into PDL fibroblasts and secrete collagen, which may interconnect with the adjacent bone and cementum and induce cementum/PDL-like tissue formation during transplantation in the mice model (Handa et al. 2002). Interestingly, the proliferation, immunity, and morphological and mineralizing properties of DFSCs can be detected after more than 30 passages and eventually lead to cementum/PDL-like tissue formation (Guo et al. 2012). On the other hand, in amalgamation with treated dentin matrix (TDM), DFSCs are able to regenerate a root-like tissue for the replacement of the alveolar fissures in a mice model, which refers to the ability of DFSCs to form tooth roots (Guo et al. 2012). Recent studies have shown that DFSCs stimulated by Hertwig's epithelial root sheath cells are able to form periodontal tissue by epithelial and mesenchymal interaction (Bai et al. 2011). It is also suggested that the BMPs and enamel-matrix derivatives may be necessary for the cementoblastic and osteoblastic differentiation of DFSCs (Wu et al. 2008; Kémoun

et al. 2007). The complex factors that emphasize the differentiation of DFSCs are unclear; however, DFSCs are still considered valuable for tooth regeneration.

12.3.6 Tooth Germ Progenitor Cells (TGPCs)

A distinct source of stem cells, TGPCs, is found in the dental mesenchyme of the late bell stage of third molar tooth germ development. They showed a highly extended activity and the ability to in vitro differentiation into lineages of the three germ layers (i.e., osteoblasts, neural cells, and hepatocytes). They positively impact tissue regeneration in liver disease (Ikeda et al. 2007). Compared to other dental MSCs, TGPCs can be differentiated into hepatocyte-like cells. TGPCs is an ideal remedial ingredient for the treatment of liver disease and liver regeneration. In a study, TGPCs were transplanted into a carbon tetrachloride (CCl4)-treated liver injured rat model and found successful engraftment of the TGPCs by PKH26 fluorescence at 4 weeks after transplantation. In CCl4-treated rats, TGPCs have been found to prevent the progress of liver fibrosis and to facilitate the rehabilitation of liver function. These positive results suggest that multipotent TGPCs are a good contender for cell-based therapy for liver disease, which offers incomparable potential for the development of treatments for tissue repair and regeneration (Egusa et al. 2012a, b). A substantial therapeutic effect of engrafted TGPCs was reported to prevent the inclination of liver fibrosis (Ikeda et al. 2007). TGPCs were proliferated for 60 passages and were found to retain their expansion rate and morphology. They express MSC markers (i.e., STRO-1, CD29, CD44, CD73, CD90, CD105, CD106, CD166) (Liu et al. 2015). They also tend to pluripotent gene expression (i.e., sox2, nanog, oct4, c-myc, and klf4) and portentous a mesenchymal phenotype (Ikeda et al. 2007; Yalvac et al. 2010, Yalvaç et al. 2010).

12.3.7 Natal Dental Pulp Stem Cells (NDP-SCs)

In 2010, the first NDP-SCs were isolated and described from the two natal teeth of a healthy newborn baby girl (Karaöz et al. 2010). NDP-SCs are homogeneous dental pulp-related stem cells isolated only from the natal dental pulp. Dental pulp was used and a single-cell suspension was made using collagenase, and a very few number of cells were found to adhere to plastic in cultures that looked like fibroblasts that became flattened at later passages, which were described as NDP-SCs. They have in vitro multilineage differentiation potential and can be differentiated into osteoblastic, adipocytic, chondroblastic, myoblastic, and neuro-gliallic cells (Karaöz et al. 2010; Akpinar et al. 2014). They showed a higher proliferation rate compared to SHEDs and DPSCs. NDP-SCs exhibit CD13, CD29, CD44, CD73, CD90, CD146, and CD166 markers but do not show CD14, CD34, CD45, and HLA-DR antigens like other DSCs (Karaöz et al. 2010; Akpinar et al. 2014). Significantly, NDP-SCs reveal the level of identification of ESC markers (i.e., Nanog, Rex-1, and Oct-4, also the transcription factors Sox-2 and FoxD3), signifying the pluripotency of

NDP-SCs. Additionally, NDP-SCs showed affinity to immune reaction on the osteogenic markers (i.e., COL1, OCN, ON, OPN, BMP-2, and BMP-4), some myogenic markers (i.e., desmin, myogenin), the chondrogenic marker COL2, and neural markers (i.e., nestin, vimentin, GFAP, and βIII-tubulin) in standard medium (Karaöz et al. 2010). The protein expression of human NDP-SCs resembles more like the SHED proteome than that of DPSCs, and it identified 61 proteins that were initially been expressed by all three DSCs (Akpinar et al. 2014). Moreover, the identified proteins play a role in cellular architecture and are involved in folding machines and protein-related replication, synthesis, and degradation, thus inferring the presence of cellular self-renewal and progression.

12.3.8 Gingival Mesenchymal Stem Cells (GMSCs)

Gingiva is a special oral tissue connected to the alveolar bone of the tooth socket, which plays as a biological mucosal barrier and various integral constituents of the oral mucosal immune system (Zhang et al. 2009). It consists of three layers, intermingling of an epithelial layer, a basal layer, and a lower thin spinous layer like a basal dermis. It has excellent wound healing and regenerative capability, exhibited by a rapid reconstruction of tissue with or without slight intimation of scarring compared to the common scar formation present in the skin (Irwin et al. 1994). GMSCs were first secluded and reported from the spinous layer of human gingiva in 2009 (Zhang et al. 2009). A healthy gingival tissue obtained from a residue or waste tissue was used following a routine dental procedure. Typically, gingival tissues are incubated with dispase to detach the epithelial and lower spinous layers. Tissues are then enzymatically crushed and processed using collagenase (Zhang et al. 2009; Gao et al. 2014). GMSC isolation may involve the use of collagen and dispase together, initially, they remove the first enzymatically processed cell suspension to remove epithelial cells, and then they are isolated by additional incubation using the same enzyme solution (Tomar et al. 2010; Jin et al. 2015). GMSCs were identified to have fibroblast-like morphology, uniformly adhered to the plastic (Zhang et al. 2009). These cells exhibited constant phenotypes and maintained a normal karyotype and telomerase action in prolonged cultures (Tomar et al. 2010). GMSCs are favorable for the expression of surface markers CD13, CD29, CD44, CD73, CD90, CD105, CD146, STRO-1, Oct-4, and SSEA-4 antigens and negative for the hematopoietic markers CD14, CD34, and CD45, and HLA-DR (Zhang et al. 2009; Gao et al. 2014; Tomar et al. 2010). Remarkably, GMSCs were found to display immunoregulatory properties; specifically, they inhibit lymphocyte proliferation and express immunosuppressive factors (i.e., IL-10, iNOS, and COX-2) in response to the inflammatory cytokine interferon γ (IFNγ) (Zhang et al. 2009). GMSCs have recognized the possibility of multilineage differentiation of in vitro osteogenic, chondrogenic, and adipogenic cells (Zhang et al. 2009; Gao et al. 2014; Mitrano et al. 2010). It was also stated that endothelial and neural differentiation occurs in vitro if GMSCs are stimulated through a suitable culture medium (Zhang et al. 2009). A connective tissue-like collagen fiber

regeneration was reported in GMSCs. However, osteogenic differentiation in transplanted GMSCs was not observed. The possibility of in vivo bone regeneration using GMSCs was also described. GMSCs were cultured with HA/TCP particles in an osteogenic medium and then implanted in immunocompromised mice. The implants in a subcutaneous tissue exhibited the presence of high mineralized tissue (Tomar et al. 2010).

12.3.9 Oral Mucosa-Derived Stem Cells (OMSCs)

The oral mucosa contains the accumulated squamous epithelium, called the "oral epithelium," and an underlying connective tissue termed the lamina propria. To date, two distinct human adult stem cell types have been identified in the oral mucosa. One is the oral epithelial progenitor cell/stem cell, known as a subset of small oral keratinocytes ($\leq$40 mm). Although these cells appear to be unipotent stem cells (i. e., developing only on the epithelium, possessing clonogenicity, ex vivo regeneration of a highly stratified well-organized oral mucosa), they are recommended to use for inter-oral grafting. Another type of stem cell in the oral mucosa was recognized in the gingival lamina propria. A multipotent neural crest stem cell is known as an oral mucosal stem cell (OMSCs) that can be reproducible and differentiated into in vitro three germ-layer lineages from the lamina propria of the gingiva (Marynka-Kalmani et al. 2010). The innate stemness of these cells is defined as the high reprogramming capability of gingiva-derived fibroblastic cells through induced pluripotent stem cell (iPSC) generation. The multipotent OMSCs are easy to isolate, clinically abundant, and exhibit rapid ex vivo expansion that provides important assistance as a stem cell source for prospective clinical applications (Egusa et al. 2012a, b).

12.3.10 Alveolar Bone-Derived Mesenchymal Stem Cells (ABMSCs)

BMSCs are found in the osseous tissue of the alveolar bone. Matsubara et al. described human alveolar bone-derived mesenchymal stem cells (Matsubara et al. 2005). ABMSCs express a spindle-shaped fibroblastic morphology, plastic adherence, and cluster formation. They respond positively to the release of surface markers CD73, CD90, CD105, and STRO-1 and negative expression to hematopoietic markers CD14, CD45, and CD34 (Matsubara et al. 2005; Mason et al. 2014; Park et al. 2012a, b). They can be distinguished into osteoblast-like lineages by high alkaline phosphatase (ALP) expression (Matsubara et al. 2005). The treatment of human BMSCs with dichloromethane fraction of Dipsaci Radix (Kim et al. 2011), interferon-induced transmembrane protein 1 (Kim et al. 2012a), and nicotine (Kim et al. 2012b), low-frequency pulsed electromagnetic fields (Lim et al. 2013a), low-intensity pulsed ultrasound (Lim et al. 2013b), low fluid dynamic shear stress (Lim et al. 2013c), and orbital shear stress (Lim et al. 2014) have shown to increase osteogenesis. They exhibit differential potential for chondrogenic and adipogenic cell differentiation (Pekovits et al. 2013). Studies have shown that

bioceramics can provide a good scaffold for ABMSC attachment, expansion, transfer, and differentiation for bone tissue engineering (Kim et al. 2013). In vitro ABMSC passaging displays distinct osteoblastic lineage differentiation and ALP expression (Matsubara et al. 2005). In addition, ABMSCs can be easily harvested during dental implant surgery (Park et al. 2012a, b). These cells' source gives an advantage to ABMSCs over others cell types appropriate for clinical trials and confirms the practicability of using ABMSCs in clinical applications for treating bone defects (Park et al. 2012a, b).

12.3.11 Periosteum-Derived Stem/Progenitor Cells

The periosteum is the external surface of a bone tissue, which is comprised of two distinct layers with up to five histologically varied functional regions. Primarily fibroblasts and elastic fibers form the outer layer, and MSCs, osteoblasts, fibroblasts, osteogenic progenitor cells, microvessels, and sympathetic nerves develop the inner area. The osteogenic capacity of the PSCs was reported to form an in vitro mineralized extracellular matrix (Fell 1932). Moreover, many studies have reported the perspective of periosteal osteogenesis, along with long bone and periosteum growth, vasculature growth, periosteum interaction, and the osteogenic capacity of periosteum. However, the heterogeneous cell detached from the periosteum appears to endure osteogenic differentiation significantly and can be differentiated into osteoblasts, adipocytes, and chondrocytes, expressing the typical MSC markers. It was also reported that clonal populations derived from unicellular cells of periosteal cells in adults possess mesenchymal multiplicity and differentiate into osteoblasts, chondrocytes, adipocytes, and skeletal myocytes in vitro and in vivo (De Bari et al. 2006). It was speculated that expended periosteum-derived cells may be conducive to functional tissue engineering, particularly bone regeneration. A comparative analysis of canine MSCs/progenitor cells displayed that periosteum cells had a higher in vivo probability than BMSCs and alveolar bone cells derived from the ileum. Phenotypically maxillary and mandibular periosteum cells were like maxillary tuberosity-derived BMSCs, and both cells produce an ectopic bone following subcutaneous implantation in mice. It has also been shown that human periosteal cell proliferation was faster than marrow stromal cells, and subcutaneous transplants of periosteal cells treated with collective recombinant growth factors form an additional new bone than that of BMSCs in mice. In addition, periosteal grafts were available to confirm the structure of the cortical bone. In contrast, bone marrow grafting inspired the formation of cancellous bones with bone marrow-like structures in the rat calvarial defect model, suggesting that the source of transplanted cells may affect the structural features of the regenerated bone (Agata et al. 2007).

12.3.12 Salivary Gland-Derived Stem Cells

The salivary glands originate from the endoderm and are composed of a combination of acinar and dental epithelial cells with exocrine function. After the ligation of the

salivary gland duct, acinar cells become apoptotic, and later the duct epithelium develops. The stem cell responsible for the development of all epithelial cells in the gland is yet to be identified. Thus, the characteristics of stem cells in the salivary glands were attempted using isolated tissue cell cultures (Kishi et al. 2006). It was identified that the salivary gland stem/progenitor cells from the rat submandibular gland were highly proliferative and expressing markers for the acinar, duct, and myoepithelial cell lineages. In vitro differentiation of salivary gland duct cells, mucin, and amylase-producing cells was described. In addition, stem/progenitor cells can be isolated from swine and human salivary glands. However, initial cultures of isolated cells mainly comprise numerous cells with different origins, such as parenchymal cells, stromal cells, and blood vessel cells, which makes it challenging to select salivary gland stem cells. In fact, cells such as nascent MSCs isolated from human salivary glands (e.g., stromal tissue), which show embryonic and adult stem cell markers, may be directed to differentiate into osteogenic, chondrogenic, and adipogenic cells (Gorjup et al. 2009). For a stem cell population that can be genuine stem cells for the salivary gland, cells labeled with a specific marker-bearing or induced reporter protein need to be selected (Egusa et al. 2012a, b). Interstitial transplantation of these cells has been reported to successfully rescue the salivary function of the irradiated gland, suggesting that these are the favorable stem cell source targeting patients with irradiated head and neck cancer for future treatment (Neumann et al. 2012). Therefore, adult salivary gland stem cells are expected to be suitable for autologous transplantation treatment alternative for tissue engineered-salivary gland or direct cell therapy.

12.4 Stem Cell Therapy in Dentistry

Stem cell therapy in regenerative dentistry is generally thought to revive damaged dental tissues and completely restore dental anatomy and physiology. The function of externally managed dental stem cells exceeds their differentiation potential and swaps lost cells due to trauma or pathology. In Fig. 12.3, the schematic illustration is representing the pathway of the therapeutic application process of stem cell-based therapy in dentistry. A dental stem cell application can facilitate a reparative microenvironment, enhance the activity of endogenic stem/progenitors' cells in the target site, and lead to an adequate dental tissue repair condition by activating and organizing properly organoactive scaffold endogenetic stem and progenitor cells, thus circumventing exogenous stem cell application (Hayashi et al. 2015; Mitsiadis et al. 2012). This innovative application exhibits a great prospect of regenerating and repairing dental tissues by stimulating endogenous dental stem cells and progenitor cells. The objectives of employing stem cells for the regeneration and repair of different dental tissue are described below.

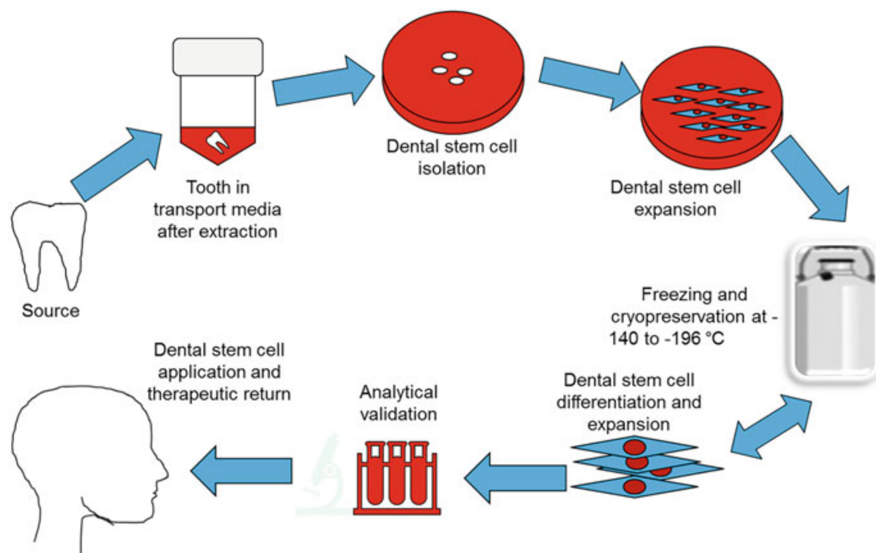

Fig. 12.3 Schematic illustration of stem cell-based therapy in dentistry

12.4.1 Dentin-Enamel Regeneration

The regeneration of the pulp-dentin complex is primarily influenced by different dentin formations (i.e., tertiary dentin, reactionary dentin, and reparative dentin). DPSCs are the crucial element for tertiary dentinogenesis. They undergo migration, proliferation, and differentiation into odontoblasts and form the tertiary dentin at the damaged sites (Goldberg et al. 2006). Mainly, two different regeneration processes are engaged for dentin regeneration using tissue engineering procedures. First, a device fills the material in a deep dental cavity with a partial dentin layer at the top of the pulp and later with growth factors or molecules producing reparative dentin. Second, a scaffold is employed to the open pulp with odontoblast-like cells so that it can be cultivated, resulting in replaceable dentin. DPSCs for engineering dentin tissues were cultured in a variety of scaffolds (Albuquerque et al. 2014; Wang et al. 2011a, b, 2016a, b, c; Chiang et al. 2016; Zhang et al. 2016; Song et al. 2017a, b; El-Backly et al. 2008). The signaling pathways underlying DPSC regulation of dentin regeneration are mostly unknown, limiting their operative applications in dentin tissue engineering.

Scaffold and cell combined methods were also suggested in tissue engineering for producing bioengineered dentin-enamel complex regeneration from dissociated cells. Epithelial cell rests of Malassez (ERM) activity on dental tissue regeneration was investigated by ERM subculture and seeded to scaffolds and then to the athymic rat's omentum (Shinmura et al. 2008). Specifically, ERM was co-implanted in collagen scaffolds together with dental pulp cells at the stage of crown formation, and 8 weeks later, enamel-dentin complex structures were recognized. Structures

such as enamel-like tissue and stellate reticulum were identified, where long columnar ameloblast-like cells were attached to the surface of enamel-like tissues. At the same time, comparative results were obtained with dental epithelial stem populations isolated by fluorescence-activated cell selection, where epithelial stem cell markers were used in serum-free and xenon-free conditions (Sato et al. 2009). In addition, complex dental tissue rebirth was tested using various reformations between epithelial and mesenchymal tissues and/or cells from mouse embryos in vitro cultured and replaced in vivo. Reattached tissues were grown, and this led to the formation of interconnected tooth structures in vitro. They showed normal epithelial histogenesis and allowed effective differences in ameloblast and odontoblast. Reassociations produce roots and PDLs, which are later connected to developing bones after in vivo implantation (Hu et al. 2006).

12.4.2 Pulp Regeneration

Tooth pulp usually needs to be eliminated if infected, and it is particularly challenging for root pulp that involves endodontic treatment. Thus, the recovery of pulp is a multi-force point because the current practice of substituting infected pulp with inorganic materials (i.e., resin, cement, metal, hybrid) results in a devitalization of the tooth (dead tooth). Due to the impact of the loss of the pulp vitality on infected tooth forecasts, researchers and clinicians are dedicated to establishing the regeneration of the dentin-pulp complex in endodontic treatment. Recent research and developments in stem cell treatments have paved the way for dentin-pulp regeneration. A harmonic and consistent functional regeneration of the pulp-dentin complex is not only possible by only implantable scaffolds but also an appropriate microenvironment is crucial to produce the interaction between scaffolds and cells.

Regenerative dentistry and endodontic therapies are the treatment method that depends on intracanal stem cell delivery and emphases on the restoration of pulp vitality and continuous root development (Chrepa et al. 2015; Peters 2014). Much effort was put into achieving extensive pulp regeneration in various animal models using human DPSCs (hDPSCs). However, proper regeneration requires restoration and regeneration of the pulp, including blood vessels and nerves, and allows the formation of new dentin (Peters 2014). The first experimental study using human dental mesenchymal stem cells (hDMSC) found that these cells can be distinguished on odontoblasts, which form dentin-like structures when implanted together with HA/TCP ceramic powder in immunosuppressive mice ex vivo model (Gronthos et al. 2000). Recently, an experimental study using seeded hDPSCs and SCAPs in poly-D, L-lactide/glycol scaffolds has shown the recreation of vascularized pulp tissues while replacing empty root canals of a mouse tooth (Hayashi et al. 2015; Huang et al. 2010b; Volponi et al. 2010). However, these investigational efforts were made in ectopic locations using DMSC transplantation and cannot be directly interpreted in the clinic settings as a stem cell-based therapy for complete pulp regeneration. Thus, new experimental approaches were explained, where DMSCs and/or other stem cells, conjugated using scaffolds or bioactive molecules, will be

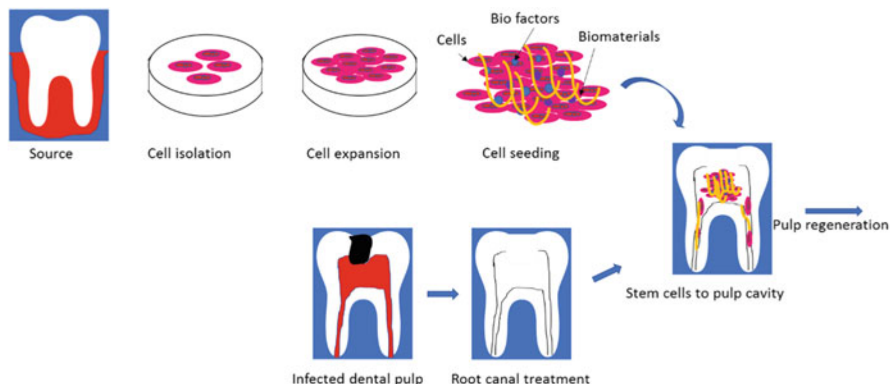

Fig. 12.4 Scheme of cell delivery-based strategy for dental pulp regeneration and pulp tissue engineering

employed to fully fill the empty pulp chamber (pulpotomy) or completely remove the pulp (pulpectomy) (Chrepa et al. 2015; Iohara et al. 2011, 2013; Lovelace et al. 2011; Zheng et al. 2012). DPSCs were simultaneously replaced with granulocyte-colony stimulating factors in pulpectomized dog teeth and were seen to recreate the whole pulp and to form new dentin (Iohara et al. 2013). Post-natal stem cells grown on external scaffolding can also be used for the regeneration of pulp tissue. To reduce immune rejection, the patient's own cells can be used for the regeneration of the pulp. Likewise, BMPs were also used for facilitating the pulp regenerative response. Although these approaches were found to improve tissue regeneration, their real efficacy to achieve a durable repair is still unclear. For example, fibrotic tissue in pulp regeneration cannot maintain an enduring therapeutic effect where fibrous pulp tissue can be able to go through degeneration over time or be replaced with bones. Current reproductive endodontic methods applied in clinics are using bleeding techniques. In this technique, a blood clot acts as a scaffold and delivers MSCs into the root canal of partially or full necrosed tooth (Chrepa et al. 2015; Deepak and Nandini 2012; Lovelace et al. 2011; Sonmez et al. 2013). Bleeding and clot formation in the root canals are facilitated via over-instrumentation, and the canal is irrigated with antimicrobials. However, care should be taken with this approach since the composition and concentration of cells trapped in the clot cannot be predicted. The scaffolding should contain bone morphogenetic protein, growth factors, nutrients, and antimicrobials to aid in cell regeneration (Rai et al. 2013; Murray et al. 2007). Three-dimensional cell printing for precise orientation of cells and gene therapy that introduces therapeutic protein into the canal are newer approaches that require further research (Rai et al. 2013). However, stem cell-based endodontic therapy in dentistry is still in the experimental stage which is yet to be considered at the treatment stage (Peters, 2014). In Fig. 12.4, a diagrammatic representation shows the pulp regeneration approach that may apply in pulp/root canal therapy.

12.4.3 Cementum Regeneration

The cementum forms a thin acellular layer encircling the root neck, and a dense cellular layer covers the root apex area. Hertwig's epithelial root sheath cells are known for the secretion of the acellular cementum in early cementogenesis and dental follicle-derived cementoblast for cellular and reparative cementum development. As a part of the periodontium, it is always subjected to be affected by periodontitis. The regeneration of cementum in regular clinical treatment is relatively low. However, the regenerated cementum should be like the acellular extrinsic fiber cementum to support the periodontal attachment. Regenerated and renewed cementum is mostly cellular intrinsic fiber cementum and expresses uncertain attachment to PDLs, and the interfacial tissue bonding seems to be feeble (Foster et al. 2012; Matalová et al. 2015; Foster et al. 2007). Several cementum-specific proteins (i.e., cementum-derived growth factor, cementum attachment protein (CAP), and cementum protein-1 (CEMP1)) were found to enhance regeneration of cementum and bone at the damaged sites by inducing different signaling pathways responsible for mitogenesis, an increase of cytosolic Ca^{2+}, and activation of the protein kinase C cascade and promoting the migration and adhesion of progenitor cells, thus resulting in the cementoblastic and osteoblastic differentiation and the formation of a mineralized extracellular matrix (i.e., cementum) (Arzate et al. 2015). In culture, the stem cells in the PDLs, gingiva, and alveolar bone, which are sources of cementoblastic progenitors, were able to produce cementum specific markers and cementum-like mineralized nodules (Bosshardt and Schroeder 1996; Bar-Kana et al. 1998). In vivo study found that PDLSCs, stem cells from the dental follicle (DFSCs), and adipose-derived stem cells (ADSCs) were able to differentiate into cementoblasts and regenerate cementum-like tissue and PDL fibers as well as periodontal vessel regeneration (Zhu et al. 2015; Lemaitre et al. 2017). Similarly, TDM can persuade and support DFSCs for developing new cementum-periodontal complexes and dentin-pulp-like tissue, which implies successful root regeneration. DFSCs combined with TDM exhibit the formation of a rich extracellular matrix in the histological examination after implanting into the mice's dorsum subcutaneously (Yang et al. 2012a, b). Thus, replacing these stem cells in periodontal defects will be an effective exercise for cementum regeneration (Crossman et al. 2018). On the other hand, co-culturing different cells also could promote cementoblast differentiation. The co-culture of PDLSCs with apical tooth germ cells proves the characteristics of the genus of cementoblast, such as morphological changes, more significant expansion, improved ALP activity, and expression of cementum-associated genes and mineral nodules. Another in vivo study showed generations of structures such as new cementum and PDLs using tissue-regeneration capacity and PDLSCs. They found a layer of mineralized tissue resembling PDL-like collagen fibers attached to the new cementum.

The "cell sheet" strategy (Owaki et al. 2014) was also proposed for cementum regeneration. The three-layered PDL cell sheets supported by polyglycolic acid sheets were placed on the surface of the exposed roots, and the three-walled infrabony wound was filled with macroporous beta-tricalcium phosphate (β-TCP).

Ligament-like tissues were grown near the cementum and the new bone, which consisted of collagen fibers (Iwata et al. 2009). The effect of nanomaterials on the differentiation of cementoblast and the mechanisms were also well reported (Mao et al. 2015). The hydroxyapatite bioceramics promoted cell proliferation, expression of osteogenic and cementogenic markers (i.e., CAP, CEMP1, Runx2, ALP, and OCN), and ALP activity, which suggested that hydroxyapatite could be a promising grafting material for the repair of cementum and bone tissue. However, further in vivo assessments are needed to assess and ensure the effectiveness of periodontal regeneration. Therefore, cementum-specific proteins can adjust the renaissance of periodontal homeostasis and cementum structure. Recent studies have shown some novel methods to promote cementum regeneration and repair, such as differentiation of cementoblast and cementogenic progenitors themselves, joint-culture with the released cytokines of other specific cells, cell sheet applications, and biomaterials made of micro-nano-hybrid surfaces.

12.4.4 Periodontal Regeneration

The periodontium is typically made up of a combination of tissues that surround and support the teeth to maintain the teeth in the tooth socket. The PDL fibers attach the cementum to the alveolar bone to stabilize the teeth in the alveolar socket and to withstand occlusal loads. PDLs play a vital role in tooth function. Periodontal inflammation leads to an inflammatory disease (periodontitis) that affects the periodontium and causes irreversible damage to connective tissue attachment and supporting alveolar bone. Thus, PDL regeneration is an imperative requirement. Hence, the result is the regeneration of highly organized collagen fibers to restructure vertically and firmly to connect the reconstituted cementum and new bones (Zhu et al. 2015). The new ligaments and bone formation and proper connections between these tissues, bone, and tooth roots are therefore a great challenge for cell-based replacement of a functional periodontium. Periodontitis can alter the cell biology in the pathological periodontium. Depending on the availability of MSCs, the periodontium has only a limited capacity for regeneration. Various MSCs remain and are responsible for tissue homeostasis. They serve as a source of renewable progenitor cells to make other necessary cells throughout the life of adults. In addition, recent studies have shown that periodontal stem cells can be transplanted into periodontal defects without any distortion or inflammation, or immune response. Thus, the regeneration of periodontal tissues depends on the practical application of locally derived regenerative progenitor cells for defects in tissue homeostasis and consistent differentiation into PDLs, cementum, and osteoblast cells (Bartold et al. 2006). New PDL-like tissues were successfully generated by delivering the stem cells to the defect sites (Caton et al. 2011), including the delivery of PDLSCs (Shi et al. 2018), BMMSCs (Du et al. 2014), adipose-derived stem cells (ADSCs) (Mohammed et al. 2018), and iPSCs (Duan et al. 2011).

Recently, stem cell-based effective periodontal therapy was strongly reported. Several animal studies have supported stem cell transplantation in periodontal

lesions for periodontal regeneration. In addition, cell sheet engineering was recently developed as a scaffold-free method for cell delivery (Chen et al. 2012). PDLSCs are the first choice for periodontal regeneration for regenerating typical PDL-cementum tissues. In vivo, the PDL-cementum complex was demonstrated by grafting monolayered or layered PDLSCs (Iwata et al. 2009; Flores et al. 2008a, b). Different trial studies have also been reported regenerating periodontal tissues in rats, dogs, and pigs by transplanting PDLSC sheets (d'Aquino et al. 2009; Hoashi et al. 2009; Flores et al. 2008a, b). After being restored by PDLSC sheets in a mesial dehiscence model in an immunodeficient rat, a thin layer of cementum-like tissue formation and the insertion of new fibrous structures were observed, which indicates that cell sheet transplantation has a perspective and can be used for periodontal regeneration (Flores et al. 2008a, b). In another study, PDLSC sheets were found to promote the regeneration of periodontal tissue and cementum in a beagle dog; it was also reported to have found new blood supply to collagen fibers, inserted vertically into the newly formed cementum and bone (Hoashi et al. 2009).

Similarly, in a minipig periodontitis model, periodontal regeneration was found to use autologous and allogeneic PDLSC replacements (Ding et al. 2010). Besides animal models, autologous periodontal ligament progenitor cells with bone grafting components have shown their therapeutic advantage in humans during transplantation into bone defects (Feng et al. 2010). Periodontal defects were reconstructed; clinical trials and general studies have demonstrated the fortification and effectiveness of autologous PDL cells in periodontal therapy (Feng et al. 2010). Moreover, recent clinical trials showed that autologous PDLSC therapies are harmless and effective for treating periodontal intrabony defects (Chen et al. 2016). PDLSCs are less likely to reject immune and have become a reputable resource for periodontal therapy. In addition, PDLSCs have a more extraordinary potential for periodontal regeneration than other mesenchymal stem cells. A conceptually simple method of periodontal regeneration involves engineered cell sheets to facilitate the replacement of human PDL cells (Hasegawa 2005).

Improved periodontal tissue regeneration in immunocompromised mice was reported using human PDLSCs, which gives enough potential indications for future cell-based dental therapies (Seo et al. 2004). The possibility of regenerative activity of PDLSCs, DPSCs, SHEDs, and BMSCs in the treatment of inflammatory periodontal diseases was also proved in the miniature swine and dog models (Ding et al. 2010; Du et al. 2014; Fu et al. 2014; Khorsand et al. 2013). To improve stem cell-based therapeutics, various growth factors such as platelet-derived growth factors (PDGFs) and BMPs were used. PDGFs were shown to increase periodontal tissue regeneration (Howell et al. 1997; Lynch et al. 1989), while BMPs strengthen the formation of cementum and alveolar bone (Selvig et al. 2002). However, BMPs might have undesirable effects on periodontal tissues and exaggerate tooth ankylosis. Commercially available amelogenin extracts were found successful for periodontal tissue regeneration in a clinical setting, though their mechanism of action is still indistinct (Veis et al. 2000). PDLSCs were cultured and stimulated osteogenically and later seeded on a biphasic calcium phosphate (BCP) scaffold (Shi et al. 2018). The PDLSC-seeded scaffolds were transplanted to the dog model.

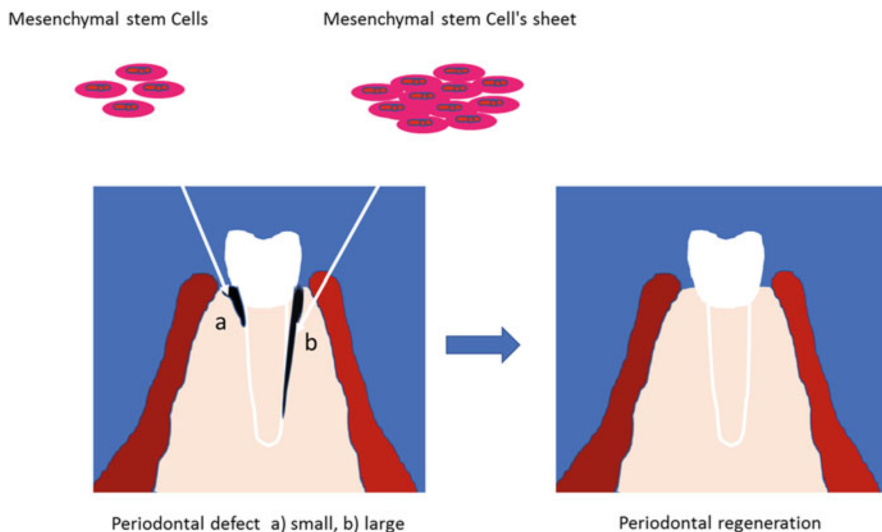

Fig. 12.5 Stem cell-based periodontal therapy

The results indicated that the PDLSC-seeded BCP promoted effective regeneration of a periodontal tissue along with new bone formation. However, various bone substitute materials are said to have been transplanted into periodontal lesions, which produce a long junctional epithelium (LJE) but could not revive a real periodontium (Bosshardt and Sculean 2009). An LJE formation can only reduce periodontal pocket depth, but there is no regeneration of PDL fibers. On the contrary, proper periodontal tissue regeneration requires properly organized fibers attached to adjacent cementum and bones (Siaili et al. 2018; Singh 2013). In another study, a barrier membrane was used to accommodate the gap between the defects of the root surface to increase the proliferation of PDLSCs, PDLs, and bone formation (Siaili et al. 2018). Animal studies have shown periodontal regeneration histologically (Bosshardt and Sculean 2009). Therefore, guided tissue engineering can provide soft tissue regeneration without downgrowth in bone defects, resulting in the regeneration of periodontium (Bottino et al. 2012).

The regeneration of alveolar bones in periodontal lesions is another key challenge for clinicians. The first clinical trial described the use of autologous hDPSCs for the regeneration of alveolar bones in conjugation with collagen scaffolds was reported several years ago (d'Aquino et al. 2009). Similarly, a noteworthy and constant clinical result was described in another study on a patient suffering from severe periodontitis. Autologous bone marrow mononuclear cells (BMMNCs) were successfully used to regenerate alveolar bones, enclosed in thermo-reversible gelation polymer scaffolds confirmed by clinical and radiographic assessments in a 3-year follow-up trial (Sankaranarayanan et al. 2013). Figure 12.5 shows a common method that can be considered for both small and large defects in regeneration therapy using stem cells.

12.4.5 Whole Tooth Regeneration

The implant is the latest modern state of the art in tooth replacement. A metal screw-like rod is placed in a predrilled shack in the bone, and on the top, a ceramic, metal, or plastic crown is placed. The minimum amount of jawbones should be present for implant placement because they are directly connected to the bone without PDL support. Here, the masticatory force is transmitted directly to the bone, which is thought to be one of the main reasons for implant failure. Insufficient bone (i.e., chronic periodontitis, chronic osteomyelitis, postmenopausal osteoporosis) is a challenge for implants, which requires bone grafts for implant placement. Thus, the ultimate goal in dentistry is to utilize a process for replacing lost teeth using a biological system; that may be a cell-based implant rather than a bare-metal one. The least obligation for biological replacement of a tooth is to produce basic functional elements of teeth (i.e., root, PDLs, and blood vessels and nerves). Inconsistently, the visible tooth crown is less important in biological replacement as it can be replaced well using a synthetic tooth crown (metal, ceramic, and resin crown), which can be ideally reproduced for size, shape, and color. Therefore, the challenge in the biological tooth replacement is the biological root. Thus, the regeneration of the tooth root is a credibly more realistic and clinically applicable approach. Regeneration of a tooth requires signaling interaction between epithelial and mesenchymal cells. Any scaffolds designed to achieve odontogenesis should contain both epithelial and mesenchymal stem cells or cells that are capable of differentiating into these germ layers (Shete et al. 2015). Though both prenatal and postnatal tooth germs can be used for regeneration, the use of prenatal tooth germ is preferred since it has a higher propensity toward the development of normal tooth structure (Majid et al. 2016).

Research in this field showed a root/periodontal complex constructed using PDLSCs, SCAPs, and HA/TCP scaffolds, which was able to support an artificial crown and perform normal tooth function in a swine model (Ikeda and Tsuji 2008; Ode et al. 2010; Sonoyama et al. 2008; Yang et al. 2012a, b). Using DFSCs in combination with a dentin matrix-based scaffold, cell sheet technology was also successfully used to reconstruct the roots of teeth. The entire tooth replacement is estimated to be one of the outstanding breakthroughs in dentistry. Dental tissue engineering was established using various stem cells from mice in vivo to form tooth structures. A mouse model showed a fully functional tooth replacement by a bioengineered tooth germ reconstructed from epithelial and mesenchymal progenitor/stem cells in a collagen gel in the alveolar bone. The bioengineered tooth erupted, occluded, and displayed perfect tooth structure, mineralized hard tissue, and response to injurious stimulation (i.e., mechanical stress, pain) (Ikeda et al. 2009). Recently, using the same cell source, in vivo reconstruction of the "bioengineered tooth unit" was demonstrated in a murine model. Unexpectedly, the unit showed the growth of an adult tooth, PDLs, and alveolar bone. It was a fully functional tooth with bone regeneration in a vertical alveolar bone defect mouse model (Ikeda and Tsuji 2008; Yang et al. 2012a, b; Oshima and Tsuji 2014). Taken together, these findings suggested a new concept in tooth regeneration therapy. Thus, it was

assumed that the transplantation of a bioengineered tooth not only an entire tooth regeneration but also a treatment for clinical cases accompanied by a severe alveolar bone defect (Egusa et al. 2012a, b). In fact, bioengineered teeth were also studied using human stem cells and were found to form teeth in an ectopic site with a lack of some vital tooth components like real crown morphology and comprehensive root formation. However, animal tests using bioengineered methods have shown the possibility of functional teeth with whole roots (Oshima and Tsuji 2014). Recent ex vivo research has confirmed that the re-assembling of iPSC-derived neural crest cells and mouse odontogenic epithelial tissues has resulted in a whole tooth generation (Otsu et al. 2014). iPSC technology is likely to open new prospects in regenerative dentistry. However, more technological improvements may be needed as these results have not yet been found as proponents with human cells. Various human DMSCs are still in the experimental stage. Time, on the other hand, is a great challenge for dental regeneration as the entire process of odontogenesis in humans takes more than 7 years. This long-term physiological method can discourage people from replacing lost teeth with bioengineered teeth. A simplified schematic representation of tooth regeneration is shown in Fig. 12.6.

12.4.6 Orofacial Tissue Regeneration

Because of the complexities in growth and structure, there was no leeway of conducting clinical trials about regeneration therapy for multifaceted oral tissues and organs in the orofacial region (i.e., head, neck). However, there is progress based on an animal study known as the promising technique for reviving these tissues. To date, prosthetic and functional correction of the orofacial soft and hard tissues is necessary following significant defects occupying lesions, trauma, or surgery (Shete et al. 2015; Rouabhia 2015). However, oral keratinocytes might be seeded onto 3D scaffolds to create tissue-engineered oral mucosa. Collagen seeded with myoblasts could be used to reconstruct orofacial muscles along with growth factors and platelet-rich plasma (Majid et al. 2016). On the other hand, an iliac bone graft with adipose-derived cells was used for the correction of defects of the calvarium. These adipose-derived cells can also be used as scaffolds for soft tissue repair. Bone marrow-derived stem cells, along with DPSCs that differentiate into odontoblasts in a collagen sponge scaffold, are used for the correction of large defects (Mouli et al. 2012; Shete et al. 2015).

12.4.7 Tongue Regeneration

The tongue is the organ for speech, swallowing, and airway safety. Loss of tongue or tongue tissue by surgical resection intensely affects the quality of human life. The transformation of tongue defects was a continuing challenge in dental practice. Interestingly, an animal study in tongue regeneration showed to reconstruct tongue defects and restore speech, swallowing, and air protection (Luxameechanporn et al.

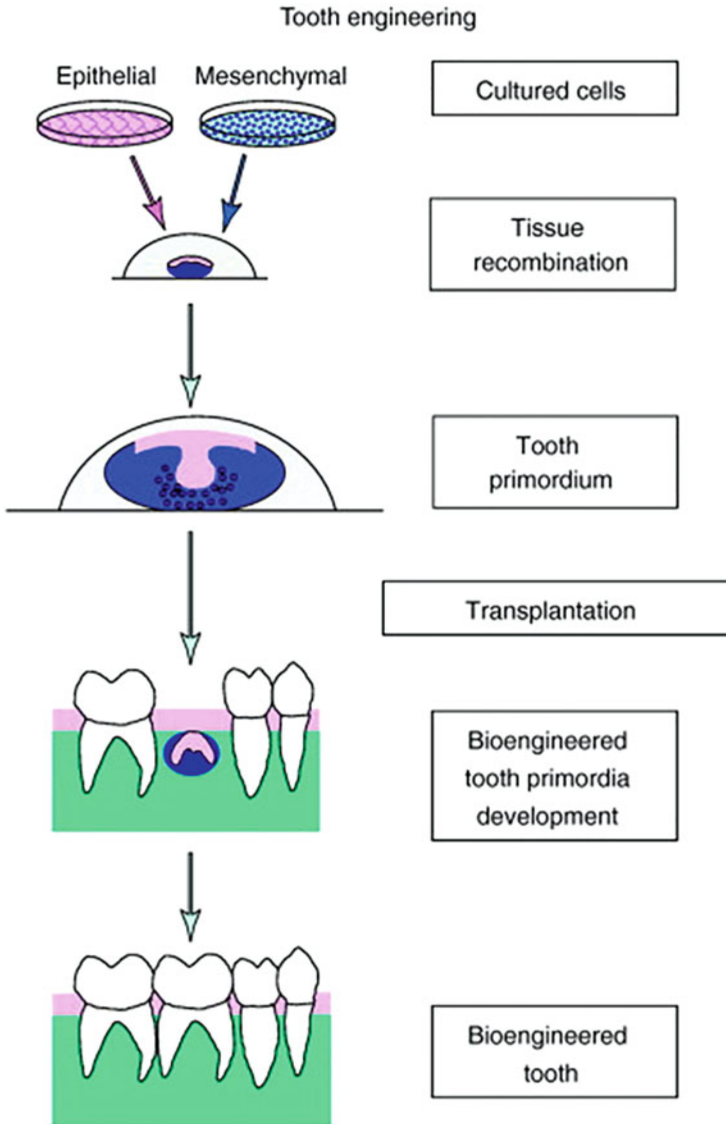

Fig. 12.6 Diagrammatic representation of the generation of biological replacement teeth. (Reproduced with courtesy—Trends Cell Biol**.** 20:715)

2006; Bunaprasert et al. 2003). A mouse model has been reported to have a cell-based reconstruction of the tongue by implanting myoblast-progenitor cells in a hemi-glossectomized tongue for muscle regeneration (Luxameechanporn et al. 2006). However, the effective regeneration of the tongue is quite complex. A

study revealed that the application of cyclical strain to BMSCs animates the attainment of an aligned myotube assembly (Egusa et al. 2013). Further advanced analysis in stem cell engineering is suggested to develop regenerative methods for injured or resected tongue reconstruction and to reestablish its effectiveness to improve quality of life (Egusa et al. 2013).

12.4.8 Salivary Gland Regeneration

Salivary gland regeneration is a challenging and exciting topic for head and neck oncology specialists. To date, two regenerative technologies were performed for the restoration of the function of salivary glands: first, tissue engineering for obtaining an artificial salivary gland and, second, application of stem cells in the damaged salivary gland tissue. The report found that stem cells like MSCs were applied to restore the functioning of damaged salivary glands (Nanduri et al. 2011). It has also been reported that BMSC transplantation in a mouse tail vein may repair the function of irradiated salivary glands (Sumita et al. 2011). It can be considered that stem cell transplantation can be used to repair damaged salivary glands and replace their function. However, the detailed regeneration process should be studied and clarified to determine whether the donor repairs damaged host cells by replacing stem cells/progenitor cells or activating the remaining host cells.

12.4.9 Mandibular Condyle Regeneration

Traumatic or arthritic defect in the temporomandibular joint disk or condyle may result in lifetime difficulty, pain, and distressed masticatory function in individuals. Tissue engineering and regeneration therapy may shed light and improve the quality of life of a patient. Reports are evident to reconstruct condylar defects. In an animal study using the goat model, successful reconstruction of condylar osteochondral defects was studied by the use of a combination of cartilage tissue engineering and distraction osteogenesis. In this study, cartilage-derived progenitor cells in a hydrogel were used. A human-like mandibular condyle was plotted from chondrogenically and osteogenically persuaded rats, where the BMSCs were wrapped in a biocompatible polymer. In another study, BMSCs were induced to the rabbit for regenerating mandibular condyle, where BMSCs were differentiated into chondrogenic and osteogenic cells. These findings may guide us to accept the concept of stem-cell-based tissue engineering in degraded articular condyles in the traumatic or rheumatic arthritis cases (Egusa et al. 2012a, b; El-Bialy et al. 2010).

12.4.10 Bone Regeneration

Bone loss due to periodontitis results in mobility, dislocation, and ultimate tooth loss. Therefore, the restoration of bone loss is another crucial issue in daily clinical

dental treatment. In the research field, the osteogenesis process was well documented. Bone grafting (Anushi and Suresh 2015), scaffolds (Zhou et al. 2019), stem cells (Chen et al. 2019), and introduction of growth factors for bone regeneration were well-established therapies in bone restoration. Bone grafting is proven in clinical application using autogenous bone (Sakkas et al. 2017), allogeneic bone grafts (Keith et al. 2006), xenografts (Liu et al. 2016), and synthetic alloplastic materials (Zhang et al. 2006; Belal et al. 2005; Zhang and Xu 2005). On the other hand, translational models using adipose stem cells (ASCs) targeted the mesodermal potential of ASC in bone regeneration; bone repairs including mice, rabbits, canines, and human ASCs were reported in the last decade. The formation of the first in vivo osseous tissue using ASC was reported in 2004 (Hicok et al. 2004). HA/TCP cubes were planted in conjugation with osteogenically stimulated human ASCs, and subcutaneously transplanted in athymic mice. The tissue was compatible histologically with osteoid and produced 80% scaffolds. It was also well established that human ASCs existed at the newly generated osteoid. In another study, 84% of cranial bone defects in dogs were reported to be cured by dexamethasone-induced ASC implantation, and 25% cured for acellular defects. In acellular defects, it shows fibrous tissue instead of bone tissue, which was also identified around ASC-seeded implants (Cui et al. 2007). Osteogenically primed ASCs placed in palatal defects resulting in uninterrupted bone formation were also reported (Zuk 2013).

Different absorbable and injectable scaffolds were described for bone regeneration applications (Tsai et al. 2016; Simon et al. 2002). Calcium phosphate cement (CPC) paste is one of the injectable scaffolds used in these applications (Brown and Chow 1986; Takagi et al. 2003). After injection in a bony defect, this paste becomes hard in situ and forms a scaffold by a dissolution-precipitation reaction at 37 °C (Xu et al. 2017). In particular, cell seeding in perforated CPC scaffold processes weak seed function and common cell infiltration into the scaffold (Villalona et al. 2010). On the other hand, it is not reasonable to directly blend the cells with the paste, which is injurious to the cell because of the mixing pressures, ionic exchanges, and/or pH variations of the CPC paste setting circumstances. Considering these disadvantages, an encapsulated stem cell delivery system was developed using absorbable and injectable alginate-microfibers/microbeads (Alg-MB/MF), which protect the stem cells during CPC paste manipulation and injection (Wang et al. 2016a, b, c). In this system, cell health, proliferation, and differentiation were secured, and the microbeads degrade within 3–4 days and release the encapsulated cells in the designated site (Grosfeld et al. 2016; Song et al. 2017a, b). It was reported that six different stem cells (i.e., human bone mesenchymal stem cells (hBMSCs), hDPSCs, MSCs derived from embryonic stem cells (hESC-MSCs), human umbilical cord MSCs (hUCMSCs), human induced pluripotent stem cell-MSCs derived from the bone marrow (BM-hiPSC-MSCs) and from the foreskin (FS-hiPSC-MSCs)) were seeded in an injectable CPC condensing in hydrogel microfibers and microbeads and seen to have cell proliferation, osteogenic differentiation, and high osteogenic gene expressions in 7 days (Mitsiadis et al. 2011; Wang et al. 2015; Zhao et al. 2010). It was reported that cell-synthetic bone matrix minerals were enhanced by culture over time, indicating the ability of excessive bone regeneration potential,

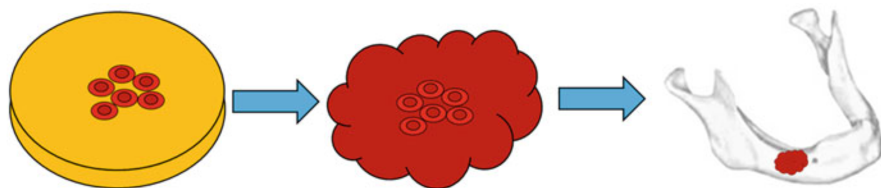

Fig. 12.7 Stem cell application in bone repair and regeneration

such as the gold-standard hBMSCs (Wang et al. 2016a, b, c). On the other hand, a study of hBMSC-encapsulated Alg-MB-CPC paste in a defective rat bone has reportedly revealed three times more new bone formation than the control group, where an osseous bridge was also produced in the bone defects (Song et al. 2017a, b). In another study, an injectable, load-bearing, and absorbable stem cell-MB/MF-CPC construction was reported to exhibit a promising cell delivery system that highly enhances periodontal bone regeneration and repair (Xu et al. 2017). Hybrid poly(ethylene glycol)-co-peptide hydrogels were also reported to be promising in endogenous regeneration and suggested as an updated therapeutic approach (Schweikle et al. 2018). One study described a tri-culture system with hiPSC-MSCs, human umbilical vein endothelial cells (HUVECs), and pericytes to provide pre-vascularization to CPC scaffolds (Zhang et al. 2017). It is learned that the vessel-like structure was formed in vitro and advance angiogenic and osteogenic marker expressions, along with bone matrix mineralization, were found. For the regeneration of periodontium and periodontal tissue, innovative nanomaterials were studied in combination with other scaffold materials and biologics (Besinis et al. 2015; Batool et al. 2018; Mitsiadis et al. 2012; Polini et al. 2013; Xu et al. 2012). It is also essential to establish vascularization in periodontal regeneration (Acar et al. 2016; Du et al. 2015). In one study, BMSCs were transferred with BMP-7 and planted in nHA/PA scaffolds and implanted into an in vivo mandibular defect rabbit model and seen to have a quick response in regeneration (Li et al. 2010, 2017). In another study, gold nanoparticles (GNPs) were incorporated into CPC and reported to exhibit improved cell adhesion, proliferation, and osteogenic induction on CPC (Xia et al. 2018). Therefore, it was advised to apply synthetic materials and nanoparticles to functionalize scaffolds for composite with other bioactive additives to increase bone and tissue regeneration for periodontal repair. This application flow may be considered in bone regeneration (Fig. 12.7).

12.5 Prospects of Dental Stem Cells in Medicine

Dental stem cells can potentially be employed for medical treatments. Several reports are confirming the application of dental stem cells in general medical research and application such as in cardiac therapies (Gandia et al. 2008), liver disease (Egusa et al. 2012a, b; Ikeda et al. 2007), brain tissue regeneration (Nosrat et al. 2001), bone regeneration (Graziano et al. 2008a, b), and muscular dystrophy

therapies (Kerkis et al. 2008). SHEDs can be used to generate cartilage (Fecek et al. 2008) and adipose tissue (D'Andrea et al. 2008). The first advanced animal study for bone grafting was announced in 2008, thereby reconstructing large cranial bone defects in rats by human DPSCs (de Mendonca et al. 2008).

12.6 Dental Stem Cell Banking

Since DSCs have several clinical advantages and are applied in various regenerative medicine, DSCs' reservations for medical applications document the concept of "tooth banking." Teeth and surrounding tissue complexes are the sources of stem cells. Once stem cell-linked tissues (e.g., pulp, apical papillae, PDLs, follicles, gingiva, and the tooth itself) are recovered from the patient, they can be stored for many years to retain their reproductive potential for future regenerative therapies. DSCs are stored using cryopreservation or magnetic freezing (Oh et al. 2005; Gioventù et al. 2012; Tamaoki et al. 2010). Stem cell banking is currently available, and even a few of these banks freeze not only cord stem cells but also dental stem cells of deciduous teeth. Typically, during teeth shedding, the extracted tooth is stored in a special kit provided by the stem cell bank company and shifted to their specialized labs for cultivating dental stem cells. Each child's cells are confidentially stored in the bank until they are required by him/herself or any member of the family (Arai et al. 2004). It is still in the early stages of tooth banking and thus is not very popular at present. The trend is mainly progressing in developed countries and getting acceptance. Stem-cell banking companies such as "Store-A-Tooth" (Provia Laboratories, Littleton, Massachusetts, USA) and "StemSave Inc." (New York, USA) are intensifying their presence globally. BioEden (Austin, Texas, USA) has international laboratories in the UK and Thailand. Hiroshima University has started their first commercial dental bank as an enterprise organization called "Three Brackets" (Kaku et al. 2010) in collaboration with Taipei Medical University, and Nagoya University, Japan, also brought a dental bank to Japan. Advanced Centers for Tissue Engineering Limited, Tokyo, Japan, also has a reputation for tooth banking. In Europe, Norwegian tooth bank is a collaborative project of the Norwegian Institute of Public Health and the University of Bergen. They collected and installed exfoliated primary teeth from 100,000 children in Norway in 2008. In South Asia, Stemade Biotech introduces the concept of DSC banking in India. The usefulness of stem cell banking in dentistry should be evaluated carefully. Furthermore, legislation is mandatory for banking systems as it provides bio-insurance for future use, which is highly questionable. Checks and audits must be controlled whether the banking institution can manage well in the future and maintain well in terms of quality for future use in cryopreserved cells and tissue replacements.

12.7 Perspectives and Conclusion

Several stem cell lines, including imperative erraticism and strength, have been identified from human adult teeth in recent years. Considerable variation of cells separated from the same dental stem cell pool can affect clinical outcomes. As a result, identification and treatment of stem cell subpopulations with increased capacity are necessary before applying cell-based treatment to clinical settings. In addition to the choice of the dental stem cell population, various factors, such as wound size and depth, surrounding tissue conditions, and distribution methods, are also likely to affect the success of therapy. Stem cell-based tissue engineering claims fine instrumentation of three elementary components: scaffolds, cells, and signaling pathways (Rosa et al. 2012). At this stage, there is still a long way to go to understand the processes, which control the information, fate, and function of stem cells after applying them to a diseased or injured dental pulp and/or periodontal tissues. Although applications that use dental stem cells for pulp and periodontal regeneration were well reported in animal studies, long-term clinical trials and follow-ups are still to be performed. Moreover, translational and preclinical stem cell research in clinical trials is very sturdy due to technical safety, regulatory, and ethical concerns. Patients will not benefit from this regenerative treatment until most of the complications and conditions described above are determined and potential clinical limitations will be thoroughly examined and taken utterly.

Regenerative medicine is growing every day, and it endures to appeal to imperative academic interest worldwide. So it is of utmost importance to train and educate a new generation of researchers, although it was not well thought out in the early period. A course titled “Introduction to research in regenerative medicine” was initiated by the University of Virginia to facilitate research skills and confirm basic knowledge of stem cell and tissue regeneration research (Tavakol et al. 2018). Similarly, regenerative dentistry is developing as a dedicated field of education and research to provide a “biological alternative” to the replacement and regeneration of oral and dental tissues that have historically been repaired with prostheses or recent dental implants. Regenerative dentistry combined biomimetic scaffolds, proper growth factors, and MSCs with common surgical measures. So future oral and dental practitioners need to be better trained on biological procedures to improve their expiates with the most up-to-date ideas related to stem cell-based guided regenerative dentistry (Marrelli et al. 2014). Recent studies have reported on the possibility of regenerative properties of MSCs found from oral and dental tissues. Generally, human dental tissues are discarded as waste in most cases after oral procedures. However, many dental tissues are well known as rich and readily available sources of adult MSCs. “Disposable tissue medicine,” also known as “waste medicine,” is the latest subdivision of regenerative medicine to look carefully at these newly exposed MSC sources, such as human periapical inflammatory cysts or other resected tissues. In addition, the health status of donors should be taken seriously when treatment engages human-origin cells. In practice, it was hypothesized that donor health variables might also affect the biological characteristics of their MSCs (Morsczeck et al. 2008; Tatullo et al. 2016). Thus, it

is highly expected from researchers and clinicians to have deep concerns in isolation, preservation, and the use of stem cells in tissue engineering to achieve the outcome.

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Ian Situ Tissue Engineering: A New Dimension

13

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Abstract

Tissue engineering has evolved to provide ways to construct tissues primarily aiming at replacing lost or damaged tissues or improving function. It has been classically developed using ex vivo means in which cells are generally cultured with biomaterials and subsequently engineered constructs are transplanted into the body. However, this approach is associated with several challenges that have limited its successful translation to the clinic. With in situ tissue engineering, it is possible to stimulate internal body regenerative potential by using biomaterials, biomolecules, and genes, which can reduce risks and challenges associated with

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ex vivo tissue engineering. In addition, in situ tissue engineering may potentially accelerate the clinical application of the technology and may lead to the development of more effective regenerative therapeutics through a collaborative multidisciplinary approach.

Keywords

Biomaterials · Guided tissue regeneration · In situ · Regeneration · Tissue engineering

13.1 Introduction

Millions of people suffer from tissue loss or organ failure (Langer and Vacanti 1993). Organ transplantation is needed to replace failed organs, but this is limited due to the shortage of organ donors (Giwa et al. 2017). Therefore, scientists have been seeking ways to create biological substitutes by using tissue engineering (Kurniawan 2019a; Ashammakhi et al. 2021a). Tissue engineering was developed to enable the production of functional tissue constructs for the purpose of providing graft-like structures that can be used to replace or treat tissue defects or failed organs (Rouwkema et al. 2011). It has conventionally relied on seeding scaffolds with cells (Zhang et al. 2016; Gunther et al. 2015) or recently developed three-dimensional (3D) bioprinting of cell-laden constructs (Potyondy et al. 2021; Tavafoghi et al. 2021; Davoodi et al. 2020; Erdem et al. 2020; Shao et al. 2020) ex vivo, followed by the transplantation of engineered constructs to target sites in the body. However, engineered constructs often fail because cells die following transplantation due to the lack of vascular supply (Ashammakhi et al. 2020a; Chandra et al. 2020). The need to use cells from various sources and expand and seed them outside the body is associated with increased risks and concerns (Belk et al. 2020; Marks et al. 2016). On the contrary, in situ tissue regeneration is reliant on stimulation of body innate potential for regeneration, which can be achieved without the need for ex vivo procedures, and it can be achieved using biomaterials, biomolecules, genes, or cellular products (Ashammakhi et al. 2021b; Sengupta et al. 2014; Abdulghani and Mitchell 2019). In addition to reduced challenges and risks of complications, costs can also be reduced, and the path to regulatory body approval and clinical translation can be accelerated (Yang et al. 2020; Wissing et al. 2017; Gaharwar et al. 2020; Xia et al. 2021). Various approaches to in situ tissue engineering are presented and discussed in this chapter. Current challenges and new ideas for future developments are also highlighted.

13.2 Tissue Engineering

13.2.1 Concepts and Evolution of Tissue Engineering

The term "tissue engineering" was first coined at a National Science Foundation (NSF)-sponsored meeting in 1988 (Skalak and Fox 1988). Later, in 1993, it was defined by Langer and Vacanti as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes for the repair or regeneration of tissue or organ functions (Langer and Vacanti 1993). After that, the number of studies in this area has exponentially increased (Viola et al. 2003). For instance, a study conducted by Cao et al. in 1997 drew much attention to tissue engineering. In this study, a three-dimensional a polymer construct containing cartilage cells was implanted in the subcutaneous tissue of a mouse, and the cartilage tissue formed in the sahpe of human ear was reported (Cao et al. 1997). Moreover, the skin substitute TransCyte, made by culturing foreskin fibroblasts on nylon mesh to improve wound healing, was approved by the Food and Drug Administration (FDA) in 1997. Tissue engineering was considered as a promising strategy for the engineering of various organs and tissues, ranging from musculoskeletal tissues such as bone (Cao et al. 2020) and cartilage (Yang et al. 2020) to cardiovascular tissues such as heart valves and blood vessels (Wissing et al. 2017). FDA-approved treatments developed by tissue engineering approach have made significant progress in the past two decades (Ashammakhi et al. 2021a; Hoffman et al. 2019).

Tissue engineering has proposed a new concept for repair, regeneration, and replacement of tissues with constructs made using cells, scaffolds, and signaling molecules (Langer and Vacanti 1993). Because these components could be made available in laboratories, this concept potentially is a safe and reliable source for clinical use and can contribute to addressing organ deficiency (Smits and Bouten 2018; Kurniawan 2019b). Cells for tissue engineering can be autologous primary cells or stem cells (Khademhosseini et al. 2020; Bajada et al. 2008; Vapniarsky et al. 2015). The use of autologous primary cells helps to avoid the risk of developing an adverse immune response. However, they have disadvantages, such as the need for an invasive procedure for their retrieval, which can be more of a problem, especially in the elderly or people with underlying diseases (Fodor 2003; Koh and Atala 2004). The use of stem cells with very high proliferation capacity is an excellent option to circumvent these limitations. These cells respond to chemical and mechanical signals from their microenvironment and differentiate into various tissues. Complete differentiation or removal of all stem cells before transplantation should be ensured because of the risk of uncontrolled or abnormal growth and tumor formation (Bedel et al. 2017; Howard et al. 2008).

Signaling molecules that include growth factors, peptides, and small molecules are used to guide cell behavior and promote them to regenerate new tissue. Growth factors are polypeptide molecules that interact with cell surface receptors and direct cellular behavior, such as proliferation and differentiation, by activating signaling cascades. For instance, growth factors such as bone morphogenetic proteins (BMPs) secreted by osteoblasts, chondrocytes, and osteoprogenitor cells are critical not only

for regulating bone formation and repair but also for maintaining the bone mass during postnatal growth. By interacting with BMP receptors at the cell surface, they activate intracellular signal transduction and cause the expression of bone-specific genes (Wu et al. 2016). However, the main drawbacks of using growth factors are high cost, immunogenicity, short half-life, and need for supra-physiological doses (Subbiah and Guldberg 2019; Caballero et al. 2019; Ashammakhi 2018). Short peptides derived from therapeutic proteins are less immunogenic than growth factors due to their small size, but they are also not stable. Small molecules can be natural or synthetic non-peptide molecules with low molecular weight. Because of their small size, which is less than 1 kDa, they are stable and non-immunogenic. Moreover, they are often uncharged and hydrophobic. So they can easily penetrate the phospholipid bilayer cellular membrane and activate signaling pathways of transcription and gene expression, thus directing cellular behavior (Balmayor 2015).

Scaffolds are artificial matrices that can provide a foundation for cells to adhere and perform their activities such as proliferation, migration, and protein synthesis. The main purpose of scaffold design is to produce structures similar to those of the extracellular matrix (ECM) of native tissue. These 3D constructs with interconnected pores can be in the form of hydrogels, fiber-based structures, sponges, or other structures that are made of natural, synthetic materials or hybrids. They may contain ceramics, polymers, or a combination of both. Scaffolds must be biocompatible and conventionally biodegradable. Besides, their degradation time must be proportional to the formation of new tissue. It has been proven that their physicochemical properties can be used to control and guide cellular behavior (Xia et al. 2021; Khademhosseini et al. 2020; Haider et al. 2020; Mabrouk et al. 2020).

13.2.2 Approaches: Ex Vivo Versus In Situ Tissue Engineering

The human body has an innate ability to repair, regenerate, and renew via stem cells that are resident or migrate to damaged sites (Fig. 13.1). However, this inherent regeneration mechanism is not always sufficient. Therefore, tissue engineering can be employed to improve the self-regeneration capacity of the body (Andreas et al. 2014). Ex vivo and in situ tissue engineering are two main strategies for the repair of damaged tissues, which are beyond repair.

In the ex vivo strategy, the process of tissue engineering is performed in the laboratory and outside the body in such a way that scaffolds are combined with cells with or without biological molecules in vitro. The starting point of this strategy is to obtain tissue-matched cells, either from the patient or from other individuals as stem cells. Then, the cells are expanded in vitro followed by their seeding into a scaffold. After that, the cells are stimulated to produce their own ECM. Eventually, these engineered tissue constructs are implanted into the body to produce desired tissues (Kurniawan 2019b). However, this strategy is limited due to major drawbacks such as donor tissue morbidity, lack of reliable and reproducible cell sources, the need for large quantities of immune-compatible cells, and challenges of in vitro cell culturing such as loss of cellular phenotype. Furthermore, these expensive engineered

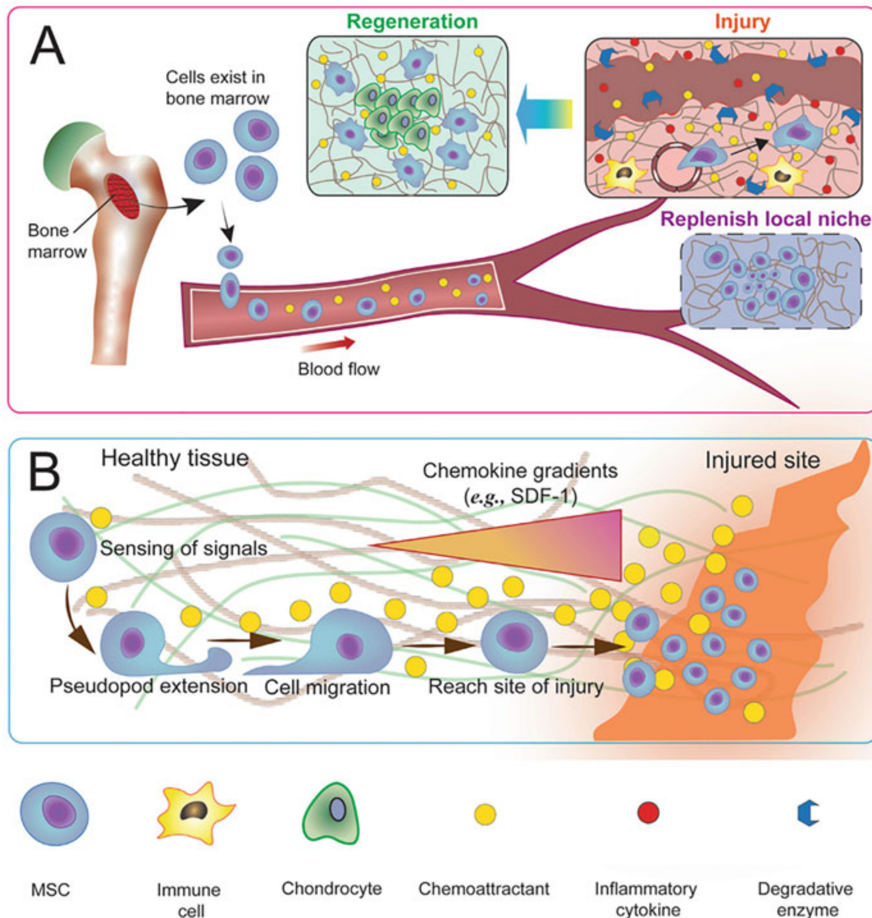

Fig. 13.1 Schematic representation of two principal modes of endogenous stem/progenitor cell (ESPC) mobilization, migration, and recruitment (illustrations not to scale) in response to guidance gradients (e.g., chemokines and growth factors). (**a**) In general, ESPCs can mobilize from a central cell niche (e.g., bone marrow), disseminate throughout the body via the blood flow, and recognize and interact with microvascular endothelial cells in desired tissues or organs, where these cells replenish the local niches during homeostasis or reach the sites of injury and participate in tissue regeneration. (**b**) The second mode of ESPC mobilization, which is known as interstitial migration, requires that stem/progenitor cells migrate within the extracellular matrix and recognize and obey extravascular guidance gradients to reach the injured site and participate in tissue regeneration. In contrast to mode (**a**), this trafficking mode occurs independent of blood flow and requires active amoeboid movement. (Reproduced from Yang et al. 2020, with permission from Elsevier)

constructs may elicit an immune response after implantation. On the other hand, in in situ tissue engineering, the body itself forms the desired tissue with higher chances of success of the tissue engineering process eventually (Cao et al. 2020; Smits and Bouten 2018). Hence, in situ tissue engineering strategy or endogenous regeneration

Table 13.1 Ex vivo vs. in situ tissue engineering

Ex vivo	In situ
Requires a compatible cell source	Utilizes the body's inherent regenerative capacity
Complex cell culture processes	Improved shelf life
High cost	Low cost
Immune reaction	Fewer regulatory hurdles
Donor-site morbidity	Scalable and consistent quality

was proposed by scientists to overcome the challenges associated with ex vivo tissue engineering (Barrilleaux et al. 2006). In situ tissue engineering eliminates the need for ex vivo cell manipulation and its complex culturing processes, with the consequent reduction in costs. Moreover, endogenous repair and regeneration are more compatible and reduce the risk of immune rejection. In situ tissue engineering could also induce neovascularization throughout the scaffolds by providing adequate stimuli. Because there is no need to use cells in this approach, regulatory hurdles are reduced, and as a result, the clinical translation of this strategy can be faster (Table 13.1) (Yang et al. 2020; Cao et al. 2020; Kurniawan 2019b; Andreas et al. 2014; Jakob et al. 2012).

13.3 In Situ Tissue Engineering

13.3.1 Concept and Approaches

In situ tissue engineering field has gained much attention due to its advantages such as being a minimally invasive treatment, ability to integrate to the native tissue, ability to treat complex defects, low cost, and ease of administration, which results in enhanced patient compliance (Yang et al. 2014; Di Bella et al. 2018; Avti et al. 2012; Hakimi et al. 2018). In situ-forming hydrogels involve the sol-gel transition of polymeric precursors containing a variety of polymers in the presence of various hydrogel crosslinking agents including nontoxic chemical crosslinking, physical ionic crosslinking, enzymatic crosslinking under physiological conditions, and supramolecular chemistry (Ghavami Nejad et al. 2020). This category of tissue engineering requires several factors, such as chemical stability, nontoxic networking agents, and controllable gelation time and kinetics (Yang et al. 2014). Numerous natural and synthetic polymers have been suggested for the synthesis of hydrogels. Natural polymers, including polysaccharides (such as alginate, chitosan, hyaluronic acid, and gums) and proteins (such as gelatin, collagen, and fibrin), have attracted much attention because of their inherent bioactivity, biocompatibility, biodegradability, non-toxicity, abundant sources, and low cost (Bao et al. 2020). However, their molecular weight inconsistency from batch-to-batch processes, low mechanical stability, and possible immunogenicity are their main drawbacks, which lead to preferring synthetic polymers such as polylactide (PLA), poly(lactide-*co*-glycolide) (PLGA), polycaprolactone (PCL), poly(ethylene glycol) (PEG), poly(vinyl alcohol)

(PVA), polyacrylamide (PAM), polypropylacrylamide (PNIPAM), and polymethacrylate (Abdulghani and Mitchell 2019; Li et al. 2015). Synthetic polymers are well-known for their high mechanical stability, low immunogenicity, and tailored structure but lack sufficient bio-functionality. To combine the advantages of natural and synthetic materials, hybrid hydrogels (Wang et al. 2015; He et al. 2017; Ashammakhi et al. 2019a), nanobiocomposite hydrogels (Olate-Moya et al. 2020; Yang and Yuan 2019), interpenetrating network (IPN) hydrogels, and slide-ring hydrogels were developed (Jiang et al. 2017; Bin Imran et al. 2014).

The development of biomaterials and polymer chemistries has enhanced our knowledge and use of various biomaterials and various material solidification approaches that employ local microenvironment pH, temperature, and ions (Hoare and Kohane 2008). The development of biomaterial-based approaches can utilize the immune response in a favored manner by using cellular reprogramming to direct cells toward tissue regeneration (Sadtler et al. 2016; Dziki et al. 2016). As a result, combinatorial transcriptional "code" can lead to the recruitment of endogenous stem cells. This approach can modulate the differentiation of immune cells and cytokines and enhance endogenous progenitor contribution to regeneration by the aid of chemotactic signaling. It can also help to mitigate immune rejection of exogenously delivered cells considerably. These immune-mediated approaches can be facilitated by designing smart and stimuli-responsive biomaterials (Piras et al. 2006; Ashammakhi and Kaarela 2017; Lu et al. 2016). These stimuli-responsive biomaterials can stimulate regeneration by interacting with the immune system and regulating the healing kinetics through their inherent biochemical and biophysical characteristics. These biochemical properties include the release of signaling factors, such as small biomolecules to direct cellular responses by activating specific genes or pathways. However, these signaling factors need to be considered and designed carefully based on the targeted tissue microenvironment. For example, in vascularized tissues such as bone and heart, biomolecules should facilitate angiogenesis, whereas in avascular tissues such as cornea and cartilage, they should suppress it. The release of growth factors and mineral ions can also promote cell differentiation toward specific cell lineages and modulate targeted tissue regeneration (Ashammakhi 2018). For example, calcium ions can activate calcium-sensing receptors to trigger chemotaxis and differentiation processes (Hofer and Brown 2003). On the other hand, the biophysical properties of biopolymers such as topography, degradation, structure, and stiffness can influence the local microenvironment by altering the number of cells and concentration of ions or enzymes by in situ injection therapy. The stiffness and topological features of the polymeric scaffold, microparticles, and hydrogels can alter cell adhesion, spreading, and fate (Reilly and Engler 2010; Engler et al. 2006; Wimpenny et al. 2012). The porosity of the biomaterial determines cellular infiltration and vascularization (Griffin et al. 2015). The rate of degradation of the in situ synthetic matrix should be synchronized with the rate of tissue regeneration. Because degradation alters other physical properties such as porosity, morphology, roughness, and 3D framework of the scaffold, new tissue should allow load transfer and provide mechanical integrity eventually to fully replace functions provided by the scaffold. To match scaffold degradation with the

neo-tissue generation, strategies such as varying structural parameters and biological and biochemical functionalization can be used (Gaharwar et al. 2020; Nikkola et al. 2015). In addition, magnetic nanoparticles can also be used to influence stem cells, and exerted physical effect can be used to direct their differentiation (Khademhosseini et al. 2020; Ashammakhi et al. 2018a; Du et al. 2017), which opens new avenues for the use of nanoparticles for in situ tissue engineering.

Several in situ tissue regeneration approaches, including those applied to the bone, cartilage, skin, tendons, and other tissues, can be categorized as top-down and bottom-up approaches. In both approaches, the selection of appropriate materials and processing techniques need to be carefully selected to fulfill the demands of simplicity, adaptability, portability, and ease of manipulation over a short period of time. Generally, in situ regenerative technologies can be divided into non-computer-aided and computer-aided approaches, which is also known as additive manufacturing.

13.3.1.1 Non-computer-Aided Approach to In Situ Tissue Engineering

This approach aims to generate a non-predefined pattern of a construct that can be used to fill a tissue defect. For example, spinning and spraying technologies can be used to form nanofibers or droplets/particles to produce biomimetic scaffolds by employing polymers, ceramics, or their combinations (Nikkola et al. 2015, 2008; Yang et al. 2018; Ndreu et al. 2008; Will et al. 2016; Araujo et al. 2010). In electrospinning, for example, a polymer precursor is fed into a capillary and the extrusion of the spinning solution in the presence of high voltage is carried out. Upon reaching a desired value of electric field between the collector and the generated droplet at the tip of the nozzle, a jet stream of charged viscous solution is directed toward the collector and forms nano-scale non-woven fiber constructs. Several processing parameters such as applied voltage, solvent type, polymer concentration, feed rate, needle diameter, and distance between the tip of the nozzle and the collector influence the diameter, orientation, and structure of the forming fibers. Single nozzle, coaxial nozzle, and dual nozzle enable the production of simple fibers, core-shell fibers, and hybrid fibers. Furthermore, the incorporation of different collectors allows the control over fiber orientation in the resulting constructs (Ashammakhi et al. 2012, 2007a, 2006, 2007b) (SkinCare n.d.). The basic elements used in the electrospraying method are similar to those used in electrospinning, consisting of a high voltage supply, a metallic nozzle, a collector, and a syringe pump. Here, an electric charge between two electrodes makes the liquid jet breaks into fine droplets. The higher the electric charge, the smaller is the size of generated droplets. Several types of solvents, such as ethanol, water-ethanol, and organic solvents, have been used for producing particles based on their evaporation and polymer solubility (Boda et al. 2018). Consequently, in situ spraying and spinning are becoming promising approaches, and portable devices are being developed for the formation of fibrillar or aggregated particles onto tissue defect sites (Dias et al. 2020).

Investigation of in situ gelling matrices in the form of 3D polymeric networks has increased tremendously due to their capacity to carry biomolecules, oxygen, and nutrients and support cellular functions, namely, proliferation, migration, and

differentiation (Park et al. 2012). Crosslinking of polymers for in situ gelling matrices can be achieved via physical and chemical stabilization techniques (Ghavami Nejad et al. 2020). These networks may be composed of either conventional or smart biomaterials, which can be engineered to trigger regenerative processes while exhibiting stimuli-responsive, shape memory, and self-healing properties (Ashammakhi et al. 2021; Rammal et al. 2021; Mantha et al. 2019). Reversible and irreversible self-assembly of building blocks for the production of in situ-forming matrices can be achieved by physical mechanisms such as thermal, ionic, electrostatic, or peptide self-assembly crosslinking, or by chemical mechanisms such as click chemistry, Schiff-base reaction, enzyme-mediated crosslinking, and photo crosslinking (Fig. 13.2) (Yang et al. 2014).

Other methods of in situ tissue engineering include guided tissue engineering (GTR) (Hutmacher et al. 1996; Kellomäki et al. 2002), in which tissue regeneration can be achieved by protecting the tissue defect site from invasion by scar tissue (Fig. 13.3) (Elgali et al. 2017). Other theories behind the function of this method include the local concentration of important cytokines. This method has been widely investigated and applied to different tissue types that include guided nerve regeneration (Bell and Haycock 2012), guided periodontal ligament regeneration (Villar and Cochran 2010), and guided bone regeneration (Elgali et al. 2017; Ashammakhi et al. 1995a; Ashammakhi 1996; Vesala et al. 2002; Asikainen et al. 2005). Membranes made from biodegradable or non-degradable materials are used to protect such tissue defects. Biodegradable membranes need no subsequent removal procedure, and they guide the formation of material-native tissue hybrid neomembranes (Ashammakhi 1996; Ashammakhi et al. 1995b), and they are gradually replaced by native tissues. GTR membranes can be used alone or with additive elements such as osteoconductive materials (Kellomäki et al. 2000; Puumanen et al. 2005) in case of bone regeneration or drugs and cytokines such as growth factors. They can also be combined with tissue materials such as grafts (Ashammakhi et al. 1995a). With the rising interest in in situ tissue engineering, guided tissue regeneration will gain a new momentum, and accumulated literature should be of great benefit to support future research and advances in this area.

13.3.1.2 Computer-Assisted Approach to In Situ Tissue Engineering

Additive manufacturing, also known as three-dimensional (3D) printing, is a state-of-the-art technique that relies mainly on printing artificial acellular constructs. Among the available options, light-based (infrared [IR], ultraviolet [UV], or visible light) and extrusion-based systems are more readily available, either in the form of portable devices or a robotic approach, controllable movements along three axes. BioPen is a good example of an extrusion-based portable device, which can be used with various inks to support rapid in situ gelation and consists of a bioink chamber, a multi-inlet nozzle, a motorized extruder, and a light source (Di Bella et al. 2018; Cathal et al. 2016). Gelatin methacryloyl (GelMA), which is a photocurable polymer, can be transited from sol to gel within 1 s upon exposure to light irradiation in the presence of photoinitiators and appropriate physiological conditions (Erdem et al. 2020; O'Connell et al. 2020). GelMA printability and mechanical properties

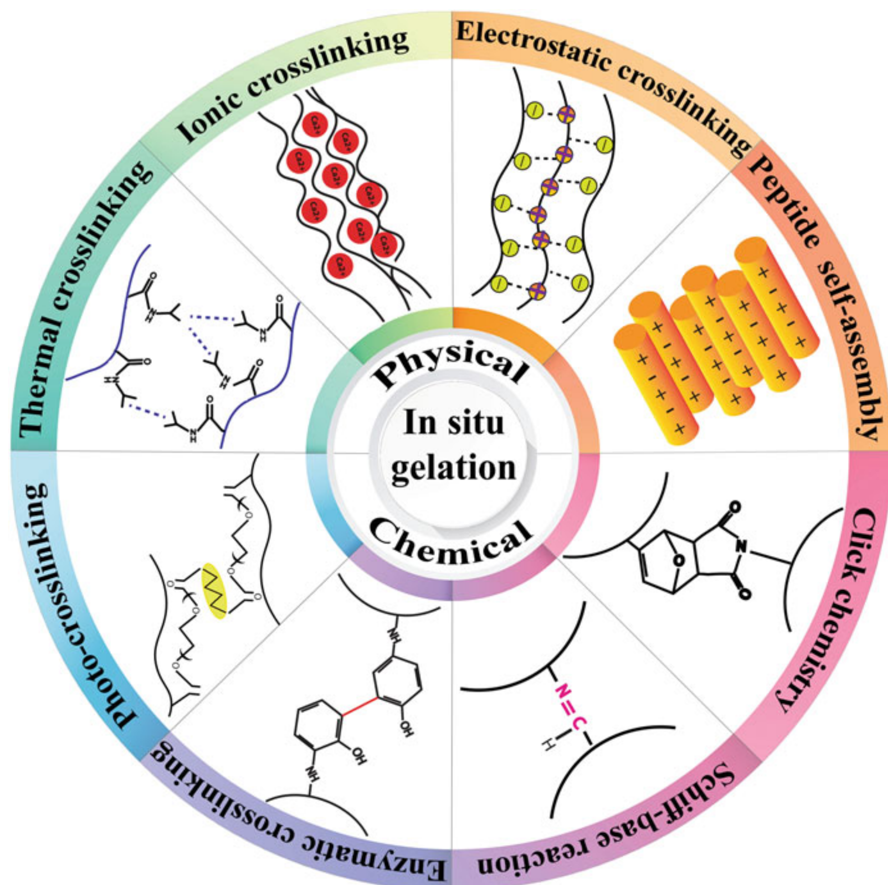

Fig. 13.2 Various in situ gelling mechanisms, consisting of physical crosslinking including (**a**) thermal, (**b**) ionic, (**c**) electrostatic crosslinking and (**d**) peptide self-assembly, and chemical crosslinking including (**e**) click chemistry, (**f**) Schiff-base reaction, (**g**) enzyme-mediated crosslinking, and (**h**) photo crosslinking

of the final hydrogel can be increased by the incorporation of rheological modifiers such as silicate particles and methylcellulose. In contrast to handheld devices, robotic-assisted bioprinting can better be used to produce complex architectures via computer-aided design (CAD), thus better mimicking complex native structures (Ma et al. 2020; Li et al. 2017). A study introduced the integration of wound imaging with additive manufacturing in order to improve personalized medicine and significantly reduce surgeon's intervention (Ding and Chang 2018; Ashammakhi et al. 2019b). However, clinical translation needs to be undertaken. Developing a suitable bioink formulation and gelation mechanism are two important steps that determine the performance of in situ-forming hydrogels. Therefore, attempts have been made to optimize bioink composition, crosslinking efficiency, gelation time, stiffness, and

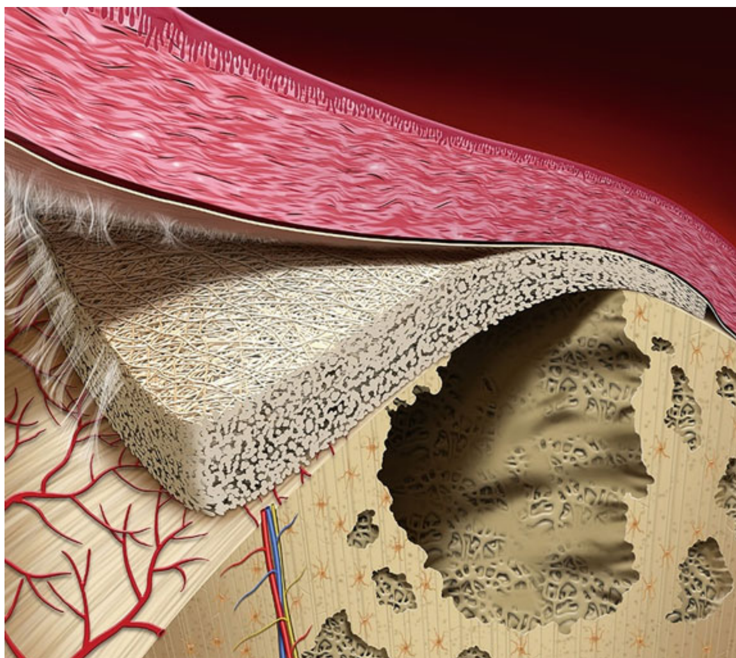

Fig. 13.3 Schematic illustration of the principle of guided bone regeneration (GBR). (Reproduced from Elgali et al. 2017, under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution)

shape retention without postprocessing manipulation (Dias et al. 2020; Agostinacchio et al. 2021; Jang et al. 2018).

13.3.2 Biomaterial-Based In Situ Tissue Engineering

The elimination of the need to recapitulate the exact tissue microenvironment for engineering tissues might be the greatest advantage of in situ regenerative approaches. In situ tissue regeneration requires the selection of appropriate biomaterials. Biomaterials suitable for in situ tissue engineering could be divided into synthetic, natural, and hybrid materials (Murdock and Badylak 2017). In all cases, biomaterials must be biodegradable and nontoxic and do not lead to adverse immune responses (Cao et al. 2020; Murdock and Badylak 2017). In situ-forming hydrogels have recently received considerable attention due to their desirable features such as injectability, minimal invasiveness, and complete defect filling (Sontyana et al. 2018). In one study, silk fibroin-based injectable hydrogels were reported to be of great help for in situ bone regeneration. After 4 and 8 weeks of hydrogel injection, in situ bone regeneration in implanted hydrogel occurred at 220% faster than that seen in untreated cases (Fig. 13.4) (Shi et al. 2017).

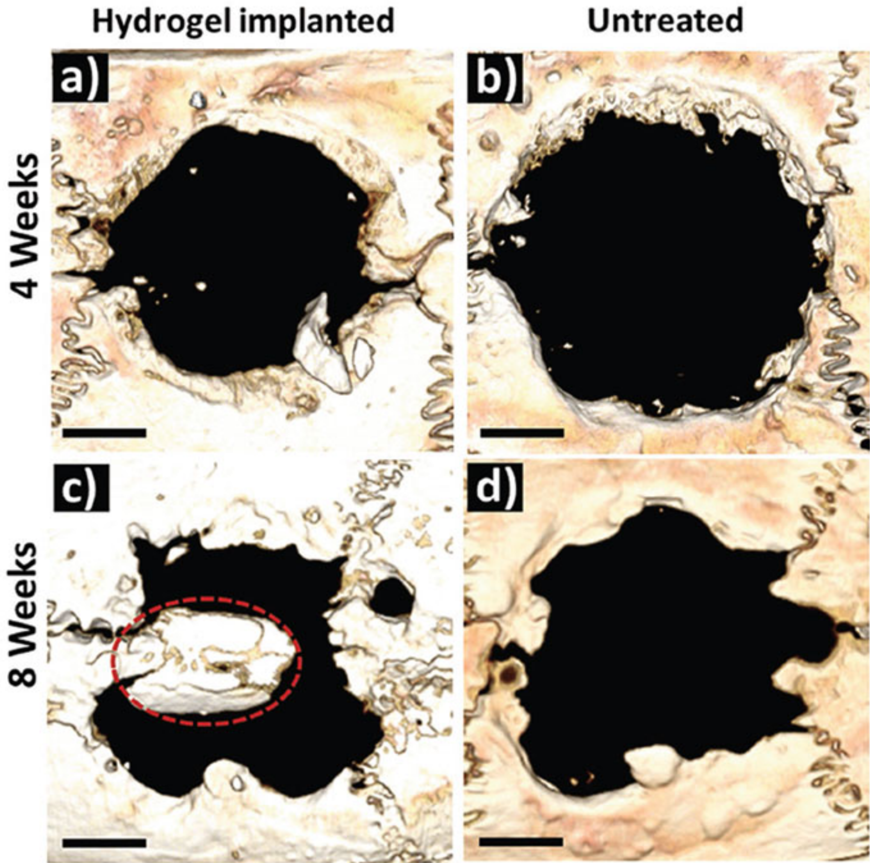

Fig. 13.4 (**a**–**d**) Micro-CT showing accelerated bone repair in hydrogel implanted group, as compared to untreated group. Reproduced from Maiz et al. 2020, under the terms and conditions of the Creative Commons Attribution (CC BY) license, http://creativecommons.org/licenses/by/4.0/.

To treat skin wounds, an electroconductive based on quaternized chitosan-g-polyaniline (QCSP) and benzaldehyde group functionalized poly(ethylene glycol)-co-poly(glycerol sebacate) (PEGS-FA) was developed (Zhao et al. 2017). Its use for the treatment of experimental skin defects in mice for 15 days indicated that hydrogels with optimal cross-linker concentration enhance wound healing (higher expression of EGF, TGF-β, and VEGF).

In situ tissue engineering using injectable hydrogels is also considered to be suitable for the regeneration of the heart tissue (Maiz et al. 2020). A recent study reported improved cell and tissue migration with the use of silk fibroin photo-lyogels containing microchannels as a platform for in situ tissue engineering (Baptista et al. 2020). In another study, an injectable and self-healing hydrogel based on

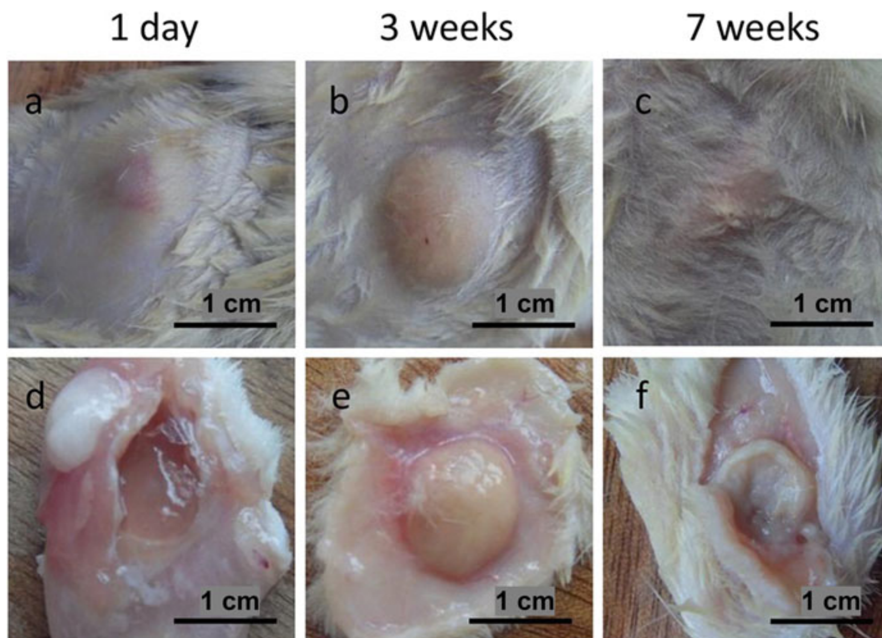

Fig. 13.5 Carbohydrate-based hydrogel was subcutaneously injected in rats using a syringe for 1 day, 3 weeks, and 7 weeks. (**a–c**) The natural state of the hydrogel after injection; (**d–f**) the rats were sacrificed, and the injection site was carefully cut open. (Reproduced from Lu et al. 2015, with permission from the American Chemical Society)

carbohydrates was developed, and cells were encapsulated in the hydrogel. It was found that hydrogel was biodegradable and helped in tissue repair (Fig. 13.5) (Lu et al. 2015). Another important aspect of in situ tissue engineering is the need of proper vascularization in targeted tissue (Markowicz et al. 2005; Shahabipour et al. 2020).

13.3.3 Biomolecule-Based In Situ Tissue Engineering

Living systems are influenced by various complex biomolecules produced by cells and living organisms. Biomolecules have a wide range of structures and sizes with different functions in the body. They are normally classified according to their biochemistry into carbohydrates, proteins, lipids, and nucleic acids (NCERT 2019; Karp et al. 2016). Proteins are the most abundant biomolecules of living systems. They have many roles and virtually control all cell activities. As enzymes, they accelerate the rate of metabolic reactions. As structural elements, they provide mechanical support. As hormones, growth factors, and gene activators, they play essential signaling and regulatory functions in the body. As antibodies and as membrane receptors and transporters, they can be used for special targeting or

signaling purposes. For example, the use of peptide molecules such as anti-VEGF-receptor 2 (anti-VEGF-R2) aptamer and arginine-glycine-aspartic acid (RGD) adhesion peptide-incorporated hydrogel system was investigated for use in wound healing (Roy et al. 2020). The synergistic effect of RGD peptides and VEGF-R2 aptamer enhance cell attachment, migration, and survival, and they can; therefore, be useful for future application for in situ wound healing. The incorporation of RGD peptides and BMP has been frequently reported in other studies (Lin et al. 2016; Wang et al. 2017). Hyaluronic acid modified with bisphosphonate or hydrogel nanocomposites based on hyaluronic acid and dextran have been recently reported for in situ bone regeneration as noninvasive stem cells and BMP-2 delivery systems for bone regeneration. Results indicated that the bioactivity of BMP-2 is preserved for more than 4 weeks, resulting in better osteogenesis both in vitro and in vivo (Ensrud 2013; Zhang et al. 2020). To treat osteochondral defects, a biocompatible, injectable hydrogel based on dextran functionalized with ureido-pyrimidinone (UPy) was developed (Hou et al. 2015). In this study, two hydrogels were mixed together, where one hydrogel was loaded with chondrocytes for cartilage regeneration and the other with BMP-2 for bone regeneration. After 8 weeks of implantation, cartilage-bone tissue interface was successfully developed. The results of a platelet-derived growth factor-AB (PDGF-AB) and collagenase-loaded system on in situ meniscal defect repair were also reported to be successful (Qu et al. 2017). A pH-switchable super-molecular hydrogel (polyethylene glycol derivative functionalized with UPy) loaded with growth factors (hepatocyte growth factor [HGF], and insulin-like growth factor-1 [IGF-1]) was used for cardiac tissue regeneration (Bastings et al. 2014). The hydrogel is liquid in basic pH environment and rapidly forms a hydrogel when it is in tissue neutral pH environment. In vivo results in pigs demonstrated its ability to reduce scar collagen in a chronic myocardial infarction.

Furthermore, there are other small or macro-biomolecules, which are used in tissue engineering. For example, kartogenin (KGN) is a small molecule that induces chondrogenesis and can be used for in situ cartilage tissue engineering (Dehghan-Baniani et al. 2020). Dendrimers, also known as synthetic macromolecules, represent another group of small molecules. For example, a synthetic dendritic polyglycerol sulfate-based in situ-forming hydrogel system was investigated, and it was proposed as a promising tool for future in situ cartilage tissue engineering (Dey et al. 2016). Another in situ injectable hydrogel system containing thiol functionalized poly(amido-amine) dendrimer and oxidized dextran was also investigated and proposed for potential use for in situ tissue engineering (Li et al. 2016a).

13.3.4 Genetics-Based In Situ Tissue Engineering

13.3.4.1 Approach

Polynucleotide-Based In Situ Tissue Engineering

Nucleic acids represent an important category of biomolecules used for in situ tissue engineering. They can be classified into two types: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Since they are long-chain polymers of nucleotides, they are also called polynucleotides. Gene-activated scaffolds for in situ bone regeneration and vascularization were reported to be successful (Lin et al. 2020; Sun et al. 2020). The activation of SOX-5, SOX-6, and SOX-9 transcription factors using gene-activated scaffolds for in situ chondrogenesis and inhibition of endochondral ossification was also reported (Raftery et al. 2020; Madry et al. 2020). In situ release of recombinant adeno-associated viral vectors on cartilage regeneration was also investigated for effective gene therapy and cartilage repair (Madry et al. 2020). Among important RNAs is RNA interference (RNAi), which silences the expression of specific genes and can be obtained through the delivery of microRNAs (miRNAs) or small interfering RNAs (siRNAs). However, the delivery of RNAi molecules is not easy. Moreover, RNAi biomolecules can be degraded under some circumstances. Although viral delivery vectors are mostly used for the expression of RNAi, it is risky due to mutagenesis and immunogenicity. Recently, improvement of the stability of mRNA by chemical modifications has attracted interest in developing mRNA-based therapies (Gaharwar et al. 2020).

Transcription Factors

By the delivery of lineage-determining transcription factors, cell state can be reprogrammed, and lineage-specific differentiation is induced. However, challenges associated with this approach include difficulties in preserving integrity and protein activity. These challenges can be overcome using retroviruses, lentiviruses, adenoviruses, and plasmids by integrating transgenes into the genome of the host. For instance, reprogramming of somatic cells into pluripotent cells can be performed by using four important transcription factors, including OCT4, SOX2, KLF4, and MYC expression using retroviral transduction (Gaharwar et al. 2020).

13.3.4.2 Mechanism

For in situ regenerative engineering, genetic material can be delivered directly into the target site. Target genes integrate into the host genome of endogenous stem cells and transform the transfected cells to boost tissue regeneration. Target genes are included in vectors to protect them from DNase and lysosomal digestive enzymes. Two types of vectors are commonly used, viral and nonviral vectors (Ji et al. 2011).

Viral Delivery

The use of viral vectors is the usual method to introduce a therapeutic gene into cells. There are different kinds of viral delivery systems that can be used for gene transfection (Santos et al. 2011; Cucchiarini et al. 2016; Partridge and Oreffo

2004). DNA-based viral vectors used for gene delivery are usually more durable and integrating into the genomes. DNA-based viral vectors include lentivirus, poxvirus, adenovirus, adeno-associated virus, retrovirus, human foamy virus (HFV), and herpesvirus. RNA-based viral vectors for gene delivery are able to directly transcribe the infectious RNA transcripts. RNA-based gene delivery is not constant, and it is not permanent. For RNA-based gene delivery, human foamy virus, oncoretroviral vectors, and lentiviral vectors can be used (Sung and Kim 2019).

Retrovirus

Retroviruses are widely used for DNA delivery into cells, such as hematopoietic marrow cells, meniscal cells, hepatocytes, keratinocytes, and endothelial cells (Partridge and Oreffo 2004). By integrating retroviral vectors into the host cell genome, transgenes of the host cell can be maintained for much longer times. Yet such integration may cause mutagenesis and activation of tumor genes. These vectors are used in the transduction of dividing cells and have low efficacy (Cucchiarini et al. 2016).

Adenovirus

Adenoviruses are capable of producing a high-titer virus and great efficiency of transfection both in dividing and nondividing cell lines. Helper-dependent adenoviral vectors (HDAds) and adenoviral–retroviral hybrids result in long-term expression. The disadvantage of this type of gene delivery is the immune response and inflammation it induces (Partridge and Oreffo 2004).

Lentiviral Vectors

The benefits of lentiviral vectors are greater than other viral approaches due to their integration into the genome of nondividing cells and high transduction effect. However, there still remain concerns regarding their mutagenesis potential (Cucchiarini et al. 2016). The viral transfection methods, including lentiviral and adenoviral vectors, are demonstrated in Fig. 13.6.

Recombinant Adeno-Associated Virus (rAAV) Vectors

rAAV vectors have less immunogenicity than adenoviral vectors and are more efficient than nonviral and retro-/lentiviral vectors for transducing both dividing and nondividing cells and have long-lasting transgene expression. They remain stable in their host and have the ability of gene transfer in situ through the extracellular matrix because of their small size. Due to these reasons, rAAV vectors have been widely used in clinical applications (Cucchiarini et al. 2016).

Herpes Simplex Virus (HSV) Vectors

HSV vectors deliver long transgenes into various cell types, including nondividing cells, but these kinds of vectors are toxic and have a temporary expression of the transgene (Cucchiarini et al. 2016).

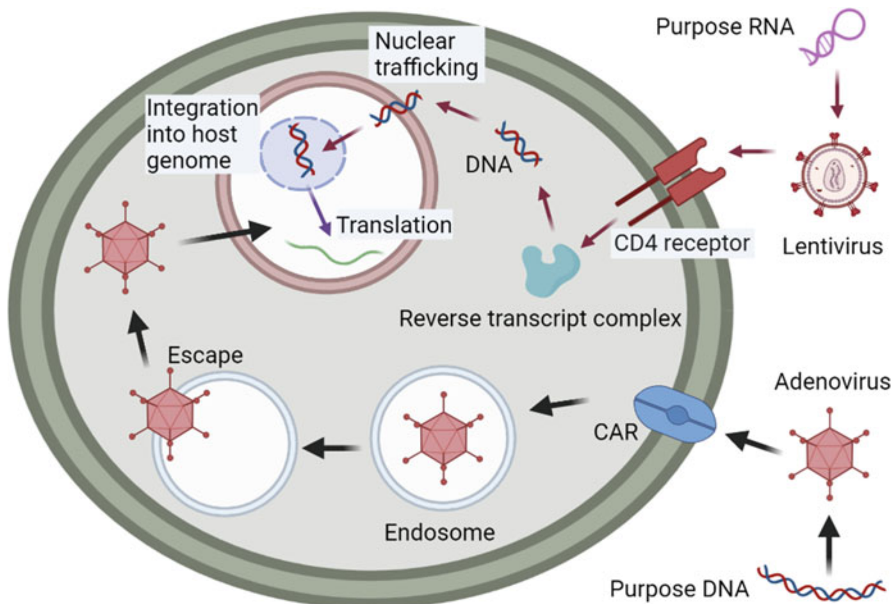

Fig. 13.6 Lentiviral and adenoviral based transfection methods. This is an open access article distributed under the Creative Commons Attribution License, which allows unrestricted use, distribution, and reproduction. (Redrawn from Chen et al. (2010), using Biorender.com)

Nonviral Delivery

Because of host immune reactions, toxicity, and risk of mutagenesis, the use of nonviral vectors has been increasing. These vectors can be used repeatedly, but they have low and transient transgene expression (Partridge and Oreffo 2004). Nonviral gene delivery is usually performed by using plasmid DNA, which includes the protein-gene encoding under the transcriptional control (Santos et al. 2011).

Chemical Methods

Chemical methods involve the use of natural or synthetic materials for genetic material delivery into the cell. Nonviral vectors have high structural and chemical versatility; they can be manipulated and adjusted to improve gene delivery and expression efficiency (Santos et al. 2011).

Physical Methods

Physical methods represent an ineffective way of gene delivery into cells, including naked DNA. They also include cell manipulation, enabling improved gene delivery, like nano- and microinjection, electroporation, and molecular vibration (Santos et al. 2011).

13.3.4.3 Advantages and Limitations

Ease of production and use, the durability of expression of therapeutic genes, and the simplicity and safety are the most important advantages of the gene activation method. Viral vectors have high efficiencies in gene delivery and transduction, and they allow prolonged expression of genes. The function of the viral vector is dependent on the characteristics of the parental viral vector, which are not yet well understood. Also, for producing viral vectors, the development of complex procedures based on cell culture and infection must be undertaken to obtain virions that contain therapeutic nucleic acids. Some viral vectors persuade inflammation and immune response after injection into the host (Giacca 2010). Clinical use of viral vectors is limited because of disadvantages such as transformation, mutagenesis, inflammation, and immune reactions. Furthermore, they have limitations in the delivery of exogenous DNA and large and big productions (Santos et al. 2011; Partridge and Oreffo 2004). On the other hand, nonviral gene delivery methods have less immunogenicity and risk of infectious disease transmission. They are flexible in the molecular size of DNA and are very cheap. However, chemical methods have shown lower transfection efficiency, temporary expression of the gene, and high toxicity compared to viral vectors (Santos et al. 2011).

13.3.5 Cell-Product-Based In Situ Tissue Engineering

More recently, it was thought that the therapeutic effect of stem cells might also be attributed to their secreted extracellular vesicles (EVs) (Lamichhane et al. 2015). Therefore, studies that explore the use of stem cell-derived EVs have been increasingly published (Lamichhane et al. 2015; Tsiapalis and O'Driscoll 2020; Liu and Holmes 2021; Kanada and Ashammakhi 2021). With the help of EVs, the need for cell delivery and attending risks and complications can be eliminated (Kanada and Ashammakhi 2021; Allan et al. 2020). In one study, exosomes were loaded into a methylcellulose-chitosan hydrogel and injected in a wound, where they resulted in enhanced cell proliferation and skin remodeling in diabetic mice (Wang et al. 2020).

13.4 Current Challenges and Future Directions

In situ tissue engineering has shown significant progress over the recent years by using biomaterials, biomolecules, genetics-based platforms, and cellular products. In particular, pre-vascularized tissue constructs, acellular organs, and microfluidics can be introduced as novel approaches in this field (Sengupta et al. 2014). Further, dynamic four-dimensional (4D) printing (Ashammakhi et al. 2018b) can be considered for further research and application for in situ tissue engineering in the future (Momeni et al. 2017; Ashammakhi et al. 2019c). The promising therapeutic outcome of the in situ toolbox has led to the development of a new paradigm to mitigate the current limitations of tissue engineering. However, there are still challenges in using

in situ platforms that need to be addressed to enhance its successful clinical translation in the future.

Advances in in situ tissue engineering require profound insight into the tissue healing dynamics, which involve biophysical and biomechanical factors acting at the defect site in order to establish an efficient tissue engineering tool and achieve successful regeneration. A full understanding of regeneration processes and the fate of endogenous stem cells and their migration is needed (Li et al. 2016b). Therefore, cell labeling followed by their long-term tracking would be a useful strategy to monitor engineered platforms and cell homing. This also requires the development of appropriate animal models (Zhang et al. 2017; Jahed et al. 2020). Also, novel in vitro models that investigate the use of human macrophages to stimulate the immune response should be considered. This can be achieved by using organ-on-a-chip (OoC) models, which will obviate the need for the use of other species that have different physiology (Elmusrati and Ashammakhi 2018; Ashammakhi et al. 2018c, 2019d, 2020b). It was proved that many factors such as scaffold morphology and stiffness and biochemical cues that are released following degradation affect the immune response, which should be taken into consideration in vitro (Guo et al. 2019), and new methods can also be useful in this regard (Ashammakhi et al. 2020c; Jiang et al. 2021; Tanataweethum et al. 2020; Bhatia and Ingber 2014). Providing appropriate conditions that can simulate the body environment with specific biophysical and biomechanical features can help to regenerate tissues in a more controlled and efficient fashion. This requires that advanced and intelligent designs of bioreactors be developed. Such approaches will help to predict and guide the formation of new tissues in the native microenvironment using in situ regenerative approaches, which can be fine-tuned at a later stage (Ashammakhi et al. 2019e). Since in situ tissue engineering relies on the body's inherent ability to regenerate, interindividual, tissue, and site variations in regenerative potential should be considered and investigated. Besides, the body regeneration capacity is affected by the immune system, which can also vary with age, health status, and medications, factors which should also be considered.

13.5 Conclusions

The use of in situ regeneration relies on leveraging body innate regenerative potential by using different tools to stimulate and enhance this ability, such as biomaterials, biomolecules, and cell products. In contrast to the ex vivo, the need for an external source of cells for transplantation is not required. In situ tissue engineering can be an effective method to advance regenerative therapeutics and enhance their clinical translation. Indeed, stimulating and enhancing specific pro-regenerative microenvironment will lead to the development of more effective and minimally invasive therapeutics. Although much progress has been made in the field of in situ regeneration of some tissues, many other tissues still need investigation to develop novel therapeutics. To make impactful advances in in situ tissue engineering a multidisciplinary approach based on the cooperation between

biochemists, biologists, biomedical engineers, and clinicians, as well as sustained funding are required.

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Chitosan for Wound Healing in the Light of Skin Tissue Engineering and Stem Cell Research

14

Ruchi Chawla and P. K. Dutta

Abstract

Chitosan, as a cationic biopolymer, is widely recognized for its applications in the field of biomedical research, especially in skin tissue engineering (STE). The arena of wound healing in the light of STE and stem cell research has attracted significant attention over the last two decades. Specifically, chitosan and its hybrids are being used as ideal biomaterials for wound healing application because of their biodegradable, biocompatible, nontoxic, antimicrobial (antibacterial and antifungal), and hemostatic properties. Their use in various physical forms, including hydrogels, fibers, membranes, films, and sponges, aids in different stages of wound healing and improves the overall healing process in full-thickness as well as chronic wounds, which has been exhibited in various animal models. The incorporation of stems cells, especially mesenchymal stem cells (MSCs) derived from the bone marrow, human umbilical cord, synovial membrane, and adipose tissue, in chitosan-based hybrids is another path-breaking development that has led to substantial enhancement in the wound healing process via secretion of pro-regenerative cytokines and enhanced collagen deposition. Thus, the research on wound healing application of chitosan and its derivatives in light of STE and the role of stem cells has been compiled in this book chapter, focusing on authors' and other researchers' original research, including both published and students' PhD thesis work.

Keywords

Chitosan · Wound healing · Skin tissue engineering · Stem cells

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14.1 Chitosan (CS): A Biopolymer for Wound Healing Applications

CS as a cationic biopolymer is widely recognized for its applications in the field of biomedical research. Structurally, it is a linear amino polysaccharide that consists of randomly distributed D-glucosamine and *N*-acetyl-D-glucosamine units held together by β-(1–4)-linkages (Domard 2011; Dash et al. 2011). The source of CS is chitin, which ranks as the second most plentiful natural polysaccharide on earth (cellulose being the first) and constitutes the framework material of the exoskeleton of crustaceans (chiefly crabs and shrimps) and insects and the cell wall of fungi and yeast. CS is obtained when chitin is subjected to deacetylation via chemical (alkali treatment) or enzymatic processes (Fig. 14.1) (Cheung et al. 2015). The degree of deacetylation (DD) has a remarkable effect on the properties of the biopolymer, and therefore, it is commercially available in different forms with variable DD (50–95%). The molecular weight of CS (~300 to 1000 kDa available commercially) is another factor that has a significant effect on its properties (Liu et al. 2011b).

The physicochemical properties of CS, which are mainly governed by its DD and molecular weight, include crystallinity, solubility, and degradation, which in turn depend on the employed production method and process parameters (Yang et al. 2008). One hundred percent deacetylated CS displays maximum crystallinity (Levengood and Zhang 2014). The solubility of CS is restricted in basic and physiological pH, whereas acidic medium favors the same. In an acidic medium, the addition of a proton to the amino group (pK_a 6.5) on the deacetylated subunits creates positive charge density, which renders the biopolymer soluble (Kumirska et al. 2011). Hence, the solubility of the polymer in an acidic medium is directly proportional to the degree of deacetylation. Due to its cationic nature, CS has the ability to interact electrostatically with the anionic mucin macromolecules (sialic acid) found in the mucus membrane, which leads to the increase in its mucoadhesive property (Dhawan et al. 2004). Similarly, the interface between positively charged CS and anionic blood cells is accountable for the hemostatic activity of the biopolymer. The positive charge on amino groups is responsible for the reorganization and opening of tight junctions of the cell membrane. Consequently, CS can also be used as a permeation enhancer (Smith et al. 2004). Besides, it is known to possess significant antimicrobial effect, which may be due to (1) changes in cell wall permeability of microbes in the presence of CS's protonated amino group causing leakage or restricting the absorption of vital materials, or (2) inhibition of the

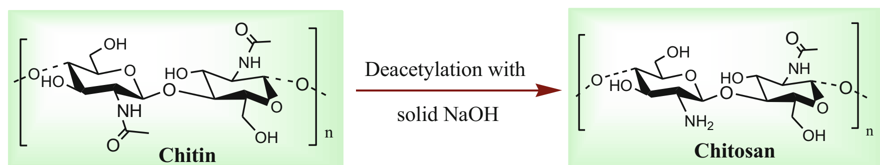

Fig. 14.1 Preparation of CS from chitin (alkali treatment)

synthesis of microbial RNA because CS binds to the cell DNA (Chung and Chen 2008). This antimicrobial activity of the polymer is greatly affected by its molecular weight (Kingkaew et al. 2014).

The tensile strength of CS in the wet state is directly proportional to its DD, whereas no such effect was observed in the dry state (Mima et al. 1983). With regard to molecular weight, higher tensile strength is exhibited by films fabricated from high-molecular-weight CS in comparison to those prepared from low-molecular-weight CS (Huei and Hwa 1996). For targeted application in tissue engineering (TE), the rate of degradation of a scaffold is a significant characteristic to consider, and in the case of CS-based scaffolds, the rate of degradation varies inversely with the DD. It has even been observed that an increase in the molecular weight of CS can lead to delayed degradation (Tangsadthakun et al. 2007).

CS also exhibits analgesic property as a consequence of the protonation of amino groups by the protons discharged at the inflammatory area (Okamoto et al. 2002). Its biodegradability can be attributed to the presence of glycosidic bond, which is cleaved by proteases, mainly lysozyme (Aranaz et al. 2009; Dutta et al. 2011). Cell adhesion and keratinocyte proliferation are known to decrease with the increase in the degree of acetylation (Barnes et al. 2007). Although CS is endowed with such incredible features, it lacks sufficient mechanical properties (Kim and Lee 2016). However, the solubility and mechanical strength issues of CS can be easily tackled by its derivatization and amalgamation with other polymers owing to the unique structural features of the polymer (Dutta et al. 2004). The aforementioned versatile properties of CS have led to the extensive utilization of this biopolymer and its derivatives in STE and wound healing (Chandy and Sharma 1990; Archana et al. 2013a, b, 2015).

14.2 Wound Healing, Skin Tissue Engineering (STE), and Stem Cell Research

Tissue engineering (TE) is the application of the principles of life sciences, engineering, and materials science to restore, regenerate, maintain, or improve functions in damaged tissues. It is a vital element of regenerative medicine that utilizes biomaterials, cells, and biochemical factors individually or in combination for the healthy functioning of tissues (Jiang et al. 2014; Lee et al. 2014). Using TE, scientists have been successful in engineering a variety of tissues in the body, including skin, nerve, and heart tissues, with varying degrees of success.

The prime function of the skin, the largest organ in the body, is to serve as a defense barricade against external mechanical, biochemical, and environmental factors. Adult mammalian skin broadly consists of three stratified layers: the epidermis, dermis, and subcutaneous fat tissue (often called the hypodermis). Any break or defect in the skin caused by physical and thermal damage is known as a wound (Lazarus et al. 1994). It may be classified into superficial, partial-thickness, and full-thickness wounds based on the layers involved; superficial wounds involve merely the epidermis, partial-thickness wounds encompass both dermis and epidermis, and

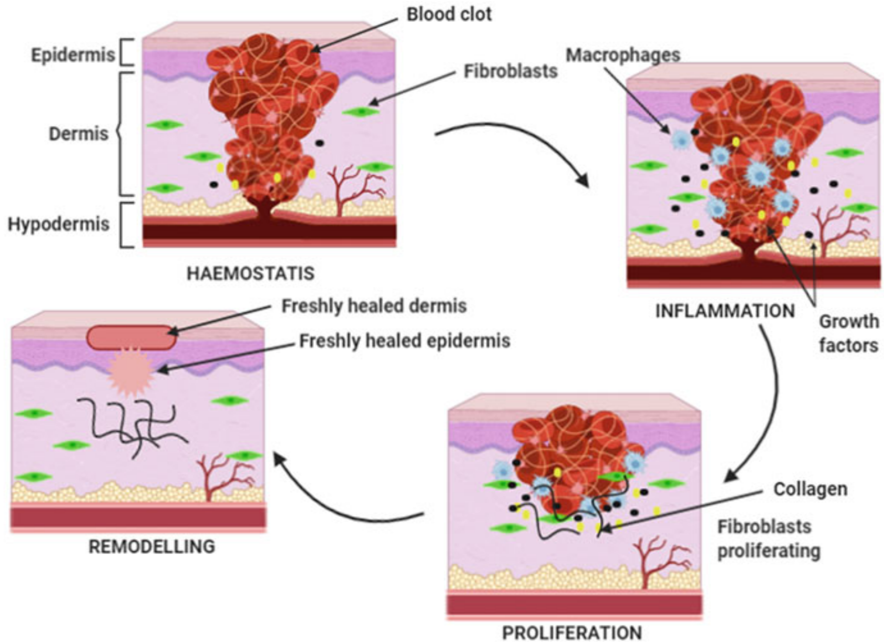

Fig. 14.2 The various phases of the wound healing process

full-thickness wounds include the hypodermis (Bolton and Van Rijswijk 1991; Krasner et al. 1993; Boateng et al. 2008; Archana 2014). Wounds can also be classified into acute and chronic wounds. Acute wounds are due to mechanical, thermal, or chemical injuries, and they heal totally with the least scarring, generally in 8–12 weeks (Percival 2002), while chronic wounds are mainly due to diabetes, malignancies, and persistent infections, and they heal gradually (not healed beyond 12 weeks even) (Harding et al. 2002).

Wound healing is an intricate progression involving interactions of cells, matrix components, and biological factors (Clark 1998; Ferguson and O'Kane 2004; Miller and Nanchahal 2005; Werner and Grose 2003; George Broughton et al. 2006). It comprises overlapping phases of hemostasis, inflammation, proliferation, remodeling, and maturation. The inflammation phase is characterized by transmigration of leukocytes and secretion of cytokines (slightly soluble peptides), and the proliferation phase is marked by reepithelialization, migration of keratinocytes, and wound contraction (Fig. 14.2) (Rhett et al. 2008). The various phases of wound healing are aided by the application of wound dressings, which are getting better day by day in terms of wound care owing to the great advances in the field of STE. An ideal wound dressing should impart a moist environment, avert infection, and aid in the complete restoration of damaged tissues. It should be elastic, biocompatible, porous, and nontoxic and should upregulate growth factors necessary for reepithelialization (Babu 2000; Lin et al. 2001; Khil et al. 2003; Ulubayram et al.

2001). To develop tissue-engineered constructs that meet all these requirements for improving the process of wound healing is a hot topic of research, and skin repair constitutes a chief area of study in the domain of TE.

Satisfactory skin regeneration in the instance of third-degree burns and chronic wounds still remains a challenge. One of the most extensively used reconstructive techniques in these cases is skin grafting (Shevchenko et al. 2010), which involves the relocation of skin from a healthy region to the damaged region. Skin grafts can be categorized into autografts (collected from patients only), isografts (from a genetically similar donor, i.e., twins), allografts (from different person), xenografts (from animals), and prosthetic grafts (substitution of damaged tissues by synthetic materials like plastic, metal, and ceramic) on the basis of source or donor site. Human autografts and allografts have limited availability, and allografts are expensive too, which limit their use in instance of extensive third-degree burns. Xenografts are more readily available in comparison to allografts, but they fail in leading to permanent revascularization. The development of ideal artificial skin substitutes (prosthetic grafts) is an apt solution to foster wound healing in severe cases (Leung and Fish 2009). Engineered skin should have all the elements crucial to facilitate the process of wound healing, and it must retain the traits of innate skin with limited scarring. Therefore, the ultimate goal of current research in STE is to develop tissue-engineered skin that compliments the traits of the autologous skin graft (MacFarlane 2006).

The utilization of stem cells (SCs) in TE is one of the major biological advances of the century, which is rapidly changing the face of health care. The main clinical objective of SC therapy in wound care is to enhance the quality of wound healing by speeding up the healing process, limiting scar formation, and achieving ideal regeneration of the skin (including the appendages) (Butler et al. 2010). As per the findings of McCulloch and Till (Chen et al. 2009), SCs are the cells that possess capability for self-renewal, asymmetric replication, and differentiation into other cells, which can lead to the construction of different tissues and organs. They are responsible for the replenishment of the lost cells all through an organism's lifespan. Their unlimited replication provides a population of "sister" SCs, which maintains the steady count of aging somatic cells (ones that become apoptotic with time). Their therapeutic potential in wound healing is mainly due to their ability to secrete pro-regenerative cytokines (Duscher et al. 2016). The main types of SCs that are used for wound healing and regeneration of injured skin include embryonic SCs, induced pluripotent SCs, and adult SCs (Dash et al. 2018). However, the application of SCs for skin regeneration still remains a challenge in terms of the selection of the optimum source of SCs, method of processing and administration, and their use in the real clinical situation (Burd et al. 2007). CS and its derivatives are extensively used for STE, especially wound healing applications owing to the various characteristics of the versatile natural polymer described above. CS-based tissue-engineered products for wound healing can be broadly divided into acellular (without SCs) and cellular (with SCs) categories as outlined in Fig. 14.3 and described in the following sections.

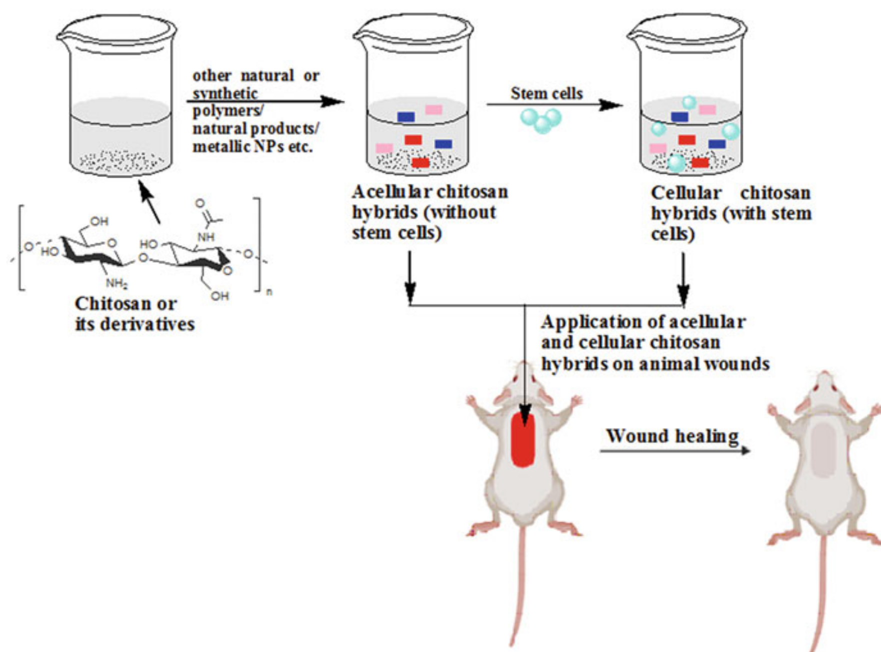

Fig. 14.3 A pictorial representation of the synthesis and use of acellular (without stem cells) and cellular (with stem cells) CS hybrids for wound healing application

14.2.1 CS for Wound Healing in the Light of Skin Tissue Engineering (Acellular, Without SCs)

CS and its derivatives find extensive applications in tissue engineering, including engineering of skin, bone, cartilage, blood vessels, nerves, ligament, and tendon tissues (Wang et al. 2012; Kumar et al. 2020; Jiang et al. 2014). This versatile natural polymer has been combined or co-grafted with other natural/semisynthetic/synthetic polymers, natural products, metallic nanoparticles (NPs), etc., to enhance its properties associated with tissue engineering (Qi et al. 2010; Liu et al. 2011a; Azizi et al. 2014; Khairkar and Raut 2014; Archana et al. 2013; Kim et al. 2006). Specifically, concerning wound healing and STE, a number of reviews are available in literature devoted to different aspects of this application of the polymer (Archana et al. 2014, 2016; Khan et al. 2020). In 2017, the reports on nanocomposites based on CS merged with ZnO, TiO_2, and silver NPs for antimicrobial wound healing applications were reviewed by Lee and coworkers (Bui et al. 2017). These metallic NPs show excellent results in wound healing when combined with CS scaffolds as evident in the recent reports based on ZnO (Sun et al. 2019; Sakthiguru and Sithique 2020), Ag (Luna-Hernández et al. 2017; Hernández-Rangel et al. 2019) and CuO (Dutta et al. 2021).

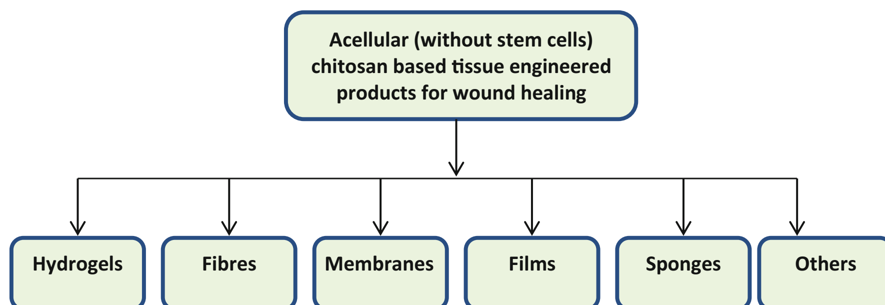

Fig. 14.4 Various forms in which acellular (without stem cells) CS hybrids are processed for wound healing

In the next subsections, a systematic analysis of the recently reported CS-based hybrid materials is presented on the basis of the physical form (hydrogel, fiber, membrane, film, and sponge; Fig. 14.4) in which they are used as potential candidates for application in the field of wound healing and STE. The reports included in this section do not involve the use of SCs (acellular).

14.2.1.1 Hydrogels Based on CS

CS-based hydrogels are regarded as ideal materials for improving wound healing because of their good moistening, biodegradable, biocompatible, nontoxic, antimicrobial, and hemostatic nature. These hydrogels are known for suppressing extreme inflammation and infection in chronic wounds, which in turn promotes wound healing. A number of reviews that are dedicated to CS-based hydrogels for therapeutic applications (Xu et al. 2018b; Sahranavard et al. 2020), especially wound healing (Khan et al. 2020; Liu et al. 2018), can be found in literature.

Recently, CS-based hydrogels impregnated with metallic NPs of Ag, ZnO, and copper have been reported, which have better mechanical and antimicrobial properties and aid in the process of healing of different types of wounds, including burn and diabetic wounds (Table 14.1).

Other than metallic NPs, natural products like marine peptides from tilapia (Ouyang et al. 2018), α-tocopherol (Ehterami et al. 2019), and cordycepin (Song et al. 2019); drugs like phenytoin (Cardoso et al. 2019), unfractionated heparin, or bemiparin (Cifuentes et al. 2020); and alkali lignin (a major by-product of the paper-producing industries) (Ravishankar et al. 2019) have been used in combination with CS/CS-based hydrogels to yield good results in wound healing. CS has also been used in combination with other polymers, viz., cellulose (Huang et al. 2018), poly(*N*-vinyl-2-pyrrolidone) (PVP) (Rasool et al. 2019), polyacrylamide (Xue et al. 2019), and recombinant human collagen peptide (Deng et al. 2020a), as excellent hydrogel-based wound dressings.

Table 14.1 Metallic NPs reinforced CS-based hydrogels for wound healing

S. no.	CS-based hydrogels	Metallic NPs reinforced on hydrogel	Characteristics of metallic NPs reinforced on hydrogels	Reference
1.	CS hydrogel	Silver NPs	• Ultrahigh mechanical properties • High antibacterial properties • Increased rate of reepithelialization and collagen deposition	Xie et al. (2018a)
2.	Keratin-CS nanocomposite hydrogel	Nano zinc oxide	• Increased swelling property • Increased bactericidal activity • Quicker skin cell construction along with collagen development	Zhai et al. (2018)
3.	Polyvinyl (alcohol)/CS hydrogel	Nano zinc oxide	• Increased porosity and wound fluid absorption • Increased elastic modulus and tensile strength with decreased elongation at break point • Good antibacterial properties, biocompatibility, and in vitro wound healing	Khorasani et al. (2019)
4.	CS-polyethylene glycol (PEG) hydrogel	Silver NPs	• Superior porosity, degree of swelling, and water vapor transition rate • Better antimicrobial and antioxidant properties in vitro • Improved wound healing ability in vivo in diabetic rabbits	Masood et al. (2019)
5.	CS/polyvinyl alcohol (PVA) hydrogel	Cerium oxide NPs	• Better antibacterial activities • Healthy human dermal fibroblast viabilities	Kalantari et al. (2020)
6.	CS/PVA hydrogel	Copper NPs	• Increased antimicrobial activity • Efficient care for open wounds in the early stages of wound healing and averting the incidence of prolonged open wounds	Lemraski et al. (2021)

14.2.1.2 Fibers Based on CS

Deng et al. fabricated diclofenac potassium-reinforced sutures based upon CS/PEG/PCL-keratin blends and evaluated them on the basis of their physical, thermal, and mechanical properties. The best tensile strength of the sutures was observed when the blend ratio was taken as 80:19:1 (w/w) of PCL, PEG, and CS-keratin. Drug-loaded (30 wt%) fabricated composite demonstrated high cell viability and accelerated wound healing in vitro cytotoxicity testing (Deng et al. 2020b).

Batista and coworkers developed a new route for the fabrication of CS-alginate aerogel fibers and evaluated their potential for wound healing applications. The emulsion-gelation method produced the fibers via the synthesis of polyelectrolyte complex hydrogels of CS and alginate, followed by solvent exchange and drying

with supercritical CO_2. Fluffy, lightweight, and mesoporous aerogel fibers were obtained with a specific surface area and a specific pore volume of 162–302 m^2/g and 1.41–2.49 cm^3/g, respectively. Aerogel fibers exhibited good antibacterial activity and accelerated wound healing (Batista et al. 2020).

14.2.1.3 Membranes Based on CS

CS-based-asymmetric membranes (Miguel et al. 2019) and electrospun CS membranes holding bioactive and therapeutic agents (Augustine et al. 2020) have recently been reviewed for enhanced wound healing application. Tamer et al. prepared CS–hyaluronan composite membranes and incorporated a mitochondrially targeted antioxidant—MitoQ—into them. These three-component membranes exhibited higher roughness (which enhanced tissue membrane interaction during the healing processes), lower cytotoxicity, and superior healing properties in injured rabbit and rat skin compared to control membranes (Tamer et al. 2018). Another report on membranes composed of CS and hyaluronic acid, including new arginine derivatives with thiazolidine-4-one scaffold for wound healing, was published in the same year (Iacob et al. 2018). These membranes revealed microporous structure, biocompatibility, superior swelling degree, better hydrophilicity, and satisfactory healing effects on the burn wounds induced in rats.

Considering the good antibacterial properties, Ag (Shao et al. 2019) and OH-CATH30 (Zou et al. 2020) NPs were incorporated into CS-based nanofibrous membranes to enhance their wound healing potential. Recently, Adalberto Enumo Jr. and coworkers developed novel CS-based membranes containing curcumin incorporated in pluronic copolymers for effective wound healing (Enumo et al. 2020). Other recent reports on CS-based membranes for wound healing application include glycerol-plasticized CS/PVA blends (Bano et al. 2019), CS/PVA/bioglass nanofibrous membranes (Chen et al. 2019), gelatin/CS/cinnamaldehyde crosslinked membranes (Kenawy et al. 2019), poly(ε-caprolactone) (PCL)/quaternized CS-graft-polyaniline-based nanofibrous membranes (He et al. 2020), and CS-based composites with urethane cross-linkage (Rahmani et al. 2020), which have demonstrated accelerated wound healing in chronic/full-thickness wounds.

14.2.1.4 Films Based on CS

In 2013, we fabricated the blends of CS, PVP, and TiO_2 and evaluated their wound healing efficacy. The mechanical property studies of nanocomposite indicated that the incorporation of TiO_2 NPs increased its strength. In vivo evaluation suggested that the developed nanocomposite film accelerated healing of excision-type wounds on adult male albino rats and superior results were obtained compared to conventional gauze, soframycin skin ointment, and CS-treated groups (Archana et al. 2013b). Later, our group was also successful in fabricating films composed of CS, PVP, and silver oxide NPs (CPS films), which again exhibited excellent efficacy (better than cotton gauze, 100% CS, and other reported CS-based dressings) toward wound healing when study was carried out on adult male albino rats (140–180 g). These films were further characterized for their morphological, mechanical, and antibacterial properties and their thermal behavior. The CPS film demonstrated

good swelling capability with lesser chances of wound dehydration, better antibacterial activity owing to the presence of good antibacterial agents (CS and silver oxide), and transparency, which helped in frequent monitoring of the wound without removal of the film from the wound location. The cytotoxicity study was performed in L929 cell lines by the alamarBlue assay, and it was found that cell viability decreased significantly when the concentration of NP was increased (Archana et al. 2015).

An attractive modification of CS films by γ-ray degradation for wound healing was reported by Madian and coworkers. At low γ-dose, more elastic and highly biodegradable films were obtained due to the accumulation of ions between the CS chains, which made a bulk network due to hydrogen bonds (Madian et al. 2018). To exploit the antibacterial properties of silver, several silver-containing CS-based nanocomposite films have been fabricated with proven antimicrobial wound dressing potential (Rahimi et al. 2019; Shah et al. 2019; Mohamed and Madian 2020). Other than these, the formulation of a number of CS or CS-based films for accelerated wound healing has been reported recently, as outlined in Table 14.2.

14.2.1.5 Sponges Based on CS

CS-based two-component to multi-component sponges have been fabricated to achieve effective wound healing in the cases of chronic/full-thickness/cutaneous wounds. The recent reports include the examples of CS–sodium hyaluronate–resveratrol-containing sponges (Berce et al. 2018), CS–silica hybrid sponge (Park et al. 2018), ampicillin-grafted CS sponges (Wu et al. 2018a), carboxymethyl konjac glucomannan-crosslinked CS sponges (Xie et al. 2018b), *Curcuma zedoaria* polysaccharide with platelet-rich plasma exosomes compiled on CS/silk hydrogel sponge (Xu et al. 2018a), sponges of carboxymethyl CS grafted with collagen peptides (Cheng et al. 2019), CS–gelatin–curcumin sponge (Naghshineh et al. 2019), quaternary ammonium CS NPs/CS composite sponge (Xia et al. 2020), and alginate/CS/fucoidan sponges (Hao et al. 2020).

14.2.1.6 Other CS-Based Hybrid Materials

Considering the curing effects of CS, pectin, and TiO_2, our group was successful in preparing an effective ternary CS–pectin–TiO_2 nano dressing material. Further, we characterized it by Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis, differential scanning calorimetry, transmission electron microscopy, and scanning electron microscopy. IR spectrum of the dressing material was indicative of polyelectrolyte complex formation between pectin and CS. The morphological study indicated that TiO_2 NPs were well distributed into the dressing material and the tensile strength of the blend increased with the decrease in pectin content (1:1) and the incorporation of TiO_2 NPs. The prepared nano dressing exhibited enhanced antimicrobial activity against all five pathogens and excellent blood compatibility. It also demonstrated biocompatibility, high swelling behavior and water vapor transmission rate, and good hydrophilic nature. Decreased cytotoxicity was observed using NIH3T3 and L929 fibroblast cells, and superior results were obtained compared to conventional gauze and CS when in vivo open excision-

Table 14.2 CS-based films for wound healing

S. no.	CS or CS-based films	Loaded/ surrounded drug or NPs	Bioassay	Results	Reference
1.	CS-PVP-bentonite nanocomposite film	–	3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide (MTT) assay on L929 and NIH3T3 cells and animal model	• Suitable surface morphology and high thermostability • Improved tensile strength, pH, and porosity • Better antibacterial activity and collagen deposition • Accelerated healing rate of 97% with less scarring	Shanmugapriya et al. (2018)
2.	CS-gelatin hydrogel films	Lupeol	MTT assay with NIH/3T3 fibroblast cells	• Smooth, flexible, and non-brittle films with excellent swelling ability • Equilibrium water content and water vapor transmission rate were found to be 85.40% and 2228 $\pm$ 31.8, respectively • Excellent antioxidant and antibacterial properties • Acceptable cell viability and non-toxicity	Patel et al. (2018)
3.	Hydroxypropyl CS modified by soy protein isolate (SPI)–nanocomposite films		MTT assay, live/dead assay, full-thickness skin wound model in rats	• Tensile strength of the film in a tunable range • Good cytocompatibility and hemocompatibility • Films with 50% SPI content had the best healing speed and skin regeneration efficiency	Zhao et al. (2018)

(continued)

Table 14.2 (continued)

S. no.	CS or CS-based films	Loaded/ surrounded drug or NPs	Bioassay	Results	Reference
4.	Hydroxyapatite-CS nanocomposite films	Fluorescent erbium III	Human lung fibroblast cells (WI-38)	• Strong antimicrobial activity • Excellent biocompatibility • Good biodegradation and mineralization behavior	Banerjee et al. (2018)
5.	CS grafted PVA films	Silk fiber	Bacterial strains of *S. aureus* and *E. coli*	• Thermally stable • Enhanced antibacterial and antifungal properties	Sheik et al. (2018)
6.	CS film	A mixture of bioactive compounds	Streptozotocin-induced diabetic rat model	• Provided a moist wound environment • Exhibited good antioxidant activity, proliferative effect, and biocompatibility • Stimulated wound contraction and accelerated the wound healing process	Colobatiu et al. (2019b)
7.	CS film using PEG as plasticizer and PVA for improving the bioadhesive properties	A mixture of bioactive compounds	Bacterial strains of *S. aureus*, *E. coli*, and *P. aeruginosa*	• Film thickness of 0.09 mm • Solubility in water was 44.9% • Higher swelling degree of 2157.4% • Efficient only against *P. aeruginosa*	Colobatiu et al. (2019a)
8.	CS film	Retinoic acid-loaded solid lipid NPs	Diabetic mouse model	• Great drug entrapment efficiency (98.0%) and low polydispersity index	Arantes et al. (2020)

				• Homogeneous distribution of the drug and controlled drug release	
				• Accelerated closure of wounds	
				• Lower leukocyte infiltrate in the wound bed, better collagen deposition, and reduced scar tissue	
9.	CS films	*Mansoa hirsuta* fraction (MHF), rich in acid triterpenes	In vivo wound treatment	• Amorphous, thermally stable, and dispersal of MHF in the CS matrix	Rodrigues Pereira et al. (2020)
				• Enhanced mechanical performance and thickness of film	
				• Improved healing, reepithelization, fibroblast proliferation, and collagen synthesis	
10.	CS film	Fluorescent carbon dots	–	• Reduced wettability	Kandra and Bajpai (2020)
				• Sevenfold decrease in the water absorption capacity	
				• Decreased bovine serum albumin adsorption	
				• Antioxidant property	
11.	CS/gelatin blend films	Tannic acid and/or bacterial nanocellulose	In vivo tests on Wistar rats	• No chemical reaction between the constituents	Taheri et al. (2020)
				• Desirable dispersion of nanocellulose in the matrix	
				• Amount of wound contraction is at least 15% higher for the film-treated wounds	

(continued)

Table 14.2 (continued)

S. no.	CS or CS-based films	Loaded/surrounded drug or NPs	Bioassay	Results	Reference
12.	CS/pectin films	ZnO NPs	MTT assay	• Swelling property and water retention capacity in the extent of 189–465% and 230–390%, respectively	Soubhagya et al. (2020)
				• Better compression strength and controlled degradation in the presence of lysozyme	
				• Biocompatible films with enhanced rate of fibroblast proliferation and migration	
				• Demonstrated antimicrobial property	
13.	Halloysite nanotubes modified CS nanocomposite films	–	–	• Substantial improvements in tensile strength, elongation at break, and thermal stability	Xie et al. (2020)
				• Decreased swelling rate	
				• Improved water vapor transmission rate	
14.	CS films	JR19, an *N*-acyl hydrazone subunit	In vitro tests (agar diffusion and MTT assay)	• Higher strength, flexibility, and water absorption capacity	Wanderley et al. (2020)
				• Small contact angle and greater surface roughness	
				• No cytotoxicity and better cell viability	

type wound healing efficiency evaluation was carried out in adult male albino rats (Archana et al. 2013a).

Electrospun CS-based nanofibrous mats function as excellent wound dressings because of the various advantages associated with them, viz., greater surface area, smaller pore size, biodegradability, biocompatibility, hemostatic/antimicrobial activities, and drug delivery ability. Recently, Ranjith et al. provided an overview of the latest research on CS-based nanofibrous wound dressings as vehicles for the delivery of therapeutic agents. They also discussed the recent advances in the use of nanofibrous mats as dual therapeutic/multi-therapeutic delivery agents for wound healing applications (Ranjith et al. 2019). Choudhary and coworkers fabricated nano-biocomposites scaffolds (film and sponge) through the integration of a graphene–silver–polycationic peptide (GAP) nanocomposite into CS. CS-GAP100 nano-biocomposites sponge and film exhibited exceptional porosity, fluid absorption, blood clotting capability, mechanical strength, and poor degradation. Consequently, both of them demonstrated excellent wound-healing property (Choudhary et al. 2020).

14.2.2 CS for Wound Healing in the Light of Stem Cell Research (Cellular, with Stem Cells)

CS-based hybrids in combination with SCs have proven to be potential candidates for enhancing the wound healing process. The incorporation of SCs, especially mesenchymal SCs (derived from the bone marrow, human umbilical cord, synovial membrane, and adipose tissue), into CS-based hybrids accelerates the wound healing process via secretion of pro-regenerative cytokines and enhanced collagen deposition. A systematic description of the reports on SCs incorporated CS scaffolds for wound healing (Fig. 14.5) has been covered in this section.

14.2.2.1 Mesenchymal Stem Cells (MSCs)

MSCs are the most extensively used SCs in combination with various CS scaffolds for wound healing owing to their multilineage differentiation, high occurrence, ease of isolation and characterization, and their ability to migrate to injury spots (Phinney 2012). They can be collected from various locations, viz., bone marrow, adipose tissue, amniotic fluid, and dermis. These cells have a role in all the phases of the wound-healing process and improve wound healing by various mechanisms, including immune modulation and generation of growth factors (Balaji et al. 2012). The effect of MSCs and CS gel on the healing of full-thickness skin wound in albino rats was studied by Sadik et al. and they found that MSC-treated groups manifested better and rapid healing of wounds than the control or CS-treated groups (El Sadik et al. 2015). Recently, rosuvastatin calcium and MCS-loaded CS scaffolds were implanted in induced wounds in albino rats, which resulted in enhanced skin regeneration and lack of scar formation (Maged et al. 2019). Shukla and coworkers evaluated (Shukla et al. 2021) the wound healing potential of Velgraft®, a ready-to-use biodegradable artificial skin substitute prepared by supplementing biopolymer of

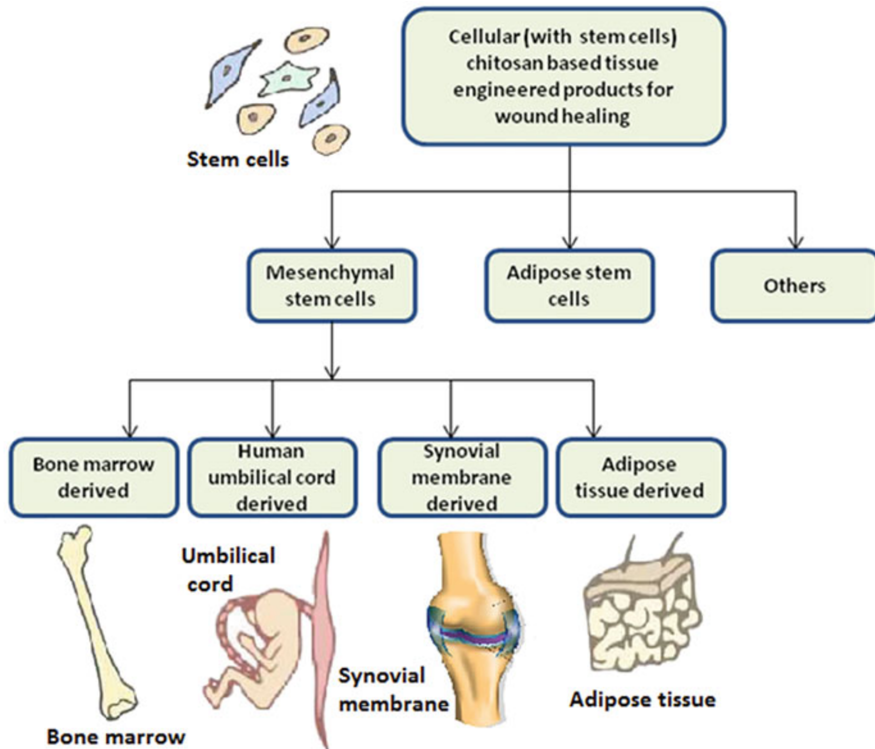

Fig. 14.5 Classification of various stem cell-incorporated CS scaffolds for effective wound healing based on the type and source of stem cells

CS and gelatin with MSCs, on excision wound in goats (*Capra hircus*). They concluded that Velgraft facilitated wound healing by improved angiogenesis and vasculogenesis. In the next subsections, a brief discussion on the application of MSC-incorporated CS scaffolds for wound healing is presented, and various reports have been classified on the basis of the source from which the MSCs were harvested.

14.2.2.2 Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSCs)

BM-MSCs are characterized by their ability to self-renew, rapid multiplication, and differentiation into various tissues by asymmetric replication (Ayatollahi et al. 2011; Pittenger et al. 1999). They are also called stromal progenitor cells. These advantages generate better results in the wound healing process compared to tissue-engineered skin substitutes (Chen et al. 2009). In the early inflammatory phase, these cells migrate to the spot of injury, where they control the proliferation and transmigration of the dermal mesenchymal and epithelial cells (Singer and Clark 1999). BM-MSCs also act as the chief source for the replacement of lost cells (Hocking and Gibran 2010; Pelizzo et al. 2015). They are known for their therapeutic potential in the treatment of ischemic wounds (Cha and Falanga 2007; Fu et al.

2006; Luo et al. 2010). These cells have also been used in conjunction with CS scaffolds on several occasions with encouraging results.

During the inflammatory phase of wound healing, a harsh microenvironment rich in reactive oxygen species (ROS) is induced, which results in the low retention of transplanted SC. To overcome this problem, Lin et al. developed a novel CS/acellular dermal matrix SC delivery system, which acted as a barrier, effectively scavenging the ROS and protected MSCs from oxidative stress resulting in lesser ROS-induced cellular death. Consequently, this MSC-loaded delivery system was successful in enhancing in vivo transplanted SC retention and improved cutaneous wound healing (Lin et al. 2020).

Abolgheit and coworkers recently reported that the collagen–CS scaffolds seeded with MSCs or their secreted extracellular vesicles (EVs) enhanced wound healing. The study was conducted on 32 adult male Sprague-Dawley rats by inducing a full-thickness skin wound on their back. EV- and BM-MSC-nursed groups exhibited enhanced macrophages count, more considerable collagen deposition, and improved collagen alignment in comparison to the control group (unprotected wounds). MSC-treated group showed lower amounts of collagen deposition in comparison to EV-nursed group (Abolgheit et al. 2020). Previously, Tong and coworkers constructed three-dimensional collagen–CS sponge scaffolds (CCSS) using the cross-linking and freeze-drying method, and BM-MSCs were seeded into them followed by hypoxia pretreatment, which resulted in accelerated wound closure via the reduction of inflammation and enhanced angiogenesis in diabetic rats with hindlimb ischemia. BM-MSCs secreted their own collagen and showed a considerable upregulated expression of proangiogenesis factors in the three-dimensional CCSS (Tong et al. 2016).

Impaired diabetic wounds are one of the foremost clinical complications caused by continuing microbial infections, extended inflammation, and inadequate angiogenic responses. To address this problem, *S*-nitroso-*N*-acetylpenicillamine (SNAP)-loaded CS/PVA hydrogel with nitric oxide-releasing ability was developed, and its efficiency in improving wound healing potential of BM-MSCs was evaluated. Experimental studies on induced wounds on diabetic rabbits exhibited that the use of SNAP preconditioned BMSCs and nitric oxide-releasing hydrogels majorly accelerated the healing procedure in comparison to the control group owing to improved collagen deposition and epithelial layer generation (Ahmed et al. 2020). However, previously, it has been reported that CS scaffold dressings, whether alone or in combination with MSCs, worsened wound healing and full-thickness wounds treated with MSCs only exhibited improved generation of granulation tissue and epithelialization in rabbits (Rajabian et al. 2017).

An interesting example of the use of photoactivated porous CS films supported by BM-MSCs to facilitate sutureless tissue repair was recently reported. The CS-based adhesive films were functionalized with oligomeric CS (10%) to improve their erodibility and to make them porous for superior tissue binding. The tested physical properties of the films, i.e., mass loss of ~20% after 7 days, swelling ratios of ~270–300%, percentage elongation of ~90%, and tensile strength and Young's modulus of ~1 MPa, were apt for maintaining the viability and multipotency of

BM-MSCs over the duration of culture, which was indicative of the potential applications of these films in STE and wound healing (Ruprai et al. 2019).

A sequence of silk fibroin/CS (SF/CS) scaffolds was synthesized using the freeze-drying method and evaluated for their use in TE. The rat BM-MSCs were loaded in these scaffolds, and their properties like 3D structure and degradation ability were tuned by changing the total concentration of the precursor solution and the blending ratio between SF and CS. The optimized SF/CS scaffold exhibited low inflammatory response and assisted the differentiation of BM-MSCs, which can accelerate the process of wound healing (Li et al. 2017). Kong and coworkers reported a fast and safe fabrication of a free-standing CS/alginate nanomembrane, which promoted BM-MSC adhesion and proliferation when transplanted to mouse dorsal skin with the full-thickness burn. The BM-MSC-loaded nanomembrane also accelerated wound contraction and epidermalization (Kong et al. 2016).

14.2.2.3 Human Umbilical Cord-Derived Mesenchymal Stem Cells (hUCMSCs)

hUCMSCs are more promising than other MSCs owing to their simple collection method, more rapid self-renewal features, and lower immunogenicity (Ding et al. 2015; Shi et al. 2018; Li et al. 2015). It has been proven that hUCMSC treatment leads to substantial promotion of wound repair by the enhancement of neovessels, secretion of several cytokines and growth factors, reepithelialization, and formation of skin appendages after epithelialization. Moreover, no major complications or side reactions are linked with the treatment involving hUCMSCs (Detamore 2013; Qin et al. 2016). With regard to CS, enhanced full-thickness cutaneous wound healing by thermosensitive CS-based hydrogel-encapsulated hUCMSCs was reported by Xu and coworkers. They fabricated a functional injectable thermosensitive hydrogel (glycerol phosphate sodium/CS/cellulose nanocrystals, GP/CS/CNC). The incorporation of CNC to the GP/CS hybrid improved the gel speed and its mechanical properties and also lowered its degradation rate. The hydrogel-hUCMSC combination promoted collagen deposition and decreased the secretion of inflammatory factors like TNF-α and IL-1β (Xu et al. 2019).

14.2.2.4 Synovial Mesenchymal Stem Cells (SMSCs)

MSCs obtained from the human synovial membrane, i.e., synovial mesenchymal stem cells (SMSCs), are characterized by their "tissue-specific" feature, which aids in the regeneration of connective tissue (Shao et al. 2015; Jones and Pei 2012). They can spread out quickly in culture, maintaining a steady molecular profile and retaining multipotentiality over at least ten passages (De Bari et al. 2003). Taking advantage of these characteristics of SMSCs, Li et al. fabricated hydroxyapatite/CS (HAP-CS) composite hydrogels reinforced with exosomes obtained from miR-126-3p-overexpressed SMSCs (SMSC-126-Exos) for diabetic chronic wound healing. In vivo tests demonstrated that the HAP-CS-SMSCs-126-Exos efficiently promoted wound surface reepithelialization, accelerated angiogenesis, and hastened collagen maturity (Li et al. 2016). Later, the group of Tao and Guo reported that CS wound dressings incorporating exosomes derived from microRNA-126-overexpressing

SMSCs provided sustained release of exosomes and healed full-thickness skin defects in a diabetic rat model. They found that SMSCs were capable of promoting fibroblast proliferation but were unsuccessful at elevating angiogenesis. They could promote angiogenesis in SMSCs by transferring the angiogenic capacity of endothelial progenitor cells to SMSCs using gene overexpression technology. In vitro studies showed that SMSC-126-Exos-embedded CS wound dressings accelerated the proliferation of human dermal fibroblasts and microvascular endothelial cells (HMEC-1) depending on the dose of the medication (Tao et al. 2017).

14.2.2.5 Human Mesenchymal Stem Cells Isolated from Adipose Tissue (AD-MSCs)

Ruiz and coworkers designed and evaluated MSC-seeded CS/glycosaminoglycan quaternary hydrogel scaffolds for wound healing applications. The stability and physical properties of hydrogel scaffolds were investigated, and the scaffold with the best suitable properties was encapsulated with AD-MSCs. The viability of AD-MSCs remained above 75%, and the number of living AD-MSCs in the scaffold reached a steady state up to ~100% at the 5th and 7th days (Soriano-Ruiz et al. 2019). The impact of AD-MSCs and keratinocytes on gelatin/CS/β-glycerol phosphate (GCGP) nano-scaffolds was studied by Lotfi and coworkers in full-thickness wounds of rats. They observed that the rate of wound closure was enhanced along with higher expressions of vascular endothelial growth factor, collagen type 1, and CD34 in the keratinocyte-AD-MSC scaffold (KMS)-treated group, which is indicative of the huge potential of the nano-scaffold for wound healing (Lotfi et al. 2019).

14.2.2.6 Adipose-Derived Stem Cells (ASCs)

Adipose-derived stem cells (ASCs) are derived from adipose tissue and have characteristics similar to BM-MSCs. These pluripotent SCs can potentially repair and regenerate damaged tissues leading to faster healing of even chronic wounds and aid in scarring treatment. In conjunction with CS, these ASCs are known to increase the repairing and healing potential of damaged tissues (Gimble et al. 2007; Zuk et al. 2001). In 2014, Gomathysankar et al. reviewed various CS scaffolds in combination with keratinocytes and ASCs as a vehicle for delivering cells increasing the proliferation of keratinocytes to achieve complete wound healing (Gomathysankar et al. 2014). Recently, the same group covered various skin substitutes in wound healing and the stimulatory effects of ASCs for the proliferation of keratinocytes on CS in a book chapter (Gomathysankar et al. 2018).

Cheng and coworkers fabricated a thermosensitive gelatin/CS hydrogel that enhanced the viability of the seeded ASCs, and the slow degradation of gelatin resulted in sustained release of ASCs from the hydrogel. It was observed that ASC-encapsulated hydrogel accelerated in vivo angiogenesis in both chick embryo chorioallantoic membrane assay and mice wound model. Therefore, the authors concluded that CS/gelatin hydrogel represented an effective delivery system with significant clinical potential in treating ischemic diseases (Cheng et al. 2017). Recently, Wu and coworkers investigated the wound-healing potential of ASC-seeded silk fibroin (SF)/CS in streptozotocin-induced diabetic rats and found

that the ASC-seeded SF/CS film treatment significantly enhanced wound healing in treated animals in comparison to control and SF/CS film-treated groups (Wu et al. 2018b).

14.2.2.7 Other Stem Cells

Human amnion-derived mesenchymal stem cells (HAMSCs) were impregnated on oleoyl CS-based nanofiber mats, which exhibited accelerated wound healing in full-thickness excisional wounds. It was demonstrated that both acellular and HAMSC-incorporated cellular nanofiber mats promoted remarkable wound contraction with better skin tissue regeneration due to enhanced collagen synthesis, reepithelialization, and initiation of epithelial cell stratification compared to the control group (Datta et al. 2017).

14.3 Conclusion and Future Perspective

Wound healing is an intricate dynamic process that is governed by the interaction among the cells, growth factors, and extracellular matrices to decide the outcome of the healing process. Any disarray can fail wound healing and can even transform acute wounds into chronic wounds. CS hybrids are widely used for wound healing because of their excellent biocompatibility and biological activity. The incorporation of SCs into these hybrids leads to better results in wound healing owing to the secretion of pro-regenerative cytokines and enhanced collagen deposition. SC-based therapeutic wound dressings, particularly adult MSC-based CS hybrids, are known for promoting scarless wound healing. However, harvesting and isolating MSC with high purity is challenging, and understanding of cellular and molecular mechanisms that form the basis of SC action is essential to overcome these limitations. Subsequently, improvement in the methods of SC delivery and recognition of the ideal source are required for better clinical utilization of these cells in wound healing. Technical advances can lead to future more innovative applications of CS-based wound care products to satisfy the unmet needs of acellular and cellular STE.

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Dynamic Interactions Between Stem Cells and Biomaterials

15

Jasmeet Kaur Virdi and Prasad Pethe

Abstract

The cellular microenvironment has been known to direct the cell behaviour through biochemical and mechanical signalling. Different biomaterials have been fabricated to study the impact of biophysical cues on proliferation and stem cell differentiation in vitro. Stem cells have immense promise in regenerative medicine. Therefore, there is a pressing need to understand the interdependency of biophysical signals and biochemical signals in regulating stem cell potency and differentiation. In this chapter, we explore the different types of biomaterials commonly used for studying mechanobiology in stem cells and highlight the primary mechanism and pathways behind extracellular matrix (ECM)-mediated cellular response. Furthermore, we discuss how the understanding of stem cell mechanobiology influences the fields of tissue engineering and regenerative medicine. We also touch upon the importance of mechanobiology in cancer. In short, we have tried to convey to our readers that although current expansion and differentiation methods use biochemical molecules alone, it is crucial to understand that biophysical cues from the stem cell microenvironment can also regulate the proliferation and differentiation of stem cells.

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Mechanobiology · Stem cells · Pluripotent stem cells · Biomaterials · Tissue engineering and regeneration

15.1 Introduction

Human pluripotent stem cells (hPSCs), which include both human-induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs), have a unique ability to differentiate into cells of three germ layers and have unlimited expansion potential; hence, they can be used for tissue engineering. Multipotent stem cells, for example, mesenchymal stem cells and hematopoietic stem cells, are often used for various clinical researches, and there are several clinical trials conducted with these cells. However, most applications remain at the clinical trial stage due to the non-functionality of transplanted cells, cell death after transplantation, deposition of cells into the lungs, or teratoma formation (Lodi et al. 2011; Naji et al. 2019). This can be due to sudden changes in the microenvironment from in vitro to in vivo. Many researchers have been trying to study interactions between stem cells and their surrounding microenvironment to overcome this.

In vivo, stem cells reside in a specific microenvironment, also known as "niche." This niche maintains an equilibrium between stem cell self-renewal and differentiation and is unique to every stem cell type. The critical regulatory components within the niche include dynamic and complex interactions between cells, macromolecules of extracellular matrix (ECM), biochemical components such as signaling molecules and hormones, and biophysical components such as ECM stiffness, pressure, shear fluid flow, stress, and strain (Pelham and Wang 1997; Vining and Mooney 2017). While the role of biochemical factors is well established, recent scientific literature points to evidence which indicates that the mechanical and biophysical signals generated from the extracellular milieu affect stem cell proliferation and differentiation (Gerardo et al. 2019; Gungordu et al. 2019). All cells, including stem cells and cancer cells, respond to mechanical cues. In stem cells, biophysical signaling control stem cell differentiation and self-renewal; and, in cancer cells, these signals lead to tumor invasiveness and metastasis (Lee et al. 2019; Choudhury et al. 2019). All these recent developments have led to the emergence of a new discipline—mechanobiology, which combines physical forces with the biological phenomenon.

The emergence of biomaterials has facilitated to artificially recreate biophysical signals experienced by cells under in vivo conditions. These biomaterials can be employed as a carrier for the transplantation of stem cells or to recruit endogenous progenitor cells at the site to repair and reconstruct damaged tissues or organs. A common hurdle in the use of biomaterials in regenerative medicine is the immune response. After transplantation, the biomaterials are extensively infiltrated by immune cells. These cells facilitate in removing cellular debris caused by injury; however, they can evoke inflammatory responses, which might hinder tissue repair and cell differentiation (Mokarram and Bellamkonda 2014). The development of new strategies has made biomaterials more sophisticated with respect to

biocompatibility, biological cues, and the potential to reduce damage by an immune response and facilitate in vivo tissue development and direct repair.

In this chapter, we have explored the mechanical and functional interactions between stem cells and their microenvironment. We begin with a brief overview of the importance of ECM in mechanobiology, along with the fundamental molecular mechanisms and the emerging field of biomaterials for stem cell culture. We touch upon cancer mechanobiology and the implications of stem cell mechanobiology and regenerative medicine. We finally provide a perspective on the use of biomaterials to create a modified 3D microenvironment for stem cell culture, which will provide a model to uncover fundamental aspects of mechanobiology and hold tremendous potential in cell-based therapies.

15.2 Unique Tissue-Specific ECM Stiffness in Normal Physiology

The ECM is composed of fibrous proteins such as collagen, fibronectin, elastin, vitronectin, laminin; proteoglycans, and glycoproteins secreted by cells and matricellular-associated proteins such as CNN family, osteopontin, fibulin, periostin, and secreted protein acidic and rich in cysteine (SPARC); however, the ratios of these proteins vary between tissues (Yue 2014; Mouw et al. 2014). Therefore, each tissue has different stiffness, which is defined as elasticity or Young's modulus (E) and is measured in a unit called pascal (Pa). For instance, bone ECM is primarily made up of collagen, which makes it stiff, and the estimated stiffness is approximately within the range of 100 kilopascal (kPa)–1 gigapascal (GPa). On the other hand, brain ECM has low fibrous proteins and higher amounts of proteoglycans compared to bone with E of approximately 1 kPa (Fig. 15.1) (Ruoslahti 1996; Wells

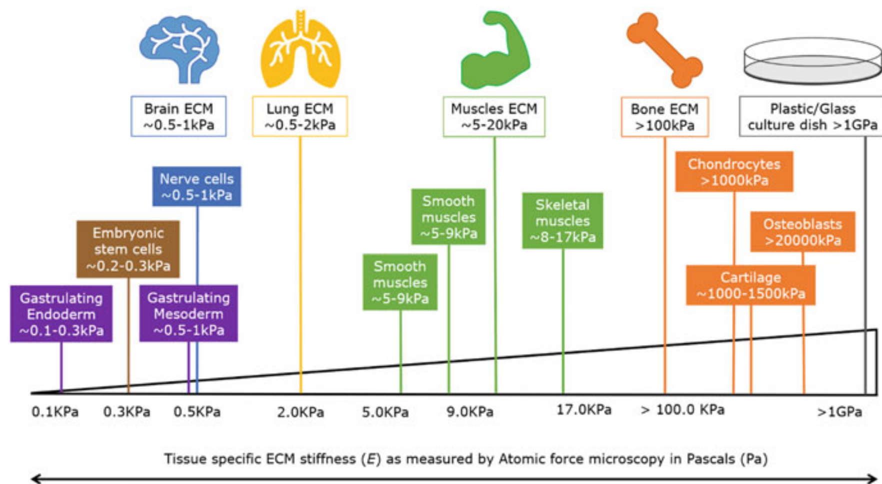

Fig. 15.1 Diagrammatic representation of the varied ECM stiffness range reported in different tissues measured by atomic force microscopy (AFM). The stiffness is defined in Young's modulus or elastic modulus (E) and measured in pascals (Pa)

2008; Budday et al. 2015). Such variations in tissue ECM have led researchers to develop scaffolds that mimic the biological ECM stiffness and properties.

Our understanding of how mechanical signals direct molecular signaling during embryo development and in in vitro differentiation is constantly evolving. The role of ECM in generating mechanical cues has been explored extensively, as the matrix is crucial in regulating cellular functions (Pelham and Wang 1997; Vining and Mooney 2017). Other than providing physical support for growth attachment, the ECM also regulates cell shape, growth, proliferation, differentiation, and migration. Numerous studies have reported that changing the mechanical properties of the matrix, such as stiffness, affects cell morphology, growth, differentiation, migration, and gene expression (Pelham and Wang 1997; Lo et al. 2000; Justin and Engler 2011; Toh et al. 2012, Ireland and Simmons 2015).

15.3 Biomaterials and Their Types

Traditionally used synthetic scaffolds from 2D polystyrene surfaces to 3D constructs provide only support to the cultured cells. Recent advances in tissue engineering have shown exciting results with various biomaterials of suitable physical and chemical properties in recreating complex in vivo microenvironment in the laboratory. Based on their source and properties, these biocompatible materials can be categorized as natural, semisynthetic, and synthetic biomaterials, with stiffness similar to the stiffness of the biological tissue (Virdi and Pethe 2021).

Natural biomaterials are synthesized using polymers such as chitin, agarose, collagen (Chevallay and Herbage 2000), alginate, and hyaluronic acid hydrogel (Toh et al. 2012) because of their similarity with native ECM. Another advantage is that they are highly biocompatible with binding sites for cells, thereby supporting cell growth. However, natural polymers are not consistent in composition, are not easy to modify, and have limited mechanical properties. To overcome these disadvantages of natural polymers, synthetic substrates have been synthesized using polyacrylamide (PA) gels (Engler et al. 2004), polydimethylsiloxane (PDMS) (Goffin et al. 2006), polyethylene glycol (PEG) hydrogel (Gilbert et al. 2010), and polyvinyl alcohol (Muduli et al. 2017), which provide better mechanical properties than natural biomaterials. The synthetic biomaterials provide a range of various stiffness similar to the stiffness of the biological tissue, have high reproducibility, and are well defined. However, synthetic polymers provide limited cell-ECM interactions as they lack the functional group to allow cells to attach.

To overcome the drawbacks of natural polymers and synthetic biomaterials, a semisynthetic hydrogel, for example, gelatin methyl acrylate (GelMa) (Guilak et al. 2009), was designed, which has the biocompatibility of natural polymer and mechanical properties of synthetic biomaterials. To enhance the clinical application of scaffolds, it is important to achieve a xeno-free, chemically-defined system for stem cell culture other than hydrogels. In this regard, other scaffolds such as artificial nano- and micro-patterned substrates (Théry 2010), flexible micropillars (Halder et al. 2012), and electrospun nanofibers (Maldonado et al. 2015; Zhu et al. 2019)

have been synthesized to study the effect of substrate stiffness on stem cell growth, differentiation, and migration.

15.4 Immunomodulatory Biomaterials

As we have introduced above, biomaterials being a foreign material may provoke an immune response, which might hinder tissue repair and regeneration. To address this limitation, researchers are synthesizing new biomaterial designs, which incorporate immunosuppressive molecules or signaling molecules that facilitate activation of the desired phenotype within the host immune cells (Dziki and Badylak 2018). These types of biomaterials are known as immunomodulatory biomaterials. Specific and durable immunomodulation can be achieved by manipulating the surface property of the biomaterials such as topology, surface charge, and ligands; this can induce activation of a desired immune cell phenotype (Stabler et al. 2019). For instance, following the implantation in murine subcutaneous implant and volumetric muscle injury model, flow cytometry analysis identified macrophages (F4/80^{+}), CD11c^{+} dendritic cells, CD3^{+} T cells, and CD19^{+} B cells within the microenvironment of the ECM bioscaffold (Sadtler et al. 2017). The authors have shown that the biomaterial microenvironment changes the polarization of the migrating immune cells upon implantation, causing them to alter the signals generated by microenvironment. This immunomodulatory effect of the biomaterial on the immune cells and the host tissue environment may help in improving the therapeutic capability of the biomaterials. Numerous similar studies that use ECM-based biomaterials show a dynamic interaction between a variety of the immune cells or between stem cells and immune cells, which promotes tissue repair (Brown et al. 2012; Sadtler et al. 2016; Dziki et al. 2018).

15.5 Biomaterials Influence Stem Cell Proliferation and Functionality

In order to design the biomaterial that allows stem cells to be transplanted for clinical use, it is important to study some key aspects such as (1) the traction forces exerted by the cells on the biomaterial, (2) stem cell growth and proliferation, and (3) the changes in the stem cell functionality and differentiation capacity when grown on biomaterial.

The synthetic hydrogel substrates are synthesized using one or more polymers, which forms an interconnecting network with the help of a cross-linking agent. The mechanical properties such as hydrogel substrates can be manipulated by changing concentrations of polymer and cross-linking agent. For example, in PA-gel substrates, altering the ratio of acrylamide to bis-acrylamide cross-linker allows variation in Young's modulus, which thereby affects cell behavior (Tse and Engler 2010). Human mesenchymal stem cells (hMSCs) cultured on stiff PA substrate with $E \sim 25$–40 kPa, which resembles bone ECM stiffness, differentiate toward osteoblast

lineage as indicated by the gene expression analysis, whereas, on soft PA substrate ($E \sim 0.1$–1 kPa) resembling brain ECM stiffness, the hMSCs differentiate toward neural lineage (García and Reyes 2005; Engler et al. 2006). Muscle stem cells self-renew when cultured on substrates mimicking the stiffness of muscle tissue ($E \sim 12$ kPa), and these cells contributed to muscle regeneration when transplanted in mice (Gilbert et al. 2010). Morphologically, stem cells appear flattened on the stiff substrate and spherical with reduced spreading and stress fiber formation on soft substrate (Deroanne et al. 2001; Engler et al. 2004). These studies reveal varying responses of stem cells toward their microenvironment, and substrate stiffness indicates an important role of substrate matrix in regulating cell behavior.

PA-gel substrate functionalized with glycosaminoglycan (GAG) peptides shows better cell attachment. Following this observation, the research group demonstrated that stiff PA-GAG substrate ($E \sim 10$ kPa) promotes pluripotency of human ESCs as evidently observed from the expression levels of pluripotency marker proteins octamer-binding transcription factor-4 (OCT4) and stage-specific embryonic antigen-4 (SSEA4) (Musah et al. 2012); however soft substrate ($E \sim 0.7$ kPa) selectively differentiated stem cells toward neuronal lineage. The same research group noted an interesting observation that even in the absence of neuronal inducing factor, hPSCs grown on softer substrate appeared neuronal-like phenotype and expressed high levels of tubulin beta 3 chain (TUJ1) protein, a neuronal specific-marker (Musah et al. 2014). A similar observation was reported by another group that used other biomaterials as well of different stiffness (Chen et al. 2020). These studies indicate that substrate stiffness alone can influence hPSC differentiation when cultured with an optimal mechanical microenvironment, independent of soluble signaling factors. Therefore, it can be said that the mechanical signals have a profound contribution on early embryo development and differentiation.

As explained above, when mimicking various physiological stiffness like neural ($E \sim 1$ kPa), muscle ($E \sim 12$ kPa), and bone ($E \sim 30$ kPa) tissues, substrates can induce respective lineage-specific differentiation of MSCs. In addition to cellular function, substrate stiffness also influence cell migration. Cell migration is important in numerous physiological processes such as wound healing, organogenesis, immune response, tumor metastasis, and morphogenesis; thus, it is crucial in regeneration tissue engineering and cancer therapy. Many studies have demonstrated stem cells migrate toward the stiff substrate, whereas neurons show a preference for softer regions (Tse and Engler 2010; Vincent et al. 2013; Flanagan et al. 2002; Hadden et al. 2017). The mechanical properties of the ECM influence the factors known to regulate cell migration, such as the integrin-cytoskeletal interaction and cytoskeletal stiffness. The cells sense the change in the matrix through an active tactile exploration mechanism and respond by exerting contractile forces (Lo et al. 2000). To understand the migration of stem cells on matrix stiffness, MSCs were treated with focal adhesion kinases (FAK) inhibitor and siRNA targeting transcriptional factor Yes-associated protein (YAP) gene. They observed reduced cellular motility of treated cells compared to untreated cells, indicating that FAK and YAP control the movement of cells from the soft region toward the stiff region (Wang et al. 2001; Hadden et al. 2017; Lachowski et al. 2018).

15.6 Mechanobiology: Mechanism of Interactions (Molecular Mechanisms)

Mechanobiology is the study of the relationship between a cell and its microenvironment. The interactions between the cell and the microenvironment mainly occur at the interface. The properties of biomaterials such as hydrophilicity, surface charge, roughness, softness, and chemical composition affect the transplantation success. To improve the interaction between cell and scaffold, the physical, chemical, and biological properties of the biomaterials need to be optimized according to the cell type. Before seeding the cells onto a scaffold, surface modification is necessary to facilitate cell adhesion and growth. Surface modification can be either coating the surface with extracellular membrane protein or modifying the surface using functional moieties, hydrophobic or hydrophilic molecules (Shi et al. 2015; Elosegui-Artola et al. 2017).

A cell senses its external environment via membrane-bound receptors, focal adhesions to the ECM, adhesion junctions between neighboring cells, and gap junctions. The perturbation of protein conformation by mechanical forces influences the cytoskeletal organization, which triggers a series of intracellular signaling pathway resulting in inactivation or inhibition of gene expression, morphology, and motility (Discher et al. 2005; Guilak et al. 2009). Integrin-based adhesion complexes are one of the key molecular players closely associated with actin filaments. Focal adhesion complex, Ras homologous (Rho) GTPases, myosin light chain kinases, and Rho-associated kinases (ROCK) form a link between integrins and actin filaments. The activated focal adhesion complex comprises talin, vinculin, paxillin, alpha-actinin, p130cas, FAK, and SRC formed near cell surface integrin receptor (Geiger et al. 2009). The cells are able to sense the substrate stiffness, topology, surface area, and dimensionality of the scaffold by means of integrin molecules and focal adhesion complexes (del Rio et al. 2009; Amano et al. 2010; Donato et al. 2010; Ciobanasu et al. 2013; Janoštiak et al. 2014; Elosegui-Artola et al. 2017).

In brief, integrins are transmembrane ECM proteins and mechanoreceptors as they sense the change in the ECM, thereby mediating the mechanotransduction by focal adhesions, which link integrins to cytoskeleton (Hynes 2020). A traction force is generated in the actin cytoskeleton, which activates the downstream signaling and translocates the signal into the nucleus. These traction forces are also exerted on the integrins and focal adhesions, thus maintaining them in the isometric tension (Bershadsky et al. 2003). External stresses generate a mechanics-based positive feedback loop by increasing tension on the cell surface receptor and activating G protein Rho and its target ROCK. Stiff substrate results in an increase in kinase activities of ROCK, FAK, and extracellular signal-related kinases (ERK1/2), causing osteogenic differentiation of MSCs. Inhibition of ROCK and FAK leads to downregulation of osteogenic markers during osteogenic induction (Shih et al. 2011). Taken together, this implies that stiff substrates affect the regulation of

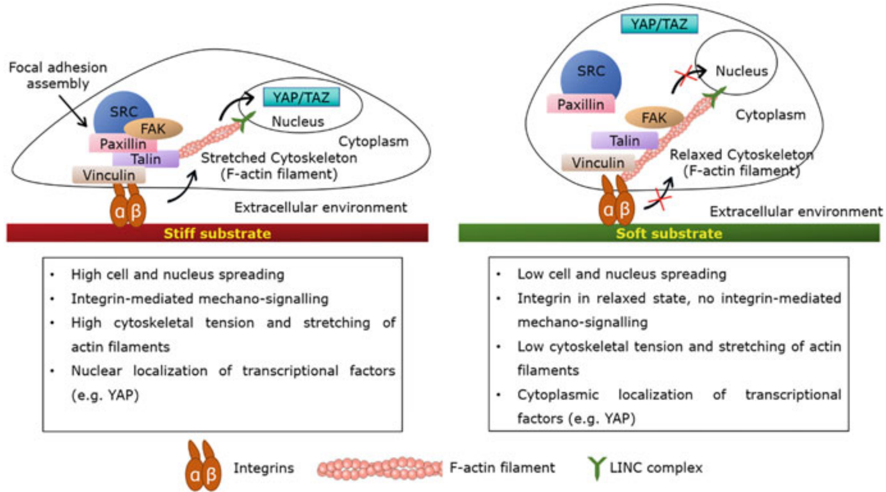

Fig. 15.2 Schematic representation of the effect of stiff and soft substrate on cell morphology and function via integrin-mediated mechano-signaling. On stiff substrate, a cell receives biophysical cues from integrin-based focal adhesion complex, which increases the cytoskeletal stress via stretching of F-actin filaments. The stretching of LINC complex due to stiff substrate and stretched F-actin causes nuclear localization of transcriptional factors such as YAP. Conversely, on the soft substrate in the absence of less integrin activity, the focal adhesion complex is not formed, leading to less cytoskeletal tension and less stretching of actin filaments, thereby leading to cytoplasmic localization and no substrate-dependent nuclear localization of the transcriptional factors

ROCK-mediated FAK and ERK1/2, and these signals regulate the transcriptional factors, thereby determining the fate of MSCs.

The mechano-sensitive transcriptional coactivators such as myocardin-related transcription factor (MRTF) (Speight et al. 2016), nuclear factor kappa B (NF-κB) (Kumar and Boriek 2003), nuclear factor erythroid 2-related factor 2 (NRF2) (Escoll et al. 2020), YAP, and beta-catenin (Gumbiner 1995; Huber et al. 1996) bind to their respective DNA-binding proteins and activate specific genes. The nuclear or cytoplasmic localization of these transcriptional factors is controlled by nuclear envelope receptor—linker of nucleoskeleton and cytoskeleton (LINC) complex (Guilluy et al. 2014; Driscoll et al. 2015) (Fig. 15.2). Apart from integrin-ligand binding, several studies have suggested that the cells produce nano-length projections that sense the surface for optimum spreading. Thus, different nano-topographical features guide cell migration and spreading on the scaffold with different topographies. The fact that cellular orientation and alignment can be controlled by topographical cues was demonstrated as early as 1911 by Robert Harrison (1911). To date, the biomaterial-based scaffold has undergone many surface modifications and alternations and has emerged as a powerful tool for mimicking in vivo microenvironment.

15.7 Biomaterials as Promising Tools for Tissue Engineering and Regenerative Medicine

From the aforementioned considerations, it can be evident that mechanobiological processes in stem cells will impact the development of innovative therapeutic methods for tissue engineering and, eventually, regenerative medicine applications. The successful outcome of any stem cell-based regenerative medicine critically depends on cell survival after transplantation and to maintain tissue homeostasis mainly by differentiating into the respective lineage. To attain this, it is crucial to maintain optimal physiologically similar culture conditions in vitro for stem cell maintenance, proliferation, and quick differentiation when required. For instance, culturing the resident liver stem cells (RLSC) on polyacrylamide gel substrate having a stiffness of 0.4 kPa has shown to help in differentiation of RLSC into hepatocytes within 24 h, whereas RLSC cultured on a stiff substrate of stiffness 80 kPa resulted in only initial hepatocyte-specific transcriptional activity (Cozzolino et al. 2016). This variation in differentiation potential is due to culturing cells on soft stiffness—which is similar to healthy liver tissue stiffness (0.3–6 kPa)—rather than using normal stiff TCP. Similarly, instead of 2D culture system, Schoonjans and colleagues developed a synthetic 3D culture system using polyethylene glycol (PEG) hydrogels with a matrix stiffness of 1.3 kPa. This 3D culture system mimicking physiological liver stiffness provided better efficiency of live organoid derivation from mouse and human hepatic progenitors (Sorrentino et al. 2020). These studies show that clinically relevant human progenitor/stem cells cultured in physiologically relevant mechanical environments open perspectives for liver organoid-based clinical applications.

An interesting study focused on regenerating complex neural tissue such as motor neurons through modulating substrate stiffness because during embryo development, biophysical cues from the surrounding microenvironment along with soluble morphogens like sonic hedgehog (SHH) and retinoic acid (RA) play an important role in morphogenesis. Sun et al. (2014) and colleagues synthesized a system with PDMS with a stiffness range of $E = 1.0$–1200 kPa for generating motor neurons (MN) derived from hPSCs. Their findings suggest that soft substrate ($E = 1$ kPa) support early MN differentiation of hPSCs compared to stiff substrate ($E = 1200$ kPa). In addition, the yield and purity of functional MNs improve four- to tenfold on soft substrate compared to stiff substrate (Sun et al. 2014). Thus, culturing hPSCs on a synthetic cell culture surface with controlled mechanical properties (such as substrate stiffness) improved the efficiency of hPSC differentiation into motor neurons. Such advances open new doors in the therapeutics of motor neuron-associated neurodegenerative (Sun and Fu 2014).

An electrospun nanofibrous vascular scaffold made up of poly(L-lactide) (PLLA) was embedded within PA hydrogel on the outer surface. This nanofibrous polymer system had stiffer matrix near the polymer and was less stiff away from the polymer and was used as a graft for cell regeneration in vivo. Multipotent neural crest stem cells (NCSCs) generated from hiPSCs were embedded within the graft and implanted in rat carotid arteries. The stiffer matrix of the polymer scaffold with

$E = 50$ kPa or higher supported the differentiation of NCSCs into smooth muscle cells (SMCs). The soft matrix area of the scaffold with $E = 15$ kPa supported the differentiation into glial cells. The results suggests that the mechanical properties of substrate play a significant role in designing biomaterials for tissue engineering (Zhu et al. 2019).

hiPSCs are traditionally generated by genetic reprogramming of adult somatic cells using biochemical signals (Takahashi and Yamanaka 2006). Fascinatingly, Grãos and colleagues demonstrate that MSCs can be reprogrammed into iPSCs by biophysical cues alone. They showed that human umbilical cord MSCs (huMSCs) exhibit PSC phenotype when cultured on soft PDMS substrate with $E = 1.5$ kPa and 15 kPa compared to stiff TCP ($E \sim 1$ GPa). huMSCs undergo chromatin modeling and show enhanced expression of pluripotency-related markers *OCT4*, *SOX2*, and *NANOG* in response to the soft substrate. Soft substrate allowed huMSCs to acquire relaxed nuclei, small FA, fewer stress fibers, and high euchromatic and lower heterochromatic content and expression of pluripotency specific genes. In short, their results suggest that substrate stiffness influences several phenotypic features of iPSCs and colonies and that soft substrate favors iPSC reprogramming (Gerardo et al. 2019). Such milestone studies indicate that substrate stiffness is a critical biophysical cue that influences stem cell differentiation into the specific lineage. Such studies also highlight the importance of biomaterials in tissue engineering and a promising platform for improving tissue engineering and regenerative applications.

15.8 Mechanobiology in Cancer Cells

Mechanobiology is one of the driving forces in guiding cell motility and tissue development during embryonic development. This cellular and tissue mechanobiology approach has been used by many researchers in understanding cancer development and tumor invasion. One of the key mechanisms by which cancer cells evade therapy is metastasis, and it has been hypothesized that the tumor cells might rely on mechanical forces for invasion and migration. The tumor microenvironment is an aggregation of cancer-associated fibroblasts (CAFs), vascular cells, immune cells, an abundance of extracellular matrix proteins, and hypoxic conditions (Choudhury et al. 2019; Sahai et al. 2020). Hypoxia and hypervascularization are directly and indirectly associated with ECM realignment and shear stress (Wang et al. 2017).

The ECM is a fundamentally essential component of the tumor microenvironment that interacts closely with cancer cells. Apart from providing necessary growth factors for tumor growth (Briquez et al. 2015), the ECM also helps in transmitting signals integrins (Canel et al. 2013). Additionally, upregulation of ECM remodeling molecules, such as transforming growth factor-beta (TGF-β), is linked to the development of desmoplasia in tumors (Papageorgis and Stylianopoulos 2015). Desmoplasia is the development of dense fibrous and connective tissue around tumor growth, usually characterized by increased synthesis of total collagen, fibronectin, glycoproteins, mainly tenascin C, proteoglycans, and a sizeable stromal cell

population that amasses within the tumor. The increased production of tumorigenic and inflammatory growth factors transforms a large number of fibroblasts into CAFs. It has been proposed that the multifunctional cytokine TGF-β activates the transformation of fibroblasts into CAFs, which produces more ECM fibers, eventually causing desmoplasia (Papageorgis and Stylianopoulos 2015). The ECM stiffness of the fibrotic/cancer tissue is around 1.08–68 kPa (Kawano et al. 2015) and has shown to upregulate alpha-smooth muscle actin (α-SMA) expression, a proven CAF marker. Another known transcriptional factor that facilitates CAF generation and maintenance is YAP/TAZ, which activates only during high actomyosin contractility and high stiffness (Goffin et al. 2006; Calvo et al. 2013). YAP has been shown to regulate the expression of specific cytoskeletal proteins, including anillin actin-binding protein, myosin regulatory light polypeptide 9, and diaphanous related formin 3, which induces CAF (Calvo et al. 2013).

During cancer progression, uncontrolled cell proliferation results in an increase in tumor mass. This leads to a difference between the ECM stiffness of tumorous tissue and normal tissue. For instance, Samani et al. (2007) reported that the mean Young's modulus of normal breast tissue is 1.9 kPa, whereas that of fibroadenoma was 11.42 kPa and that of invasive ductal carcinoma was 22.55 kPa. Multiple in vitro reports show that the stiffness of the tumor tissue and matrix directly correlates with tumorigenesis and metastasis (Zaman et al. 2006; Tilghman et al. 2010; Gkretsi and Stylianopoulos 2018; Jang et al. 2020). A breakthrough study published by Weaver and colleagues proves the hypothesis that mechanical signals mediate malignant transformation. They showed that culturing non-tumorigenic mammary epithelial cells on stiffness mimicking tumor-like stiffness induces cell proliferation, dysplasia and activates oncogenic epithelial signaling pathways. They also found that transformed cells maintain a functional link between integrins and Rho-dependent cytoskeletal tension, and in the presence of ROCK or integrin adhesion pharmaceutical inhibitors the malignant behavior of tumors was tempered (Paszek et al. 2005).

Cancer stem cells (CSCs) have been shown to reside within the tumor, and these cells have the ability to self-renew and differentiate into several cell types, which proliferate uncontrollably. Thus, CSCs sustain the growth of cancerous mass. The cancer stem cells are hard to eliminate due to their efficient DNA repair mechanisms, relative slow growth rate, and the high number of channel proteins to efflux drugs out (Turdo et al. 2019; Hirschmann et al. 2004; Fujiwara et al. 2021). Cancer stem cells lead to relapse of cancers after treatment (Eyler et al. 2008), and hence, it is necessary to investigate these cells including their mechanobiology machinery. In summary, understanding how cancer cells sense the mechanical signals and converted them into biochemical pathway may usher in new ways to control cancers. Given the similarities between the biology of stem cells and cancer cells (Shackleton 2010; Rahman et al. 2016), researchers are exploring the functional and mechanistic similarities between stem cell mechanobiology and cancer mechanobiology, with the aim of understanding the former using the latter as a guide (Fig. 15.3).

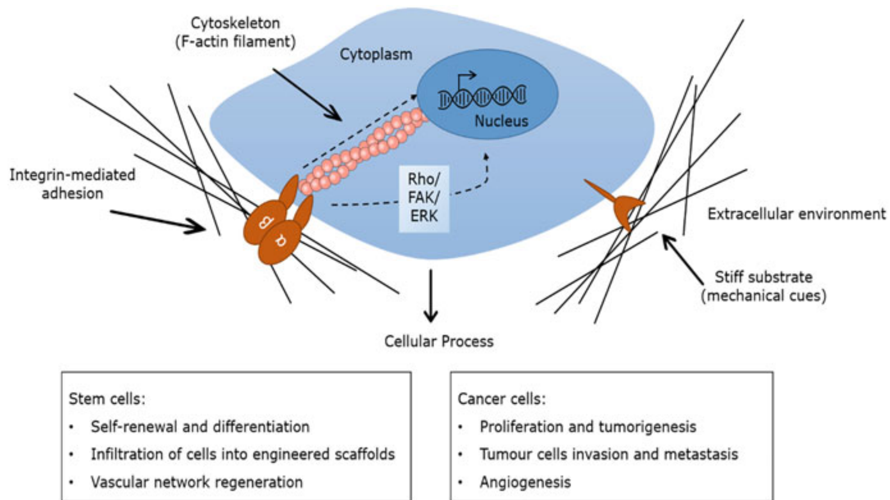

Fig. 15.3 The similarities between the ECM-cell mechanobiology of stem cells and cancer cells

15.9 Concluding Remarks and Perspectives

Many advances in fabricating biomaterials for regenerative medicine have been reported in recent decades. Fundamental properties of biomaterials and of cell responses to biochemical and biophysical cues have been described via structural and functional studies. In this chapter, we have briefly described various properties of biomaterials and their impact on cellular behavior. For detailed information on the physical, chemical, and functional properties of the biomaterials, the authors recommend some extensively detailed reviews by Amani et al. (2019) and Cun and Hosta-Rigau (2020). The existing knowledge on ECM-cell interactions has been mainly derived from 2D in vitro studies. Although the 2D culturing system is convenient and has uncovered several crucial aspects about mechanobiology and biomaterials in cell migration, adhesion, proliferation, and differentiation, it does not mimic the in vivo microenvironment, which is 3D. It is becoming increasingly evident that the cells have a distinct behavior in the 3D microenvironment than that seen in 2D microenvironment. These facts have led to the use of a 3D culture system to mimic the physiological environment required for stem cell differentiation and the generation of organoids (Pepelanova et al. 2018; Bailey et al. 2019). hPSCs cultured on 3D scaffold have already been used to develop neuronal (Levenberg et al. 2003), liver (Baharvand et al. 2004), and cartilage (Hwang et al. 2006; Bai et al. 2010) tissue equivalents, along with rudimentary vascular networks (Ferreira et al. 2007).

Other than 3D culture, 3D bioprinting can be used to fabricate well-organized cell-laden scaffolds, which can be used to repair or regenerate damaged tissue

(Antich et al. 2020; Jeong et al. 2020). Further advancement is organ-on-a-chip technology, which helps in generating self-organizing miniature organs from stem cells that replicate the functional and structural characteristics of cells present in in vivo microenvironment (Park et al. 2019). This organ-on-a-chip method has been employed in cancer cells to understand the disease progression and predict drug-induced responses (Sun et al. 2019). The studies discussed herein demonstrate the significance of the extracellular microenvironment in determining cellular behavior. They also highlight the importance of developing novel biomaterials to provide cells with biophysical cues which will help in cell-based therapies and regenerative medicine. Although much is yet to be unraveled about the influence of mechanobiology on stem cells, the newer discoveries give us insight into a promising future but also raised certain fundamental questions, such as the following: How much of the mechanical information is needed for the desired response from stem cells to form complex tissues? Can the biomaterials transplanted cause uncontrolled proliferation of the surrounding tissue? How cells generate their own mechanical forces during embryogenesis? With such diverse materials and methods for synthesizing biomaterials, it becomes crucial to understand how much of the material complexity is required for the desired stem cell response. We envision that the current research will help pave the way in understanding mechanobiological influence on stem cells and have major implications on tissue engineering and regeneration approaches.

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Hydrogel-Based Therapies for Cardiovascular Diseases

16

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Abstract

Cardiovascular diseases (CVDs) are a cluster of disorders related to abnormalities of the heart and blood vessels. Myocardial infarction (MI), rheumatic heart disease, atherosclerosis, thrombosis, pulmonary embolism, stenosis, and ischemic heart failure are some common examples of CVDs. CVDs are a leading cause of mortality, accounting for 31% of deaths worldwide. Existing therapies provide only short-term relief and fail to address the underlying causes behind the progressive deterioration in cardiac health during CVDs. Towards this, hydrogel-assisted technologies in combination with nanotechnology-based approaches have explored appealing platforms for treating CVDs. Hydrogel

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systems offered a unique ability to deliver therapeutic agents (pharmacological drugs, genes, growth factors) and cells (like stem cells) precisely at the site of injury, thereby improving therapeutic efficacy and reducing drug-related side effects. Hydrogel systems have been extensively investigated for drug delivery, tissue engineering, and recently emerging theranostic application in treating CVDs. In this chapter, we have discussed different types of hydrogel systems that are promising for treating CVDs and elaborated on various hydrogel-based technologies developed for treating a variety of CVDs. We have also discussed the clinical progress in this area and highlighted several technical challenges which should be addressed before considering the translation of such upcoming technologies.

Keywords

Smart hydrogels · Drug delivery · Cardiac tissue engineering · Cardiovascular diseases

16.1 Introduction

Cardiovascular diseases (CVDs) are disorders related to blood vessels, the heart, and heart valves. In these diseases, the cardiovascular system develops functional or structural deformations (Harsh 2010a, b). The current treatment for CVDs primarily involves therapeutics such as key pathway inhibitors, nitrates, various blockers, membrane stabilizing agents, antiplatelet agents, and blood thinners (Shah et al. 2004). In acute and complicated CVDs, interventional procedures such as heart bypass surgery, angioplasty, and balloon valvuloplasty have been performed routinely (Ewy 1992; Tripathi 2013; Vahanian 2001). However, various side effects are associated with existing medications, such as unwanted bleeding, sensitivities, dizziness, and liver damage (Tripathi 2013). Nonadherence to medications like antiplatelet drugs or blood thinners in high-risk patients can lead to failure of treatment. Cost affordability becomes one of the major challenges in the long-term treatment of CVDs. Lack of follow-up and nonadherence to medications can lead to the recurrence of the diseased condition (McClellan et al. 2019).

To overcome the limitations possessed by the current treatments, hydrogels have emerged as a potential technology for a wide variety of applications in drug delivery and tissue engineering. Typically, hydrogels are 3D polymeric networks having high water content. Cross-linking of the polymeric network is attributed either to various forces such as hydrogen-bonding and ionic bonding or to molecular entanglements, which leads to reversible or physical gel formation. Covalently cross-linked hydrogels are chemically stable in nature (Hoffman 2012). Hydrogels are widely explored for biomedical applications due to their biocompatibility, high water content, flexibility, and tunable rheological properties. Porosity enables the

encapsulation of large amounts of drugs and biomolecules, including growth factors, low-molecular-weight peptides, and even cells like stem cells. The release pattern of the loaded molecules can be deployed upon adjusting the cross-linking ratio of the materials, which helps in reducing systemic toxicity. Also, hydrogels provide an extracellular matrix (ECM)-mimicking environment, thus playing a vital role in cardiac tissue engineering applications (Liao et al. 2020; Patanarut et al. 2010).

This chapter comprehensively focuses on the design and role of hydrogels in the treatment of CVDs. Considering hydrogels as a promising approach for CVDs, this chapter affords insights into recent advances, clinical progress, and challenges that lie ahead.

16.2 Hydrogel Technologies

16.2.1 Design and Fabrication Method

Hydrogel fabrication techniques can be broadly grouped into three categories: (1) physical technique, (2) chemical techniques, and (3) microfabrication techniques. A few important design parameters to be considered in designing hydrogels for CVD applications include biocompatibility, biodegradability, porosity, mechanical strength, nonimmunogenic, and patient compliance. These parameters can be controlled by changing the degree of cross-linking, which, in turn, depends on the composition and the techniques used in the fabrication of the hydrogel.

Chemical cross-linking techniques are based on the chemical reaction between the functional groups that are present in the polymer chains. The reaction of a carbonyl with an amine group leads to Schiff's base reaction, whereas that between azides and alkynes is known as click chemistry, and the nucleophilic addition of a carbanion to an unsaturated carbonyl group is known as Michael addition. The properties of hydrogels that are synthesized by these chemical reactions can be controlled by varying the concentration of different polymers. Physical cross-linking techniques involve reversible intermolecular interaction. The most common interactions are ionic interactions (polymers with cations/anions such as Ca-alginate, chitosan-tripolyphosphate, and the like) and electrostatic interactions (anionic polymers such as alginate, chondroitin sulfate, and cationic polymers such as chitosan). The advantages of physical cross-linking techniques are their stimulus-responsiveness and self-healing properties due to the weak interaction forces between them, but their mechanical properties limit their applications. Hence, they are often combined with other crosslinking techniques (UV/chemical).

Microfabrication techniques are computer-aided advanced fabrication techniques that allow precise control of the 3D architecture up to a micron scale. The most common techniques are bioprinting and micropatterning. Bioprinting can be used to fabricate cell-laden 3D constructs for cardiac tissue engineering. The materials used as bioink should have high viscosity, rapid gelation kinetics, and cell-supporting properties. For instance, alginate is a commonly used bioink material. Micropatterning has also been explored as a fabrication technique to generate

high-resolution microchannels on the surfaces of hydrogels which can be used to guide proliferation, cell adhesion, and orientation; this would eventually be resulting in improved functionality. A study demonstrated that microfabricated hydrogels promoted the alignment of cardiomyocytes in comparison to the non-patterned ones (control); they also supported synchronous beating in response to an electric field (Annabi et al. 2013).

16.3 Hydrogels Types

16.3.1 Hydrogel Types Based on Composition

Based on material composition, the hydrogels can either be biodegradable (e.g., alginate, chitosan, gelatin) (Li et al. 2012) or nonbiodegradable (PEG, pNIPAM) (Dobner et al. 2009) in nature. The nondegradable hydrogels have to be surgically removed once they perform their function and can act as a depot delivery system but often have a possibility of eliciting an immune response (Singh and Peppas 2014; Takeda et al. 2015; Van Hove and Benoit 2015). Biodegradable hydrogels, on the other hand, are degraded by physiological reactions. They can, therefore, be used for drug delivery and/or tissue engineering purposes such that their rate of degradation matches that of the native tissue regeneration (Lueckgen et al. 2019; Sedlačík et al. 2011; Tan and Marra 2010).

Cell-free hydrogels are scaffolds that mimic the 3D microenvironment of the native tissue. It can incorporate biochemical cues such as growth factors or bioactive molecules and provide biophysical support to aid the regeneration of the damaged tissue (Mills et al. 2020; Tibbitt and Anseth 2009; dos Santos et al. 2019; Kim et al. 2017). Cell-laden hydrogels are tissue-engineered constructs that are incorporated within cells that are specific to the tissue to be regenerated and/or within stem cells that differentiate into the specific cells based on the microenvironment (Loessner et al. 2016; Yang et al. 2017). Inspired by the self-healing property of native tissues such as bone, skin, and muscles, self-healing hydrogels have been developed for biomedical applications. These hydrogels can autonomously repair, maintain their integrity, and retain their mechanical properties in response to any damage inflicted on them (Hager 2017). Such hydrogels are synthesized using dynamic covalent or non-covalent bonds because they can reform their bonds after rupture. Due to the high mechanical loading conditions (pulsatile movements) present in the cardiac tissue, hydrogels used in cardiovascular therapy are more susceptible to damage; hence, self-healing hydrogels have been widely explored for such applications (Talebian et al. 2019). For instance, a recent study developed chitosan-graft-aniline tetramer-based self-healing conductive injectable hydrogel for cardiac therapy. The self-healing ability was demonstrated using microscopy and rheology analysis. On increasing the strain from 1% to 300%, the storage modulus decreased from 2000 to 15 MPa, whereas it reverted to the original value on unloading the forces. The hydrogels, when co-cultured with H9c2 myocardial cells and C2C12 myoblasts, indicated a tunable release rate of the cells, which was confirmed by inverse

fluorescence microscopy. The injectability and biodegradability (complete degradation in 45 days) were confirmed by the subcutaneous injection into Sprague-Dawley rats. The results showed the potential of self-healing hydrogels as cell delivery carriers for cardiovascular therapy (Dong et al. 2016).

The low mechanical properties and limited functionality of the conventional hydrogels have led to the development of nanocomposite hydrogels (Zhao et al. 2020). Nanocomposite hydrogels are hydrogels that incorporate NPs to achieve superior properties and multifunctionality. For instance, carbon-nanotube reinforced gelatin methacrylate (GelMa)-based multifunctional cardiac scaffolds demonstrated outstanding mechanical (~3-fold increase in compressive modulus) and electrical properties (~10-fold decrease in electrical impedance). Given hydrogel system also indicated significant improvement in cardiomyocyte adhesion, proliferation, and differentiation ($p < 0.05$) in comparison to GelMa hydrogels (Shin et al. 2013).

16.3.2 Hydrogel Types Based on Function

The major applications of hydrogels in CVD therapy include their use as drug delivery carriers, scaffolds, and tissue-engineered constructs for cardiac regeneration. The unmet need for donors in cardiac transplants and the limited regenerative capability of cardiomyocytes have led to the development of hydrogel-based scaffolds for cardiac regeneration. Hydrogels mimic the ECM microenvironment of the native tissue, and they can provide transient biophysical supports and/or biochemical cues that can signal the differentiation of stem cells into the required cell lineage. Various natural and synthetic biopolymers such as alginate, chitosan, gelatin, and poly(lactic-*co*-glycolic acid) (PLGA) have been explored for cardiac tissue engineering (Li and Guan 2011; Hasan et al. 2015). These polymers can be used alone or in combination to achieve superior properties. For instance, Shin et al. developed reduced graphene oxide (rGO)-GelMA-based hybrid hydrogels for application in cardiac tissue engineering. The incorporation of rGO into GelMA hydrogels resulted in enhanced mechanical properties and reduced electrical impedance. Cardiomyocytes seeded on these composite hydrogels showed more than 90% cell viability up to a concentration of 5 mg/mL (Shin et al. 2016). Similarly, gelatin-gellan gum hydrogels seeded with human-induced pluripotent stem cells (iPSCs)-derived cardiomyocytes were developed. Young's modulus of the hydrogels was comparable to that of the native rabbit heart tissue. Beating studies carried out using an in-house-built phase-contrast video analysis software showed that the human iPSC-derived cardiomyocytes, when cultured for 24 h, recovered their spontaneous beating (Koivisto et al. 2019).

Hydrogels have also been used for drug delivery carriers to maximize and retain the active ingredient at the target site. For instance, a mechanically responsive and inflammatory macrophage-targeted dual-responsive drug delivery system for atherosclerosis was developed. Simvastatin was used as a model drug, and the drug release rate was seen to have increased by 49.3% on increasing the occlusion area from 0% to 75%. Targeting studies that were carried out on an apoE−/− mice using Cy7 as a

fluorescent tracer demonstrated a distinct fluorescent signal in the cardiac region (Yao et al. 2020).

16.3.3 Advanced Forms of Hydrogel Technologies

There is a consistent increase in developments in hydrogel technologies such as stimuli-responsive hydrogels, theranostic hydrogels, and injectable hydrogels. With these advances, precision in spatiotemporal release profiles can be achieved, unlike conventional forms of hydrogels. Besides, diseased conditions can be monitored in real-time using progressive hydrogels such as theranostic hydrogels. In subsequent sections, we briefly discussed these advanced forms of hydrogels.

16.3.3.1 Stimuli-Responsive Hydrogels

Stimulus-responsive hydrogels (SRH) are also referred to as "smart hydrogels." They show phase transitions such as "random coil to helix," "collapse transitions," as well as "coil-to-globule transition" upon application of various stimuli such as pH, temperature, and ROS, thus allowing the release of drugs from hydrogel matrix (Alvarez-Lorenzo et al. 2020). These triggers can be either external such as temperature and ultrasound or internal such as pH. Due to their ability to control the spatiotemporal release of loaded biomolecules or drugs in response to triggers, these hydrogels increase the specificity of the therapy and also contribute to minimizing side effects (Bastings et al. 2014; Koetting et al. 2015; Willner 2017).

Triggering a fibrinolytic cascade in response to internal cues such as thrombin, which plays a key role in the coagulation pathway, is a potential approach in case of thrombotic events. For example, Du, Li et al. developed tissue plasminogen activator (t-PA)-loaded "thrombin"-responsive antithrombotic hydrogels using thrombin-cleavable peptide. The hydrogel was prepared using acrylamide by radical polymerization. The peptide, Gly-dPhe-Pro-Arg-Gly-Phe-ProAla-Gly-Gly was used as a crosslinker which is degraded at the Arg–Gly linkage due to thrombin. A degradation study using quartz crystal microbalance (QCM) indicated that the rate of degradation that was affected by the thrombin concentration and zero-order degradation along with a surface corrosion pattern were observed. At the same time, t-PA release was found dependent on the concentration of thrombin as well as the degree of cross-linking. The fibrinogen-thrombin clot assay indicated the t-PA that is released from the hydrogel due to thrombin trigger leads to the conversion of plasminogen to plasmin which leads to lysis of the clot. These results were supported with in situ agarose plate assay, in which linear clot lysis was observed with time due to linear release of t-PA in the presence of thrombosis (Du et al. 2016).

Similarly, Jin et al. developed ultrasound-responsive nanogels that were loaded with urokinase for thrombolysis. Hollow nanogels were synthesized using glycol chitosan with aldehyde capped poly(ethylene glycol) (OHC-PEG-CHO) by ultrasonic spray. It was observed that uPA loaded nanogels were of a size 200–300 nm. The study performed on Sprague-Dawley rats indicated an increase in the half-life (t1/2) of the uPA encapsulated in hydrogels up to 40 min in comparison to naked

uPA at 18 min. Also, these nanogels were found responsive to the diagnostic ultrasound at 2 MHz, which can be rendered for imaging-guided delivery. Under sonication, thrombolysis that was similar to naked t-PA was achieved by uPA-loaded nanogels. This also demonstrated the retained bioactivity of the uPA in the nanogels (Jin et al. 2012).

16.3.3.2 Theranostic Hydrogels

"Theranostics" is a unique strategy by which diagnostics and therapy are achieved in a single platform. With this newer approach, simultaneous estimation, progression of therapy, and real-time monitoring are possible. To date, several nanocarriers such as mesoporous silica NPs, metallic NPs and lipid NPs have been studied for theranostic applications. Currently, hydrogels/nanogels are under the limelight for theranostic applications (Chambre et al. 2018; Vijayan et al. 2020; Yang et al. 2019; Ahamad et al. 2020a). Hydrogel-based theranostics showed potential as a bridge between diagnosis and therapy; however, in vivo application and safety is still a challenge (Sierra-Martin and Fernandez-Barbero 2015). Theranostic hydrogels are not much explored in the case of CVDs as compare to other diseases such as cancer, and there is a lot of scope for the application of theranostics in CVDs (Jaiswal et al. 2014; Wang et al. 2020; Han et al. 2020). Melvinsdottie et al. synthesized theranostic hydrogel (hyaluronic acid/gelatin modified with aldehydes and hydrazides, using 100 mg/mL iohexol) for post-MI to prevent Left ventricular (LV) remodeling. In vivo studies on Yorkshire pigs indicated successful delivery of the hydrogel through the intramyocardial route with ^{201}Tl SPECT/CT imaging. The end-diastolic volume (EDV) and the end-systolic volume were reduced on the 7th day after hydrogel therapy to 9.1% and 10.0%, respectively, in comparison to the respective increase of 8.9% and 27.3% in the control groups, which indicates reduced LV remodeling (Melvinsdottir et al. 2020). These results indicate the potential application of theranostic hydrogels in CVDs therapy.

Target-specific theranostic hydrogels can be an attractive approach to increase the specificity of therapy (Shi et al. 2018). For example, Hong et al. synthesized pH-responsive macrophage targeting alginate-based theranostic cisplatin-loaded nanogels (TANgel). Macrophages are known to play a pivotal role in the pathogenesis of atherosclerosis (Barrett 2020). Here, cisplatin was used as an antiproliferative drug for macrophages and cross-linking agents due to the presence of Pt^{2+} Near-infrared (NIR) fluorophore ATTO655 was incorporated into the system as an imaging agent. Cell lines like HDMVECn and the macrophage cell line J774A.1 were used for the cellular study. IC50 of TANgel was obtained at 0.12 μM than free cisplatin which was 0.21 μM. This result is attributed to the higher uptake of nanogel and in addition, the protection of cisplatin from detoxification due to chemical reactions involving thiols. In contrast, no cytotoxicity was detected up to 2.5 μM cisplatin, which is equal to the treatment of several normal cells. When compared to free dye molecules, the emission of TANgel was strong. This is attributed to J-aggregation, which lends additional excitation as well as emission wavelengths at a more extended NIR wavelength range in the case of conjugated NIR dye ATTO655 in TANgel. This enables selective imaging of macrophage cells by NIR

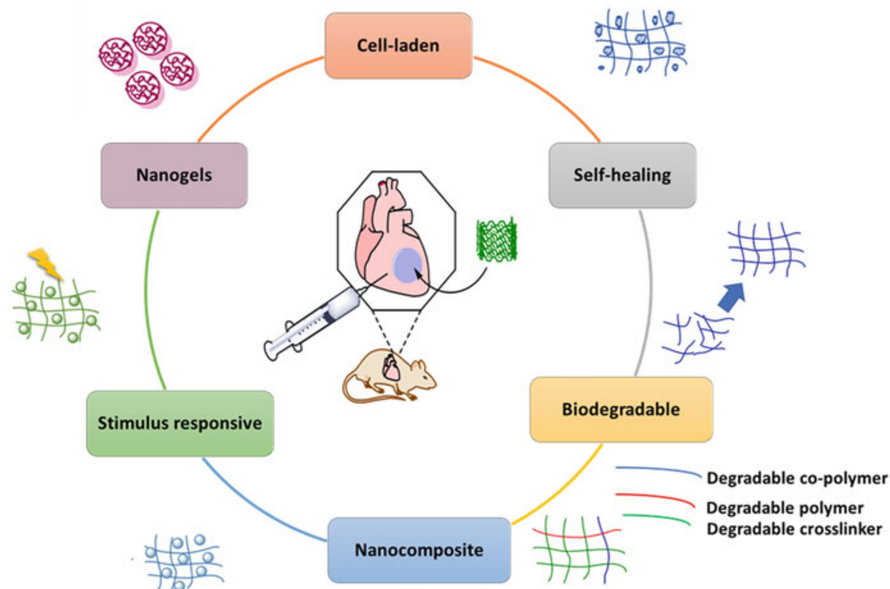

Fig. 16.1 Different types of hydrogels for cardiovascular diseases treatment. (Reproduced under Creative Commons Lincences)

fluorescence imaging in atherosclerotic lesions (Hong et al. 2018). In Fig. 16.1, we have summarized different types of hydrogels for the treatment of CVDs.

16.4 Hydrogel Interventions for Treating CVDs: The Focus of Drug Delivery

16.4.1 Hydrogels for Atherosclerosis

The low-density lipoproteins (LDLs) play a major role in the pathology of atherosclerosis. Accumulated oxidized LDL (ox-LDL) in the intima of the artery is responsible for initiating inflammatory responses and recruiting macrophages in atherosclerosis. Therefore, therapies that address oxidation are essential in the case of atherosclerosis. Basak et al. developed metalloproteinase responsive paraoxonase-1 (PON-1) enzyme-loaded polyethylene glycol (PEG) cross-linked nanogels. PON-1 is an anti-inflammatory and anti-oxidizing enzyme. RAW 264.7 macrophage cells were used for cellular studies. Similar inhibition of LDL oxidation was observed in nanogel-PON-1 and native PON-1, which indicated the viability of the PON-1 that was released from the nanogel. The ROS scavenging assay indicated a similar dose-dependent ROS reduction activity in native PON-1 and PON-1 laden nanogels. Additionally, empty nanogels were showed ROS scavenging activity, which suggests the ROS scavenging ability of the nanogel composition itself.

However, there is a need for further in vivo studies to better understand such an interesting strategy for the treatment of atherosclerosis (Basak et al. 2020; Schwenke and Carew 1989).

Apart from ox-LDL, effector and regulatory T cells (Tregs) also contribute to the progression of inflammation (Pastrana et al. 2012). Effector T cells are responsible for recruiting monocytes into plaque and secretion of interferon-γ and IL-6, leading to activation of antigen-presenting cells (APCs). However, regulatory T cells are responsible for the secretion of anti-inflammatory cytokines like IL-35 and TGF-β. They also suppress effector T cells, maintaining balance in systemic immune tolerance as well as homeostasis. Dysfunction of Tregs also marks the progression of the atherosclerotic condition (Ou et al. 2018). Hence Treg modulation can be a promising strategy in the treatment of atherosclerosis (Ou et al. 2018; Dietel et al. 2013; Tabas and Lichtman 2017). In one of the study, Yi et al. synthesized an anti-inflammatory injectable filamentous hydrogel depot (FM) to achieve sustained release of the bioactive form of vitamin D (aVD)-loaded poly(ethylene glycol)-block-poly(propylene sulfide) (PEG-b-PPS) filomicelles (MC) to study regulated anti-inflammatory and regulatory T-cell induction response. Filomicelles were prepared by the thin-film hydration method using pre-synthesized VS-PEG_{45}-b-PPS_{44} and PEG_{45}-b-PPS_{44}. Further FM depots were prepared by adding eight-arm PEG-thiol to FM solution 1.1:1 molar ratio of thiol:vinyl sulfone. Upon oxidation, drug-loaded micelles were released due to morphological transitions. Using small-angle X-ray scattering (SAXS) and cryo-transmission electron microscopy (Cryo-TEM), the change in cylinder-to-micelle conversion was investigated. ApoE-/-mice were injected through the subcutaneous route with a free aVD, PBS control, and aVD-loaded FM-depot (8 μg aVD/kg/month). A significant rise in the level of Foxp3+ Tregs was observed for at least 2 months, which indicates the sustained release and elevated Treg response in aVD-loaded-FM-depot treated group. This study established the fact that an injectable filamentous hydrogel depot (FM-depot) can be a promising platform for sustained delivery of therapeutics in CVD immunotherapy (Yi et al. 2020).

Upon an increase in inflammation, endothelial dysfunction occurs in atherosclerosis. This initiates the adhesion and migration of inflammatory cells into subendothelial areas, which leads to the transformation of macrophages within lipid-loaded foam cells. The formation of foam cells is an important step in the atherosclerosis development cascade that is driven by inflammation. Therefore, inhibition of inflammation can be a practical approach in atherosclerosis (Jung et al. 2018; Moore et al. 2018; Shang et al. 2020). In one of the approaches, self-assembling peptides were used for the atherosclerosis treatment. A peptide derived from IGF-1 and modified using an anti-inflammatory drug has been studied by Shang et al. The insulin-like growth factor (IGF-1) exhibits antioxidant and anti-inflammatory properties. Due to similar IGF-1 bioactivity, the ability of anti-inflammatory action, and self-assembling, of IGF-1 derived peptide was used to fabricate the nanofibers. Shang et al. developed bifunctional supramolecular nanofibers, which can prevent atherosclerosis by increasing plaque stability as well as reducing inflammation in apoE−/− mice. In this study, a small peptide that was

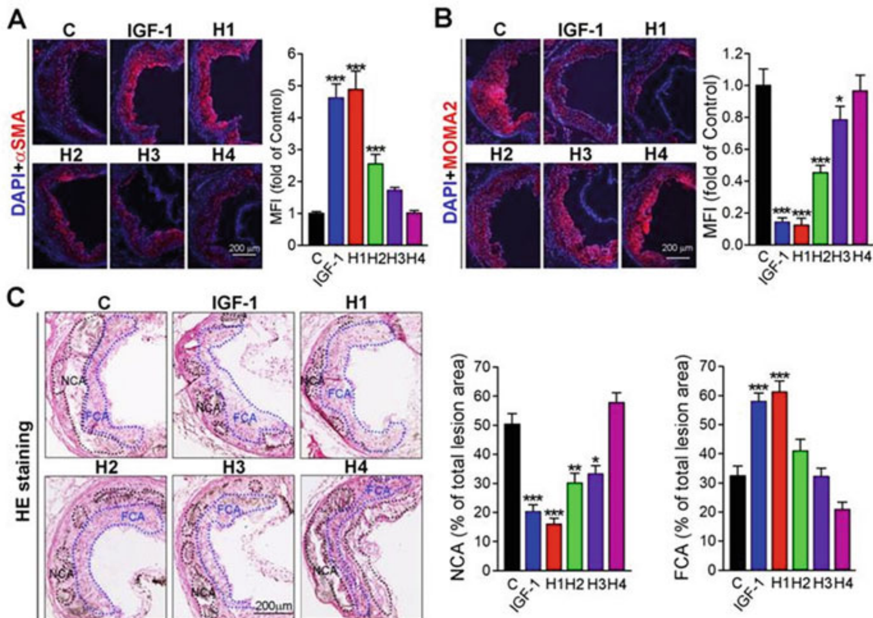

Fig. 16.2 Plaque stability analysis of aortic root cross sections from apoE−/− mice (**a**, **b**). Immunofluorescence staining of aortic root cross sections for expression of αSMA and MOMA2 with quantitative analysis of positive areas (**c**). Hematoxylin/eosin (H&E) staining of aortic root in mice with quantitative detection of necrotic core area as well as fibrous cap area. (Images **a**–**c** adapted with permission from Shang et al. Theranostics, 2020 (Shang et al. 2020). Copyright© 2019 with permission from Ivyspring International Publisher)

composed of tetrapeptide SSSR from the C-part growth factor (IGF-I) and naproxen (anti-inflammatory compound) along with an influential self-assembling D-peptide $^{D}F^{D}F$ were used to prepare bifunctional supramolecular nanofibers/hydrogel. The apoE$^{-/-}$ mice were injected via the subcutaneous route (S.C.) with the various hydrogels or natural IGF-1 once a week, and they were concurrently given a more-lipid food for 16 weeks. Posttreatment, the progression of atherosclerotic lesions and stabilization were investigated. Increased in αSMA and decreased MAMO2 expression indicated an increase in vascular smooth muscle cells (VSMCs) and a decrease in macrophage content in the immunofluorescence staining of the aortic root cross-sections from hydrogel treated apoE−/− mice. Hematoxylin and eosin (H&E) staining study indicated that Npx-$^{D}F^{D}$FGSSSR (hydrogel 1, H1) improved stability of plaque by decreasing the necrotic core of the plaque that contained lipid primarily and by enhancing the fibrous cap containing VSMCs as compared to other hydrogel groups (H2, H3, H4) (Fig. 16.2). Also, a study of the en-face aorta section showed a remarkable reduction in atherosclerotic lesions by ~53% and reduction in plaque by ~61 as compared to control groups. Finally, the IGF-1 resembling peptide indicated comparative activity to IGF-I (in vivo) and

prevented atherosclerosis by decreasing the areal extent of portion and increasing the stability of the plaque (Shang et al. 2020).

16.4.2 Hydrogels for Thrombosis

“Thrombosis” is the formation of a blood clot within the blood vessel. Vascular injury due to surgery or reputing of atherosclerotic plaque activates coagulation cascade and platelets form the clot, obstructing blood flow. Embolism is another complication associated with thrombus rapture. Anticoagulant therapy, including medications like aspirin, urokinase, is clinically used. However, rapid renal clearance, bleeding-related disadvantages are very commonly associated with anticoagulants (Mackman et al. 2020). Therefore, a drug delivery platform for safe administration of medications and having the ability to initiate therapeutic activity based on the mechanism of negative feedback upon abnormal coagulation signal can be a promising approach for thrombosis. Based on this approach, Xu et al. developed the self-regulated polymeric nanogel for the release of hirudin in response to a pathological cascade that leads to the formation of a thrombus. The single emulsion polymerization method was used for synthesis. Hirudin is an anticoagulant drug that is used in the treatment of thrombosis. The cross-linked nanogel enhances the stability, rate, and extent of the drug reaching systemic circulation and decreases its in vivo elimination. Encapsulation of hirudin also prevents its degradation due to the enzyme that is present in the plasma. The thromboplastin-induced pulmonary embolism model was used for the study of in vivo anticoagulant activity of recombinant hirudin variant 3 loaded clot-targeted thrombin-responsive nanogels (HV/ctNGs). Mice were injected by intravenous route with the cy5.5-labeled HV/ctNGs and control groups. A lower amount of fibrin deposition was observed in the case of HV/ctNGs treated mice, and a decrease in the clot area was overserved by 7%, in comparison to the saline-treated group. A ferric chloride-induced carotid arterial thrombosis mouse model was used to study the clot-inhibiting activity of HV/ctNGs. A low level of clots and embolism was observed upon treatment of HV/ctNGs in comparison to the control groups. The interaction between surface ligand and fibrin enhanced the accumulation of hirudin in the thrombi. Thus, the research concluded that the developed formulation effectively inhibits clot formation in the mouse models with thrombosis and pulmonary embolism (Xu et al. 2020). Similarly, for anticoagulant therapy, bioresponsive polymer-based hydrogels are used because of their advantages, such as precise control on release of therapeutic moieties, biodegradability, and site-specific release. Thrombin is one of the key enzymes in the coagulation pathway. A thrombin responsive platform can serve as a potential approach for thrombosis (Du et al. 2016; Maitz et al. 2013). Maitz et al. developed bio-responsive hydrogel to deliver heparin in response to thrombin. The hydrogel was fabricated using a multiarmed poly(ethylene glycol) (starPEG) and heparin, which were covalently bonded by the thrombin-sensitive peptide sequence Phe-Pip-Arg-Ser. The prepared hydrogel is responsible for the quantitative reduction of blood coagulation for longer hours in the response of procoagulant stimuli as well

as during the daily incubation of fresh, non-anticoagulated blood. This concept may help the development of materials that permit the efficient, sustained use of biomolecules and drug compounds (Maitz et al. 2013).

Apart from polymeric hydrogel, peptide-based hydrogels are widely explored due owing to their biocompatibility, design flexibility to design, ability to self-assemble, and most importantly, ability to encapsulate hydrophobic drugs (Du et al. 2015; Hamley 2017). Li et al. developed a naphthalene acetic acid-phenylalanine-phenylalanine-glycine hydrogel (Nap-FFG) that was loaded with tanshinone IIA (Tan@Nap-FFG). Tanshinone IIA (Tan IIA) is a hydrophobic drug having anticoagulant and antioxidant properties. Tan@Nap-FFG exhibited decreased ADP-driven platelet aggregation and oxidative damage associated with hydrogen peroxide. An in vivo study performed on male Sprague-Dawley rats showed low toxicity and improved pharmacokinetic parameters. The plasma retention time of Tan IIA was prolonged, leading to the sustained release of Tan IIA, which significantly improved the duration of antioxidant and anticoagulant activity (Li et al. 2020).

Implanted medical devices, stents are another reason for thrombotic clot development (Jaffer et al. 2015; Gorbet and Sefton 2004; Courtney et al. 1994). The addition of long-lasting antithrombotic biomaterials compounds can prevent thrombosis. The hydrogel had been used as a coating material for such implanted devices and also studied for nowadays thrombosis treatment (Liu et al. 2014; Chen et al. 2008). For example, Mannarino et al. formulated composite hydrogel comprising a poly(vinyl) alcohol (PVA) that was coated using poly (acrylic) acid (PAA) and subjected to heat to form a physically cross-linked hydrogel compound. Mechanical and swelling characteristics can be monitored by the heat and temperature exposure period to enhance the matrix's cross-link density. An in vitro study, using the simulated model for 162.6 days, showed that composite hydrogels could preserve mechanical properties and surface functionality post-accelerated aging. The composite PVA/PAA hydrogel demonstrated a 97% decrease in platelet adhesion when studied using in vitro blood loop model. It also showed a decreased speed in the occlusion of the tip because of thrombosis, which is in contrast to the marketed catheter products. Finally, this more effective thromboresistant hydrogel may have a significant impact as an innovative material of biological origin for use in vascular access to restore patient well-being (Mannarino et al. 2020).

16.4.3 Hydrogels for Myocardial Infarction

Thrombosis and emboli develop hypoxia in the cardia tissue, which leads to MI. The direct injection of biomolecules or cells at the infarcted heart site is one of the approaches to treat MI. The biomolecules injected in the free form are subjected to degradation, which leads to an increase in dosing and injection frequency. The encapsulation of these molecules into suitable carriers is one strategy to deliver these molecules at the site of action without degradation and subsequent loss of actives. The injectable hydrogels can transport drug compounds, cells, or engineered tissue locally to the affected part to regenerate functional cardiac tissue and, thereby,

offer a potential alternative for treating MI. They serve as a perfect and innovative delivery system that can overcome the limitations of current therapies (Lin and Metters 2006; Liu et al. 2017). Based on this approach, Shaghiera et al. developed injectable thymosin β4 (Tβ4) loaded collagen-chitosan-based hydrogels to treat MI. Tβ4 is a 43-amino acid peptide and has angiogenic and cardioprotective properties. The stimulation of angiogenesis was observed in the histopathological study. The evaluation of MI indicated epicardial heart cell migration. Further, there is a need for in vivo evaluation of therapeutic efficacy. This study also demonstrated that, for negatively charged moieties or biomolecules, the chitosan-collagen hydrogel could be a potential carrier. In comparison, further research is needed to optimize the collagen-chitosan ratio for more effective hydrogel synthesis due to poor interconnecting pores (Shaghiera et al. 2018).

Post MI neovascularization triggers in the body to reduce the hypoxia and reduce the damage. Therefore, to improve the vascularization, intervention should release the angiogenic growth factors for a longer time as the process of vascularization takes more than a week (Carmeliet and Jain 2011; Pal et al. 2020; Tous et al. 2011). Thus, Pal et al. developed VEGF-mimetic QK peptide-loaded biohybrid, dual-crosslinked poly(*N*-isopropyl acrylamide)-based injectable hydrogel (PNGQK200) with shear-thinning property for vascularization. In vitro studies indicated that endothelial cells had been successfully trapped and spread homogeneously into the 3D hydrogel matrix environment with substantially increased vascularization in the QK peptide presence after 3 days of culture. PNGQK200 and control samples were injected into IgG immunocompromised mice intraperitoneally with 250 μL bolus. An initial experiment on mice indicated an enhanced generation of blood vessels in the case of PNGQK200 hydrogel as compared to control samples. In the case of hydrogel with QK and without QK, mean numbers of blood vessels per 40× magnification field were found to be 2.2 ± 0.7 ($n = 3$) and 1.3 ± 1.0 ($n = 2$), respectively. This study indicated the potential of a minimally invasive approach for vascularization (Pal et al. 2020).

Considering complexities associated, multimodal and co-delivery approaches are attractive strategies for MI. The hydrogels act as a platform for co-delivery of biomolecules, synthetic drugs, and drug- or gene-loaded NPs (Jang et al. 2011; Paul et al. 2014). In one of the approaches, adipose-derived stem cells (ADSCs) and plasmid DNA-encoding eNOs (endothelial nitric oxide synthase) NPs laden injectable conductive hydrogels were synthesized by Wang et al. using tetraaniline-polyethylene glycol diacrylate (TA-PEG) and thiolated hyaluronic acid (HA-SH). The study performed on male Sprague-Dawley rats indicated cell loss compensation and mitigation in inflammation due to ADSCs. Neovascularization was promoted due to the upregulation of NO expression. This is attributed to the positive action of eNOs nanoparticles given through hydrogels. Also, enhancement in electrical communication was observed (Wang et al. 2018). Similarly, astragaloside IV nanoparticles (AST NPs) and gold nanorods encapsulated within phenylboronic acid hyperbranched macromer-based conductive hydrogel were given in MI. Gold nanorods aided electrical stimulation during myocardial

dysfunction, and left ventricular remodeling was inhibited by astragaloside IV NPs after MI in rats (Chen et al. 2020).

Similarly, Wu et al. synthesized chitosan-based injectable hydrogel loaded with vascular endothelial active factor (VEGF) and bone morphogenetic protein 9 (BMP9)-loaded silk fibroin (SF) microspheres. For in vivo study, the composite hydrogel was administered by the intramyocardial route. The study indicated a quicker release of VEGF initially over the first few days and a comparatively slow and regulated release of BMP9. It results in the development of blood vessels in the initial stages and prevents myocardial fibrosis in the prolonged stage. Hydrogel loaded with both VEGF and B/SF microspheres (Gel + B/SF + V) showed significant reduction in infraction site size, which was $11.6 \pm 0.4\%$ as compared to blank gel, PBS, gel + B/SF, and gel + V group which was $21.5 \pm 0.7\%$, $22.1 \pm 0.9\%$, $17.8 \pm 0.4\%$, and $16.6 \pm 1.3\%$ respectively. In vivo study indicated the cumulative impact of the VEGF and BMP9 on the management of MI and improvisation operation of heart, suggesting its promising role of dual drug-loaded hydrogel in heart therapy (Wu et al. 2021).

Injection of hydrogels in the ventricular wall has been shown promising results in cardiovascular therapy, while regurgitation of hydrogels into the circulation upon endocardial injection remains one of the challenges in hydrogel-based therapies. Precise injection methods can be one of the methods to improve hydrogel injection therapies. To address the concern regarding leakage of hydrogel into the circulatory system, an alternative approach was explored by Zhu et al. in which a thermoresponsive injectable hydrogel based on *N*-vinylpyrrolidone with *N*-isopropylacrylamide (VP gel) with robotic injection devices were used to facilitate the injection strategy. The hydrogel was synthesized using *n* *N*-isopropyl acrylamide with *N*-vinylpyrrolidone, whereas the newer injection system was equipped with cooling liquid running parallel to the thermoresponsive hydrogel. Till the site of injection, the polymer solution was kept cool using a cooling fluid. Further, phase transition of the injected polymer solution was observed in the targeted LV wall region. The compatibility of injections at the ventricular wall at predecided locations with depth was confirmed using beating porcine hearts and ex vivo. With an injection device, one deeper and one shallow injection were given at 3.5 mm and 6.8 mm deep, respectively, on the porcine heart at 37 °C. After 5 min of the injection site dissection, hydrogel deposits were observed orienting along the circumferential direction and ellipsoid with long axes, which was 8.2 mm long and 2.7 mm wide for deeper and 10.8 mm long with 3.1 mm wide for shallow deposit. The smooth injection was given without hydrogel clotting due to the functioning of the cooling system. Due to faster gelation of hydrogel, no leakage of hydrogel was noted. This mechanism was attributed to the warm surround of the myocardium. Further, in vivo assessment is necessary in such cases to establish a therapeutic correlation. Combining hydrogels or engineered biomaterials with a robotic delivery platform can be a potential technique to achieve therapeutic interventions in patients who have a high risk of heart failure (Zhu et al. 2016).

16.4.4 Hydrogels for Ischemic Heart Disease

The imbalance between oxygenated blood demand and supply in the myocardial region leads to cardiac disability, known as ischemic heart disease (IHD). The myocardial infarction is one of the forms of IHD. Cardiac function loss due to left ventricular remodeling leads to heart failure (Baig et al. 1998; Song et al. 2016). Conventional treatments are based on relieving symptoms. There is a need for new therapies which will be based on repairing infracted heart tissue. Intramyocardial injection of a hydrogel loaded with bioactive molecules is one of the alternative approaches to conventional therapies to treat infarcted heart and ischemic conditions. Song et al. developed sericin-based in situ injectable hydrogel, and its therapeutic potential is investigated in the C57BL/6 mice model. Sericin is a protein and major constituent of silk. The developed sericin-based hydrogel injection into the MI portion reduces the development of infarct size and scar. Overall improvement in wall neovascularization and thickness were observed with a decrease in inflammatory responses and apoptosis induced by MI. Echocardiographic evaluation on C57BL/6 mice after 6 weeks indicated an increase in ejection fraction (EF) as well as fractional shortening (FS) of LV in the group treated with sericin hydrogel as compared to control groups. Restoration of blood pumping function was confirmed with this study. The potential anti-inflammatory action of sericin hydrogel was evaluated in vivo. Study results indicated selective inhibition of the expression of the interleukin (IL-18), pro-inflammatory cytokine tumor necrosis factor (TNF-α), and chemokine (CCL2) in the case of sericin hydrogel-treated mice. Reduction in TNF-α expression is attributed to the suppression of TLR4-MAPK/NF-jB pathways. However, sericin demonstrates vasculogenic action by aiding the migration of human umbilical vascular endothelial cells (HUVECs) in the scratch area and tubular formation. It also facilitates the expression of VEGF by activating the phosphorylation of ERK. Furthermore, by blocking the activation of caspase 3, sericin protects endothelial cells and cardiomyocytes from apoptosis. Hence, this variety of biochemical activities of sericin resulted in cardiac function recovery. Thus, this research concludes that sericin serves as a potential biomaterial in vivo for ischemic myocardial repair, which can be given in injectable hydrogel form (Song et al. 2016).

Similarly, Rufaihah et al. formulated polyethylene glycol-fibrinogen (PF)-based injectable hydrogel loaded with vascular endothelial growth factor (VEGF) with angiopoietin-1 (ANG-1) (PF-VEGF-ANG1) to treat ischemic hearts, given hydrogel system is explored for controlled delivery of both bioactive moieties to improve myocardial repair and function. *The* study was performed in a rodent model with male Wistar rats, and in posttreatment weeks, the degree of fibrotic scar and arteriogenesis was evaluated. Both VEGF and ANG-1 were followed control release for 30 days. A prominent increase in ejection fraction values were observed in the case of 1% PF-VEGF-ANG1 as compared to 1% PF-treated rat groups, which were 79.1% and 64.1%, respectively. Cardiac tissue sections of PF-VEGF-ANG1 were observed with minimum fibrotic scars and higher muscle salvage. Significant cardiac function restoration, maximum cardiac muscle preservation level, and arteriogenesis were observed in rats treated with PF-VEGF-ANG1. Research has also shown that

PF hydrogels alone can to provide mechanical support to the cardiac muscle by decreasing myocardial remodeling and also offers the pro-angiogenic benefit of the VEGF and ANG1 sustained release, resulting in a post-MI restorative effect. This combination of bioactive molecules like growth factors and hydrogel with inherent mechanical supportive ability can be a significant development in the treatment of ischemic hearts (Rufaihah et al. 2017).

Though delivery of growth factors and pro-angiogenic cytokines by encapsulating into hydrogel is a potential approach in the case of ischemic heart disease, some of the factors like viscoelastic properties and mechanical nature of hydrogel impact the delivery of bioactive molecules. In an attempt to solve this problem, Steele et al. developed highly self-healing and shear-thinning, multilayer catheter-injectable hydrogel consisting of hydrophobically modified hyaluronic acid using dodecylamine (HA-C_{12}), which is cross-linked with poly(ethylene glycol)-block-poly(lactic acid) core-shell NPs (PEG-PLA). The hydrogel was synthesized using 2 wt% HA-C12 and 10 wt% NPs loaded with ESA, where 16 μg of HGFdf and 25 μg ESA were encapsulated per hydrogel. This hydrogel system is designed to achieve dual-stage protein-engineered cytokine release in order to minimize abnormal left ventricular remodeling post-MI. Two bioactive moieties were encapsulated in the given hydrogel system which are a dimeric fragment of the hepatocyte growth factor (HGFdf), which is known for anti-fibrotic, anti-apoptotic as well as pro-angiogenic properties, and analogue of stromal cell-derived factor 1α (ESA) which is a very effective chemoattractant for CD34+ stem cells. The sustained release of ESA and HGFdf from distinct portions of the hyaluronic acid-loaded gel provides prolonged and distinct advantages because of the accurate drug release timing. Adult male Wistar rats were used as small animal models whereas, male Dorset sheep were used as a large animal model for in vivo analysis. Echocardiographic studies in small animals indicated improvement in inner diameter of left ventricle at end-systole of animals treated with HG + HGFdf + ESA which was 5.87 ± 0.80 mm as compared to other control groups which was 6.92 ± 0.79 ($p < 0.009$), 7.45 ± 1.05 ($p < 0.001$), and 6.75 ± 0.61 ($p < 0.039$) for HGFdf + ESA, PBS, and HG, respectively. Furthermore, these outcomes from small animal studies were converted into a preclinical large animal model. A significant reduction in infarct size was observed in the case of a large animal model, which was found to be $15.9 \pm 2.1\%$ ($p < 0.01$) as compared to only HG-treated animals ($20.3 \pm 3.5\%$) and PBS-treated animal ($31.4 \pm 6.1\%$). This study demonstrates the potential of hydrogel in the case of spatiotemporally controlled delivery of two bioactive molecules with distinct release profiles to target different regenerative pathways for the treatment of ischemia (Steele et al. 2020).

16.5 Hydrogel-Assisted Tissue Engineering for CVDs

In cardiovascular diseases, particularly in MI, the hypoxic condition damages the tissue, kills cardiomyocytes, and manifests into the structural and functional modification of the ventricular wall and the chamber of the heart (Heusch 2019). The adult

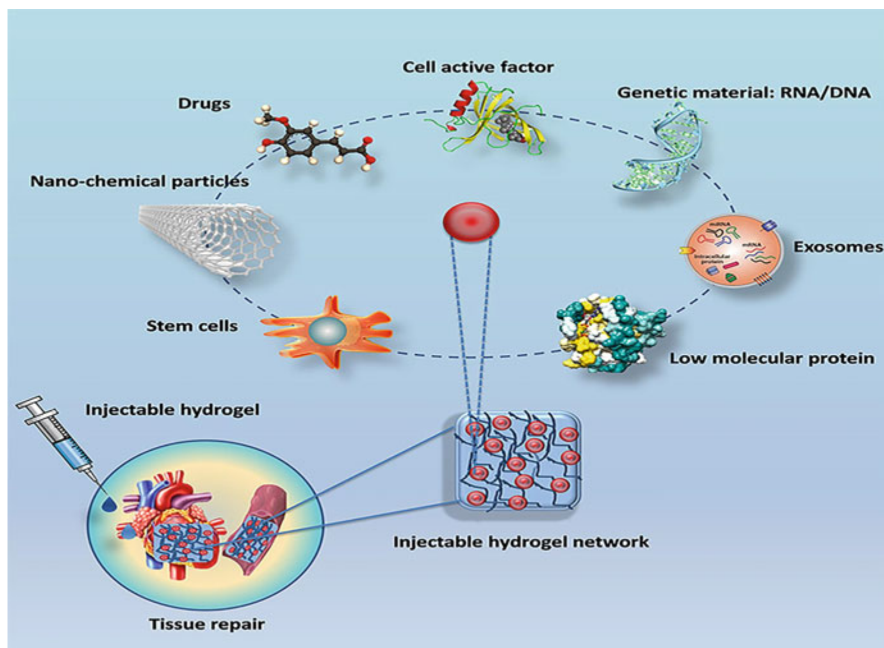

Fig. 16.3 Types of active materials encapsulated in the injectable hydrogels for tissue engineering. (Image adapted with permission from Liao et al. Frontiers in Bioengineering and Biotechnology (Liao et al. 2020). Copyright © 2020 with permission from Frontiers)

cardiomyocytes in the heart have a limited capacity for regeneration. Therefore, a post-MI episode limits the regeneration and restoration of cardiac tissue to function normally (Kikuchi and Poss 2012). Therefore, there is a dire need for new treatment strategies, such a regenerative medicine, to repair damaged hearts. For treating late post-MI condition where structural and functionally damaged infarcted tissue is prominent, the regeneration of cardiac tissue approach had gained a lot of interest (Eschenhagen et al. 2012). For successful cardiac tissue regeneration, the availability of ECM is essential, which was not achieved by NPs; hence researchers worked on developing biomimicking 2D or 3D scaffold. Various hydrogels such as injectable hydrogels are frequently used in the tissue engineering platforms for the controlled release of bioactive drugs like growth factors, exosomes, and proteins to repair damaged cardiac tissue (Tan and Marra 2010; Lee and Mooney 2001) (Fig. 16.3). Tissue-engineered constructs are often encapsulated with cardiac cells or iPSCs for successful engrafting at the damaged site and subsequent differentiation into cardiac tissue.

Since the first description of engineered heart tissue, researchers formulated hydrogels, nanofibrous (optimum diameter and pore size <100 nm) scaffolds, and layer-by-layer (LBL) assembly nanocomplexes (5–1000 nm). Numerous examples of nanofibrous and LBL assembly nanocomplexes for tissue regeneration in various animal models reported elsewhere (Kikuchi and Poss 2012; Huyer et al. 2015;

Prabhakaran et al. 2011). Although generating an artificial scaffold substitute for cardiac tissues remains challenging (Garcia and Garcia 2016). Thus, to address these limitations, researchers developed various types of hydrogels and tested them for cardiac tissue regeneration applications (Peña et al. 2018; Saludas et al. 2017). For example, Landa et al. demonstrated that injectable in situ gellable calcium-cross-linked alginate hydrogel (10–50 cP) is one of the potential acellular strategies for cardiac dysfunction and remodeling in the Sprague-Dawley rat model of MI (Landa et al. 2008). The microscopic examination showed the alginate hydrogel occupied an average of 45% of the area of the infarct in 1 h after injection. The gel remains at the site for 4–6 weeks. Further, echocardiography studies showed improved scar thickness from 0.14 ± 0.01 to 0.16 ± 0.01 cm ($p < 0.01$) and an increase in diastolic and systolic anterior wall thicknesses when compared with control treatment groups. The histopathological study of the 2-month posttreated heart shows myofibroblasts populated in the scar tissue at the injection site and the increased scar thickness. This study provides a proof of concept of injectable, alginate hydrogel implants enabling conservation of cardiac function after old and recent MI (Landa et al. 2008).

Synthetic polymer-originated hydrogels have the desired mechanical strength but lack intrinsic groups for bioavailability and cell adhesion surfaces. Thus, some synthetic polymers need surface modifications for delivering cells entrapped hydrogel for cardiac tissue engineering (Saludas et al. 2017; Wang et al. 2009). For example, Wang et al. developed α-cyclodextrin/poly(ethylene glycol)-b-polycaprolactone-(dodecanedioic acid)-polycaprolactone-poly(ethylene glycol) (α-CD/MPEG-PCL-MPEG) (10% w/v) hydrogel to localize bone marrow-derived stem cells (BMSCs) into the infarcted zone of rabbit model (Wang et al. 2009). Post 30 days of treatment, fluorescence images of the infarcted site showed increased infiltration of the BMSCs into the infarcted zone treated with cells + hydrogel (2150 ± 235 mm^{-2}) as compared to the cell group (845 ± 156 mm^{-2}). Further, the cell + hydrogel-treated group shows significantly reduced infarct size as compared to the cell-treated group ($p < 0.05$). Echocardiography results show a reduction in the dilatation and preservation of the LV function, which could be due to increased infiltration of BMSC into the infarcted site (Wang et al. 2009). Apart from this, hybrid hydrogels were developed to overcome the limitations presented by both natural and synthetic polymers. Ideally, these hybrid hydrogels would have cell-recognizable binding sites as well as desirable mechanical properties for cardiac tissue engineering (Huyer et al. 2015; Prabhakaran et al. 2011; Saludas et al. 2017; Toh and Loh 2014). For example, A. Rufaihah et al. developed VEGF-loaded hybrid hydrogels of polyethylene glycol linked to a fibrinogen backbone to improve neovascularization cardiac recovery after MI (Rufaihah et al. 2013). These are representative examples that demonstrate the potential application of conventional hydrogels in cardiac tissue engineering. However, conventional hydrogels, including hybrid hydrogels developed as patches, can only be placed into the heart or as gel via epicardial deposition, which demands major intervention procedure (Peña et al. 2018; Moorthi et al. 2017). This could limit the user-friendliness and, thus, clinical translation potential of the conventional hydrogels (Moorthi et al. 2017). To overcome the shortcomings of the conventional hydrogel, researchers developed

injectable hydrogel using stimulus-responsive polymers, which would respond to stimulus and form a gel.

SRH is an emerging strategy for cardiac tissue engineering. For example, Fujimoto et al. developed a thermo-responsive injectable polymer (poly (NIPAAm-co-AAc-co-HEMAPTMC)) to prevent progressive adverse remodeling of ventricular wall postischemic cardiomyopathy event (Fujimoto et al. 2009). The poly(NIPAAm-co-AAc-co-HEMAPTMC) is synthesized using *N*-isopropylacrylamide (NIPAAm) and acrylic acid (AAc) with hydroxyethyl methacrylate-poly(trimethylene carbonate) (HEMAPTMC) at 86/4/10 molar ratio. The in vitro gelation characterization of poly(NIPAAm-co-AAc-co-HEMAPTMC) shows the immediate transition of the sol-gel occurred upon solution treatment at a bath of 37 °C. Further incubation for 10 min, a very flexible gumlike material was observed. Further, the poly(NIPAAm-co-AAc-co-HEMAPTMC) was injected into the left ventricular wall of the infarcted rat and PBS used as a control group. The histological study of LV myocardial wall of post-8-week treated rats shows extensive fibrous tissue as well as the reduction in tissue thickness found in the anterior-lateral wall and aneurysm formation in the anterior wall of the PBS treated rats; hydrogel-injected rat group shows the thickened tissue of the ventricular wall. The ultrasound imaging analysis shows no change in % fractional area change and end-diastolic area in the rats treated with hydrogel; in the case of PBS, both parameters show significant change as compared to pre-treatment values ($p < 0.05$). This study shows that injected thermo-responsive hydrogel injection prevents ventricular dilation as well as increased contractile function in a chronic infarction rat model (Fujimoto et al. 2009). Similarly, pH-responsive and self-healing supramolecular hydrogel carrier developed using UPy-hydrogelator polymers to deliver human recombinant IGF-1 and human recombinant HGF to achieve a reduction of infarct scar in a pig MI model (Bastings et al. 2014). However, other stimuli such as ROS and cytokines have yet not been explored for the same.

Apart from the SRH, hydrogels with conductive, elastomeric, and oxygen-releasing properties had been studied for cardiac tissue engineering applications as cardiac tissue possesses these properties. Such types of hydrogels were developed by adding various NPs or modifying polymer properties. For example, McGann et al. demonstrated the application of resilin-like polypeptide (RLP)-polyethylene glycol hydrogels for repair myocardial disfunction (McGann et al. 2013). Similarly, for making hydrogel mechanically tuneable and conductive carbon nanotubes (CNTs) gelatine methacrylate (GelMA), hydrogels in vitro showed improved cardiomyocyte adhesion and maturation (Shin et al. 2013). In similar lines, Fan et al. developed oxygen-releasing microspheres of poly(lactic-co-glycolic acid) shell, and PVA/H_2O_2 core was loaded into the NIPAAm/HEMA/AOLA (86.0:10.5:3.5). The effect of oxygen release study in MI rats shows significantly stimulated tissue angiogenesis and increased cardiac function (Fan et al. 2018). For cardiac tissue regeneration hydrogels, the injectable type of SRH will have a significant advantage over other scaffolds as it can be injected into the heart with minimally invasive methods. SRH provides better control over hydrogel matrix formation; however,

further research is warranted to achieve stiffness similar to cardiac tissue, gelation time, and repose to stimulus.

16.6 Clinical Progress

Owing to the tuneable physicochemical properties and the ability to be delivered via a minimally invasive procedure like injections, hydrogel-based platforms made pivotal clinical success in treating various CVDs (Mandal et al. 2020). Terminal heart failure, MI, ischemia, dilated cardiomyopathy, left ventricular remodeling, ventricular dysfunction, VAD implantation, and blood leakage during surgery are some of the critical medical conditions where hydrogel interventions showed promising outcomes on human patients. For this purpose, researchers investigated a wide variety of biomaterials, wherein several natural materials like alginate, silk-fibroin, hyaluronic acid, and tissue-derived acellular matrix (ECM) and synthetic materials like polyethylene glycol, poly(lactic-co-glycolic acid), polyacrylic acid (PAA), methacrylic acid (MAA), and cyclohexylamine (CHA) showed most promising clinical success (Liao et al. 2020; Song et al. 2016). These materials have been widely used for developing injectable hydrogels for cardiac tissue repairing (Hasan et al. 2015). The CVDs particularly is one of the most appealing areas wherein many hydrogel-based technologies have already been approved for treating patients and numerous others are being evaluated in clinical trials (Table 16.1). For instance, LoneStar's Algisyl-LVR® is the first hydrogel developed using alginate to be approved for terminal heart failure patients (Anker et al. 2015; Mann et al. 2016). Similarly, VentriGel® is developed from connective tissue obtained from pig heart that can be lyophilized and milled in the form of dry powder and then reconstituted in a buffer to be easily injected into the heart muscles via a minimally invasive procedure without needing any surgery (Traverse et al. 2019). In Table 16.1, we have described many such examples of hydrogel technologies that showed excellent clinical performance in treating a variety of CVDs.

16.7 Challenges and Way Forward

In recent years, there has been notable progress in hydrogel-assisted technologies, and hydrogels are being explored in various diseases, including CVDs, whereas despite progress in hydrogel synthesis technologies, parameters like surface modifications and degradation reaction rate are needed to critically monitored as they are highly susceptible to variability. Similarly, immunological responses are needed to be considered for better compatibility. In the case of responsive hydrogels, in vivo safety is a significant concern due to interference of complex biological environment (Abdollahiyan et al. 2020; Sood et al. 2016; Xue et al. 2019; Ahamad et al. 2020b). Hydrogels are considered an excellent choice for depot preparation. Also, hydrogel as a carrier for one or more drugs or nanoparticles, especially by localized delivery, is an attractive approach to achieve the synergistic effect of

Table 16.1 Clinical progress on hydrogel technology for treating cardiovascular diseases

Hydrogel intervention	Target CVD	Procedure	Key outcome	Reference/ clinical trial No.	Stage (No. of participants)
ECM-based hydrogel derived from decellularized porcine myocardium (VentriGel) (Traverse et al. 2019)	MI, heart failure, and patients with evident left ventricular remodeling	Trans-endocardial injection of VentriGel within 60 days to 3 years after first MI incidence	Demonstrated safety and feasibility of delivering VentriGel in post-MI patients	NCT02305602	Phase I (15)
Alginate hydrogel (Algisyl-LVR™ device (implants) (Anker et al. 2015; Mann et al. 2016)	Heart failure and dilated cardiomyopathy	Intramyocardial injection of Algisyl-LVR with standard medical therapy	No device-related complications in all treated patients. Alginate hydrogel with standard therapy showed superior efficacy over standard therapy alone	NCT01311791	Phase II Phase III (78)
Engineered heart muscles (EHMs) derived from iPSC-derived cardiomyocytes and stromal cells in collagen-type 1 hydrogel (Tzatzalos et al. 2016)	Terminal heart failure	Biological Ventricular Assist Tissue (BioVAT) for implantations	Study yet to start	NCT04396899	Recruiting for Phase I and Phase II (53)
Amiodarone releasing hydrogels	Patients undergoing lung transplantation	Intraoperative instillation to prevent postoperative atrial fibrillation (POAF). Hydrogel delivered through a CO_2 driver along the pulmonary vein and arterial anastomoses	Incidence of POAF was significantly reduced	NCT03221764	Phase II (20)
Hybrid biotherapy involving autologous-human cardiac derived stem cells (hCSCs) with controlled release of	Congestive heart failure, ischemic cardiomyopathy,	Transplantation procedure	Clinical study yet to start. Previously tested in the MI pig model, the controlled release of bFGF in	NCT00981006	Phase I (6)

(continued)

Table 16.1 (continued)

Hydrogel intervention	Target CVD	Procedure	Key outcome	Reference/ clinical trial No.	Stage (No. of participants)
basic fibroblast growth factors (bFGF) using a gelatin hydrogel sheet (Takehara et al. 2008)	ventricular dysfunction		combination with cell transplantation significantly augmented the formation of new vessels and contractibility in MI heart		
High molecular weight PEG-based synthetic FDA-approved hydrogel (CoSeal™ or BioGlue®) to be used as a surgical sealant/ adhesive during heart surgery	To prevent blood leakage during heart surgery or LVAD implantation	Applied using dual-syringe applicator directly at the site of leakage to mechanically sealing the area	Reduces microemboli and also prevents tissue adhesion during heart surgery. It provides superior anastomotic suture line sealing	NCT01605019, NCT01244321	Phase II (30)

therapeutics (Cao et al. 2019; Cheng et al. 2017). Yet, there is a challenge of precision in different release rates for more than one drug which is needed to be investigated (Xue et al. 2019).

Although hydrogels are considered as an ideal system for protein, biomolecules delivery owing to special features like hydrophilicity, controllable porous nature by the degree of cross-linking. However, in affinity-based hydrogels, there is a limited number of suitable proteins. For example, heparin-based affinity hydrogels can cause abrupt leakage of loaded proteins due to competitive binding to plasma heparin leading to disruption of protein-heparin interactions in gel matrix (Bae et al. 2013; Engelberg and Dudley 1961).

Overall, hydrogel-assisted therapies are one of the cutting-edge technologies which is ideal for drug delivery as well as regenerative medicine. While exhaustive optimization of formulation with critical parameters like degree of crosslinking, surface modification can be done to avoid severe complications upon administration of hydrogels. However, there are several challenges like patient compliance; scale-up feasibility is needed to be addressed to the clinical applicability of hydrogel-assisted therapies. In the future, ongoing progress on the above mention challenges of hydrogels as well as introducing newer fabrication techniques can open doors to more recent opportunities for CVDs.

16.8 Summary

The chapter explains and discusses the recent advances, clinical progress, and challenges ahead in the use of hydrogel systems for treating CVDs. Particularly the chapter described different approaches and fabrications methods used to develop hydrogels, ranging from conventional hydrogels scaffolds, injectable hydrogels, stimulus-responsive hydrogels, and nanogels. The hydrogels as a therapeutic delivery agent for CVDs such as atherosclerosis, thrombosis, MI, and other CVD complications where the prime aim is the restoration of dysfunctional cells and not the killing the diseases cells (like in the case of oncology). Along with delivery applications, examples of hydrogels had shown an important role in cardiac tissue engineering and improving and restoring cardiac tissue function. The clinical trials of hydrogels have shown the potential of hydrogel for CVDs treatment. The advancement of hydrogels to SRH, injectable hydrogels, and nanogels have shown a potential to overcome limitations presented by hydrogel scaffolds. However, further research is warranted in the case of all types of hydrogels to achieve stiffness similar to cardiac tissue, gelation time, and repose to stimulus to be optimally suitable for clinical use in CVD treatment.

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Decellularized Extracellular Matrix for Stem Cell Culture

17

Takashi Hoshiba

Abstract

Many decellularized extracellular matrices have been developed and applied to various bioindustrial applications. Similar to these applications, decellularized extracellular matrices have started to be used for stem cell engineering. There are numerous efforts to unveil the effects of decellularized extracellular matrices on stem cell functions, and it has been demonstrated that decellularized extracellular matrices impact various stem cell functions, particularly differentiation, stem cell maintenance, and recovery of lost stemness (rejuvenation). In this chapter, stem cell functions on the decellularized extracellular matrices are summarized following a brief comparison of sources and a description of decellularized extracellular matrices preparation and characterization. Additionally, the present problems of decellularized extracellular matrices in stem cell applications are outlined in this chapter.

Keywords

Decellularized extracellular matrices · Stem cell culture · Stem cell differentiation · Stemness

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17.1 Introduction

Stem cells are a promising cell source for various bioindustrial applications (e.g., tissue engineering, regenerative medicine, and pharmacological studies) (Liu et al. 2020; Mohammadian 2018). The technology of regulating stem cell functions, including differentiation and stemness maintenance, is the key to their achievements. There are many efforts to regulate stem cell functions. One of the approaches is improving the culture conditions (Liu et al. 2020; Chen et al. 2011; Ng et al. 2008). Among the culture conditions, cell culture substrate is an important factor as well as a culture medium. Many substrates for stem cell culture have been developed with synthetic polymeric materials (Olivares-Navarrete et al. 2017; Duffy et al. 2014) and isolated proteins (Chen et al. 2011; Nakagawa et al. 2014), and stem cell functions are regulated to some degree.

In our body, stem cells are surrounded by a specific microenvironment, the so-called "stem cell niche." The stem cell niche is composed of cells and extracellular matrix (ECM) (Mercier et al. 2002; Meran et al. 2017). In particular, the ECM plays pivotal roles in regulating many cell functions, such as cell adhesion, growth, migration, differentiation, and responses to soluble factors, through the activation of various intracellular signaling pathways (Harburger and Calderwood 2009; Geiger and Yamada 2011). It has been tried to reconstitute native ECM as culture substrates for regulating stem cell functions. ECM is constituted with many proteins and carbohydrates. However, there are over 300 types of ECM proteins (Hynes and Naba 2012), and their combinations vary according to cell/tissue/organ types and developmental and pathological stages (Naba et al. 2012; Lu et al. 2011; Manabe et al. 2008). Moreover, not all ECM molecules have been identified, and the complete compositions of the ECM have not yet been identified. Therefore, it is very difficult to reconstitute native ECM by combining identified ECM proteins and other conventional chemical methods. For this reason, the decellularization technique has been used to reconstitute native ECM as culture substrates in vitro. Currently, ECM reconstituted by the decellularization technique (decellularized ECM: dECM) are commercially available for clinical applications (Nakamura et al. 2017; Crapo et al. 2011), and dECM have started to be used as in vitro ECM models for cancer research (Hoshiba and Tanaka 2016; Hoshiba 2018, 2019; Castelló-Cros et al. 2009). dECM are now used as culture substrates for stem cell culture to induce specific stem cell functions. In this chapter, I summarize the methods for dECM preparations after comparing dECM sources. Additionally, stem cell functions exhibited on dECM are reviewed. Finally, the potential applications of dECM as culture substrates are discussed.

17.2 Preparation of dECM for Stem Cell Culture

17.2.1 Sources

dECM are generally prepared from two sources: ECM in tissues/organs and ECM produced by cultured cells. Both of them have advantages and disadvantages for the preparation, characteristics, and feasibility of stem cell culture (Table 17.1). Therefore, the sources of dECM should be carefully selected according to their purposes. Generally, dECM derived from tissues and organs can possess compositional, macro- and microstructural, and mechanical similarities with native ECM if decellularization is properly performed. Additionally, tissue-/organ-derived dECM can be obtained at larger scales than cultured cell-derived dECM. These are the most significant advantages of tissue-/organ-derived dECM to cultured cell-derived dECM. However, tissue-/organ-derived dECM tends to show significant batch-to-batch differences due to the individual differences in sources compared with cultured cell-derived dECM. More importantly, for stem cell culture, it is difficult to isolate ECM in limited regions (e.g., stem cell niche) as tissue-/organ-derived dECM due to the difficulties of their identification and isolation.

In contrast to tissue-/organ-derived dECM, cultured cell-derived dECM can be prepared with smaller batch-to-batch differences than tissue-/organ-derived dECM if the culture conditions (e.g., the compositions of a culture medium, initial culture substrates, and cell passages) are properly controlled. Additionally, cultured cell-derived dECM can mimic ECM in limited regions, such as the stem cell niche, which is the most significant advantage of cultured cell-derived dECM to tissue-/organ-derived dECM. However, compositional, macro- and microstructural, and mechanical similarities of cultured cell-derived dECM tend to be lower than those of tissue-/organ-derived dECM. Therefore, these similarities should be checked more carefully when cultured cell-derived dECM are used as ECM models. Additionally, cultured cell-derived dECM are challenging to prepare at large scales even though the batch-to-batch differences can be controlled at a small level. Tissue-/organ-derived dECM

Table 17.1 Comparison of dECM sources

dECM source type	Advantages	Disadvantages
Tissue-/organ-derived dECM	– Similar to native ECM compositions, macro-/microstructures, and mechanical properties	– Limitation of ECM source supply
		– Difficult to use for large-scale in vitro analyses due to large batch-to-batch differences
		– Difficult to isolate limited regions from tissues and organs
Cultured cell-derived dECM	– Usable for large-scale in vitro analyses due to small batch-to-batch difference	– Difficult to obtain dECM similar to the native ECM compositions, macro-/microstructures, and mechanical properties
	– Easy to obtain dECM derived from limited regions (e.g., stem cell niche)	

tend to be used for stem cell differentiation and for the reconstitution of engineered tissues and organs with stem cells. On the other hand, cultured cell-derived dECM tend to be used to maintain and differentiate stem cells and are mainly applied for in vitro applications. The above functions and applications of dECM are further reviewed in the following sections.

17.2.2 Preparation

17.2.2.1 Decellularization

The decellularization process is critical for many properties and functions of the dECM. Decellularization is performed by various methods: treatment with detergents (e.g., sodium dodecyl sulfate, sodium deoxycholate, Triton X-100), chemical methods (e.g., alkaline or acid treatments and chelating), physical treatments (e.g., hypotonic and hypertonic pressures, ultrahydrostatic pressure, and freeze-thawing), enzymatic treatments (e.g., DNase, RNase, and proteinases), and their combinations (Gilbert et al. 2006; Keane et al. 2015; Nakamura et al. 2017; Crapo et al. 2011). For the decellularization of tissues and organs, the solution for decellularization can be perfused through their blood vessels, enabling decellularization while keeping macro- and microstructures of original tissues and organs (Ott et al. 2008; Uygun et al. 2010). For this structural advantage, whole tissue-/organ-derived dECM have been developed as scaffolds for the in vitro reconstruction of new tissues and organs. In addition to whole tissue/organ decellularization, tissues and organs can be decellularized after physical treatments, such as cutting and milling, for fabrication.

17.2.2.2 ECM Formation by Cultured Cells

For the preparation of cultured cell-derived dECM, ECM should be formed by the cells prior to decellularization. ECM formation is influenced by various factors: culture medium compositions (influencing ECM compositions and formation rates) (Satyam et al. 2014; Furuyama and Mochitate 2000; Furuyama et al. 1999), initial culture substrates (influencing ECM compositions, formation rates, and macrostructures) (Mochitate et al. 2020; Hoshiba and Tanaka 2015; Prewitz et al. 2013), and cell types (influencing ECM compositions) (Hoshiba and Tanaka 2015; Hoshiba et al. 2011a, 2012a) (Fig. 17.1). ECM formation conditions should be carefully optimized for cultured cell-derived dECM because the formed ECM strongly influences cell functions. Additionally, decellularization (described above) and post-decellularization (described below) processes impact the functions of cultured cell-derived dECM. Detailed effects of ECM formation conditions are discussed in a previous review (Hoshiba 2017).

17.2.2.3 Modification

Prepared dECM are often modified with several methods. ECM components and ECM fragments (e.g., ECM-derived peptides) can be easily modified by immersion in these molecule-containing solutions (Mahara et al. 2019). Additionally,

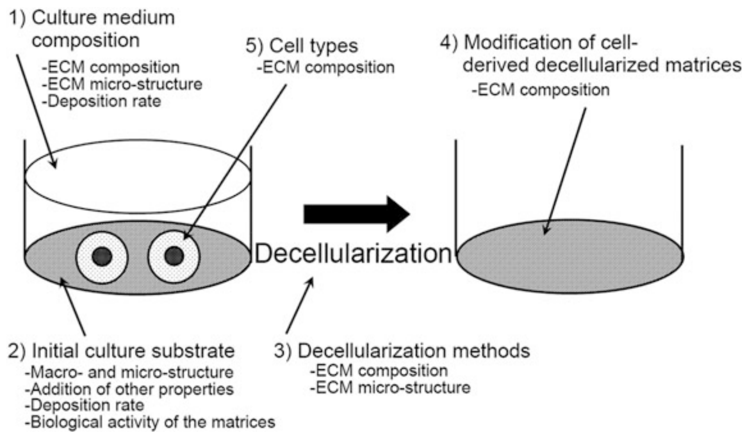

Fig. 17.1 Schematic illustration of the simple decellularization process. (Reproduced from Hoshiba (2017) by permission from RSC publishing)

treatments with enzymes, such as proteinases and enzymes degrading carbohydrates, can remove specific ECM components (Hoshiba 2018; Hoshiba et al. 2011b). Cross-linking of the dECM can change the mechanical properties to modulate mechanical signal transduction (Kim et al. 2018b). These modifications can improve dECM functions and can add new functions to the original dECM. Additionally, dECM is sometimes treated with pepsin for solubilization. Solubilized dECM can be used as a supplement to the culture medium (Crapo et al. 2012) and coating materials to the substrate surface (Aguado et al. 2016). More importantly, the solubilized dECM form gels after neutralization, making it possible to use solubilized dECM as injectable gels (Singelyn et al. 2012; Freytes et al. 2008).

17.2.2.4 Fabrication

Tissue-/organ-derived dECM can maintain the macro- and microstructures of original tissues and organs, which is an advantage for tissue and organ reconstruction. On the other hand, it is usually required for the fabrication of dECM into desirable shapes, including patch and tube shapes. dECM are sometimes solubilized and incorporated in other polymeric materials. Then, hybrid materials are fabricated into several shapes (Gao et al. 2017; Baiguera et al. 2014). According to the recent progress of three-dimensional (3D) printing technology, the combination of dECM and 3D printing technology has been applied to fabricate dECM into desirable shapes (Dzobo et al. 2019; Kim et al. 2018a). For this purpose, solubilized dECM are used as bioinks. For cultured cell-derived dECM, initial culture substrates act as templates and then determine dECM shapes. Therefore, initial culture substrates are prepared by 3D printing technology to form desirable shapes of cultured cell-derived dECM and initial culture substrate complexes (Hoshiba and Gong 2018). Moreover, initial culture substrates as templates can be removed if they are prepared with degradable materials, such as poly(lactic-*co*-glycolic acid) (PLGA) (Lu et al. 2011a, 2011b).

17.2.3 Characterization

Cell removal confirmation should be the first step for dECM characterization. Additionally, it is necessary to examine how ECM components and structures are retained in the prepared dECM. A summary of methods for these characterizations is shown in Table 17.2. Additionally, a general characterization of the materials is required.

17.2.3.1 Confirmation of Cell Removal

Detection of cellular components is always performed to confirm cell removal. In particular, cell nuclei and/or nuclear DNA are generally detected for the confirmation of cell removal. For tissue-/organ-derived dECM, three criteria are proposed for the confirmation of cell removal: (1) no visible nuclei are detected in tissue sections stained with DAPI or hematoxylin, (2) <50 ng dsDNA per mg ECM dry weight, and (3) <200 bp DNA fragment length (Crapo et al. 2011). However, these criteria have not been fully discussed, and a consensus should be built in the field. On the other hand, there are currently no criteria to confirm cell removal for cultured cell-derived dECM. For the cultured cell-derived dECM prepared in 2D culture, cell removal is easily confirmed by microscopic observation. For the dECM prepared in 3D culture, it seems proper to follow the criteria for tissue-/organ-derived dECM. In addition to

Table 17.2 Frequently used characterization methods of dECM

Purposes	Principle	Methods
Confirmation of cell removal	DNA/cell nuclei detection	– Staining with hematoxylin and Hoechst 33258
		– DNA content measurement
	Intracellular protein detection	– Actin staining with fluorescent-labeled phalloidin
		– Immunocytochemistry of cytosolic proteins
Compositional analysis	Detection of non-nucleic components	– Eosin staining
	GAGs detection	– Alcian blue and toluidine blue stainings
	Collagens detection	– Sirius red and azan stainings
	Specific proteins/ carbohydrates detection	– Immunohistochemical analysis with antibodies
		– Staining with lectins
	Proteomics (exhaustive research)	– Mass spectrometry
Structural analysis	Structure observation	– SEM
	Basement membrane detection	– TEM
	Fibril alignment	– Fast Fourier transform analysis

Reproduced from a previous review (Hoshiba 2019) with permission from the Multidisciplinary Digital Publishing Institute (MDPI) under Creative Commons 4.0

nuclear detection, the detection of cytosolic proteins, such as cytoskeletal fibrillar actin, is often used. The detection of these components was performed by immunohistochemical analyses.

17.2.3.2 Confirmation of ECM Component Retention

The confirmation of ECM components is usually performed by immunohistochemical analyses with antibodies against specific ECM proteins and carbohydrates and with lectins against specific glycosaminoglycans. In addition to the detection of specific ECM components, denatured collagen can be detected with some collagen hybridizing peptides that might be used for the quality control of dECM (Li et al. 2012; Hwang et al. 2017). Additionally, several histochemical analyses, such as Sirius red staining and alcian blue staining, were performed. Recently, mass spectrometry has been used for exhaustive testing of ECM components (Piccoli et al. 2018).

17.2.3.3 Other Characterization

To examine the ECM microstructure, observations are usually performed with scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In particular, the basement membrane, a special architecture of the ECM, can be identified as an electron-dense region by TEM. TEM should be used to check the integrity of the basement membrane after decellularization (Furuyama and Mochitate 2000). Additionally, fast Fourier transform analysis has been used to study fibril alignment (Harris et al. 2018).

17.3 Stem Cell Functions on dECM

Many studies have developed dECM for the culture of various stem cells. These studies reported that the dECM influenced many stem cell functions (Fig. 17.2). In particular, stem cell differentiation, stemness maintenance, and recovery from loss of stemness (rejuvenation) are important for bioindustrial applications with stem cells. In this section, the behaviors of stem cells on the dECM are summarized from the viewpoints of these functions.

17.3.1 Stem Cell Differentiation

The regulation of stem cell differentiation is the key for stem cell-based bioindustrial applications, including regenerative medicine and tissue engineering. Therefore, dECM have been applied for the regulation of stem cell differentiation. Many dECM derived from both tissues/organs and cultured cells have been developed. The partial lists of these dECM are shown in Tables 17.3 and 17.4. Proper ECM composition is required to fully exert cell functions (Hynes and Naba 2012). Indeed, it has been reported that the functions of somatic cells are strongly induced on the dECM originating from the same sources (Hoshiba et al. 2011a; Sellaro et al. 2007).

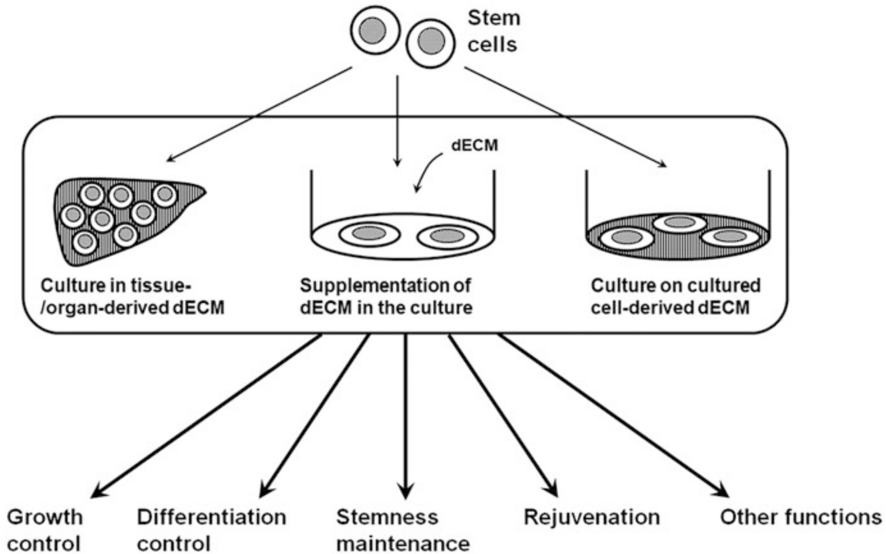

Fig. 17.2 Representation of the impact of dECM influencing many stem cell functions

Therefore, tissues/organs/cultured cells that are the same as the targets of differentiation are frequently used as sources for dECM preparation.

17.3.1.1 Differentiation on Tissue-/Organ-Derived dECM

Tissue-/organ-derived dECM have been applied for the differentiation culture of both multipotent somatic stem cells and pluripotent stem cells (i.e., embryonic stem (ES) cells and induced pluripotent stem (iPS) cells) (Table 17.3). Myoblasts were cultured on skeletal muscle-derived dECM (Chaturvedi et al. 2015; Stern et al. 2009). The dECM supported the growth of a murine myoblast cell line, C2C12, even in a serum-free medium (Chaturvedi et al. 2015). Moreover, myotube formation was promoted on these dECM.

Human liver stem-like cells (HLSCs), which can differentiate into hepatocytes, were also cultured in whole liver-derived 3D dECM (Navarro-Tableros et al. 2015). The dECM can support liver stem cell viability with EGF and FGF and can induce differentiation into hepatocytes. Additionally, some HLSCs differentiated into epithelial cells and tubular structure-formed endothelial cells. The reconstructed tissues with HLSCs and dECM showed urea production activity. Therefore, it is expected that this reconstructed tissue can be used as a liver-like tissue. When neural stem cells (NSCs) were cultured on brain-derived dECM, contradictory results were reported (Waele et al. 2015; Crapo et al. 2012). Waele et al. reported that an NSC line that was originally established on brain-derived dECM suppressed the differentiation of neural cells (Waele et al. 2015). On the other hand, Crapo et al. reported that PC12 cells (an NSC model cell line) on brain-derived dECM promoted differentiation into neural cells (Crapo et al. 2012). It is not clear why these opposite

Table 17.3 Partial list of tissue-/organ-derived dECM and their effects on stem cell differentiation

Stem cell for culture	Tissue/organ sources for dECM preparation	Effects	References
ES cells	Lung with trachea	Site-specific differentiation into lung cell lineages	Cortiella et al. (2010)
	Mammary tissue	– Functional mammary gland formation	Bruno et al. (2017)
		– Teratoma formation suppression	
	Muscle	Cardiac differentiation with the abilities of electrical stimulated response and normal adrenergic response	Hong et al. (2018)
	Kidney	Renal differentiation	Batchelder et al. (2015)
	Heart	Cardiac differentiation	Higuchi et al. (2013)
iPS cells	Liver	Hepatic differentiation	Hirata and Yamaoka (2017), Jaramillo et al. (2018)
	Amnion membrane	Male germ cell differentiation	Ganjibakhsh et al. (2019)
MSCs	Bone	Promotion of osteogenic differentiation in both in vitro and in vivo conditions	Lee et al. (2016)
	Tendon	– Tenogenic differentiation	Yang et al. (2013)
		– Suppression of osteogenesis	
	Brain	Neural differentiation	Baiguera et al. (2014)
	Cartilage	Chondrogenic differentiation	Yin et al. (2016)
	Spleen	– Hepatic differentiation	Xiang et al. (2016)
HLSC	Liver	– Hepatic differentiation	Navarro-Tableros et al. (2015)
		– Endothelial and epithelial cell differentiation	
NSCs	Brain	Suppression of neural differentiation	Waele et al. (2015)
	Brain, optic nerve, spinal cord, urinary bladder	Neural differentiation	Crapo et al. (2012)
Myoblasts	Skeletal muscle	Proliferation and myogenic differentiation	Stern et al. (2009), Chaturvedi et al. (2015)
Hair follicle stem cells	Dermis tissue	Hair bud-like structure formation under the co-culture condition with dermal papilla cells	Leirós et al. (2014)

(continued)

Table 17.3 (continued)

Stem cell for culture	Tissue/organ sources for dECM preparation	Effects	References
Testicular cells	Mammary tissue	Functional mammary gland formation	Bruno et al. (2017)
Retinal progenitor cells	Retina	Maturation of retinal progenitor cells	Kundu et al. (2016)

results were obtained after using similar brain-derived dECM. It is possible that the differences in cell types used in the studies, decellularization methods, region of the brain for decellularization, the provided form of dECM to the cells, and other culture conditions led to these conflicting results.

Tissue-/organ-derived dECM have also been applied for the differentiation culture of pluripotent stem cells, such as ES and iPS cells. Higuchi et al. prepared heart and liver-derived dECM, and these dECM were applied for the cardiac differentiation culture of ES cells (Higuchi et al. 2013). Cardiac differentiation marker expression was higher on heart-derived dECM than liver-derived dECM. This report suggested that dECM from different sources have different effects on stem cell differentiation. Additionally, tissue-/organ-derived dECM have been used for ES/iPS cell differentiation to reconstruct new tissues and organs with minor cell populations in vitro because these pluripotent stem cells can differentiate into almost any cell type. This is one of the biggest challenges in tissue engineering and regenerative medicine with stem cells. Bruno et al. prepared dECM from mammary tissues and solubilized the dECM (Bruno et al. 2017). Then, ES cells were transplanted in cleared mammary fat pads with solubilized dECM. The solubilized dECM inhibited teratoma formation in all transplanted cases but directed differentiation into mammary epithelial cells with gland-like structures.

Cortiella et al. tried to culture ES cells in dECM derived from the whole lung with the trachea (Cortiella et al. 2010). The dECM supported cell viability and growth and, surprisingly, the dECM directed site-specific differentiation into lung and tracheal cells. In the upper tracheal region, there were no occlusions, and the cells expressing cytokeratin were lined along the tracheal wall to form a sheet structure. Additionally, cytokeratin-18-expressing cells (i.e., ciliated epithelial cells) were found in the upper tracheal region. Clara cell protein 10-expressing cells (i.e., Clara cells) were found in the lower tracheal region. In the distal lung regions, pro-surfactant protein C-expressing cells (i.e., type II pneumocytes) formed hollow epithelial cyst-like structures. Moreover, CD31-expressing cells (i.e., endothelial cells) were found in lung and tracheal regions, suggesting that the reconstructed tissues were vascularized. This report suggests that tissue-/organ-derived dECM provide powerful platforms for the reconstruction of large tissues and organs with ES/iPS cells. However, further studies are required for cell seeding methods, teratoma formation suppression, and site-specific differentiation in other tissue-/organ-derived dECM. For the reconstruction of new tissues and organs with ES/iPS cells,

Table 17.4 Partial list of cultured cell-derived dECM and their effects on stem cell differentiation

Stem cell for culture	Cell sources for dECM preparation	Effects	References
ES cells	Pancreatic β RIN5F cells	– Differentiation into insulin-secreting β cells with RIN5F cell's conditioned medium (Also mentioned the differentiation into kidney tubule cells and cardiomyocytes on similar dECM)	Narayanan et al. (2014)
	804G bladder carcinoma cells	– Pancreatic lineage commitment – Differentiation into insulin-secreting β cells	Kaitsuka et al. (2014)
	Embryonic kidney HEK293 cells expressing laminin-511	– Pancreatic lineage commitment – Differentiation into insulin-secreting β cells	Higuchi et al. (2010)
	Spontaneously differentiated embryoid bodies	Early differentiation into mesoderm	Goh et al. (2013)
iPS cells	SV40- and hTERT-immortalized periodontal ligament cells	Differentiation into periodontal ligament stem cell-like cells	Hamano et al. (2018)
	NSC-derived from ES cells	Neural differentiation	Yan et al. (2015)
	Retinal pigment epithelial cell line (ARPE19)	Retinal pigment epithelial differentiation	McLenachan et al. (2017)
MSCs	MSC-derived osteoblasts	Osteogenic differentiation	Datta et al. (2005, 2006)
	Chondrocytes	– Chondrogenic differentiation – Hypertrophy suppression	Cheng et al. (2009), Choi et al. (2010)
	MSCs differentiating at early osteogenic stage	– Osteogenic differentiation – Adipogenic differentiation suppression	Hoshiba et al. (2009, 2012b)
	MSCs differentiating at early adipogenic stage	– Adipogenic differentiation – Osteogenic differentiation suppression	Hoshiba et al. (2010, 2012b)
	MSCs differentiating at early chondrogenic stage	Chondrogenic differentiation (Also shown the suppression of chondrogenesis in dECM derived from MSCs differentiating at the late chondrogenic stage)	Cai et al. (2015)
	MSC	– Differentiation into hepatocyte-like cells – Decreasing intracellular ROS level	He et al. (2013)
NSCs	Embryonic fibroblasts	Differentiation into basal forebrain cholinergic neurons	Yang et al. (2017)

(continued)

Table 17.4 (continued)

Stem cell for culture	Cell sources for dECM preparation	Effects	References
	Glioma C6 cells	Differentiation into neural cells	Jian et al. (2015)
Myoblasts	Myoblasts differentiating at the early myogenic stage	Myotube formation	Hoshiba and Yokoyama (2020)
Tracheal basal cells	SV40-immortalized type II alveolar epithelial cells	Differentiation into ciliated cells, Clara cells, and mucous cells	Hosokawa et al. (2007)

trials to seed cells differentiated in vitro into specific cells into tissue-/organ-derived dECM have also been performed (Takeishi et al. 2020; Ghaedi et al. 2013).

17.3.1.2 Differentiation on Cultured Cell-Derived dECM

Cultured cell-derived dECM have also been applied for the differentiation culture of somatic stem cells and ES/iPS cells (Table 17.4). Cultured cell-derived dECM are mainly used for research purposes and in vitro applications, such as pharmacological studies. In contrast to tissue-/organ-derived dECM, many stem cells have been cultured on the dECM derived from cells that are different from differentiation target cells. These trials will help to find alternative tissue/organ dECM sources for stem cell differentiation. When NSCs were cultured on glioma C6 cell-derived dECM with an inhibitor of GSK-3β, SB216763, NSCs were promoted to differentiate into neural cells (neurons, astrocytes, and oligodendrocytes) (Jian et al. 2015). MSCs were cultured on chondrocyte-derived dECM to promote chondrogenesis without hypertrophy (Choi et al. 2010). In addition to somatic stem cells, ES and iPS cells were grown on cultured cell-derived dECM. When ES cells were cultured on pancreatic RIN5F cell-derived dECM, ES cells differentiated into insulin-secreting β cells (Narayanan et al. 2014).

dECM derived from cells that are different from differentiation target cells have been used for stem cell differentiation. NSCs were cultured on embryonic fibroblast-derived dECM, and the cells differentiated into basal forebrain cholinergic neurons (Bai et al. 2018). SV40-immortalized type II alveolar epithelial cell-derived dECM have been used for tracheal basal cell culture, and tracheal basal cells are differentiated into ciliated cells (Hosokawa et al. 2007). Pancreatic lineage commitment was promoted by dECM derived from both HEK293 cells expressing laminin-511 and 804G bladder carcinoma cells (Kaitsuka et al. 2014; Higuchi et al. 2010).

17.3.1.3 dECM Mimicking Native ECM at Stepwise Developmental Stages

Stem cell differentiation proceeds step-by-step in vivo, and the compositions of the ECM surrounding differentiating cells are changed according to their differentiation stages (Hoshiba et al. 2009, 2010, 2011b, 2012b; Cai et al. 2015). It is difficult for tissue-/organ-derived dECM to be prepared as dECM surrounding differentiating

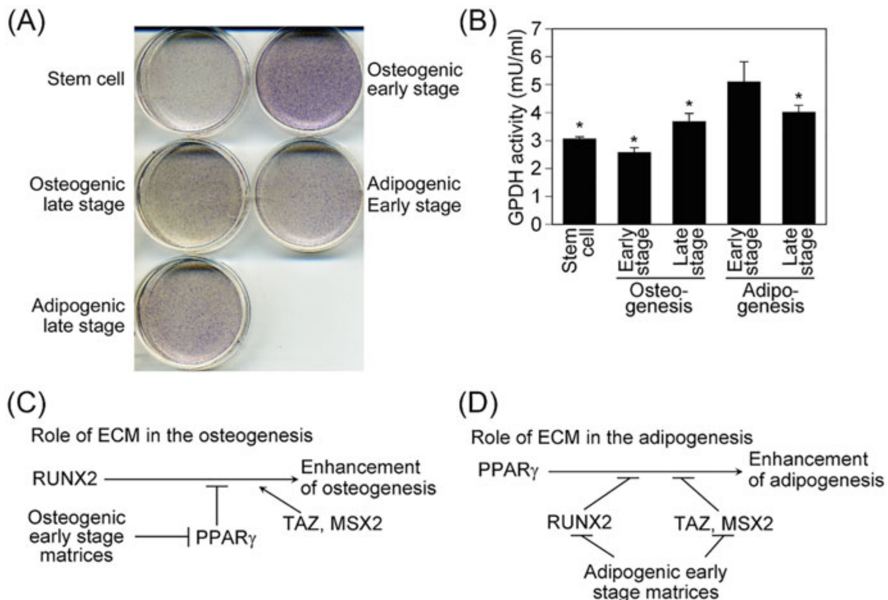

Fig. 17.3 Osteogenesis and adipogenesis of MSCs on stepwise osteogenesis-/adipogenesis-mimicking matrices. (**a**) Alkaline phosphatase staining of MSCs under osteogenic conditions on stepwise osteogenesis-/adipogenesis-mimicking matrices. (**b**) Glycerol-3-phosphate dehydrogenase (GPDH) activity in MSCs under adipogenic conditions on stepwise osteogenesis-/adipogenesis-mimicking matrices. Schematic models of the role of ECM in osteogenesis (**c**) and adipogenesis (**d**) of MSCs. (Reproduced with slight modification from the reference Hoshiba et al. 2012b by permission from Elsevier)

stem cells because such ECM is difficult to identify and isolate. In contrast to tissue-/organ-derived dECM, it is possible for cultured cell-derived dECM to be prepared as dECM mimicking native ECM surrounding differentiating stem cells. Hoshiba et al. prepared dECM derived from MSCs differentiating into osteoblasts and adipocytes at each differentiative stage, and these types of dECM are termed stepwise tissue development-mimicking matrices (stepwise osteogenesis-/adipogenesis-mimicking matrices) (Hoshiba et al. 2009, 2010). The osteogenesis of MSCs was promoted on dECM mimicking ECM at an early osteogenic stage (osteogenic early-stage matrices) but not on other dECM mimicking ECM at a late osteogenic stage (osteogenic late-stage matrices), adipogenic early- and late-stage matrices (adipogenic early/late-stage matrices), and undifferentiation state (stem cell matrices) (Fig. 17.3a) (Hoshiba et al. 2012b). On the other hand, adipogenesis of MSCs was promoted on adipogenic early-stage matrices but not stem cell matrices, osteogenic early/late-stage matrices, and adipogenic late-stage matrices (Fig. 17.3b) (Hoshiba et al. 2012b).

The mechanisms of MSC differentiation were examined from the viewpoint of transcription factor expression (Hoshiba et al. 2012b). The gene expression of an osteogenesis-promotive transcription factor, runt-related transcription factor 2 (*RUNX2*), was increased on dECM except for stem cell matrices to promote

osteogenesis. Gene expression of an osteogenic-inhibitory/adipogenesis-promotive transcription factor, peroxisome proliferator-activated receptor γ (*PPARG*), was inhibited on stem cell matrices and osteogenic early-stage matrices (Hoshiba et al. 2012b) by the suppression of canonical Wnt signaling via the binding between chondroitin sulfate and Wnt ligands (Hoshiba et al. 2009, 2011b, 2012b). These results suggest that osteogenic early-stage matrices promote osteogenesis by increasing osteogenic transcription factor expression and suppressing unexpected apoptosis (Fig. 17.3c) (Hoshiba et al. 2009, 2012b). Similar results were obtained in the case of adipogenesis. *PPARG* expression levels were similar on the dECM. However, *RUNX2* (suppressing adipogenesis) expression was suppressed on stem cell matrices and adipogenic early/late-stage matrices. Moreover, the expression of *TAZ*, an osteogenesis promotive and adipogenesis inhibitory factor, was suppressed on only adipogenic early-stage matrices. These results suggest that adipogenic early-stage matrices promoted adipogenesis by inhibiting osteogenesis (Fig. 17.3d) (Hoshiba et al. 2010, 2012b). Similar to the stepwise osteogenesis-/adipogenesis-mimicking matrices, stepwise myogenesis-mimicking matrices were prepared by differentiating myoblasts into myotubes (Hoshiba and Yokoyama 2020). Myotube formation was promoted on myogenic early-stage matrices by the suppression of inhibitor of DNA binding (ID) gene expression via the inhibition of bone morphogenetic protein (BMP) signaling activation. As demonstrated in these studies, cultured cell-derived dECM can be used as a suitable tool to examine the comprehensive roles of ECM in stem cell differentiation.

17.3.1.4 Future Problems in Stem Cell Differentiation Culture with dECM

As described above (Sect. 17.3.1.1), the cells exhibited opposite effects on stem cell differentiation on the dECM even though the dECM originated from the same source (Waele et al. 2015; Crapo et al. 2012). It is necessary to understand why the opposite effects were obtained; otherwise, unexpected side effects might occur for bioindustrial applications. However, the feasibility of dECM might be expanded to regulate stem cell differentiation if the effects of dECM can be changed by the preparation methods, cell types, and the provided form of dECM to the cells, differentiation culture conditions, etc.

There are many reports of decellularization methods, and their effects on the compositions and structures of dECM have been examined (Gilbert et al. 2006; Keane et al. 2015). The composition and structure impact stem cell differentiation. Therefore, the optimization and standardization of dECM preparation methods are required. In addition to the dECM preparation methods, the optimal methods for the preservation and sterilization of dECM should also be investigated. However, a limited number of studies have been performed on preservation (Fidalgo et al. 2018; Tsuchiya et al. 2014). Mechanistic analyses will be helpful for maximizing the effects of dECM. There are numerous reports of the mechanisms of stem cell differentiation by molecular biological methods. dECM can be modified to improve the functionalities of dECM by comparisons with these reports. In addition to molecular biological analyses, mass spectrometric analyses will improve the dECM.

17.3.2 Stemness Maintenance

Stemness is maintained in the stem cell niche in vivo (Mercier et al. 2002; Meran et al. 2017). Therefore, reconstruction of the ECM in the stem cell niche has been attempted by decellularization techniques. For this purpose, cultured cell-derived dECM has been mainly prepared to mimic native ECM in the stem cell niche.

17.3.2.1 Somatic Stem Cells

In this field, the maintenance of MSC stemness has been well investigated. Generally, MSCs spontaneously differentiate and lose their stemness during in vitro expansion culture. Spontaneous differentiation of MSCs is suppressed on undifferentiated MSC-derived dECM (Chen et al. 2007). Additionally, MSC differentiation is partially suppressed even under osteogenic and adipogenic differentiation conditions (Hoshiba et al. 2009, 2010). This suppression mechanism of MSC differentiation on the dECM is partially unveiled. BMP signaling is one of the triggers for MSC differentiation. However, activation of BMP signaling is inhibited by undifferentiated MSC-derived dECM (Chen et al. 2007; Hoshiba et al. 2009). It is speculated that BMP or BMP signaling inhibitory molecules (e.g., Chordin) are captured by ECM components, such as chondroitin sulfate, to regulate their accessibility to cells. In other words, BMP cannot activate intracellular signaling effectively when BMP is captured by the ECM and its accessibility to cells is suppressed. Alternatively, BMP signaling inhibitory molecules accumulate in the ECM via capture, leading to the effective suppression of BMP signaling.

Because the dECM can inhibit MSC differentiation, the dECM have been applied for in vitro MSC expansion culture. Chen and colleagues reported that undifferentiated MSC-derived dECM could suppress spontaneous differentiation and maintain the differentiation ability into osteoblasts and adipocytes after in vitro expansion culture (Chen et al. 2007). In particular, the differentiation ability of MSCs is maintained on undifferentiated MSC-derived dECM for a more extended period (i.e., higher passage number) than tissue culture polystyrene (TCPS). Similar to MSCs, other somatic stem cells, such as hematopoietic stem/progenitor cells (HSPCs) and umbilical cord blood-derived nonhematopoietic stem cells (UCB-NHSCs), have been cultured on the dECM. HSPCs were cultured on the bone marrow stromal cell line (MS-5)-derived dECM, and HSPCs showed effective expansion on the dECM with HSPC-specific surface markers (Tiwari et al. 2013). UCB-NHSCs were cultured on dECM derived from bone marrow stromal cells (containing MSCs) (Wu et al. 2016). UCB-NHSCs could be expanded on the dECM and exhibited differentiation ability into three germ layers, indicating the maintenance of pluripotency.

17.3.2.2 Pluripotent Stem Cells

Pluripotent stem cells, such as ES and iPS cells, have also been cultured on the dECM. Usually, these pluripotent stem cells are cultured on feeder cells (e.g., embryonic fibroblasts). Therefore, dECM have been prepared by the culture of these feeder cells. ES cells can be successfully grown on embryonic fibroblast-

derived dECM without feeder cells (Klimanskaya et al. 2005). Moreover, the ES cells possessed the ability to differentiate into three germ layers after passage culture for more than 6 months. Additionally, ES cells can be newly established on the dECM without feeder cells.

Recently, it has been reported that laminin-511/521 is important for the maintenance of pluripotent stem cells (Nakagawa et al. 2014). Therefore, cells producing abundant laminins can be used for the preparation of dECM for pluripotent stem cells. Vuoristo et al. prepared dECM with a human choriocarcinoma cell line, JAR, which produces abundant laminins and applied this JAR cell-derived dECM for iPS cell culture (Vuoristo et al. 2013). Additionally, they reported that iPS cells can be grown on JAR cell-derived dECM with the expression of undifferentiation markers and that the cells possess pluripotency. Moreover, they successfully prepared new iPS cells from fibroblasts on the JAR cell-derived dECM. Fibroblasts transfected with retroviruses containing *OCT4*, *KLF4*, *SOX2*, and *c-myc* were seeded on both JAR cell-derived dECM and Matrigel. The induction efficacy of iPS cells was similar between JAR cell-derived dECM and Matrigel. Prepared iPS cells could also be subcultured on JAR cell-derived dECM.

17.3.2.3 Future Problems in Stem Cell Maintenance Culture with dECM

Feasibility to Other Stem Cells

dECM have been mainly used for in vitro expansion and culture of MSCs. Currently, undifferentiated MSC-derived dECM is commercially available from StemBioSys, Inc. Although it is evident that dECM is helpful for in vitro expansion culture of MSCs with the maintenance of their stemness, the feasibility of dECM is not confirmed for other stem cell cultures. In particular, the passage numbers at which somatic stem cells can be subcultured are limited, and their stemness is lost during their in vitro expansion. Therefore, it is important for bioindustrial applications with stem cells to investigate whether the cells can be subcultured for a long period and whether their stemness is maintained.

Cell Sources for dECM Preparation

Tissue-/organ-derived dECM may not be suitable for stem cell maintenance culture because the concept of dECM for this purpose is to reconstitute the stem cell niche. Therefore, almost all dECM for this purpose are derived from cultured cells. These cell sources are often the same as the cells that are cultured on the dECM. This is a major problem to resolve because the availability of stem cells (particularly somatic stem cells) is limited. To solve this problem, the establishment of new cell lines is one approach (Kusuma et al. 2017). The other approach is to generate cells producing extrinsic ECM molecules. The development of alternative cell sources is a major challenge for dECM research.

Mechanisms

For bioindustrial applications alone, it is not necessary to unveil the mechanisms to maintain stemness on the dECM. However, the mechanisms will help to improve the

effectiveness of dECM. There are two approaches for the study of mechanisms: intracellular signaling analyses and ECM compositional analyses. Both are important. Intracellular signaling analyses, particularly upstream signaling that is linked with the ECM, will promote the understanding of how cells interact with the ECM. ECM compositional analyses will unveil important ECM components for stemness maintenance. If the ECM components are clarified, the development of alternative cell sources for dECM preparation will be accelerated.

17.3.3 Rejuvenation of Somatic Stem Cells

Recently, increasing evidences have demonstrated the rejuvenation of somatic stem cells by dECM (Fig. 17.4).

17.3.3.1 Rejuvenation of Passaged Stem Cells

Many somatic stem cells lose their differentiation ability after in vitro expansion. There are several trials to recover their differentiation ability using dECM derived from cells with lower passage numbers (Pei et al. 2011; Lai et al. 2010). Pei et al. cultured passage 5 MSCs on the dECM derived from undifferentiated MSCs (< passage 5), and their MSC functions on the dECM were compared with those on TCPS (Pei et al. 2011). MSCs recovered their growth ability and the expression of stage-specific embryonic antigen-4 (SSEA-4) on the dECM (Lai et al. 2010). Moreover, the intracellular reactive oxygen species (ROS) level was lower on the dECM than on the TCPS. Differentiation ability was also checked. Chondrogenic and osteogenic capabilities of MSCs expanded on the dECM were at higher levels than those on TCPS. On the other hand, the adipogenic ability of MSCs expanded on the dECM was lower than that on TCPS. These results suggest the possibility of dECM rejuvenating or recovering stem cells whose abilities were lost.

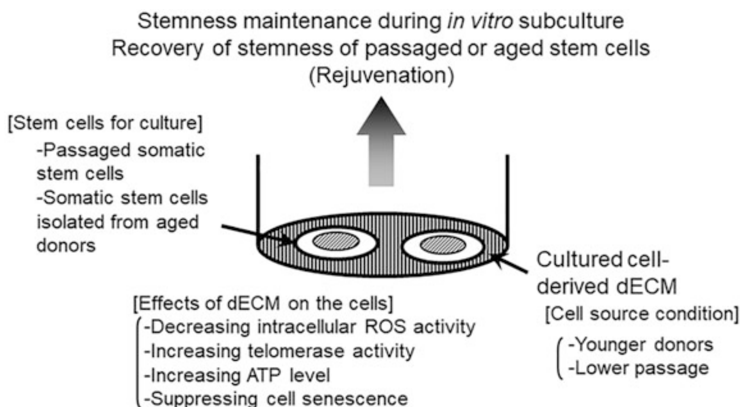

Fig. 17.4 The stemness maintenance during the in vitro subculture

17.3.3.2 Rejuvenation of Stem Cells Isolated from Aged Donors

The differentiation ability of somatic cells is also lost by individual aging; that is, the differentiation ability of stem cells isolated from aged donors is lower than that of stem cells isolated from younger donors. Stem cells isolated from aged donors have been cultured on dECM derived from more immature cells (Ng et al. 2014; Sun et al. 2011). Ng et al. cultured MSCs isolated from adult donors on the dECM derived from undifferentiated fetal and adult MSCs and fibroblasts (Ng et al. 2014). Adult MSC growth was promoted on the dECM derived from fetal MSCs (passages 3 and 4) compared with dECM derived from adult MSCs (passage 3) and fibroblasts and TCPS. Additionally, the cell size (increasing cell size is a cell senescence phenomenon) was smaller on the dECM derived from fetal MSCs (passage 3) than on the dECM derived from adult MSCs (passage 3) and fibroblasts and TCPS. Moreover, the differentiation ability of adult MSCs tended to be higher on dECM derived from fetal MSCs (passage 3) than on dECM derived from adult MSCs (passage 3) and fibroblasts and TCPS.

Sun et al. also prepared dECM derived from younger and aged donors (younger and aged dECM) and cultured MSCs isolated from younger and aged donors (younger and aged MSCs) on these dECM (Sun et al. 2011). Aged MSCs possessed osteogenic differentiation ability on younger and aged dECM even after in vivo expansion culture, although the aged MSCs lost osteogenic differentiation ability on TCPS. Comparing younger and aged dECM, both younger and aged MSCs on younger dECM exhibited higher osteogenic differentiation ability than on aged dECM. Additionally, they examined intracellular ROS levels. Intracellular ROS levels were lower on younger dECM compared with aged dECM and TCPS. Moreover, a younger dECM increased telomerase activity and ATP levels in both younger and aged MSCs. Finally, they checked the osteogenesis in vivo. The osteogenesis levels of aged MSCs were similar to those of younger MSCs when the cells were expanded on younger dECM. In contrast, aged MSCs exhibited lower osteogenic levels than younger MSCs when the cells were expanded on TCPS. These reports demonstrate that dECM derived from MSCs isolated from younger donors can rejuvenate the stemness of MSCs isolated from aged donors.

Similar to MSCs, some somatic stem cells have tried to rejuvenate with dECM. Aged tendon stem cells were cultured on the dECM derived from younger tendon stem cells (Jiang et al. 2018). Senescence-associated (SA) β-galactosidase activity was decreased in aged tendon stem cells on the dECM derived from younger stem cells to a level similar to that of younger tendon stem cells. Additionally, the gene expression levels of octamer-binding transcription factor 4 (Oct-4), stage-specific embryonic antigen-1 (SSEA-1), tenomodulin, and scleraxis recovered to levels similar to those of younger tendon stem cells on the dECM derived from younger tendon stem cells. Similar results were obtained for synovium-derived and urine stem cells (Li et al. 2014; Pei et al. 2014).

17.3.3.3 Future Problems for Stem Cell Rejuvenation with dECM

Studies of somatic stem cell rejuvenation by dECM have just begun. Therefore, there are many unclarified points. For example, there are few somatic stem cells which

were proved for rejuvenation with dECM, although various types of somatic stem cells exist in the body. It is unclear whether dECM can lead to the rejuvenation of other somatic stem cells. The feasibility of rejuvenation by dECM should be examined for other stem cells. Additionally, it is not clear which types of dECM can rejuvenate stem cells. There are various points to prepare cultured cell-derived dECM: donor age (including fetal donors), passage number, and cell types (e.g., stem cells or non-stem cells). At this point, it seems that younger stem cells with lower passage numbers are suitable to prepare dECM for rejuvenation. Additionally, it appears that aged or multiple-passaged stem and non-stem cells are not ideal for this purpose (Ng et al. 2014). Mechanism analyses have almost never been performed. Several studies have focused on intracellular ROS levels, ATP levels, and telomerase activity (Sun et al. 2011; Pei et al. 2011). However, it is unclear how these parameters are modulated by the dECM.

17.4 Future Perspectives of dECM for Stem Cell-Based Bioindustrial Applications

It has become clear that the dECM can regulate more functions of stem cells than previously thought. Therefore, there seem to be many possible applications of dECM.

17.4.1 Research Use

The dECM can be used as in vitro ECM models for comprehensive studies of ECM roles in the regulation of stem cell functions (Hoshiba et al. 2016). Single isolated ECM molecules are examined to clarify the roles of the ECM in the regulation of stem cell functions, although the ECM is composed of many molecules, and these molecules are orchestrated to activate intracellular signaling pathways to regulate stem cell functions. However, it is difficult to understand how each effect given by each ECM molecule is orchestrated and how the total effects influence stem cell functions by studies with only single ECM molecules. dECM can be helpful as an experimental control to understand how the ECM influences stem cell functions.

17.4.2 In Vitro Applications

As described above, stem cells show unique functions in the dECM. Therefore, the dECM are useful substrates for stem cell culture. Indeed, undifferentiated MSC-derived dECM is now commercially available from StemBioSys as CELLvo™. Additionally, tissue-/organ-derived dECM is commercially available from several companies (such as Xylyx Bio). They claimed that these dECM could be used for the 3D culture of stem cells. However, reports of subsequent dECM applications are very limited because in vitro applications of dECM in bioindustries

have just started. Further trials and application reports are required for their broader applications.

17.4.3 In Vivo Clinical Applications

Today, dECM have been used in the clinic, and many dECM products for clinical use are commercially available (Nakamura et al. 2017; Crapo et al. 2011). However, clinical applications of dECM with stem cells seem to remain under research. This seems to be due to the lack of dECM sources, particularly tissues and organs, and the difficulty of preparation without a large batch-to-batch difference. The solutions for these problems are required. Moreover, a few studies have been performed from the viewpoints of clinical applications. As described above, dECM showed unique possibilities, for example, site-specific differentiation and the suppression of unexpected differentiation (including hypertrophy during chondrogenesis). Additionally, clinical applications with stem cells are still developing. Therefore, it is expected that clinical applications with stem cells and dECM will be expanded in the future.

17.5 Conclusions

Almost all dECM for stem cell culture are now at the research level. In the past decade, researches on dECM for stem cell culture focused on the effects of dECM on stem cell functions as described above. These studies have unveiled the unique effects of dECM on stem cell functions, particularly differentiation, stemness maintenance, and rejuvenation. On the other hand, a few applications of these dECM are commercially available, although stem cells exhibit unique functions on the dECM. This is due to the difficulty of preparation and small dECM source supplementation. These problems should be solved to expand the feasibility of dECM for stem cell-based bioindustrial applications. Moreover, more application studies of dECM are required as well as basic researches in the next decade. Then, methods will open for stem cell-based bioindustrial applications in the future.

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Fabrication of Chitosan-Based Biomaterials: Techniques and Designs 18

Abrar Ali Sitab, Jakiya Sultana Joya, Jyoti Prokash Barman, Shanta Biswas, and Taslim Ur Rashid

Abstract

In recent decades, chitosan is emerging as a promising functional biopolymer for the fabrication of biomaterials in various forms due to having immense structural possibilities for chemical and mechanical modification and superior biocompatibility, biodegradability, antimicrobial activity, and nontoxic properties. Such extraordinary properties make chitosan-based biomaterials suitable for several biomedical applications, especially for tissue engineering, wound healing, drug delivery, gene delivery, regenerative medicine, etc. Biomaterials for these applications are designed into desirable forms such as scaffolds, sponges, gels, films, particles, and so on. These forms can be fabricated by various techniques such as solvent casting, electrospinning, emulsification, lyophilization, 3D printing, gelation, cross-linking, etc. Researchers are incessantly modifying these fabrication techniques to elicit remarkable functional properties from these available forms. This chapter highlights a detailed study on available techniques and designs of fabricating biomaterials tailored with chitosan and its composites.

Keywords

Chitosan · Biomaterials · Fabrication techniques · Designs

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18.1 Introduction

In recent years, the progress in the field of biomaterials has galvanized the researchers to study for enhancing biocompatibility and bioactivity of materials to be used in biomedical applications concentrating on their properties, benefits, limitations, and alternative resources (Jovic et al. 2019; Rao et al. 2020; Samadian et al. 2020). A material having desired biocompatibility, biodegradability, nontoxicity, cell affinity, sustainability, mucoadhesivity, and biosensitive properties is a suitable candidate for using in biomedical purposes. For fabricating biomaterials, currently, biopolymers have availed much attention than other types of materials due to their minimal toxicity, controlled reactivity, and huge abundance in nature, along with no environmental issues. Among the biopolymers commonly used in biomedical applications, chitosan has garnered scientific and economic interest owing to its prominent features.

Chitosan is a β-(1,4)-D-glucosamine copolymer derived from chitin which is the most widely found polysaccharide after cellulose (Fourie et al. 2020). This deacetylated form of chitin is usually extracted from the exoskeleton of crustacean shells, mollusks, insects, silkworm chrysalides, and microorganisms (Rufato et al. 2018). Chitosan is renowned for its lucrative characteristics, including modifiable chemical structure, antimicrobial activity, nontoxicity, biocompatibility, biodegradability, hydrophilicity, cell affinity, non-antigenicity as well as bioadherence (Kumar et al. 2009). Besides, its degradation generates inoffensive and absorbable amino sugars for the human body, and the modified structures of chitosan impart superior elasticity and flexibility along with low inflammatory responses (Gu et al. 2013). These appealing properties make chitosan preeminent for diversified biomedical applications such as tissue engineering, wound healing, systematic and local drug delivery, gene delivery, and so on (Islam et al. 2017, 2020; Nasrin et al. 2017).

Physicochemical modifications of chitosan through composite formation provoke their bioactivity as biomaterials. Thus far, native chitosan or combining chitosan with other polymers, nanoparticles, and bioceramics has been designed in the form of scaffolds, sponges, gels, particles, beads, films, membranes, etc. (Ali Khan et al. 2020). Among them, scaffolds are commonly required in tissue engineering and tissue regeneration applications, whereas gels, micro-, and nanoparticles are used as drug carriers (Parhi 2020; Sangkert et al. 2020). Furthermore, sponges, films, and membranes are applied in wound healing (Moeini et al. 2020). These functional forms can be fabricated by several common and widely used techniques, including solvent casting, spray-drying, electrospinning, 3D printing, emulsification, lyophilization, foaming, gelation, and cross-linking (Balagangadharan et al. 2017; Naskar et al. 2019; Sahranavard et al. 2020). Fabrication techniques are the major concerns for designing biomaterials because particle size, shape, morphology, porosity, mechanical strength, and adhesive properties of the obtained forms are strongly dependent on the procedures and parameters of the techniques.

As far as authors' best knowledge, many review articles and book chapters reported on the biomedical applications of chitosan. Still, none of them focused on fabrication techniques and designs of chitosan-based biomaterials (Kalantari et al.

2019; Kravanja et al. 2019; Ding et al. 2020; Islam et al. 2020; Jana and Jana 2020). Fabrication techniques and designs play the principal role in implementing chitosan for biomedical purposes. Research in this area has blossomed rapidly, and it is still a fast-growing field of study. Considering the importance of these researches, this chapter aims to agglomerate the advancements in fabrication techniques and designs of chitosan-based biomaterials. In the beginning, the chapter discusses the structure, properties, and extraction modes of chitosan. Afterward, a detailed overview of various fabrication techniques and designing methods of the available forms of chitosan-based biomaterials has been presented. In the end, some suggestions have been provided for future research directions based on the current challenges.

18.2 Chitosan

Chitosan is the second-longest naturally occurring polysaccharide obtained from chitin which is available in various plants, animals, and other nonconventional sources (Islam et al. 2020). Mostly, the exoskeletons of crustaceans, mollusks, insects, fishes, etc. are the key sources of chitin which is further converted into chitosan via various means of processing (Rahman et al. 2015). Moreover, it can be directly extracted from fungi. Chemically, chitosan is a polysaccharide composed of β-(1 → 4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units (Fig. 18.1). Chitosan is a suitable candidate for biomaterials fabrication because of its abundance, versatility, and unique properties like biodegradability, biocompatibility, nontoxicity, hydrophilicity, antimicrobial activity, and wound-healing effects

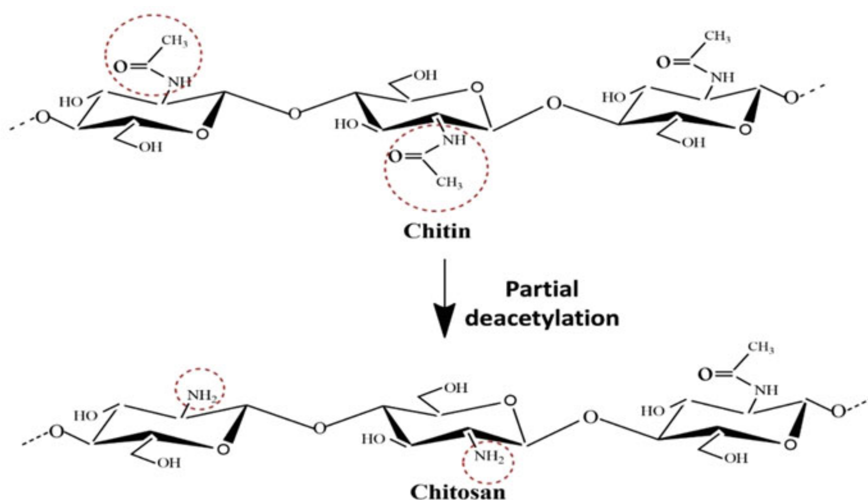

Fig. 18.1 Structure of chitin and chitosan. In chitin, *N*-acetyl-D-glucosamine units are in abundance compared to glucosamine, whereas, in chitosan, the glucosamine unit dominates which is obtained by the deacetylation (partial or complete) of chitin

(Islam et al. 2020). In addition, the eco-friendly behavior of chitosan makes this topic more featured and illustrated to future researchers.

18.2.1 Mode of Extraction and Properties

Chitosan extraction begins with the treatment of chitins. The most common method of isolation of chitin followed by chitosan extraction is based on the chemical method. Figure 18.2 illustrates different pathways of chitosan extraction. Generally, chitosan extraction needs to follow three basic steps, (a) demineralization, (b) deproteinization, and (c) deacetylation. Demineralization refers to eliminating inorganic matter (mainly calcium carbonate, calcium chloride) using dilute acids, most commonly dilute hydrochloric acid (HCl). The acid-to-material ratio, concentration of acid, duration of treatment, and process conditions depend on the presence of minerals in the sample. For example, cuttlefish pens are usually demineralized with 1 M HCl, whereas other species of crustaceans need 0.25 M HCl (Al Sagheer et al. 2009). In most common practice, the dried sample of the exoskeleton of crustaceans (prawn shell, shrimp shell, etc.) is demineralized with 1 M HCl at a ratio of 1:16 (w/w) with stirring for 3 h (Rashid et al. 2012; Teli and Sheikh 2012). In all cases, the emission of CO_2 can be an indicator of the demineralization process.

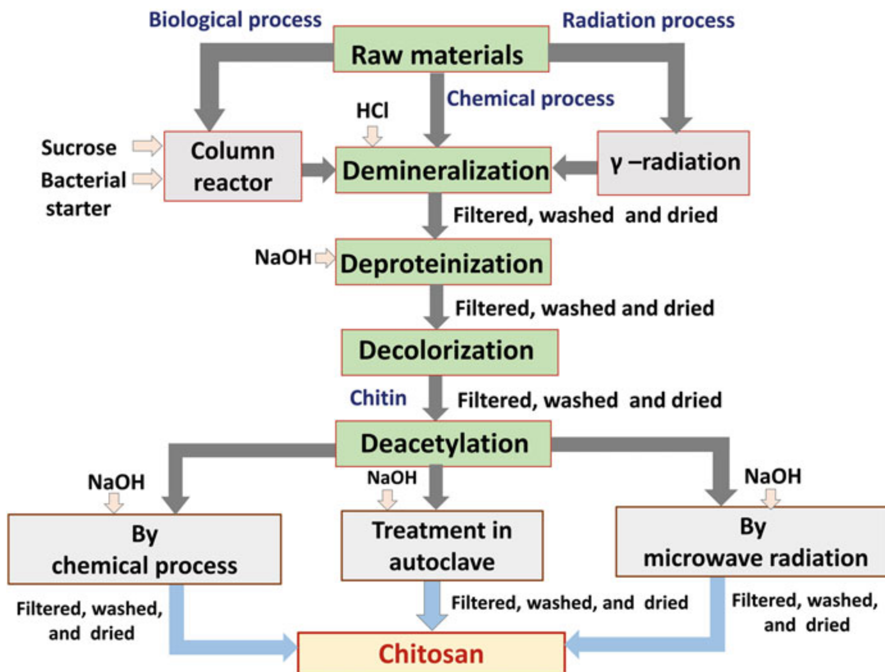

Fig. 18.2 Flow diagram of extraction of chitosan by different methods

Deproteinization is traditionally carried out by an aqueous solution of a strong alkali like NaOH or KOH. The effectiveness of alkali-based deproteinization mainly depends on the process temperature (65–105 °C) and alkali concentration. The alkali treatment is carried out repeatedly several times until the color of the solution is completely removed. The product is washed with deionized water to remove excess alkali and dried to obtain chitin. Furthermore, if there is any color in the chitin, an additional decolorization step is performed to eliminate pigments like astaxanthin and β-carotene by using various organic and inorganic solvents such as sodium hypochlorite, acetone, and hydrogen peroxide under variable process conditions (El Knidri et al. 2018). Besides that, direct oxidation of the chitin can also remove the color of the product. For example, chitin isolated from squid pens is white, whereas chitin from other sources is highly pink. Pigments, which are responsible for this pink color, are removed by a multicomponent-based oxidizing treatment ($KMnO_4$, oxalic acid, H_2SO_4). Moreover, refluxing in ethanol for several hours can also eliminate traces of protein and coloring materials from chitin (Abdou et al. 2008). Deacetylation is the final step where the chitins are converted into chitosan by treating with a strong alkali. It can be done using the microwave, autoclave, and traditional methods (No et al. 2000; Sedaghat et al. 2017). In the conventional process, the extracted chitin needs to be treated with a concentrated NaOH solution. In the case of using microwave radiation, the mixture of extracted chitin and 45–50% (w/v) NaOH is placed in a conical flask which is covered tightly and exposed to microwave radiation (Al Sagheer et al. 2009; Kumari and Rath 2014). Following the radiation process, the mixture is cooled, filtered, washed to neutral pH, and freeze-dried. In the autoclave method, the chitin is treated with NaOH solution at elevated temperature and pressure in an autoclave. According to a recent report, the highest yield of chitosan is obtained with the autoclave method and the lowest observed with the microwave method (Sedaghat et al. 2017). Chitosan extraction can also be done by introducing the sample to γ radiation (Rashid et al. 2012).

Chitosan is also extracted via the biological process. According to the method of Pacheco et al., shrimp waste is initially mixed with 10% (w/w) sucrose and bacterial starter (5% v/w) and then kept in a column reactor for 144 h at 35 °C for incubation (Pacheco et al. 2011). Noted that glucose can also be used as an alternative to sucrose (Sedaghat et al. 2017). After this fermentation process, the product is treated under mild conditions with acid and alkali solution to eliminate the remaining minerals and proteins. Finally, deacetylation of chitin takes place (Fig. 18.2) (Lamarque et al. 2005). However, in the biological process, demineralization is done by lactic acid produced by bacteria; deproteinization performed with enzyme proteases; decolorization by acetone or organic solvent; and deacetylation process by using bacteria under enzymatic environment (El Knidri et al. 2018). The chitosan obtained is dried and purified by dissolving in aqueous 2% (w/v) acetic acid (Islam et al. 2020). The insoluble part is eliminated via filtration, and the rest of the solution is neutralized with NaOH solution, which gives a white precipitate of purified chitosan. The final form of chitosan can also exist in the form of white to yellowish flakes that can be converted into beads or powders.

The protonated amino groups of chitosan at specified conditions can subsequently form ionic complexes with anionic moieties of different natural and synthetic substances such as lipids, proteins, DNA, etc. Besides, the amino and hydroxyl groups of chitosan form stable covalent bonds through esterification, etherification, and reductive amination reactions (Ibrahim and El-Zairy 2015). Chitosan behaves as a cationic electrolyte in an aqueous acidic solution when the degree of deacetylation (DD) is higher than 0.5 (Muzzarelli and Rocchetti 1985; Tolaimate et al. 2003). The DD and molecular weight of chitosan determine the physicochemical properties and application fields of chitosan. These two fundamental characteristics of chitosan depend on the sources and extraction methods. Table 18.1 shows the variation in DD of chitosan which is based on their sources and extraction methods.

18.2.2 General Biomedical Applications

The diversity of chitosan as a biomaterial depends on its significant properties at the time of interaction with the human body. Chitosan possesses some attractive properties such as biodegradability, biocompatibility, natural specific reactivity, and nontoxicity. These behaviors of chitosan make it a promising biopolymer to have a variety of applications in the field of medical, agriculture, food processing, nutritional enhancement, electrochemical devices, cosmetics, and waste treatment (Rahman et al. 2013; Rashid et al. 2014, 2017; Dey et al. 2016; Nasrin et al. 2017; Biswas et al. 2020, 2021; Hasan et al. 2020). It is also used in some sophisticated applications such as contact lenses, tissue adhesive, preventing bacterial adhesion, sutures, etc. (Zargar et al. 2015). Furthermore, chitosan becomes positively charged in acidic media, which develops strong electrostatic interaction with oppositely charged moieties. This characteristic is responsible for mucoadhesion, controlled drug release, transfection, in situ gelations, efflux pump inhibition, and permeation enhancement making chitosan to be considered as one of the most important polysaccharides for drug delivery application (Abd Elgadir et al. 2015; Choi et al. 2016; Islam et al. 2017). The strong electrostatic interaction of chitosan with negatively charged mucosal surfaces or other macromolecules such as genetic materials makes chitosan a good carrier for gene delivery in order to accelerate transfection efficiency and protect genetic materials against nuclease (Choi et al. 2016). Chitosan is also used as a wound-healing accelerator especially in the case of osteopontin and leukotriene B4, transforming growth factor b1, platelet-derived growth factor, and fibroblasts. It enhances the functions of inflammatory cells, retains biological activity, and affects the macrophage function that facilitates wound healing (Abd Elgadir et al. 2015). Due to the exceptional binding capacity and chelation ability, it is also equally important as a binder to cholesterol, fats, and metal ions under certain physiological conditions. Furthermore, the diversified nature of hydrophilicity and hydrophobicity upon forming the derivatives makes these features to be more convenient for future researchers.

Table 18.1 Dependency of DD of chitosan on sources and extraction methods

Source of chitosan	Example	Mode of extraction	DD%	References
Crustaceans	Shrimp shell (*Penaeus merguiensis*)	Chemical	88	Sedaghat et al. (2017)
	Shrimp shell (*Metapenaeus monoceros*)	Biological	96	Younes et al. (2012)
	Shrimp shell (*Penaeus monodon*)	Chemical	74	Marei et al. (2016)
	Peregrine shrimp waste (*Metapenaeus stebbingi*)	Chemical	92	Kucukgulmez et al. (2011)
	Antarctic krill (*Euphausia superba*)	Chemical	11	Wang et al. (2013c)
Insects	Honey bee (*Apis mellifera*)	Chemical	96	Marei et al. (2016)
	Beetles (*Calosoma rugosa*)	Chemical	95	Marei et al. (2016)
	Dung beetle (*Catharsius molossus*)	Chemical	95	Ma et al. (2015)
	Desert locust (*Schistocerca gregaria*)	Chemical	98	Marei et al. (2016)
	Larvae: superworm (*Zophobas morio*)	Chemical	74	Soon et al. (2018)
Fungal sources	*Penicillium chrysogenum*	Chemical	86	Dhillon et al. (2013)
	Basidiomycetes (*Agaricus* sp.)	Chemical	82	Dhillon et al. (2013)
	Zygomycete fungi (*Rhizomucor miehei*)	Chemical	81–99	Tajdini et al. (2010)
	M. racemosus	Chemical	84–97	Tajdini et al. (2010)
Fish	Fish scales (*Labeo rohita*)	Chemical	78	Muslim et al. (2013)
Mollusks	Squid gladius (*Loligo vulgaris*)	Biological	71	Abdelmalek et al. (2017)
	Squid pens (*Doryteuthis* spp.)	Biological and chemical	96	Fiamingo et al. (2016)
	Cuttlefish bones (*Sepia officinalis*)	Chemical	70	Abdelmalek et al. (2017)

18.3 Fabrication Techniques for Chitosan-Based Biomaterials

Biomaterials are substances that interact with biological systems, especially for medical purposes such as enhancing and repairing tissue functions, drug delivery, and diagnostic activities. These should possess biocompatibility, biodegradability, nontoxicity, cell affinity, sustainability, mucoadhesivity, and biosensitive features

for biomedical applications. Chitosan-based biomaterials are designed in varieties of forms, and these designs depend on fabrication techniques. Several techniques are employed for fabricating biomaterials, such as solvent casting, spray-drying, electrospinning, gelation, 3D printing, emulsification, electrophoretic deposition, etc. Thus far, these techniques are exhibiting high efficacy in fabricating various forms of chitosan-based biomaterials.

18.3.1 Solvent Casting

Solvent casting is the most inexpensive and most straightforward technique for the fabrication of plain or structured chitosan substrates. The two most common routes to casting are either by using a 2D surface or a 3D mold for producing different forms of structure. Casting the polymer solution on a 2D surface (e.g., glass petri dish) yields films/membranes, whereas casting the solution on a 3D mold produces scaffolds. To fabricate films/membranes by solvent casting, chitosan is first dissolved in aqueous acetic acid with stirring. Some fillers, reinforcing agents, or other additives are then added to the solution to attain desired properties. Finally, the solution is poured on a petri dish, and a film/membrane is obtained by evaporating the solvent from the solution in atmospheric condition or in the oven at elevated temperature (Fig. 18.3a).

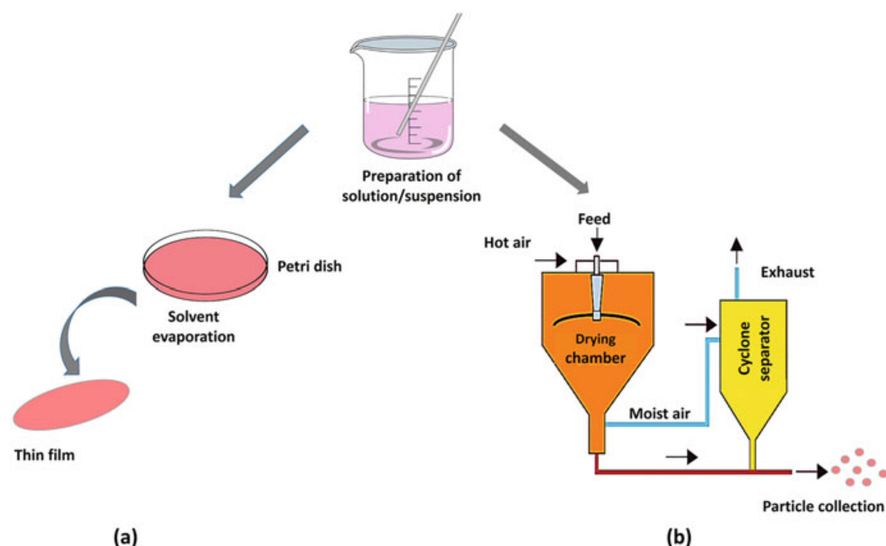

Fig. 18.3 Schematic diagram of fabrication techniques of chitosan-based biomaterials: (**a**) solvent casting and (**b**) spray-drying. (**a**) Prepared chitosan-based solution/suspension is being poured on a petri dish and dried to obtain the thin film. (**b**) The solution/suspension is being fed to a drying chamber where hot air evaporates solvent from liquid droplets of solution/suspension and particles are being collected at the bottom with the assistance of a cyclone separator

Table 18.2 A list of chitosan-based biomaterials fabricated by solvent casting and spray-drying techniques

Fabrication technique	Materials used	Final form	Application	References
Solvent casting	Chitosan, glucose oxidase, graphene, Au nanoparticles	Nanocomposite films on gold electrode	Glucose biosensing	Shan et al. (2010)
	Chitosan, distamycin, and vancomycin	Films	Preventing infection	Smith et al. (2010)
	Chitosan, aloe vera gel, glycerol	Membranes	Wound dressing	Silva et al. (2013)
	Chitosan, Au nanoparticles	Nanocomposite films	Antimicrobial coating	Regiel-Futyra et al. (2015)
	Chitosan, Tween 80, *Hypericum perforatum*	Films	Wound healing	Güneş and Tıhmınlıoğlu (2017)
	Chitosan, poly(vinyl pyrrolidone), nanocellulose	Nanocomposite films	Wound dressing	Poonguzhali et al. (2017)
	Chitosan, konjac glucomannan	Bilayer films	Wound dressing	Neto et al. (2019)
Spray-drying	Chitosan, ethyl cellulose, genipin, rifabutin	Complex microspheres	Antituberculosis drug delivery	Feng et al. (2013)
	Chitosan, Fe_3O_4, halloysite nanotubes, ofloxacin	Microspheres	Drug delivery	Wang et al. (2013b)
	Chitosan, verapamil hydrochloride	Microspheres	Intranasal drug delivery	Mouez et al. (2014)
	Chitosan, glutaraldehyde, polymeric antigen BLSOmp31	Microspheres	Nasal mucosal vaccination in sheep against brucellosis	Díaz et al. (2016)
	Chitosan, sodium tripolyphosphate, venlafaxine hydrochloride	Microparticles	Drug delivery	Aranaz et al. (2017)
	Carboxymethyl chitosan, ZnO nanoparticles	Microspheres	Antibacterial applications	Zhong et al. (2018)
	Chitosan, TiO_2 nanoparticles, glutaraldehyde	Microspheres	Sunblocking applications on skin	Morlando et al. (2018)

Chitosan-based composite films/membranes fabricated by solvent casting have various biomedical applications such as wound dressing, bone regeneration, bone substitution, etc. (Table 18.2). For example, Bakhtiari et al. developed chitosan/multiwall carbon nanotube (MWCNT) composite films for application as a bone

substituting agent (Bakhtiari et al. 2019). Firstly, they dissolved chitosan in a 2% acetic acid solution and then dispersed MWCNTs in the solution as a reinforcing agent. After homogenizing the solution, it was cast on a petri dish. At last, the film was obtained by drying in a vacuum oven at 60 °C for 24 h. Here the incorporation of MWCNTs improved thermal stability and bioactivity and reduced the degradation rate of the films. Hernández-Rangel et al. also used similar technique to fabricate chitosan/silver nanocomposite films for wound dressing (Hernández-Rangel et al. 2019). They added $AgNO_3$ into the chitosan solution and stirred it at 95 °C until the colorless solution was converted into light yellow due to the formation of Ag nanoparticles. Finally, the suspension was poured into a petri dish and dried at 60 °C for 12 h to obtain chitosan/silver nanocomposite film, which showed augmented antibacterial activity. However, uniform dispersion of Ag nanoparticles throughout the film is vital to maintain the high antibacterial activity. Tu et al. fabricated nano-hydroxyapatite/chitosan membranes for bone regeneration (Tu et al. 2017). For this purpose, they prepared a nano-hydroxyapatite slurry and then added chitosan solution to the slurry. After adjusting pH to 5 and stirring, they poured the mixture on a glass plate and dried it at 60 °C in a vacuum oven. At last, the membrane was detached from the plate by immersing into 1 M NaOH solution and washed with distilled water with subsequent drying. The membrane exhibited the ability to mineralize osteoblasts and calcify loose bone for accelerating bone regeneration.

Solvent casting can also be used to fabricate chitosan-based scaffolds. For this purpose, chitosan is first dissolved into a suitable organic solvent (e.g., aqueous acetic acid), and salts are dissolved in the same solution based on the prefixed polymer to salt ratio. Casting can usually be done by any of the two routes, i.e., (a) immersing the mold into the polymeric solution or (b) pouring the polymeric solution into the mold (Balagangadharan et al. 2017). In either way, after providing enough time for the solution to be cast on the mold, the solvent is removed by simple evaporation, vacuum drying, or lyophilization. After removal of the solvent, the salt is leached out by distilled water which leaves a porous structure on the scaffolds (Liao et al. 2002). Finally, the porous scaffolds are peeled off the mold.

Solvent casting is the simplest one among all techniques used for biomaterial fabrication. However, it is not suitable for the complex design of biomaterials. Moreover, it is challenging to maintain the desired porosity of the scaffold in solvent casting techniques. Porosity is controlled to an extent by selecting suitable salt and maintaining a required polymer to salt ratio. However, the uniform dispersion of salt throughout the polymer solution is challenging because the degree of contact between the salt particles is not precisely controllable and also due to the differences in the density of the liquid polymer solution and solid salts (Murphy et al. 2002). As a result, a highly interconnected porous structure that facilitates copious cell-to-cell interaction and cell migration cannot be achieved by solvent casting. Additionally, it becomes difficult to leach out the salt particles totally because particles get wrapped completely by the polymer matrix (Sin et al. 2010).

18.3.2 Spray-Drying

Spray-drying is a familiar technique in which fluid materials (solution, emulsion, or free-flowing slurry) are converted to dried particles by spraying the fluid feed to a hot drying medium. Though spray-drying is often considered as a dehydration technique, it can also be used for encapsulating hydrophilic and hydrophobic active compounds within various carriers. Usually, non-cross-linked and cross-linked chitosan-based microspheres and nanoparticles are produced by the spray-drying method (He et al. 1999).

The overall spray-drying method consists of three major phases: (a) atomization, (b) droplet-to-particle conversion, and (c) particle collection (Santos et al. 2017). Before the atomization phase, a feed solution is prepared by dissolving pure chitosan powder in an aqueous acetic acid solution while stirring (Naskar et al. 2019). For preparing cross-linked chitosan microspheres and nanospheres, cross-linking agents such as glutaraldehyde (Katsarov et al. 2018), formaldehyde (He et al. 1999), tripolyphosphate (Desai and Park 2005), genipin, citric acid (Naskar et al. 2019), etc. are added to the feed solution. Then, the solution is pumped and sprayed into the drying chamber through a nozzle (atomizer), and upon exiting from the nozzle tip, the droplets are atomized. Subsequently, the droplets come in contact with hot air and are converted into particles by evaporation of the solvent (Naskar et al. 2019). The particles are then collected at the bottom of the dryer by a cyclone separator or electrostatic particle collector. Figure 18.3b illustrates the overall process of the spray-drying technique. Nozzle size of the spray-dryer plays the main role in determining the types of the particles, i.e., nanoparticles and microparticles. For instance, nanoparticle fabrication can be done by a nano spray-dryer (4 or 7 μm nozzle) (Demir and Değim 2013), whereas microparticle fabrication can be done by a mini spray-dryer (700 μm nozzle) (Feng et al. 2019).

Spray-drying can be used to fabricate nanocomposite microspheres. Zhong et al. prepared carboxymethyl-chitosan/ZnO nanocomposite microspheres with enhanced biocompatibility for antibacterial purposes by spray-drying method (Zhong et al. 2018). They dissolved carboxymethyl-chitosan in deionized water and then dispersed ZnO nanoparticles into the solution by sonication. Finally, the solution was fed to a spray-dryer. Morlando et al. also prepared chitosan and chitosan/TiO_2 nanocomposite by spray-drying (Morlando et al. 2018). They dissolved chitosan and photocatalyst TiO_2 powder in 3% (v/v) aqueous acetic acid and stirred overnight. Then the solution was spray-dried at a flow rate of 100 mL/h. Later, particles were cross-linked by glutaraldehyde via the vapor phase process in a heated vacuum desiccator for 48 h at 25 °C. Since the photocatalytic activity of TiO_2 was suppressed in the particles, this nanocomposite could be used for sunblocking applications. Similarly, Ruphuy et al. prepared nano-hydroxyapatite/chitosan composite microparticles (Ruphuy et al. 2016). They prepared chitosan solution with an acetate buffer of pH 5.5 and added aqueous nanodispersions of nano-hydroxyapatite in it by fast stirring. Subsequent spray-dried microparticles mimicked bone composition.

Spray-drying is also a well-known method for the fabrication of drug-loaded chitosan-based microspheres. Desai et al. produced drug-loaded

chitosan-tripolyphosphate microspheres by spray-drying (Desai and Park 2005). They prepared chitosan solution in 1% (v/v) acetic acid and then dissolved acetaminophen (1% or 2% w/v) as a model drug in the solution with subsequent stirring at 8000 rpm for 20 min. After that, 1% (w/v) aqueous solution of tripolyphosphate as a cross-linking agent was added to the chitosan drug suspension dropwise under constant stirring for 20 min at 8000 rpm. Spray-drying was done by using a standard 0.5 mm nozzle to obtain drug-loaded microspheres. Cerchiara et al. followed a similar technique to fabricate vancomycin-loaded chitosan micro and nanoparticles for colon-targeted delivery (Cerchiara et al. 2015). They dissolved vancomycin in chitosan-tripolyphosphate solution to prepare the feed for spray-drying. Antoniraj et al. also loaded the drug in chitosan microparticles by a modified three fluid nozzle spray-drying technique (Antoniraj et al. 2020). They encapsulated quercetin and ferulic acid in chitosan microparticles with cross-linkers sodium tripolyphosphate and sodium alginate. The coat solution (chitosan with dispersed quercetin and ferulic acid) was sprayed by the peripheral nozzle, and the core solution (sodium tripolyphosphate and sodium alginate) was sprayed by the inner nozzle. Table 18.2 illustrates some other works where drugs are loaded on chitosan-based particles by spray-drying.

Spray-drying is advantageous in fabricating drug carriers because heat-sensitive substances can be dried without thermal degradation due to quick-drying and comparatively shorter exposure time to heat (Sosnik and Seremeta 2015). However, the main challenge of the spray-drying process is that a significant amount of loss of the product occurs due to the accumulation of the materials on the wall of the drying chamber (Cevher et al. 2006; Bowey et al. 2013). Also, fine particles (<2 μm) pass into the exhaust air due to the low separation capacity of cyclone separators. So for producing nanoparticles, electrostatic particle collector has a higher product yield (above 70%) than cyclone separator (Li et al. 2010b; Lee et al. 2011). However, stickiness between obtained particles may render a great problem to implement spray-drying in commercial applications; hence, further research is needed to overcome this issue.

18.3.3 Electrospinning and Electrophoretic Deposition

Electrospinning and electrophoretic deposition are two fabrication techniques that involve an electric field. Among them, electrospinning is popular for fabricating chitosan-based fibrous structures, whereas electrophoretic deposition is used to apply bioactive chitosan-based coatings on various bioimplants.

18.3.3.1 Electrospinning

Electrospinning is a process in which charged threads from polymer solutions, or melts are drawn by using high voltage. A typical needle electrospinning setup generally has three major components such as (a) a high-voltage source, (b) a capillary tube with a needle, and (c) a metal collector plate (Elsabee et al. 2012). Some critical parameters for electrospinning of a polymer solution are voltage,

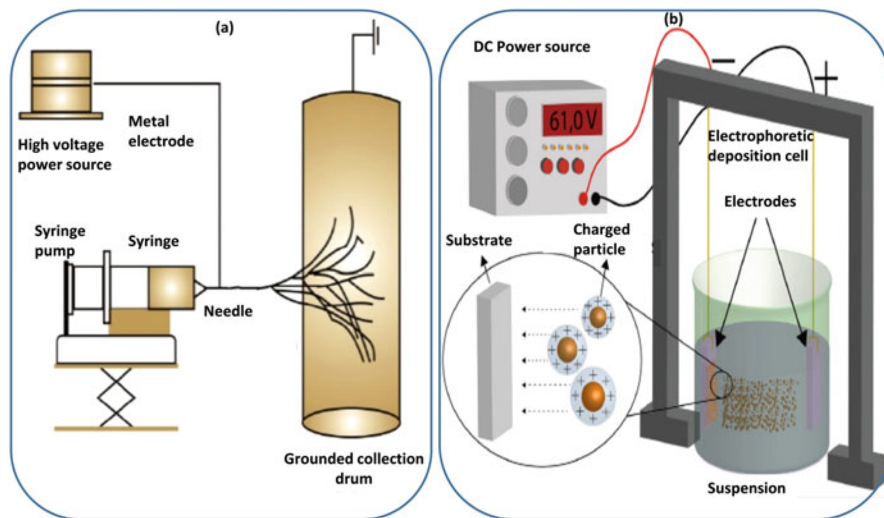

Fig. 18.4 Schematic diagrams of (**a**) electrospinning (reprinted with permission from reference Zafar et al. 2016. Copyright © 2016 Zafar et al.) and (**b**) electrophoretic deposition (reprinted with permission from reference Avcu et al. 2019. Copyright © 2019 Elsevier Ltd.) techniques. In (**a**), the high-voltage source is creating a potential difference, and a jet of charged polymer solution is being elongated towards the grounded collection drum. (**b**) shows a typical electrophoretic deposition setup where positively charged particles are being deposited on the negatively charged electrode

needle-to-collector distance, feeding rate of polymer solution, and polymer solution characteristics (Sun and Li 2011).

Electrospinning can be used to produce chitosan nanofibers. Figure 18.4a illustrates the basic methodology of the conventional needle electrospinning technique. Firstly, chitosan is dissolved in aqueous acetic acid (Geng et al. 2005). Then, the solution is pumped towards the needle capillary by a syringe pump (Agarwal et al. 2008). A DC voltage source creates a potential difference between two terminal ends of the needle capillary and the collector (Qasim et al. 2018). As a result, a jet of charged polymer solution is created towards the metal collector of the opposite charge. Due to high voltage, electrostatic repulsion begins to counteract the liquid droplets' surface tension at the needle tip, which starts to stretch the solution and deform the spherical droplet into a conical shape (Dalton et al. 2007). At a stage, the electrostatic repulsion completely overcomes the surface tension, and a jet of the charged solution is ejected from the tip of the conical protrusion; this point of ejection is known as the Taylor cone (Huang et al. 2003). Then, the charge density of the jet interacts with the oppositely charged metal plate to cause instability. High surface charge density elongates the jet by a whipping process where bending of the jet results in highly stretched fibers with simultaneous rapid evaporation of the solvent (Elsabee et al. 2012). The fibers deposit on the metal collector and can be dried under a vacuum to remove the remaining acetic acid and water from the electrospun fibers (Geng et al. 2005). The major problem for electrospinning of

chitosan in an aqueous solution is the inconsistency and structural instability of nanofibers due to the remaining solvent and the formation of chitosan salts in an acidic medium. A possible solution to this problem can be exposing electrospun fibers to water vapor and further cross-linking by using cross-linkers such as glutaraldehyde, genipin, etc. (Fadaie et al. 2019).

Coaxial and emulsion electrospinning are two modifications of conventional electrospinning of polymer solution. In emulsion electrospinning, the core-shell structure of chitosan nanofibers is obtained by using stable polymer emulsion instead of the polymer solution. Thus, the requirement for a solvent to dissolve the drug and polymer at the same time is eliminated (Zamani et al. 2013). On the contrary, in coaxial electrospinning, fibers with core-shell structures are obtained by using two coaxial capillaries in which two channels are connected to two reservoirs (Lu et al. 2016).

Chitosan is not easily electrospinnable because of higher surface tension due to internal interactions between hydroxyl and amine groups. However, this increased surface tension can be diminished by synthesizing composite nanofibers of chitosan with inorganic particles and synthetic polymers. In a study, Rijal et al. fabricated MgO/poly(ε-caprolactone)/chitosan composite nanofibers for tissue engineering applications by electrospinning process (Rijal et al. 2018). They added chitosan solution dropwise onto the solution of polycaprolactone/MgO and blended the prepared solution homogeneously. Finally, the solution was electrospun to obtain nanofibers. An alternative approach for fabricating biodegradable chitosan composites for biomedical applications can be electrospun polylactic acid/chitosan composite. Thomas et al. prepared the solutions of polylactic acid in chloroform and chitosan in acetic acid (Thomas et al. 2018). Then, the solutions were mixed homogeneously for 12 h and ultrasonicated for 20 min before electrospinning. Besides tissue engineering applications, chitosan-based composite nanofibers also have the potential to be applied in drug delivery systems. For example, Sundar et al. studied the potential of collagen/poly(*N*-isopropyl acrylamide)/chitosan nanofibrous mat to be used as a blood-contacting biomaterial for drug delivery in the field of cancer therapy (Sundar and Sangeetha 2012). After preparing the solution, they performed electrospinning with an electric voltage of 20 kV, keeping the needle tip-to-collector distance 15 cm. They also adjusted the flow rate and the rotation speed of the collector to 0.3–3 mL/h and 200 rpm, respectively.

Electrospinning can also be used to prepare therapeutic agents loaded with chitosan nanofibers. Antibacterial, antioxidant, and anti-inflammatory agents are some common therapeutic agents that can be loaded in chitosan nanofibers (Zhu et al. 2016; Poornima and Korrapati 2017; Ranjith et al. 2019). Poornima et al. synthesized ferulic acid and resveratrol-loaded chitosan/polycaprolactone composite core-shell nanofibers by coaxial electrospinning method (Poornima and Korrapati 2017). They prepared shell solution by dissolving polycaprolactone in the dichloromethane-ethanol mixture, whereas core solution was prepared by dissolving chitosan in acetic acid. Then, resveratrol and ferulic acid were added in both solutions as antioxidant and anti-inflammatory agents. Finally, coaxial electrospinning was done by controlling the voltage at 25 kV, needle tip-to-collector

distance at 10–12 cm, and feeding rate at 0.2 mL/h (core) and 0.4 mL/h (shell). A similar process was used to fabricate asiaticoside (antioxidant and anti-inflammatory agent)-loaded chitosan nanofibers (Zhu et al. 2016). Sodium alginate and polyvinyl alcohol in acetic acid were used as shell solution, while chitosan in acetic acid was the core solution.

Remarkable progress has been made in the fabrication of chitosan-based fibrous structures by the electrospinning process in recent years. However, many problems are still to be solved, such as low mechanical strength of chitosan nanofibers, cytotoxicity of spinning solvents, and difficulty to scale up the laboratory scale setup (Sun and Li 2011).

18.3.3.2 Electrophoretic Deposition

The technique in which suspended and charged particles/molecules in a solution move under an applied electric field and deposit on an oppositely charged electrode to form films is called electrophoretic deposition (Van der Biest and Vandeperre 1999). A typical electrophoretic deposition process comprises two steps such as (a) electrophoresis and (b) deposition. In the first step, charged and dispersed particles in an appropriate solvent move toward a working electrode of the opposite charge. Next, in the deposition step, particles coagulate in an ordered manner on the electrode to form rigid and homogeneous deposits (Boccaccini et al. 2010). This process can be used to develop chitosan-based composite coatings on metallic surfaces to render antibacterial and bioactive properties for biomedical applications.

A typical electrophoretic deposition cell contains a power source, electrodes (cathode and anode), and a stable suspension (Fig. 18.4b) (Corni et al. 2008; Avcu et al. 2019). To deposit native chitosan on the cathode surface by electrophoretic deposition, a solution of chitosan in dilute acetic acid is generally used. The amine groups of chitosan (Chit-NH_2) are protonated at low pH (Eq. 18.1) that facilitates its dissolution in the acetic acid solution (Zhitomirsky and Hashambhoy 2007). On the contrary, chitosan becomes insoluble, and its amine groups become deprotonated at high pH (Li et al. 2017). This transition from soluble to insoluble state happens within pH 6.0–6.5 (Yi et al. 2005). Protonated chitosan molecules of the solution move towards a negatively charged cathode and then molecules deposit on the cathode surface. Equation (18.2) and (18.3) represent reactions occurred on the cathode surface due to electrochemical decomposition of water present in the chitosan solution (Zhitomirsky and Hashambhoy 2007). Due to the production of OH^- from the decomposition of water, local pH on the cathode surface increases, and chitosan becomes insoluble and deposits on the electrode surface according to reaction (18.4) (Zhitomirsky and Hashambhoy 2007).

$$\text{Chit-NH}_2 + H_3O^+ \rightarrow \text{Chit-NH}_3{}^+ + H_2O \quad (18.1)$$

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (18.2)$$

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \quad (18.3)$$

$$\text{Chit-NH}_3{}^+ + OH^- \rightarrow \text{Chit-NH}_2 + H_2O \quad (18.4)$$

For composite coatings, co-deposition of other inorganic particles with chitosan can be utilized. Bioactive ceramic materials (bioactive glass, hydroxyapatite, etc.), carbonaceous materials (carbon nanotube, graphene-related materials, etc.), gelatin, TiO_2, boron nitride, etc. are usually used to form composites with chitosan in typical applications of composite coatings. Chitosan-based composite coatings on metallic implants, such as titanium alloys, stainless steel, etc., can boost up the bioactivity, antibacterial properties, and adhesion to living tissue (Avcu et al. 2019). Many studies have already shown the successful application of these composite coatings on bioimplants. Clavijo et al. used electrophoretic deposition to develop chitosan/bioactive glass/TiO_2 composite coating for bioimplants (Clavijo et al. 2016). Firstly, they prepared a suspension of bioactive glass in water and added chitosan to it. Then, TiO_2 powder was added to the suspension after adjusting the pH of the suspension with HCl and NaOH solution. After ultrasonication for 30 min and homogenization for 45 min, a stable suspension was obtained, which was ready for use in the electrophoretic deposition process. At last, the deposition of composite material was done on a stainless steel substrate. In another study, Singh et al. coated Ti-13Nb-13Zr alloy instead of stainless steel with hydroxyapatite/chitosan/iron oxide composite (Singh et al. 2020). After preparing suspension, electrophoretic deposition was performed for 6 min at the voltage of 15 V. Finally, the coated substrate was dried for 24 h at room temperature. Radda'a et al. also used a similar process to coat orthopedic implants by tetracycline hydrochloride loaded halloysite nanotubes/chitosan/bioactive glass composite (Radda'a et al. 2017). They determined suitable voltage and deposition time (40 V and 30 s respectively) by trial and error method. Finally, they applied the coating suspension on stainless steel foil by keeping the distance between electrodes constant at 10 mm. Abdulkareem et al., Huang et al., and Farrokhi-Rad et al. also prepared chitosan-based coatings with enhanced antimicrobial activity by electrophoretic deposition (Huang et al. 2017; Farrokhi-Rad et al. 2018; Abdulkareem et al. 2019). Farrokhi-Rad et al. prepared vancomycin-loaded chitosan/halloysite nanotubes composite coating on orthopedic implants (Farrokhi-Rad et al. 2018). Abdulkareem et al. found the antibacterial activity of polyethetherketone/chitosan/nano-hydroxyapatite composite coating (Abdulkareem et al. 2019). Huang et al. incorporated zinc to chitosan/gelatin nanocomposite coating to obtain antibacterial activity (Huang et al. 2017). All these works used a similar kind of procedure of making suspension in the first step, and then electrophoretic deposition was performed by maintaining suitable voltage and deposition time.

Electrophoretic deposition of chitosan with other materials on metallic substrates is recently gaining interest for various biomedical applications. However, this technique's major problem is difficulties in maintaining nanoscale dimensions for some composite coatings (Avcu et al. 2019). Moreover, effective biological responses and cytotoxicity of these biomaterials need to be investigated extensively.

18.3.4 Gelation and Cross-Linking

Gelation refers to the progressive linking between branched polymer chains to form a larger branched macroscopic molecule. As gelation starts, the viscosity of the polymeric system becomes very high and the system loses fluidity (Odian 2004). Linking between polymer chains can occur either by physical linking or chemical cross-linking. Physical linking involves physical bonds such as hydrogen bonding, hydrophobic association, electrostatic interaction, etc., whereas chemical cross-linking involves covalent bonds. Most of the chitosan-based biomaterials are fabricated by two distinguished techniques, ionotropic gelation, and freeze gelation.

18.3.4.1 Ionotropic Gelation

Ionotropic gelation is a technique in which the charged groups of polymer chains are physically linked by oppositely charged ions via electrostatic interactions (Pedroso-Santana and Fleitas-Salazar 2020). Ionotropic gelation can be used to obtain chitosan-based microparticles and nanoparticles. Furthermore, this method can be used for encapsulation of various bioactive compounds within the micro- and nanoparticles (Calvo et al. 1997). Chitosan having positively charged amino groups is physically linked with negatively charged molecules such as tripolyphosphate, citrate, sulfate, etc. by electrostatic interactions (Jayasuriya 2017). Tripolyphosphate is the most common counter ion used for ionotropic gelation of chitosan and shows lower toxicity than the other commonly used cross-linker, glutaraldehyde. In ionotropic gelation of chitosan with tripolyphosphate, chitosan is dissolved in acetic acid, and then, aqueous tripolyphosphate solution is added dropwise to the chitosan solution with subsequent stirring (Calvo et al. 1997). Fast stirring results in the homogeneous dispersion of tripolyphosphate anions, causing the formation of smaller nanoparticles with narrower size distribution. On the contrary, slow stirring results in nonuniform dispersion of tripolyphosphate anions, causing larger particles with wider size distributions (Fan et al. 2012). Increasing the concentration of tripolyphosphate results in decreases in particle size (Dong et al. 2013). The appearance of opalescent suspension during stirring indicates the formation of nanoparticles. Finally, the spontaneously formed nanoparticles are isolated by centrifugation.

Ionotropic gelation is used to encapsulate bioactive compounds such as proteins, antigens, drugs, etc., within chitosan nanoparticles. Amaral et al. prepared miconazole-loaded chitosan nanoparticles for treatment of vulvovaginal candidiasis fungal infections by ionotropic gelation method (Amaral et al. 2019). Pentasodium tripolyphosphate was added dropwise to the chitosan solution in acetic acid. Miconazole nitrate was added to the solution by keeping the drug concentration 2% (w/v). The obtained nanoparticles showed augmented therapeutic efficacy. Mahmood et al. also used a similar technique with subtle modifications to prepare docetaxel-loaded chitosan nanoparticles for drug delivery purposes (Mahmood et al. 2019). They prepared docetaxel in ethanol and tripolyphosphate in water in separate containers and mixed both the solutions afterward. Then, chitosan solution was added to the mixture dropwise with stirring at 900 rpm for 45 min. At last, chitosan nanoparticles

as fine pellets were separated by centrifugation. Bhattacharya used anionic surfactant polysorbate-80 instead of tripolyphosphate to prepare imatinib-loaded chitosan-based nanoparticles for colorectal cancer targeting application (Bhattacharya 2020). He added imatinib dropwise to the solution of chitosan containing polysorbate-80. After homogenization and centrifugation, the suspension was further mixed with cryoprotectant (3% D-trehalose) using a magnetic stirrer. Finally, the suspension was frozen at −80 °C for 48 h and subsequently lyophilized at −78 °C for 8 h. Other additives can also be incorporated in chitosan nanoparticles for drug vehicles. For example, collagen peptide-functionalized chitosan nanoparticles were prepared for encapsulation and delivery of cancer drug doxorubicin (Anandhakumar et al. 2017). An aqueous solution of collagen peptide along with tripolyphosphate was added dropwise to the chitosan solution while ultrasonication at room temperature. The nanoparticles were finally separated by centrifugation.

Though ionotropic gelation is an inexpensive, fast, and mild technique having high encapsulation efficiency, it has some limitations that need to be addressed. Nanoparticles obtained by ionotropic gelation show wider particle size distribution which can affect the quantity of the encapsulated drug and hamper interaction of the formulation with biological structures (Pedroso-Santana and Fleitas-Salazar 2020).

18.3.4.2 Freeze Gelation

Freeze gelation is a technique in which a polymer solution is frozen below the freezing point and subsequently immersed in a precooled non-solvent to adjust the required pH for gelation. A porous structure is generated during gelation under freezing conditions. Freeze gelation is used to fabricate chitosan-based porous hydrogel scaffolds. To fabricate scaffolds, firstly, chitosan is dissolved in an aqueous acetic acid solution and placed in a mold/glass petri dish. The solution is then frozen at −20 °C, and the frozen chitosan solution is immersed in a NaOH/ethanol solution (precooled to −20 °C) to adjust pH 13, suitable for gelation (Ho et al. 2004). The gelled chitosan is then rinsed with buffer (70% aqueous ethanol solution) and subsequently washed repeatedly by a phosphate-buffered saline solution to remove excess NaOH. Finally, the porous scaffold is dried at 25 °C for further use (Hsieh et al. 2007; Shamloo et al. 2019). Kamali et al. followed the same technique to obtain porous morphology in a chitosan/gelatin hydrogel layer of bilayer hydrogel-electrospinning scaffolds (Kamali and Shamloo 2020). The bilayer scaffolds showed higher elastic modulus and tensile strength than the single-layer scaffolds. Hsieh et al. also followed a similar technique to fabricate freeze-gelled chitosan/g-PGA scaffolds for the carrier of rhBMP-2. They dissolved chitosan and g-PGA in 0.2 M aqueous acetic acid and cooled for 12 h at −80 °C. The frozen solution was then immersed in a 3 M NaOH/ethanol solution (−20 °C) for 12 h and subsequently rinsed (Hsieh et al. 2006). The fabricated scaffolds showed enhanced and prolonged release of rhBMP-2. Some other examples of freeze gelation reported in recent years are mentioned in Table 18.3.

The main advantage of freeze gelation is that the porous structure would not be destructed during drying as gelation occurred during the freezing stage. Moreover, the method is more time- and energy-saving and has lower residual solvent content

Table 18.3 A list of chitosan-based biomaterials fabricated by ionotropic gelation, freeze gelation, electrospinning, and electrophoretic deposition techniques

Fabrication technique	Materials used	Final form	Application	Reference
Electrospinning	Chitosan, liposome, gentamicin	Nanofiber meshes	Wound dressing	Monteiro et al. (2015)
	Chitosan, phospholipid, asolectin, vitamin B12, curcumin, diclofenac	Nanofibers	Transdermal drug delivery	Mendes et al. (2016)
	Chitosan, poly (vinyl alcohol), graphene oxide	Nanofibers	Substitute as an artificial cartilage	Cao et al. (2017)
	Chitosan, starch, poly(vinyl alcohol)	Nanofibrous Mats	Wound dressing	Adeli et al. (2019)
	Poly(vinyl alcohol), chitosan, *Bidens pilosa*	Nanofibers	Antibacterial agents	Kegere et al. (2019)
	Chitosan, poly (vinyl alcohol), carboxymethyl chitosan, OH-CATH30 nanoparticles	Nanofibrous Membrane loaded with nanoparticles	Wound healing	Zou et al. (2020)
Electrophoretic deposition	Chitosan, gelatin nanospheres, vancomycin, or moxifloxacin	Composite films	Coating on bioimplants for sustained release of antibacterial drug	Song et al. (2016)
	Chitosan, gelatin nanospheres, dexamethasone	Composite films	Coating on bone implants promoting anti-inflammation and osteogenesis	Qi et al. (2018)
	Chitosan, nano-hydroxyapatite, nano-graphene oxide	Nanocomposite films	Coating on bone implants	Karimi et al. (2019)
	Chitosan, copper (II) chloride dehydrate	Complex films	Antibacterial coating on bioimplants	Akhtar et al. (2020)
Ionotropic gelation	Pluronic F-127, chitosan, gemcitabine	Nanoparticles	Oral delivery of anticancer drug	Hosseinzadeh et al. (2012)

(continued)

Table 18.3 (continued)

Fabrication technique	Materials used	Final form	Application	Reference
	Chitosan, sodium tripolyphosphate, saquinavir	Nanoparticles	Anti-HIV drug delivery	Ramana et al. (2014)
	Chitosan, ibuprofen, gellan gum, propylene glycol	Nanogel	Controlled transdermal delivery of ibuprofen	Abioye et al. (2015)
	Chitosan, collagen peptide, sodium tripolyphosphate, doxorubicin	Nanoparticles	Anticancer drug delivery	Anandhakumar et al. (2017)
	Chitosan, sodium tripolyphosphate, $ZnSO_4 \cdot 7H_2O$	Nanoparticles	Treatment of acute lymphoblastic leukemia	Saravanakumar et al. (2018)
	Chitosan, sodium tripolyphosphate	Nanoparticles	Antimicrobial applications	Shafiei et al. (2019)
	Chitosan, poly (vinyl alcohol), budesonide	Nanoparticles	Drug delivery	Michailidou et al. (2020)
Freeze gelation	Chitosan, hydroxyapatite, poly(lactic acid)	Porous composite scaffolds	Bone tissue engineering	Rogina et al. (2016)
	Chitosan, cellulose, triethyl orthoformate, K doped ZnO nanoparticles	Hydrogel membranes	Stimulation of angiogenesis in tissue-engineered skin grafts	Shahzadi et al. (2018)
	Chitosan, bioactive glass nanoparticles	Hybrid scaffolds	Bone tissue engineering	Oudadesse et al. (2020)
	Chitosan, thyroxine	Hydrogel	Stimulation of angiogenesis and wound healing	Shahzadi et al. (2020)

than the freeze-drying method and can easily be scaled up (Ho et al. 2004). However, local melting can occur during gelation due to the exothermic reaction of frozen acetic acid solvent and NaOH. So the process should be done carefully to minimize the effect of exothermic reaction (Aminabhavi et al. 2017).

18.3.5 Emulsification and Lyophilization

18.3.5.1 Emulsification

Emulsification is a modified form of solvent evaporation where tiny droplets of one liquid phase are dispersed throughout another continuous liquid phase to form a semi-stable mixture by reducing the interfacial tension between the two phases. Simple emulsification starts with a polymer solution in a polar organic solvent for biomaterial fabrication, followed by mixing with aqueous solutions. Upon mixing of this hydrophilic part, the polar solvent diffuses out of the emulsion drop, and the precipitate of lipophilic polymer compound is obtained after the final evaporation of the solvent. Yang et al. prepared soy protein isolate (SPI)-chitosan nanoparticles by following the simple emulsification principle (Yang et al. 2020). Chitosan solution in acetic acid was added dropwise to aqueous SPI solution under constant stirring until the formation of a transparent and white suspension. The SPI-chitosan nanoparticle dispersion was obtained by heating in the water bath at 80 °C. Besides this simple mode of emulsification, many researchers also focused on the technique based on multiple steps of emulsification. Wang et al. followed a three-step process to fabricate a drug-loaded chitosan-coated by poly(lactic-co-glycolic acid) nanospheres (Wang et al. 2017b). Another revised variant of the emulsification process is pickering emulsion, which emerged as a promising approach for the preparation of drug delivery systems in the biomedical field due to its property of being stabilized by colloids in the interface of oil-in-water type emulsion (Wang et al. 2017b). In this process, firstly, they formed chitosan colloids by processing chitosan solution into a high-intensity ultrasonic processor. The emulsification started with this colloidal chitosan mixing with poly(lactic-co-glycolic acid)/methylene dichloride that resulted in an oil-in-water emulsion. Among the emulsification processes, pickering emulsion is getting attention day by day due to its eco-friendly nature and high stability. Also, it has opened new avenues for designing and production of innovative materials (Sharkawy et al. 2020). Although the emulsification process has a promising biomaterial fabrication technique, very little research (listed in Table 18.4) has been conducted on its potentiality to fabricate chitosan-based biomaterials. The aqueous acetic acid-based chitosan solution can be easily used to coprecipitate chitosan with other polymers dissolved in polar organic solvents. Particles with nano to microsize range can be fabricated by this method for biomedical applications.

18.3.5.2 Lyophilization

Lyophilization is a water removal process where the solutions are frozen and solvents are sublimated to get a solvent-free structure, mostly porous by form. Pore characteristics (e.g., size, volume, morphology, etc.) of the products can be controlled by freezing temperature, solution concentration, nature of solvent and solute, etc. (Qian and Zhang 2011). As it is a low-temperature operation without toxic organic solvent, the activity of biomacromolecules and pharmaceuticals is not disrupted. So fabrication of chitosan-based biomaterials through the lyophilization process will be more significant than the other processes. In general, the simple

Table 18.4 A list of chitosan-based materials fabricated by emulsification and lyophilization for biomedical applications

Fabrication technique	Materials used	Final form	Application	Reference
Emulsification	Chitosan, poly(lactic-co-glycolic acid), CH_2Cl_2	Core-shell nanocomposites	Drug delivery	Wang et al. (2017b)
	Chitosan, soy protein isolate	Nanoparticles	Food-grade pickering	Yang et al. (2020)
Lyophilization	Chitosan, gelatin, NaCl	Scaffolds	Bone tissue engineering	Modaress et al. (2012)
	Chitosan, gelatin, silica	Nanocomposite scaffolds	Bone tissue engineering	Kavya et al. (2013)
	Chitosan, gelatin, SiO_2 powder, glutaraldehyde	Nanocomposite scaffolds	Bone tissue engineering	Kavya et al. (2013)
	Chitosan powder, carbonate apatite	Scaffolds	Tissue engineering	Ariani et al. (2013)
	Chitosan, plasmid DNA	Nanoparticles	Gene delivery, pharmaceutical application	Veilleux et al. (2016)
	Chitosan powder	Sponges	Drug delivery, tissue engineering	Berretta et al. (2017)

lyophilization operation occurs where the chitosan solution is introduced into the freeze-drying machine at a specific time and temperature. Previously, an optional neutralization reaction was performed with dilute NaOH depending on the pH of the solution.

Ariani et al. applied the lyophilization principle to fabricate a carbonate apatite-chitosan-based scaffold (Ariani et al. 2013). Deacetylated chitosan powder was dissolved in acetic acid and neutralized with NaOH solution followed by centrifugation to obtain the chitosan gels. Different amounts of carbonate apatite were added with chitosan gels and then again centrifuged to get the carbonate apatite-chitosan gels. Finally, the mixture was freeze-dried to get the material in scaffold form. Kavya et al. also used a similar technique to obtain chitosan/gelatin/nano-SiO_2 nanocomposite scaffolds (Kavya et al. 2013). Initially, a chitosan/gelatin blend was prepared by dissolving gelatin in chitosan solution followed by mixing with nano-SiO_2 powder and glutaraldehyde cross-linker. The cross-linker was used for the homogeneous linking of chitosan/gelatin blend to nano-SiO_2. This chitosan/gelatin/nano-SiO_2 blend was finally freeze-dried to form a nanocomposite scaffold. Berretta et al. applied the most straightforward lyophilization technique to get the complex chitosan sponges (Fig. 18.5) (Berretta et al. 2017). The freezing of the

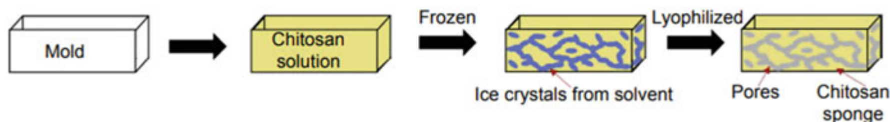

Fig. 18.5 Chitosan sponge-making process by lyophilization process. Chitosan solution is poured into the mold and frozen to form ice crystals. The frozen mixture is lyophilized to form the chitosan sponge with the required porosity. (Reprinted with permission from reference Berretta et al. 2017. Copyright © 2017 Elsevier Ltd.)

chitosan solution resulted in the formation of ice crystals. When these ice crystals were sublimated, they left behind spaces in the shape that led to the formation of lyophilized sponges. A constant cooling might produce the scaffolds more uniformly.

Among different techniques available for the fabrication of chitosan-based biomaterials, lyophilization is one of the most suitable techniques where the chemical and physical stability of the product remains for a longer period. The fabrication of three-dimensional or nanocomposite structures is easier in this process (Ariani et al. 2013). The low surface tension of solvent during this process can dominate the pore properties of the product, which may collapse in normal drying processes (Qian and Zhang 2011). The freeze-drying process extends the shelf life of the products and makes the product more convenient for transport. Because of all these features, researchers are using this technique to fabricate chitosan-based biomaterials for various applications (as listed in Table 18.4). However, further research on this eco-friendly lyophilization system is still needed to reduce the processing cost for industrial implementation. A significant reduction in operational cost would open a new horizon for future endeavors.

Beyond this emulsification and lyophilization process, there may be one similar process called micellization. The process refers to the formation of micelle involved in the back-to-back agglomeration of the hydrophobic side with the hydrophilic side. Mechanical strength and swelling efficiency of chitosan-based biomaterials depend on the uniformity of the micellization (Nasri-Nasrabadi et al. 2019). However, this process has the prospect for biomaterial fabrication but has not been extensively explored for chitosan-based biomaterial fabrication yet.

18.3.6 Precipitation, Leaching, and Foaming

18.3.6.1 Precipitation

A precipitation reaction indicates the formation of an insoluble salt when two solutions containing soluble salts are combined. In general, chitosan is dissolved in an acidic solution, and upon the addition of the precipitating agent, it can be separated from the mixture obtaining the desired pure form of chitosan as a precipitate. This precipitation technique was applied by Qiu et al. where chitosan/ZnO nanocomposite films were fabricated by pouring a solution of 3 wt% chitosan and

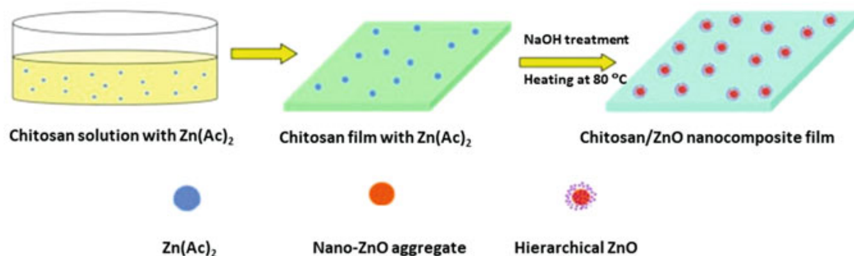

Fig. 18.6 Schematic presentation of chitosan/ZnO nanocomposite film fabrication by in situ precipitation method. Chitosan solution in acetic acid was mixed with zinc acetate ($Zn(Ac)_2$), followed by casting of the mixture as a concentrated film. Finally, the film was treated with NaOH under heat to obtain the desired chitosan/zinc oxide composite (reprinted with permission from reference Qiu et al. 2019. Copyright © 2018 Elsevier B.V.). The main advantage of the precipitation process lies in the solubility of chitosan and its derivatives into water. Chitosan itself is insoluble in water, but there are also many other derivatives of chitosan which are soluble in water and thus making the implication of this technique quite easier for the researchers

0.01 mol zinc acetate ($Zn\ (Ac)_2$) into a petri dish (Fig. 18.6) (Qiu et al. 2019). This resulted in a composite film which was further soaked in NaOH under heating conditions and finally rinsed and sterilized to get the final product. Klein et al. used the mixture of NaOH and ethanol as a coagulating agent to prepare chitosan macroparticles by precipitation method (Klein et al. 2012). The dropwise addition of chitosan solution (in acetic acid) into the coagulating solution under slow agitation forms chitosan macroparticles.

18.3.6.2 Leaching

Leaching is a separation technique to be applied to isolate the insoluble solute from the soluble one by using a solvent that is more compatible with the solute in the soluble phase than the solute in the insoluble stage. Pezeshki-Modaress et al. fabricated gelatin/chitosan films using the basic principle of leaching (Pezeshki-Modaress et al. 2014). They mixed the chitosan and gelatin solution with NaCl crystal. This NaCl acted as a leaching agent. Following evaporation of the solution, the dried product was cross-linked with acetone-water containing *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride to obtain the gelatin/chitosan films. Finally, a slow shake of the solution ended the leaching process, and the film was obtained by further lyophilization of the remaining solution. Kanimozhi et al. also used the combined technique of lyophilization and salt leaching to produce porous scaffolds of chitosan/poly(vinyl alcohol)/carboxymethyl cellulose (Kanimozhi et al. 2018). They added NaCl particles (200–500 μm) into the mixture of chitosan, poly(vinyl alcohol), and carboxymethyl cellulose as a leaching agent. The salt-leached chitosan/poly(vinyl alcohol)/carboxymethyl cellulose porous scaffolds were easily fabricated into desired shapes with a uniformly distributed and interconnected pore structure with an average pore size which leads to achieving superior mechanical strength of the scaffolds.

Leaching is the process of low capital and operating cost with a fast payback. Recently, a new leaching method called bioleaching is being introduced in different extraction fields, which uses living organisms for leaching purposes. This technique is more cost-effective than traditional chloride chemical leaching (Bobadilla-Fazzini et al. 2015). So, for the new generation of researchers, there are many scopes to explore the possibility of building up a connection between chitosan with bioleaching techniques for future biomedical applications.

18.3.6.3 Foaming

Foaming reaction refers to the formation of foam by adding an agent which normally reduces the surface tension of a liquid. In chitosan-based biomaterial fabrication, a general foaming agent or gas generating agent such as sodium bicarbonate is introduced in chitosan solution (in acetic acid) to form the foam by the following reaction:

$$CH_3COOH + NaHCO_3 = CH_3COO\text{-}Na + CO_2 + H_2O$$

Sometimes there is a necessity to fabricate biomaterials by in situ foaming, especially when the high porosity is an important criterion for the biomaterials. Highly porous matrixes with porosity close to 90% can be possible to be generated by the foaming technique (Kucharska et al. 2012). Kucharska et al. prepared in situ foamed chitosan/β-tricalcium phosphates scaffolds for bone tissue engineering following the foaming process (Kucharska et al. 2012). They added β-tricalcium phosphates powder (3–5 mm particle size) in chitosan solution (in acetic acid). The foaming agent $NaHCO_3$ was blended into this β-tricalcium phosphates/chitosan solution followed by lyophilization. The final product was highly porous chitosan/β-tricalcium phosphates scaffolds. Park et al. prepared a super porous hydrogel comprised of chitosan and glycol chitosan using the combination of foaming and lyophilization process (Park et al. 2006). Chitosan and glycol chitosan solution in acetic acid was primarily cross-linked by adding an aqueous glyoxal solution. $NaHCO_3$ was used as a foaming agent or gas-blowing agent. Foaming started when $NaHCO_3$ was added to the solution mixture due to the reaction with acetic acid. The mixture turned into super porous hydrogel by the lyophilization process.

Table 18.5 summarizes the recent reports on chitosan-based materials fabricated by precipitation, leaching, and foaming techniques.

18.3.7 3D Printing

3D printing refers to the process of transforming a digital 3D model to a physical object by computer-controlled successive material deposition, usually in a layer-by-layer manner. Polymers are commonly preferred in 3D printing due to the ease of manufacturing and handling. Chitosan can be extensively used to fabricate biomaterials by 3D printing because of its native composition and biocompatibility

Table 18.5 A list of chitosan-based materials fabricated by precipitation, leaching, and foaming technique for biomedical applications

Fabrication technique	Primary materials used	Final form	Application	Reference
Precipitation	Chitosan, coagulation solution (sodium hydroxide and ethanol), glutaraldehyde	Macroparticles	Enzyme immobilization	Klein et al. (2012)
	Chitosan, $Zn(Ac)_2$, NaOH	Nanocomposite films	Antimicrobial field	Qiu et al. (2019)
Leaching	Chitosan, NaCl, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride	Films, scaffolds	Tissue engineering	Pezeshki-Modaress et al. (2014)
	Chitosan, poly(vinyl alcohol), carboxymethyl cellulose, NaCl particles	Scaffolds	Soft tissue engineering	Kanimozhi et al. (2018)
Foaming	Chitosan, glycol chitosan,	Foamed hydrogels	Gastric retention	Park et al. (2006)
	Chitosan solution, β-tricalcium phosphates powder, sodium bicarbonate	Scaffolds	Tissue engineering	Kucharska et al. (2012)

(Kean and Thanou 2019). Some chitosan-based biomaterials fabricated by 3D printing are listed in Table 18.6. Several types of 3D printing are common in practice for fabricating chitosan-based biomaterials, such as binder jetting, material extrusion, stereolithography, indirect 3D printing, etc. Figure 18.7 depicts four types of 3D printing techniques.

Binder jetting is one of the earliest forms of 3D printing which can be defined as a modified version of 2D inkjet printing. In binder jetting, the ink nozzle not only moves side to side but also moves up and down, giving the printed material dimension of the height along with the length and width (Do et al. 2015). Instead of using ink, a 3D printer utilizes the deposition of liquid binder solution on a powder bed. At first, a layer of powder is spread on a platform by a roller system, and the liquid binder solution is deposited on the powder bed. Powder materials are then bonded by the binder solution, and the process is repeated to form successive layers of bonded powder to get desired 3D component. Finally, the excess unbounded powders are removed (Cima et al. 1995). Figure 18.7a illustrates the basic components of the 3D printing setup by binder jetting. Chavanne et al. used binder jetting to fabricate chitosan/hydroxyapatite biocomposite cylindrical scaffolds (Chavanne et al. 2013). They used 40 wt% lactic acid as a binder solution to bind chitosan/hydroxyapatite composites. After completion of printing, they immersed the scaffolds in NaOH for post-hardening. However, the main problem of using binder jetting is that only nonporous scaffolds show desired mechanical strength. Porous scaffolds fabricated by binder jetting are collapsed due to low mechanical strength when immersed in NaOH (Chavanne et al. 2013). Porosity is a vital

Table 18.6 A list of chitosan-based biomaterials fabricated by 3D printing techniques

Materials used	Final form	Application	References
β-Tricalciumphosphate, chitosan	Scaffolds	Bone tissue engineering	Haberstroh et al. (2010)
Chitosan, alginate, calcium chloride	Fibrous scaffolds	Liver tissue engineering	Colosi et al. (2014)
Chitosan, gelatin, glutaraldehyde	Hydrogel scaffolds	Liver tissue engineering	Gong et al. (2014)
Chitosan, dextran, calcium phosphate, bovine serum albumin, or vascular endothelial growth factor microparticles	Cement scaffolds	Tissue engineering	Akkineni et al. (2015)
Hydroxyapatite, polylactide-co-glycolide, hydroxypropyltrimethyl ammonium chloride chitosan	Porous composite scaffolds	Treatment of infected bone	Yang et al. (2016)
Polycaprolactone, chitosan, genipin	Hybrid scaffolds	Bone tissue engineering	Dong et al. (2017)
Methacrylamide chitosan, phenyl-2,4,6-trimethylbenzoylphosphinate, polyethylene glycol-amine modified SPIONs, iron oxide nanoparticles	Microswimmers	Delivery of therapeutics	Bozuyuk et al. (2018)
Chitosan, genipin, glycerol, or polyethylene glycol	Films	Wound healing	Hafezi et al. (2019)
Chitosan, pectin, lidocaine hydrochloride	Hydrogel scaffolds	Wound dressing	Long et al. (2019)
Chitosan, poly(L-lactide), quercetin, polydopamine	Microfibrous and nanofibrous scaffolds	Bone tissue engineering	Zhu et al. (2020)

property for scaffolds used in biomedical fields, and this issue of having low mechanical strength in porous scaffolds can be easily resolved by using material extrusion-based 3D printing.

3D printing by material extrusion is a method in which a pneumatic pressurized system is used to extrude the deposited material according to the computer-aided design (CAD) model. It is the most widely used additive manufacturing method for chitosan-based scaffold fabrication for tissue engineering. Dong et al. used material extrusion 3D printing to fabricate bioactive hydroxyapatite/chitosan/silica hybrid scaffolds for bone regeneration (Dong et al. 2018). They filled the printer cartridge with hybrid pastes and extruded the pastes keeping dosing pressure within 0.2–0.5 MPa at a constant plotting speed according to the CAD model. Finally, the prepared scaffolds were neutralized, washed with deionized water, and dried for future use. These 3D plotted hybrid scaffolds showed the required compressive strength and elasticity for human trabecular bone. Intini et al. also used a similar process to fabricate chitosan-based scaffolds for skin cell growth and wound healing (Intini et al. 2018). However, after completion of the printing step, they immersed the scaffolds in KOH solution where gelation took place and then stored in

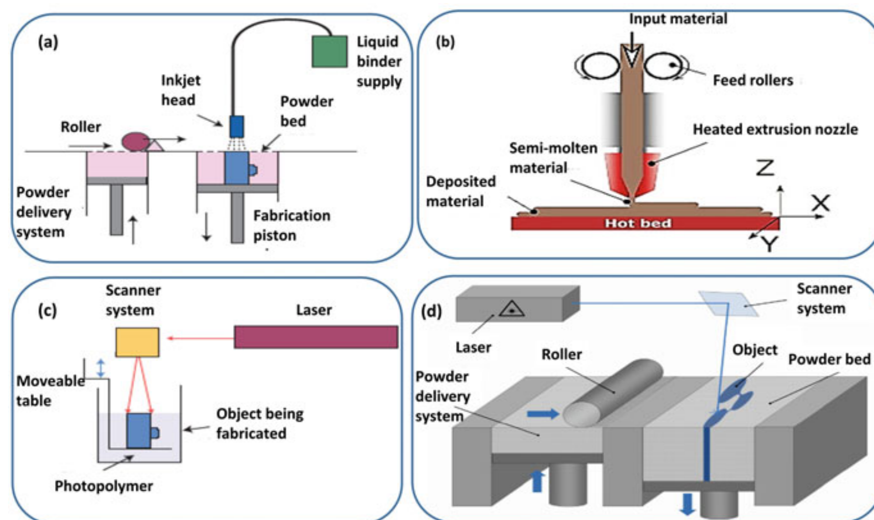

Fig. 18.7 Schematic diagrams of various types of 3D printing. (**a**) Binder jetting: printing is being done by depositing the liquid binder on a powder bed (reprinted with permission from reference Hollister 2005. Copyright © 2005 Nature Publishing Group). (**b**) Fuse deposition modeling: an extrusion-based printing where a heated nozzle is being used (reprinted with permission from reference Gomez-Gras et al. 2018. Copyright © 2017 Elsevier Ltd.). (**c**) Stereolithography: laser-based printing technique where UV laser is used to photopolymerize the liquid (reprinted with permission from reference Hollister 2005. Copyright © 2005 Nature Publishing Group). (**d**) Selective laser melting: laser-based printing technique where the CO_2 laser beam is being used to sinter the powder materials. (Reprinted with permission from reference Leitz et al. 2017. Copyright © 2016 Elsevier Ltd.)

phosphate buffer saline. This step gave the structure the required rigidity for shape retention. Fuse deposition modeling is a modified material extrusion 3D printing that utilizes a heated extrusion nozzle for thermo-responsive polymer beyond their glass transition temperature. The deposited polymer hardens and sets upon contacting the platform and the process repeats itself in a layer-by-layer manner (Do et al. 2015). Figure 18.7b demonstrates the process of fuse deposition modeling. An example of fuse deposition modeling for the fabrication of chitosan-based scaffolds can be the work of Elviri et al. (2017). They made a solution of chitosan and raffinose in acetic acid suitable for 3D printing. D-(+) raffinose pentahydrate was added to the solution as a viscosity modifier because pure chitosan solution doesn't provide suitable viscosity and hydrophilicity for fuse deposition modeling. After printing the scaffolds were immersed in KOH to attain rigidity by gelation. The obtained 3D printed scaffolds had better biocompatibility than the scaffolds produced by solvent casting.

Stereolithography and selective laser melting are two laser-based 3D printing techniques used for the fabrication of chitosan-based scaffolds. Stereolithography is a technique in which a photosensitive liquid polymer solution is smeared on a

platform and then exposed to a UV laser beam to solidify the photopolymer in a layer-by-layer manner until the CAD model is obtained (Fig. 18.7c) (Crivello and Reichmanis 2014). This method is advantageous for the fabrication of scaffolds due to the ease of creating and controlling the defined geometry of the CAD model with high resolution (Kim et al. 2010). Morris et al. fabricated chitosan/polyethylene glycol diacrylate hybrid gel ear-shaped scaffolds by stereolithography technique by using the specific molecular weight of chitosan, feed-ratios, and photo-initiator concentration (Morris et al. 2017). They used Irgacure 819 in *N*-vinyl-2-pyrrolidinone as a photo initiator and completed the stereolithography using a 405 nm laser. The obtained scaffolds showed cell adhesive properties of chitosan with the mechanical robustness of polyethylene glycol diacrylate, which is appropriate for repairing complex tissue geometries of the human ear. Other techniques associated with stereolithography can be used to fabricate chitosan-based scaffolds. For instance, Jiankang et al. used lyophilization associated with stereolithography to fabricate chitosan/gelatin hybrid scaffolds for hepatic tissue engineering (Jiankang et al. 2009). At first, they fabricated a polydimethylsilicone mold by using stereolithography and then cast the chitosan/gelatin solution on this mold. After casting the solution on the mold, they used lyophilization to prepare the scaffolds. Finally, the scaffolds were neutralized, washed, and sterilized for future use. Though stereolithography is a promising 3D printing technique for the fabrication of chitosan-based scaffolds, protonated primary amines of chitosan sometimes show reluctance to the radical formation and curing by quenching of newly formed free radicals (Morris et al. 2017). This makes the stereolithography of chitosan-based solutions challenging.

In comparison with stereolithography, selective laser melting uses a laser beam of heated CO_2 gas instead of a UV light beam. In selective laser melting, firstly, a powder layer is deposited on the platform. Then, the layer of powder is melted and sintered according to the CAD model by using a laser beam of CO_2 (Fig. 18.7d). The principal requirement for selecting laser melting is that the powder must be able to withstand the high temperature of the laser beam and resist shrinkage of the structure during the sintering process (Liu 2014). For resisting shrinkage, pre- and post-heating treatment between crystallization and glass transition temperatures plays an important role (Kruth et al. 2007). Selective laser melting has been applied to fabricate chitosan-based membranes and scaffolds. Sun et al. fabricated chitosan/polyurethane composite membrane using selective laser melting (Sun et al. 2019). They placed chitosan and polyurethane powder on a bed and used a CO_2 laser to obtain a pre-introduced pattern. The membranes showed enhanced catalytic activity and adsorption of heavy metal ions. An example of scaffold fabrication can be the work of Tsai et al. (2019). Instead of using direct chitosan powder for sintering, they sintered pre-alloyed Ti–6Al–4V using selective laser melting. The obtained scaffolds were coated with chitosan/magnesium-calcium-silicate composite to enhance bioactivity and osteoneogenesis.

Apart from these four direct printing techniques, another convenient technique for the fabrication of chitosan-based scaffolds is indirect 3D printing. In this technique, a sacrificial mold is first prepared by using a suitable 3D printing technique. After

casting chitosan-based polymer solution on the mold and subsequent freeze-drying/ particulate leaching, the mold is dissolved in a suitable solvent to obtain desired scaffold (Hassanajili et al. 2019). For instance, Lee et al. fabricated chitosan/ polycaprolactone scaffolds in gelatin mold which was prepared by 3D printing. After casting the cross-linked chitosan/polycaprolactone solution on the mold, the gelatin mold was dissolved using deionized water (Lee et al. 2013). Finally, the obtained scaffolds were washed with deionized water and lyophilized to obtain the final porous scaffold. Other direct printing methods have stringent requirements regarding powder properties and pre- or post-treatments. As indirect printing utilizes dissolvable mold, it can be crucial for overcoming the limitations of using chitosan and its derivatives in various direct 3D printing methods.

Despite the progress in fabricating chitosan-based scaffolds by 3D printing, some challenges are needed to be addressed. Integration of vascular network is one of the most critical challenges, and a microfluidic channel network can be a promising solution to this challenge. Microfluidic systems manipulate a small amount of liquids in micro-channel networks to integrate biomimetic systems into a single chip (Wu et al. 2017). These micro-channel networks provide the designable 3D architecture for cell culture and are more capable of closely mimicking native tissues to form tissue and organ-specific micro-architecture in scaffolds (Choi et al. 2007). In addition, altering traditional 3D printing with the 3D printed microfluidic system can significantly reduce manufacturing time and cost. Moreover, traditional 3D printed micro-fabrication cannot attain designs perfectly and repetitively in complex structures. Introducing a microfluidic system makes it possible to repeat the design multiple times and satisfy a wider range of commercial applications (García-López et al. 2017).

18.3.8 Other Fabrication Techniques

All the techniques mentioned above (Sects. 18.3.1–18.3.7) are the most common techniques for fabricating chitosan-based biomaterials. However, some other methods, such as wet spinning, spinning disk processing, etc., are employed for biomaterial fabrication. A polymer solution is injected through a spinneret into a coagulation bath and collected on a rotating spool in wet spinning. Finally, the coagulated fibers are rinsed and dried. Sibaja et al. fabricated chitosan/alginate fibers for biomedical applications by wet spinning technique (Sibaja et al. 2015). At first, they prepared alginate/sulfathiazole solution and then injected the solution through a spinneret to the solution of chitosan in acetic acid. Chitosan solution acted as a coagulation bath for the gelation of alginate/sulfathiazole solution. After the appropriate retention time, the fibers were taken out from the coagulation bath, washed, and dried. The incorporation of chitosan into the fibers improved their tensile properties and the resistance against the growth of *Escherichia coli*. Malheiro et al. also used wet spinning to fabricate chitosan/polycaprolactone blend fibers for tissue engineering (Malheiro et al. 2010). They prepared the solution of chitosan/ polycaprolactone and injected the solution into a coagulation bath of methanol.

Then, the fibers were neutralized with NaOH, washed, and dried for further use in fabricating scaffolds. The existence of roughness and porosity at the micron level found in the fibers could be potentially advantageous for cell adhesion. Wet spinning is becoming a convenient method for fabricating chitosan-based fibers. Along with wet spinning, recently, some other techniques are being used for fabricating nanofibers, such as self-polymerization, microcontact printing, ultrasonication and mechanical treatment, and thermally induced phase separation (Ahmed et al. 2018; Tao et al. 2020).

Another convenient fabrication technique can be spinning disk processing which is usually used to scale up the ionotropic gelation method discussed in Sect. 18.3.4.1. In this process, chitosan-based solution/suspension is fed on a rotating disk, and the dispersion is collected from the edge of the disk. Nanoparticles are obtained from the accumulated dispersion via further centrifugation. The spinning disk process has improved safety and superior particle size control with waste minimization (Loh et al. 2010). Further optimization of the technique can improve future fabrication processing and lessen the harsh processing conditions of fabricating chitosan-based biomaterials.

18.4 Designs of Chitosan-Based Biomaterials

Chitosan, being a natural cationic polymer, provides outstanding characteristics such as biocompatibility, biodegradability, antibacterial and antifungal properties, nontoxicity, hemostatic properties, antioxidant nature, protein degradability, etc. which make it an ideal component for drug delivery, wound healing, tissue engineering, gene delivery, regenerative medicines, and other biomedical applications (Ahmed et al. 2018). Hence, chitosan and its derivatives have been used to design various forms of biomaterials such as scaffolds, sponges, gels, films, membranes, beads, particles, etc., for applications in numerous biomedical fields.

18.4.1 Scaffolds

Scaffolding is the most common approach for biomaterial fabrication because of its close-to-nature structure with enhanced mechanical and biological properties. An ideal scaffold should hold the following characteristics to bring about the desired biological responses; (a) interconnecting pores of an appropriate scale to favor tissue integration and vascularization; (b) controlled biodegradability or bio-resorbability; (c) appropriate surface chemistry to avail cellular attachment, differentiation, and proliferation; (d) sufficient mechanical properties to resemble the site of implantation and handling; (e) no adverse responses; and (f) easily fabricable into a variety of size and shapes (Amoabediny et al. 2011). The three additional materials that have been successfully used for designing chitosan-based scaffolds are synthetic materials, polymeric materials, and ceramics. Depending on the fabrication methods and

construction materials, various forms of scaffolds are available such as porous scaffolds, nanofibrous scaffolds, and microsphere scaffolds, etc.

18.4.1.1 Porous Scaffolds

Porous scaffolds are three-dimensional polymeric structures with higher porosity along with homogeneous interconnected pore networks. These work like an extracellular matrix network for favoring cell attachment, proliferation, differentiation, and delivery of bioactive molecules (Liu et al. 2011). Chitosan-based porous scaffolds, due to their excellent porosity, cell compatibility, and mechanical properties, have been widely used for bone, skin, and cartilage tissue regeneration, nerve tissue engineering, wound dressing, and orthopedic applications. They are generally fabricated by freeze-drying, particulate leaching, and gas foaming methods. Among them, freeze-drying is the most common method for fabricating porous scaffolds. Some recently improvised methods have also been employed to fabricate scaffolds with high porosity. Supercritical drying technique is one of them (Liu et al. 2011). Scaffolds prepared in this method exhibit comparatively larger pore size and surface area, enhanced thermostability, and cell growth than the scaffolds prepared by conventional freeze-drying (Rinki and Dutta 2010). Porous chitosan scaffolds are twice more extensible than a nonporous one, but their tensile strength and elastic modulus can be about 10 times lower than nonporous ones (Madihally and Matthew 1999).

The incorporation of other bioactive components with chitosan enhances the porosity and various mechanical and biological properties of the porous scaffolds. Fan et al. developed collagen/chitosan porous scaffolds incorporating nano-TiO_2 hydrosol by freeze-drying technique (Fan et al. 2016). The resulting scaffolds exhibited high porosity, swelling ability, antibacterial activity against *Staphylococcus aureus*, rapid red blood cell aggregation to stop bleeding, and cytotoxicity, which made them suitable for wound repairing application. Karahaliloglu et al. also produced chitosan/silk sericin 3D porous scaffolds associated with lauric acid and zinc oxide nanoparticles for antibacterial wound dressing application (Karahaliloglu et al. 2017). The developed scaffolds were highly porous (pore size ranged from 200 to 4 μm) and exhibited good inhibition against gram-positive and gram-negative bacterial growth.

Chitosan-based porous composites are also used for bone and skin tissue regeneration. Liu et al. prepared porous chitosan scaffolds modified with phosphocreatine which exhibited high porosity (pore size ranged from dozens of microns to 200 μm), better solubility, hydrophilicity, protein adsorption, and mineralization (Fig. 18.8a) (Liu et al. 2018). These composite scaffolds possess great potential for bone repair and regeneration. Xu et al. also developed porous chitosan scaffolds with Ag-loaded strontium hydroxyapatite which exhibited excellent osteoinductivity and antibacterial activity against *Staphylococcus aureus*. The scaffolds were suitable for repairing bone defects without causing any infection (Xu et al. 2016). Another new type of chitosan magnetic porous scaffolds was developed to repair bone tumor-related defects by Yang et al. (2018). They incorporated magnetic mesoporous calcium silicate into porous chitosan scaffolds and reported that the resulting

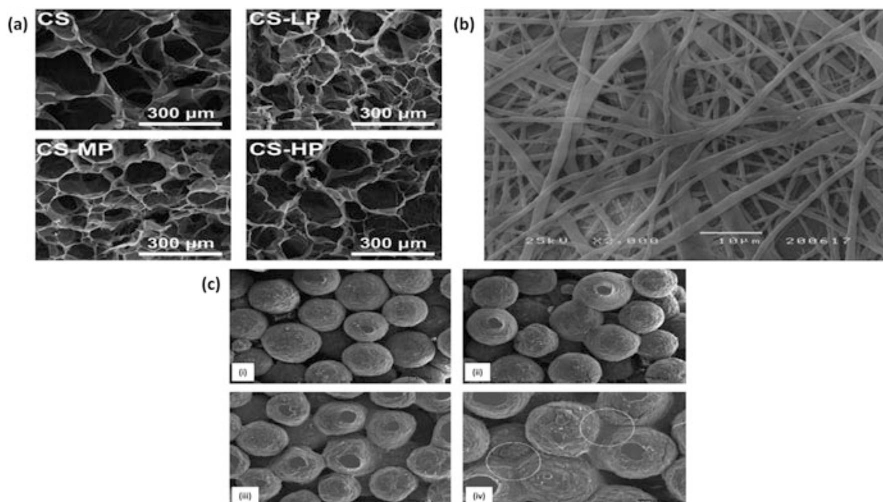

Fig. 18.8 Scanning electron microscopic (SEM) images of chitosan-based composite scaffolds. (**a**) Surface morphology of chitosan/chitosan phosphocreatine scaffolds having interconnected porous structures. The pore size of the scaffolds ranged from dozens of microns to 200 µm (reprinted with permission from reference Liu et al. 2018. Copyright © 2018 Elsevier Ltd.). (**b**) SEM image of chitosan/polycaprolactone composite nanofibrous scaffold showing fiber diameter (890 ± 364) nm (reprinted with permission from reference Gomes et al. 2017. Copyright © 2017 Elsevier B.V.). (**c**) SEM images of chitosan/poly(lactic acid-glycolic acid) microsphere scaffolds showing the morphology at (i) 100 °C, (ii) 110 °C, (iii) 115 °C, and (iv) 115 °C. The images show more excellent fusion between adjacent microspheres at elevated temperatures. Circles in (iv) illustrate pore closure among microspheres at the sintering temperature of 115 °C. (Reprinted with permission from reference Jiang et al. 2006. Copyright © 2006 Elsevier Ltd.)

scaffolds showed excellent antitumor efficacy to promote osteogenesis and synergetic photothermal chemotherapy against osteosarcoma. Chitosan porous scaffolds have also been used in cartilage tissue engineering. Vishwanath et al. blended chitosan with silk fibroin for developing a 3D porous scaffold which exhibited high porosity (pore size in the range of 71–210 µm) and compressive strength. Moreover, the assessment of glycosaminoglycan (GAG) secretion on the scaffolds made them suitable for cartilage tissue regeneration (Vishwanath et al. 2016).

However, due to their high porosity and homogeneous interconnected structure, porous scaffolds allow an extracellular matrix environment for nutrient delivery to the cells. Their porous structure sometimes restricts the homogeneous distribution of the cells. Besides, the requirements of ideal pore size for distinctive cell types are also stringent. Therefore, improvement of the interconnected porous structure is highly expected in the near future, which may advance their sectors of application.

18.4.1.2 Nanofibrous Scaffolds

Nanofibrous scaffolds are polymeric fibrous structures with fiber diameters less than 100 nm and an aspect ratio of more than 100 (Xia et al. 2003; Li and Xia 2004). Scaffolds of this type provide unique characteristics like large surface area, high

porosity, mechanical strength, and morphological uniformity, which make them capable of exhibiting superior biological functions such as resembling the nanoscale properties of extracellular matrix, transporting nutrients, promoting cell adhesion and migration, etc. (Rasouli et al. 2019). Chitosan nanofibrous scaffolds, due to characteristic uniformity, have a wide range of applications in bone, nerve, and tissue engineering, muscle and bone tissue regeneration, wound healing, etc.

The chitosan-based nanofibrous scaffold can be fabricated using electrospinning, self-polymerization, microcontact printing, ultrasonication and mechanical treatment, and thermally induced phase separation (Ahmed et al. 2018; Tao et al. 2020). Among these techniques, electrospinning is the most common and versatile one for fabricating chitosan nanofibers. Scaffolds prepared by this technique provide higher porosity, increased surface area, and the ability to mimic extracellular matrices (Croisier and Jérôme 2013). In order to improve various biological and mechanical properties of the scaffolds, chitosan is frequently blended with other polymers. For instance, Ardeshirzadeh et al. developed chitosan nanofibrous scaffolds with polyethylene oxide and graphene oxide by electrospinning which showed improved release of doxorubicin providing a new way for treating lung cancer (Ardeshirzadeh et al. 2015). Again, Shrestha et al. (2016) prepared chitosan/polyamide 6,6 biomimetic and biocompatible nanofibrous scaffolds which exhibited pre-osteoblast cell regeneration, cell viability, and higher competency to nucleate bioactive calcium phosphate nanoparticles for tissue engineering applications. Gomes et al. fabricated chitosan/polycaprolactone nanofibrous scaffolds by electrospinning process for skin tissue engineering (Gomes et al. 2017). The scaffolds showed higher elastic modulus (48 MPa), and the average fiber diameter found in the SEM image was (890 $\pm$ 364) nm (Fig. 18.8b). Dunne et al. also designed a silk fibroin/chitosan nanofibrous scaffold which offered vasculature in tissue engineering (Dunne et al. 2014).

Chitosan nanofibers exhibit better biocompatibility with stem cells which make these nanofibrous scaffolds beneficial for bone regeneration. Ho et al. reported that chitosan nanofibrous scaffolds exhibit better osteoinductive effects and initiate fast osteogenic differentiation, which enhance their use in bone defects (Ho et al. 2014). Shalumon et al. also fabricated nanofibrous scaffolds with chitosan and polycaprolactone, which exhibited better hydrophilicity and cyto-adhesion properties for skin and bone tissue engineering (Shalumon et al. 2011). Huang et al. prepared collagen/chitosan/thermoplastic polyurethanes nanofibrous scaffold for nerve regeneration application (Huang et al. 2011). The scaffolds were found to be flexible with high tensile strength and had the ability to repair vascular and nerve regeneration.

Due to the high surface area and morphological uniformity, chitosan nanofibrous scaffolds are appropriate for cell adhesion, differentiation, and proliferation. Moreover, these scaffolds' surface properties can be modified by incorporating various drugs, proteins, genes, and growth factors to improve their biomedical application. In a recent study, nanofibrous scaffolds were prepared from poly(L-lactide) acid and chitosan by coaxial electrospinning, which exhibited improve blood compatibility regarding the rate of hemolysis and platelet adhesion (Ji et al. 2013). Besides, the prepared scaffolds indicated good biocompatibility and biosecurity as drug delivery

carriers. Wang et al. (2013a) reported polycaprolactone/chitosan nanofibrous scaffolds, which can initiate osteoblasts adhesion, diffusion, and proliferation and inhibit *Staphylococcus epidermidis* colonization in the scaffolds. Sometimes nanofibrous scaffolds are designed as nanofibrous mats for the application of wound healing. Adeli et al. fabricated poly(vinyl alcohol)/starch/chitosan nanofibrous mats by electrospinning technique which showed appropriate porosity, balanced water absorption, and desired vapor transmission rate to enhance wound breathing and efficient handling of wound exudate (Adeli et al. 2019). Sometimes, nanofibrous mats are loaded with an antibacterial agent to improve cytocompatibility and antibacterial properties. For example, chitosan and poly(vinyl alcohol)-based nanofibrous mat was loaded with tetracycline hydrochloride, which showed improved cytocompatibility and antibacterial properties (Alavarse et al. 2017).

18.4.1.3 Microsphere Scaffolds

Microsphere scaffolds are generally polymer matrices having a spatial extension and temporal duration control. Being small in size, microspheres provide large surface-to-volume ratios and can be used for the controlled release of insoluble drugs. Scaffolds consisting of microspheres or nanospheres offer several advantages over the conventional ones. For example, these scaffolds provide controlled delivery of therapeutic agents and signaling biomolecules, can be designed as stimuli-sensitive drug carriers, and can be administrated as injectable and/or moldable formulations along with improved mechanical properties (Wang et al. 2012).

Chitosan microspheres are generally applied as drug carriers. These can also be employed to deliver bone morphogenetic protein-2 and insulin-like growth factor-1 (Kim et al. 2012). Besides, these can also be applied in cancellous bone defect treatment (Abdel-Fattah et al. 2007). Chitosan-based composites can manipulate various properties of microsphere scaffolds. For instance, Jiang et al. prepared chitosan/poly(lactide-co-glycolide) microspheres and sintered them into porous scaffolds (Jiang et al. 2006). The total pore volume of these scaffolds was in the range of 28–37%, with a median pore size in between 170 and 200 μm. More excellent fusion between microspheres was obtained at the elevated sintering temperature, which resulted in increased compressive properties and decreased overall pore volume of the scaffolds (Fig. 18.8c). Furthermore, the compressive modulus and compressive strength of these scaffolds are in the range for appropriate load-bearing function in bone tissue engineering. Shen et al. fabricated an injectable scaffold using two types of chitosan microspheres, with and without biomimetic apatite coatings for bone regeneration (Shen et al. 2013). Both the microspheres exhibited conducive ranges of particle diameter, density, and Rockwell hardness, though they varied in the degree of cross-linking, shape, morphology, degradation, and swelling rate. Moreover, the apatite-coated microsphere enhances the attachment, proliferation, and differentiation of cells to a greater extent than microspheres devoid of apatite. Chitosan microsphere composite scaffolds are also fabricated for nerve and skin tissue engineering applications. Niu et al. developed chitosan scaffolds loaded with silver microspheres fabricated by the lyophilization method (Niu et al. 2020). The composite scaffolds possess high porosity (66%) and

compressive strength along with antibacterial property and excellent drug release performance. These scaffolds can be used in skin tissue engineering. Another composite scaffold based on gelatin methacryloyl/chitosan microspheres was developed by Chen at al. using 3D printing, which exhibited enhanced neurite growth and extension and therefore provided an excellent possibility for nerve tissue engineering application (Chen et al. 2020). Overall, chitosan microsphere scaffolds offer suitable characteristics to slow or rapid release of drugs along with cell attachment and migration properties. However, sintering methods are sometimes not compatible with microspheres and reduce cell compatibility.

18.4.2 Sponges

Sponges are nothing but foams with a well interconnected microporous structure. Because of their unique structure, they are capable of absorbing a large volume of fluids (20 times more than their absolute weight) (Croisier and Jérôme 2013). Chitosan-based sponges with high porosity, high permeability, high swelling capacity, excellent mechanical strength, large surface area, and good antimicrobial activity have received significant attention in the biomedical field (Deng et al. 2020b). These sponges are typically fabricated by various methods such as lyophilization, gas foaming, particulate leaching, etc. Among them, lyophilization is the most common method as it produces chitosan sponges with interconnected porosity, which provides greater surface area for cells to attach and load drug solutions (Berretta et al. 2017).

Chitosan sponges are generally applied as wound dressing materials as they can absorb wound exudates during tissue regeneration. Besides, they also have applications as a filler in bone tissue engineering (Croisier and Jérôme 2013). However, the application of pure chitosan sponges as wound-healing material is restricted because of their fewer amino groups on the surface with lower hydrophilicity (Patrulea et al. 2015). Therefore, chitosan composite sponges are used in this regard. Hu et al. (2018) synthesized a composite sponge by mixing chitosan with hydroxy butyl chitosan, which significantly promoted the antibacterial ratio of *S. aureus* and *E. coli* and also exhibited better ability to enhance wound healing along with the faster generation of skin glands. Another new type of composite sponges was developed by the freeze-drying method using squid ink polysaccharide and chitosan, which showed rapid adsorption of hemocytes and a smaller volume of bleeding. These sponges exhibited good wound and burn/scalded skin healing properties along with protection from wound infection (Huang et al. 2018). Besides, the incorporation of various nano metal particles into chitosan sponges also improves the antibacterial activity and wound dressing properties. Anisha et al. (2013) synthesized composite sponges consisted of chitosan, hyaluronic acid, and Ag nanoparticles. The composite sponges exhibited interconnected porous structure with 73% porosity, whereas without incorporation of Ag nanoparticles, 63% porosity was observed (Fig. 18.9). Besides, this porous sponge exhibited high antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, methicillin-resistant

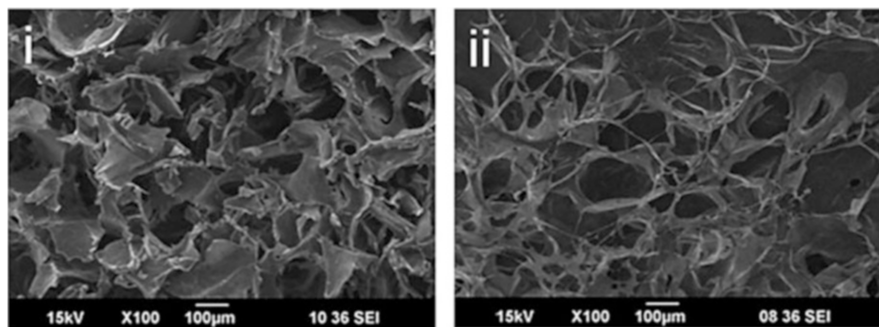

Fig. 18.9 SEM image presenting the porous morphology of composite sponges: (i) the chitosan/hydroxyapatite sponges presenting a less porous (63% porosity) and rough surface at interlayer and (ii) comparatively smoother chitosan/hydroxyapatite/Ag nanoparticles composite sponges with considerable porosity (73% porosity). (Reprinted with permission from reference Anisha et al. 2013. Copyright © 2013 Elsevier B.V.)

Staphylococcus aureus, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*. These composite sponges could be used as a wound dressing media for a patient suffering from diabetic foot ulcers caused by drug-resistant bacteria.

Another chitosan/alginic acid/zinc oxide nanostructured hydrogel sponges were developed by Bal-Ozturk et al. (2019), which showed good bacteriostatic effects on *Staphylococcus aureus* and had potency as a suitable antibacterial topical hemostat and hemorrhage control. Xia et al. (2020) also produced a novel quaternary ammonium chitosan nanoparticles/chitosan composite sponge with asymmetric wettability surfaces, which efficiently promoted chronic wound-healing reepithelialization and angiogenesis. Chitosan composite sponges have also shown application in tissue engineering because of their biocompatibility and ability to mimic extracellular matrix. Sionkowska and Płanecka (2013) fabricated three-dimensional silk fibroin/chitosan composite sponges that exhibited sufficient porosity for cell growth and mechanical strength to resist handling during implantation and can be a temporary support for the formation of new tissues and organs in tissue engineering. Chitosan and gelatin-based composite sponges also possess excellent cytocompatibility and capable of bone reproduction, making them suitable for tissue engineering application (Chaochai et al. 2016).

Chitosan-derived sponges are considered ideal for wound dressing application due to their good cell interaction, hydrophilicity, and antibacterial properties. Incorporation of other composite materials within the chitosan sponges enriches their mechanical strength, activity towards the targeted field of application. Moreover, it is possible to modify chitosan with other anti-inflammatory and pain-reducing materials to obtain sponges with numerous functions.

18.4.3 Gels

The chitosan-based gel is a semisolid continuous colloid or polymeric network that is expanded throughout its whole volume by a liquid. It can be formed by covalent cross-linkages and physical interactions among the ingredients (Slomkowski et al. 2011). The application of the gel in the biomedical sector is widespread and growing gradually. As a popular gel-forming agent, chitosan can be used to fabricate biomaterials in variable forms such as hydrogels, aerogels, nanogels, etc.

18.4.3.1 Hydrogels

Hydrogels are three-dimensional, hydrophilic, cross-linked polymeric networks that can swell and hold large amounts of water (from 10% to thousands of times of the dry mass) or biological fluids without deforming their structure (Rashid et al. 2018; Rahman et al. 2019; Rodríguez-Rodríguez et al. 2020; Akter et al. 2021). It can impart a soft consistency similar to living tissues suitable for various medical applications (Peppas et al. 2000; Ullah et al. 2015). Hydrogels also possess many other unique physicochemical properties making them compatible for varieties of biomedical applications, especially for tissue engineering (Drury and Mooney 2003), drug delivery system (Hoare and Kohane 2008), and wound healing (Kamoun et al. 2017). Throughout the years, numerous natural and synthetic polymers have been studied in hydrogel research.

As a natural cationic copolymer, chitosan presents a good deal of interest for hydrogel structures. Chitosan-based hydrogels can be fabricated either directly from native chitosan (by itself or combined with small anionic molecules) or by combining chitosan with other biocompatible polymers. The networks of hydrogels are categorized depending on the method of chitosan cross-linking and mode of preparation. Based on the extent of cross-linking action, hydrogels can be classified into two main types: (a) physically and (b) chemically cross-linked hydrogels (Hamidi et al. 2008). Physically cross-linked hydrogels can be prepared either by hydrogen bonding or by associating with small anionic molecules, polyanions, or hydrophobic interactions (Pellá et al. 2018). Boucard et al. prepared a two-layer hydrogel based on chitosan by cross-linking through hydrogen bonding (Boucard et al. 2007). The first layer showed good mechanical properties, whereas the second layer provided softness and flexibility to the material. Also, the second layer allowed the material to support wound geometry and had good mechanical properties besides gas exchange. Electrostatic interaction is another method used to produce chitosan-based physical hydrogels via cross-linking through small anionic molecules, polyelectrolytes, and metallic anions. Phosphate-bearing molecules, sodium alginate, heparin, polyacrylic acid, and polyglutamic acid are the common anions used for cross-linking of chitosan (Mohamed et al. 2017). For instance, Xiao et al. prepared chitosan hydrogel with sodium alginate, which promoted effective adsorption of compounds with varying charges and improved physical reticulation along with thermal stability (Xiao et al. 2016). In another study, a composite hydrogel based on chitosan, heparin, and poly(γ-glutamic acid) showed desired antibacterial activity, promoted cell proliferation, and imparted wound healing properties (Zhang et al. 2018).

However, the physically cross-linked hydrogel may not have high mechanical properties and may interact to environmental changes such as pH, temperature, or ionic strength (Berger et al. 2004). Moreover, the mechanical properties of these hydrogels also depend on the degree of deacetylation and molecular weight of chitosan.

On the other hand, chemically cross-linked chitosan-based hydrogels have improved mechanical properties with better chemical stability than the physical ones. Chemically cross-linked hydrogels are prepared by irreversible covalent linking of chitosan macromers by using chemical cross-linkers, photo cross-linking technique, and graft polymerization method (Moura et al. 2011). As a biocompatible cross-linking agent, genipin is widely used as a cross-linker in tissue fixation and drug delivery due to its lower cytotoxicity and improved biocompatibility (Jin et al. 2004). Genipin cross-linked chitosan hydrogels have a comparatively slower degradation rate, higher biocompatibility, and almost 10,000 times less toxicity than renowned glutaraldehyde cross-linked chitosan hydrogels (Bhattarai et al. 2010; Cui et al. 2014). Muzzarelli et al. investigated the biocompatibility, biodegradability, and pharmaceutical effects of genipin cross-linked hydrogels and reported that these hydrogels were well suited for clinical use (Muzzarelli et al. 2015). Gao et al. also studied the cellular adhesion and viability of genipin cross-linked hydrogels (Gao et al. 2014). They reported that these hydrogels did not induce any cytotoxic effects and significantly improved the cell adhesion and viability on hydrogel surfaces. Moreover, chitosan/gelatin networks cross-linked by genipin have been developed for articular cartilage tissue repair (Giri et al. 2012). In addition, chemically cross-linked chitosan hydrogels can also be applied in local tumor therapy. Olaru et al. fabricated hydrogels based on chitosan polyamine and nitrosalicylaldehyde via covalent cross-linking for local tumor therapy (Olaru et al. 2018). The prepared hydrogels exhibited an open channel architecture along with an interconnected porous structure having a pore size of around 50 μm (Fig. 18.10a). This interconnected porous structure is important in biomedical applications due to good oxygen and nutrient permeability along with good retention of the tissue fluids. Based on the techniques, photopolymerization is advantageous over other common routes of polymerization. Hydrogels formed by this advanced technology can be used for several applications, e.g., in laparoscopic devices, subcutaneous injection, or diversified surgeries (Nguyen and West 2002). Ifkovits et al. prepared a photo cross-linkable derivative of chitosan hydrogels by introducing azide and lactose in the moieties of the matrix, which can be used as a tissue adhesive in punctures (Ifkovits and Burdick 2007).

Overall chitosan-based hydrogels exhibit biocompatibility, biodegradability, and controlled release properties. Therefore, they can be encapsulated with various forms of drugs, such as nanoparticles, essential oils, solubilized drugs which enhance their application as drug delivery vehicles. However, multiple cross-linkers can interact with medications and deactivate or limit their therapeutic effectivity. Therefore, the use of chemically cross-linked hydrogels in the pharmaceutical application is restricted as it requires rigorous purification.

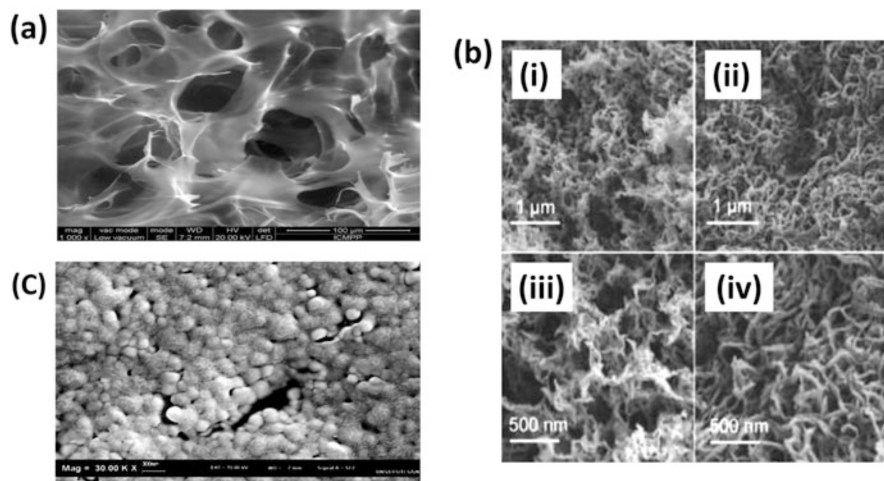

Fig. 18.10 SEM images of various chitosan-based composite gels: (**a**) chitosan polyamine and nitrosalicylaldehyde covalently cross-linked hydrogels having open channel architecture with interconnected porous structure (pore size around 50 µm) (reprinted with permission from reference Olaru et al. 2018. Copyright © 2017 Elsevier Ltd.). (**b**) Interior (i, iii) and surface (ii, iv) images of vancomycin-loaded chitosan aerogels showing a dual microporous (>50 nm) and mesoporous (2–50 nm) distribution with high porosities (>96%) (reprinted with permission from reference López-Iglesias et al. 2019. Copyright © 2018 Elsevier Ltd.). (**c**) Triclosan and flurbiprofen-loaded chitosan nanogels containing drug particles exhibited nanosized spherical structures on the background. (Reprinted with permission from reference Aminu et al. 2019. Copyright © 2019 Elsevier B.V.)

18.4.3.2 Aerogels

Aerogels are porous ultralight solid material with large surface areas. They are primarily derived from a gel, in which the liquid component of the gel has been replaced with gas without significant collapse of the gel structure. The porous microstructure in aerogel-based biomaterials is desirable for the absorption and preservation of biological fluids and transporting fluid and molecules (Zheng et al. 2020).

Chitosan-based aerogels have widespread applications depending on the surface reformations and modification in available functional groups. The unique properties of this type of aerogels, such as high surface area, high degree of polymerization, mechanical strength, high purity, and high crystallinity, make them suitable for several biomedical applications (Yahya et al. 2020). The pH-dependent solubility of chitosan allows it to process under mild conditions to form aerogels of various shapes and sizes (Rinki et al. 2011). López-Iglesias et al. reported chitosan-based aerogels loaded with vancomycin that provides a favorable pathway for the treatment of chronic wounds and also for preventing infection (López-Iglesias et al. 2019). These aerogels present remarkable antibacterial activity, with no serious adverse effects on collagenase function, and do not interfere with the normal

physiological system of wound healing. The chitosan aerogels showed a dual microporous (>50 nm) and mesoporous (2–50 nm) distribution with high porosities (>96%) and large surface areas (>200 m^2/g) (Fig. 18.10b). As a result, this chitosan-based aerogel can be loaded with different available antibiotics. For example, Wu et al. prepared ampicillin-loaded chitosan aerogel, which showed strong antibacterial activity without any leaching (Wu et al. 2018). Besides, they examined the in vitro cytotoxicity of these aerogels using human cells and found that the aerogels are biocompatible and could speed up wound healing efficiently. Gómez et al. studied a comparison of mechanical properties of chitosan-based aerogels (Gómez et al. 2018). When the aerogels were loaded with antibiotic (erythromycin), they showed a reduction in hardness and Young's modulus, unlike when aerogels were loaded with elephant garlic extract that showed an increase in the hardness and Young's modulus. Therefore, it is evident that any addition to the aerogels affects their morphology, porosity, hardness, and mechanical strength to a considerable level. Another group of researchers prepared aerogels from aqueous colloidal suspensions of chitosan and chondroitin sulfate (ChS) nano-complexes (Concha et al. 2018). The chitosan/ChS aerogels were highly neutralized, porous, ultralight, and soft with facilitated mechanical properties for application in open wounds. Besides that, the chitosan/ChS aerogels could also enhance wound bed quality and tissue granulation and suppress pain. The use of radiation is a modern technique to be used in fabricating aerogel-type biomaterials. Radwan-Praglowska et al. proposed a microwave-assisted technique for the synthesis of chitosan aerogels which promote cell proliferation significantly with adequate antioxidant property (Radwan-Pragłowska et al. 2018). Aerogels fabricated by combining genipin and chitosan can support cell adhesion and proliferation (Kumari and Dutta 2010). Furthermore, these aerogels were reported to be far less toxic than glutaraldehyde and safe for tissue engineering application.

Despite making some significant progress in the preparation and application of aerogels, some problems are still there to be solved. Aerogels are highly porous, which causes a reduction in mechanical strength. For instance, chitosan-based aerogels are often brittle. That is why controlling elasticity and strength during the preparation of chitosan-based aerogels are required for applying these aerogels in distinct fields of applications shortly.

18.4.3.3 Nanogels

Nanogels are three-dimensional hydrogel particles formed by physically or chemically cross-linked networks in the nanoscale size range (typically from tens to hundreds of nanometers) (Soni et al. 2016). They own both the characteristics of hydrogels and nanoparticles simultaneously. They are biocompatible and biodegradable materials that can absorb large volumes of water and show nanosize effects (e.g., large specific surface area and high surface energy), thus permitting fast water exchange. In recent years, chitosan-based nanogels have received considerable attention because of their excellent properties of antibacterial activity, large surface area, low immunogenicity, and stimuli responsiveness (Wang et al. 2017a).

Various properties of chitosan nanogels can be enhanced by chemical modifications in the chitosan backbone or by the addition of other therapeutic molecules/polymers/nanoparticles to the chitosan nanogel network. Chitosan backbone contains primary hydroxyl and amine groups, and these groups can be modified to yield new and superior properties (Peregrino and Seabra 2017). Zhang et al. modified chitosan nanogels by increasing the number of amino groups on the chitosan backbone, which increased antibacterial activity (Zhang et al. 2017). Polymers such as pluronic, poloxamer, agarose, etc., modify mechanical properties when incorporated into chitosan nanogels (Yap and Yang 2016; Malli et al. 2017). Nanoparticles such as graphene oxide, silver, or zinc nanoparticles etc. can be incorporated into chitosan nanogels to form composites which increase the antibacterial effect (Zhou et al. 2012; Zhao et al. 2017). Along with an antibacterial effect, nanogels can also be used as a carrier of therapeutic agents. Aminu et al. loaded triclosan and flurbiprofen on chitosan nanogels, and the nanogels showed augmented antibacterial and anti-inflammatory effects (Aminu et al. 2019). The SEM images of the nanogels containing drug particles exhibited nanosized spherical structures on the background (Fig. 18.8c). The extent of cross-linking has a significant impact on the pore sizes of chitosan nanogels. Moura et al. prepared chitosan nanogels by combined cross-linking method with glycerol disodium salt and genipin (Moura et al. 2017). The prepared nanogels exhibited comparatively larger pores (~600 μm) than the nanogels cross-linked with only the glycerol disodium salt (~250 μm). Along with glycerol, some other cross-linkers for temperature and pH-responsive nanogels are sorbitol, fructose, glucose-phosphate salts, etc. (Yang et al. 2014). Heris et al. used glycol and glyoxal (0.005%) as cross-linkers and found the degradation rate of nanogels decreasing with increasing concentration of cross-linkers (Heris et al. 2015).

Since biocompatibility is an important requirement for biomedical applications, chitosan-based nanogels should not exhibit cytotoxicity. Dang et al. examined the cytotoxicity of chitosan-based nanogels containing acid-soluble collagen, cross-linked with α,β-glycerophosphate by performing intraperitoneal injections of nanogels in mice (Dang et al. 2017). The injections were biocompatible as these did not cause hematopoiesis or damage to the liver or kidney functions of mice. Sapru et al. assessed the cytotoxicity and antibacterial activity of chitosan nanogels containing sericin cross-linked with genipin (Sapru et al. 2017). The nanogels were porous, stable ($<$6 weeks), and flexible. These nanogels showed a minimal immune response and noninflammatory behavior with increased antibacterial activity. Nanogel dimension (smaller than 5 nm), drug load capacity, drug delivery efficiency, release duration, and nanogel degradation rate create challenges for biomedical applications. Some methods carry disadvantages which are due to the unreacted molecules in nanogels, toxic residuals, and unfavorable by-products. Sometimes, less solubility of chitosan in physiological pH limits the scope of applications (Wang et al. 2017a). Table 18.7 summarizes the key properties and the advantages and disadvantages of different types of chitosan-based gels.

Table 18.7 Pros and cons of various chitosan-based gels

Types of gels	Properties	Advantages	Disadvantages
Hydrogels	• Capable of swelling and adsorbing high amount of liquids	• Exhibit flexibility similar to natural tissues for high water content and viscoelastic properties	• Physically cross-linked chitosan hydrogels exhibit low mechanical properties and may interact to environmental changes such as pH, temperature, or ionic strength (Berger et al. 2004)
	• Exhibit excellent antibacterial/antifungal activities	• Possess physiological pH and gelation temperature optimal for the human organism (Assaad et al. 2015)	• Covalently cross-linked chitosan hydrogels require an extra process of purification to remove toxic unreacted cross-linkers
	• Biocompatible and biodegradable due to their hydrophilicity and capability to degrade via human enzymes	• Injectable hydrogels allow homogenous and easy distribution of bioactive cells and molecules which reduce the risks for the surgical techniques (Moura et al. 2017)	• Genipin cross-linked chitosan hydrogels possess the risk of incompatibility with the therapeutic agent loaded into the hydrogels (Bhattarai et al. 2010)
	• Exhibit mucoadhesive characteristics via interactions between opposite charges	• Biocompatibility of chitosan-based hydrogels makes them potential wound dressing and tissue engineering materials (Falco et al. 2017)	• Production of chemically cross-linked hydrogels by irradiation requires the use of high cost and rigidly controlled equipment (Rufato et al. 2018)
Aerogels	• Porous ultralight solid material with large surface areas	• Dual microporous and mesoporous distribution make them suitable for loading antibiotics	• High manufacturing costs
	• High degree of polymerization.	• pH-dependent solubility allows treating in mild conditions	• Poor mechanical strength
	• Excellent thermal and acoustic properties	• High porosity along with antimicrobial activity provide sufficient moisture balance and pH for promoting wound healing (Batista et al. 2020)	• Large surface area may sometimes cause drawbacks in long term stability because of humidity and moisture uptake (Takeshita et al. 2021)
	• Low refractive index and dielectric constant		
	• High purity and crystallinity		
	• Adequate antioxidant property		
Nanogels	• Own both the characteristics of hydrogels and	• High swelling capacity accommodates high drug loading capacity	• Some residual surfactants or monomers

(continued)

Table 18.7 (continued)

Types of gels	Properties	Advantages	Disadvantages
	nanoparticles simultaneously		may cause toxicity (Wang et al. 2017a)
	• Can absorb large volumes of water and show nanosize effects	• By adjusting cross-linking agents pore size and drug release capacity can be regulated (Moura et al. 2017)	• Total removal of residual solvent and surfactants requires expensive techniques.
	• Provide large specific surface area and high surface energy	• Biodegradable and nontoxic	• Difficulty in controlling pore size
	• Low immunogenicity, and stimuli responsiveness	• Nanosize effects enhance better permeability through biological membranes.	
		• Excellent transport characteristics	

18.4.4 Films and Membranes

Polymer-based films and membranes are standard in various applications. Chitosan is a natural biopolymer that can be designed as films and membranes in biomaterials. Chitosan film is a thin layer of chitosan or chitosan composite, which is an opaque membranous covering, whereas a chitosan membrane is a flexible enclosing that separates two distinct environments. Chitosan-based films and membranes are most commonly applied for wound-healing, wound dressing, and drug delivery purposes, etc. Chitosan cross-linked films have remarkable applications in drug delivery devices, especially in oral mucosal delivery, buccal delivery, transdermal delivery, sublingual delivery, and periodontal delivery (Ali and Ahmed 2018).

Chitosan films exhibit excellent antibacterial and bioadhesive properties, making them a suitable candidate for use as biomaterials, especially in drug delivery, wound healing, and wound dressing purposes. These films are generally fabricated by solvent casting from chitosan salt solutions with subsequent drying (usually using the oven or infrared (IR) drying) (Khan et al. 2000). Physical and mechanical characteristics of chitosan films, such as tensile strength, water-resistant property, thermal stability, color- and moisture-retaining capacity, etc., can be improved by cross-linking and by incorporating different additives. For example, the porosity of chitosan films can be controlled into macro and micro sizes (0.5–100 μm) by incorporating silica particles or poly(ethylene glycol) (Clasen et al. 2006). Also, the incorporation of various compounds in chitosan films enhances their mechanical, biological, and wound healing properties. Chitosan films prepared with dialdehyde starch exhibit upgraded mechanical features along with improved water-absorbing capability (Tang et al. 2003). Furthermore, chitosan minocycline hydrochloride blended films speed up the healing of burns and reduces wound size (Aoyagi et al.

2007). Devi et al. prepared nanocomposite films of bentonite/chitosan for wound healing purposes which showed enhanced water absorption capacity and mechanical strength along with improved activity against gram-positive and gram-negative bacteria (Devi and Dutta 2017). Again, heterogeneous chemical modifications in chitosan films also affect the surface properties. For example, when chitosan films are attached with a stearoyl group, they become more hydrophobic and promote protein adsorption, but when these films are attached with succinic anhydride or phthalic anhydride, they become hydrophilic and promote lysozyme adsorption (Tangpasuthadol et al. 2003). Moreover, the addition of inorganic particles in these films permits a synergistic effect, which leads to high-performance healing. As an illustration, silver nanoparticles can be added to chitosan films for avoiding microorganism proliferation and increasing antibacterial activity. Li et al. prepared a complex film with chitosan, Ag nanoparticles, and zinc oxide particles which exhibited superior antibacterial activity for wound dressing (Li et al. 2010a). Also, Archana et al. produced chitosan films with poly(vinyl pyrrolidone) and silver oxide nanoparticles which showed high antibacterial activity, good swelling ability, and better wound healing capability than native chitosan (Archana et al. 2013).

Though chitosan-based films are applied advantageously in wound healing, wound dressing, and drug delivery purposes, the morphology of the films is not easily controllable during fabrication. Moreover, further research should be directed to explore new biomedical applications. Chitosan-based membranes possess hydrophilicity, biocompatibility, cationicity, and ease of modification, which make them suitable for biomedical applications. They are generally prepared by the dry-/wet-phase inversion method. These membranes can be modified into various types, including derivative, cross-linked, blended, multilayered, inorganic filler-embedded, and so on (Jafari Sanjari and Asghari 2016). Various additives can be combined with chitosan to enhance the properties of chitosan-based membranes. For instance, Behera et al. developed a chitosan/titanium composite membrane by incorporation of titanium dioxide for dressing purposes (Behera et al. 2017). Improved flexibility, crystallinity, porosity, and mechanical strength were obtained by strong O-Ti-O bonding. Membranes with 0.25% titanium dioxide exhibited a decreased apoptosis and oxidative stress and improved survival and proliferation in L929 cells. Furthermore, the membrane showed excellent antibacterial activity against *Staphylococcus aureus*. Similarly, Silva et al. blended chitosan and soy protein to fabricate membrane for skin scaffolding and wound healing (Silva et al. 2007). Chitosan-based fibrous membranes also exhibit excellent properties. Chen et al. designed composite nanofibrous membranes of poly(ethylene oxide), type I collagen, and chitosan by electrospinning (Chen et al. 2008). Cross-linking by glutaraldehyde increased the diameter of these nanofibrous membranes from 134 to 398 nm. Moreover, cross-linking increased Young’s modulus of these membranes. The membranes were better for wound dressing than viable collagen sponge wound dressing and gauze. Gu et al. cross-linked the silk fibroin/chitosan blended membranes by alginate aldehyde (Gu et al. 2013). Obtained membranes were suitable for wound dressing due to improved water permeability, water absorption, and stability. Although chitosan-based membranes have many advantages in wound dressing and wound

healing, until now no ideal wound dressing has been produced. Furthermore, the development of bacterial infections in compromised skin structure of wounds is a complication that demands more attention for further research.

18.4.5 Beads and Particles

Chitosan can be designed as beads and particles for biomedical applications, especially as drug carriers for various controlled release drug delivery such as in oral and buccal delivery, stomach specific drug delivery, intestinal delivery, colon-specific drug delivery and gene delivery, bone regeneration, and wound-healing applications (Sonia and Sharma 2011). Chitosan beads and particles are generally prepared by ionic gelation, spray-drying, complex precipitation, and emulsion-cross-linking or emulsion-droplet coalescence method (Yadu Nath et al. 2017). Beads and particles are broadly classified as (a) nanoparticles, (b) microparticles, and (c) beads. Nano- and microparticles are particles with nano- and micro size ranges, whereas beads are particles with a size range of approximately 500–700 μm (Kulkarni et al. 2007).

Chitosan nanoparticles exhibit greater importance in polymeric therapeutic delivery to the target site along with better biodistribution, specificity, and sensitivity. Nanoparticles fabricated with self-branched chitosan show improved colloidal stabilities and better cellular uptake than that of the linear polymer (Malmo et al. 2011). For instance, poly(ethylene glycol)-grafted chitosan nanoparticles exhibit sufficient gene transfer efficiency and no toxicity when tested in neuronal cells (Malhotra et al. 2011). Zhang et al. fabricated poly(ethylene glycol)-grafted chitosan by ionic gelation method, which showed excellent insulin binding capacity (Zhang et al. 2008). Grafting linear chitosan polymer and formation of nanoparticles can also be done by using tripolyphosphate instead of poly(ethylene glycol) for augmenting the rapid release of insulin (Fernández-Urrusuno et al. 1999). Banik et al. studied the function of particle size and pH in chitosan-montmorillonite nanoparticles loaded with isoniazid drug and found that swelling and mucoadhesivity had been increased with decreasing particle size and pH of the medium (Banik et al. 2012). But cytotoxicity increased with a decrease in particle size, which can be mitigated by incorporating clay into the system. Chitosan nanoparticles can also be used for bone tissue engineering. Meng et al. developed bioactive cement based on chitosan-polyglactin fiber scaffold containing chitosan nanospheres as injectable material for bone tissue regeneration (Meng et al. 2015). Some other applications of chitosan nanoparticles are bioimaging and oral nonviral gene delivery. Ge et al. reported the consequences of chitosan nanoparticles on bioimaging by investigating the effect of quantum dots for bio-detection (Ge et al. 2009). They concluded that the fluorescence of quantum yield of quantum dots in chitosan was found to be 11.8% higher than that of free quantum dots. Martien et al. developed an alternative carrier for oral nonviral gene delivery (Martien et al. 2007). They cross-linked chitosan by thioglycolic acid via amide bond formation mediated by a carbodiimide which showed improved oral delivery of therapeutic genes.

A principal use of chitosan microparticles is in drug delivery systems. The drug-releasing capacity of these microparticles in a suitable pH range can be enhanced by chemical cross-linking. For instance, Lorenzo-Lamosa et al. used chemical cross-linking to prepare a novel colonic drug delivery system consisting of chitosan core-coated microspheres (Lorenzo-Lamosa et al. 1998). These provided an enhanced release of the encapsulated drug over a prolonged and adjustable period of time at pH 7.4 with low cytotoxicity. Nano-hydroxyapatite/chitosan hybrid microparticles provide bone repairing drug delivery systems as these microparticles can be encapsulated with therapeutic and bioactive factors to increase functional properties (Ruphuy et al. 2016). Chitosan microspheres cross-linked with glutaraldehyde show poor biodegradability and higher cytotoxicity, so their application as a biomaterial is restricted. For this reason, genipin is used as a cross-linking agent as it provides better biocompatibility, biodegradability, and no cytotoxicity compared with chitosan microspheres cross-linked with glutaraldehyde (Yadu Nath et al. 2017).

Along with chitosan microspheres and nanoparticles, chitosan beads also show improved drug release and drug loading capacity. They are generally fabricated by the ionic gelation method. Chitosan beads can be incorporated with other composite or polymers for enhancing drug-bearing capacity and other mechanical and biological properties. For example, Shu et al. loaded fluorescein isothiocyanate-dextran into chitosan-tripolyphosphate beads which exhibited more than 90% loading capacity along with an increase in mechanical strength (Shu and Zhu 2000). Furthermore, chitosan beads containing phosphorus can be loaded with indomethacin (Jayakumar et al. 2006).

Chitosan beads produced under the influence of microwaves show various useful properties for biomedical applications. Piatkowski et al. synthesized microwave-assisted chitosan beads and found tunable morphology, biodegradability, and antioxidant characteristics (Piątkowski et al. 2018). Antioxidant effects of chitosan beads were increased by the addition of *Tilia platyphyllos* extract, and these beads could be applied in cell therapy and tissue engineering. Sukhodub et al. also prepared hydroxyapatite/chitosan beads loaded with C60 fullerene under the influence of microwave irradiation, where the drug release time of beads can be controlled by changing the C60 fullerene content (Sukhodub et al. 2019).

The drug release capacity of these beads was proportional to pH, and ionized phosphorus groups in beads governed the release rate. A fucoidan-shelled chitosan bead was synthesized for oral delivery to inhibit bacterial growth (Yu et al. 2015). The drug release capacity of these beads was faster in gastric fluid (pH 1.2) than intestinal fluid (pH 7.4). Besides, these beads inhibit the growth of clinical pathogens. Particle size is another important factor for drug-releasing capacity. For instance, Li et al. developed chitosan beads by continuous flow injection process (Li et al. 2020). The resulting beads were of uniform size and porous microstructure and possessed high swelling capability and good biocompatibility. As this preparation method does not need any acid/alkali treatment or cross-linking agents, hence it becomes preeminent for biomedical applications. Furthermore, these beads have the potential for hemostatic dressing application as these can induce blood coagulation and clotting. Most of the recent studies have shown that chitosan-based nanoparticle

or bead composites provide superior advantages as these render high surface area, which enhances various properties suitable for biomedical applications. Varieties of chitosan derivatives and newer formulations are being synthesized and developed day by day; more applications and modifications of the properties of chitosan-based particles are expected in the near future.

18.4.6 Other Forms of Chitosan-Based Biomaterials

All the abovementioned forms are the most common forms of chitosan and its derivatives in various biomaterials. However, there are also some other less common forms that are progressively being fabricated for biomedical applications, such as surgical sutures, microswimmers, biostrips, etc. A surgical suture is the equipment used to hold body tissues together, usually after surgery. The most important requirement for surgical suture is that it must be strong enough to hold tissues and also be flexible enough to be knotted. Along with strength and flexibility, an ideal suture should also reduce the chances of infection. Chitosan can be used to fabricate surgical sutures with good antibacterial activity and adsorption capacity. da Silva et al. loaded *N*-acetyl-D-glucosamine in chitosan filaments and found improved adsorption capacity without altering the morphology of the chitosan filaments (da Silva, da Silva et al. 2019). Also, the prolonged release of *N*-acetyl-D-glucosamine causes relief from pain and minimizes infection rate, which makes surgical sutures promising for surgical applications. Other polymers can also be blended with chitosan to fabricate surgical sutures. Deng et al. blended keratin, polycaprolactone, and polyethylene glycol with chitosan to fabricate surgical sutures, which showed rapid and prolonged release of diclofenac potassium (Deng et al. 2020a). Instead of the direct use of chitosan to prepare surgical threads, chitosan can also be coated on surgical threads made up of other materials to impart enhanced antibacterial activity and surface hydrophilicity (Debbabi et al. 2017).

Chitosan can also be used to fabricate biostrips which can electrochemically detect biomolecules such as dopamine, glucose, etc. An example can be the work of Kafi et al. (2020). They fabricated a resorbable biostrip having a sensing electrode of mesoporous chitosan/graphene oxide composite and graphene-based interconnects. The biostrips offered a limit of 10 pM dopamine detection and hence are promising for dopamine detection in the early-level diagnosis of neurodegenerative diseases. In recent years, microswimmers are gaining sparkling attention for biomedical applications due to wireless actuation, active locomotion, and precise localization capabilities (Sitti et al. 2015). Bozuyuk et al. reported chitosan-based microswimmers promising for the delivery of therapeutics due to on-demand light-triggered drug release (Bozuyuk et al. 2018). We hope that future research will open the door to broader possibilities for applying these untraditional forms of biomaterials to varieties of biomedical applications.

18.5 Limitations, Concerns, and Future Suggestions

In recent decades, researchers have given considerable efforts to develop appropriate techniques for fabricating chitosan-based biomaterials and improve existing design characteristics for enhancing their biocompatibility and physicochemical properties that are suitable for biomedical applications. However, there are still some challenges to achieve maximum efficacy of these techniques and designs, which restrict their larger-scale productions and practical implementation in the human body. These issues provide tremendous scopes for further research:

- Most of the conventional techniques for fabricating chitosan-based biomaterials have been implemented on a laboratory scale. There are many difficulties in scaling up these techniques and launching these biomaterials in the market, which provide remarkable scopes for future research. Moreover, researchers have investigated the potentiality of various chitosan-based biomaterials in biomedical applications mostly by in vitro experiments, and some of these experiments were done in animal bodies. Till now, there has been a lack of human clinical trials of these biomaterials, and this lacking opens the door of further study.
- Some major problems for fabricating chitosan nanofibers for scaffolds, mats, membranes, etc. are the inconsistency, structural instability of chitosan fibers, and high surface tension of chitosan solutions. The reason for inconsistency and instability of structure is due to the remaining solvent and formation of chitosan salts in an acidic medium. Although exposing the fibers in water vapor can substantially remove the remaining solvent, extensive investigation is needed to find out more convenient ways to reduce excess solvent without causing inconsistency and instability of fibrous structure so that the structural morphology becomes suitable for biomedical applications. Researchers are also studying on reduction of the surface tension of the chitosan solution and have been successful in many cases by adding other synthetic polymers and inorganic substances in the solutions.
- While reviewing the literature, we found that many techniques for fabricating chitosan-based nanoparticles show a wide range of size distribution which shows nonuniformity in the quantity of loaded drug and variety in drug release profile. So, further study is needed for the modification of conventional techniques to obtain narrow particle size distribution. We also found that enormous organic solvents and additives were used for the fabrication of various forms of biomaterials, but safer solvents and additives need to be further investigated. Furthermore, some high experimental requirements and complicated procedures cause a great challenge for actual practice.
- Many chitosan-based biomaterials are promising for wound dressing applications because of their superior antibacterial and antifungal activity. However, uniform recovery of the wounded area is still not achievable in many cases, which makes the recovered wound prone to antibacterial infection. So much attention is needed in this area to ensure the uniformity of healed wounds.

- Chitosan shows poor solubility in neutral and higher pH. Thus, an acidic medium is a prerequisite for solubilizing chitosan, which limits the application fields of chitosan only in an acidic medium having pH < 5. This solubility profile may cause challenges for applying chitosan-based biomaterials in many human applications. Approaches to increase the solubility of chitosan in neutral and alkaline pH could add a new dimension to make the fabrication of biomaterials more worthy.
- Modern fabrication of multifunctional biomaterials with desired properties does not rely on a single fabrication technique but instead utilizes the combination of different techniques. Moreover, another approach to attempt a new design could be the simulation of the process. Preliminary studies with simulation may be helpful to minimize the cost and duration of time for trial.

18.6 Conclusions

With the progressing developments in polymer sciences, enumerable polymers have been introduced to the scientific community. Among them, chitosan offers magnetic properties, structural modifications, and functions for biomedical and pharmaceutical applications. This chapter summarizes various fabrication techniques for designing chitosan-based scaffolds, sponges, gels, membranes, particles, etc., for biomedical purposes. Till now, all the fabrication techniques have been operated in laboratory scales; therefore, active research is going on to scale up these fabrication techniques efficiently for widespread applications. Moreover, researchers are trying to modify these fabrication techniques to obtain the expected shape of the available forms and impart valuable features to these fabricated forms like higher mechanical strength, porosity, and lower cytotoxicity, etc., for human application. The authors anticipate that further research on varying the parameters of fabrication techniques and modification of chitosan-based matrices can prove to be an excellent boon for implementing chitosan-based biomaterials for human trials in the near future.

Conflicts of Interest The authors do not have any conflicts of interest to declare.

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19 Chitosan-Based Hydrogels for Tissue Engineering

Bijoy Krishna Roy, Ishmam Tahmid, and Taslim Ur Rashid

Abstract

Hydrogel, a cross-linked polymeric material that can hold a large amount of water while maintaining its structure, has drawn gigantic attention of scientific communities for numerous applications in the realm of tissue engineering due to its structural similarity to the macromolecular-based components in the human body. Several natural polymers have been widely used in fabricating hydrogels because of their ability to provide various advantages, including biocompatibility, biodegradability, and low cost. Among the natural polymers, chitosan has been widely investigated because of its ability to impart a myriad of advantages due to unique polysaccharide structures with cationic amine groups. Given potential applications of chitosan-based hydrogels, this chapter focuses on the most recent progress that has been made concerning fabrication technologies, structural and bioactive properties of these materials, and their most significant accomplishments for tissue engineering. The chapter also highlights the newest developments of chitosan-based innovative hydrogel materials with an outlook into their future applications in the broad area of tissue engineering. An attempt has also been made to discuss the various modifications in the design of the hydrogels that boost their use for a given set of applications which would pave the way for future applied research in tissue engineering innovation.

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Hydrogel · Chitosan · Biomaterial · Tissue engineering

19.1 Introduction

Tissue engineering, which combines the knowledge of physics, chemistry, and biology, has become an exciting and revolutionary approach to treat patients who suffer from the loss or failure of an organ or tissue as a result of accidents or diseases (Lee and Mooney 2001; Muschler et al. 2004). This rapidly expanding research domain is now directed to the development of new biomaterial matrices focusing on properties, benefits, limitations, and the use of alternative resources to generate functional tissues for specific applications. Among the most studied biomaterials, hydrogels based on natural biopolymers, such as chitosan, cellulose, gelatin, alginate, dextran, xanthan, etc., have been standing out as an appealing strategy owing to their advantages, like biocompatibility, biodegradability, mechanical and thermal stability, and responsiveness. Hydrogels are excellent hydrophilic polymeric materials having high water absorption capacity maintaining a distinct three-dimensional structure. The distinctive structural and compositional similarities of hydrogels to the extracellular matrix (ECM), advantageous architecture for cellular multiplication and survival, and ease of controlling the shape, size, and morphological aspects have made them prime candidates for engineered tissue matrices (Rusu et al. 2019).

Recently, chitosan-based hydrogel materials have received a great deal of attention in tissue engineering applications due to their superior characteristics regarding biocompatibility, high mechanical and thermal stability, nontoxicity, natural abundancy, and cost-efficiency. Besides, chitosan can be degraded in vivo by several enzymes, and the degradation products are nontoxic oligosaccharides that can be excreted or incorporated into glycosaminoglycans and glycoproteins (Pellá et al. 2018). Chitosan is a cationic linear polysaccharide copolymer containing randomly distributed *N*-acetyl-D-glucosamine and D-glucosamine units obtained from deacetylation of chitin, the second most abundant naturally occurring biopolymer extracted from the exoskeleton of crustaceans and insect, from fungal cell walls, etc. (Pellá et al. 2018). The primary amine groups and hydroxyl groups of chitosan allow interactions with other materials as well as chemical derivatization. Hence, it can be easily modified to different derivatives by which the properties of this polymer can be modulated and adjusted to the required application (Racine et al. 2017). In addition, other biocompatible polymers can be combined with chitosan to fabricate hydrogels in order to achieve improved interactions with cells; the capability of carrying bioactive agents including proteins, genes, and cells; and controlled degradation through enzymatic cleavage and/or hydrolysis considering the tissue formation rate (Shariatinia and Jalali 2018). Its exceptional polycationic, antimicrobial,

antitumor, and bioresorbable properties have led to its wide range of biomedical applications, such as controlled drug delivery (Kiene et al. 2018), tissue engineering (Salehi et al. 2019), wound dressing (Chen et al. 2017), as well as biosensor fabrication (Zanini et al. 2011; Racine et al. 2017).

This chapter will summarize the use of chitosan-based hydrogels in various fields of tissue engineering. The structure, extraction, and properties of chitosan are first discussed, along with the mechanism of hydrogel formation. We will also present specific examples of chitosan-based hydrogels used for each of the major categories of applications that include bone tissue engineering, cartilage tissue engineering, intervertebral disc tissue engineering, blood vessel tissue engineering, neural tissue engineering, corneal regeneration, skin tissue engineering, periodontal tissue engineering, and tissue fixation. The future prospects and challenges will be described with adequate discussion on design and material selection for each specific application.

19.2 Chitosan

19.2.1 Structure, Sources, Extraction, Properties

Chitosan is a deacetylated derivative of chitin, a high molecular weight natural polysaccharide (Rahman et al. 2015; Ahsan et al. 2018). Next to cellulose, chitin is the second most abundant natural biopolymer, commonly found in the exoskeleton of crustaceans, insects, and fungal cell walls (Di Martino et al. 2005). It is a linear polysaccharide composed of glucosamine and *N*-acetyl glucosamine linked in a (1–4) manner, with a high degree of acetylation (Fig. 19.1). The ratio of the glucosamine to the *N*-acetyl glucosamine, referred to as the degree of deacetylation (DD), can be increased significantly, usually by the alkaline deacetylation of chitin, and the derivative obtained is known as chitosan. There are no strict guidelines for

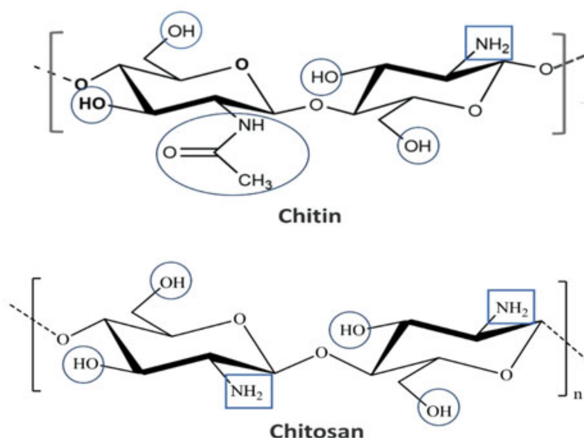

Fig. 19.1 Chemical structure of chitin and chitosan

defining chitosan; however, chitin with a degree of deacetylation (DD) of 70% or above is generally considered as chitosan (Li et al. 1992; Islam et al. 2020). Chitosan is soluble in dilute acids ($pH < 6.0$), which is facilitated by the protonation of free amino groups on glucosamine of the molecule (Di Martino et al. 2005). Depending on the source and preparation procedure, DD of chitosan ranges from 60% to 95% with a molecular weight of 300–1000 kDa (Di Martino et al. 2005; Croisier and Jérôme 2013). Even DD higher than 95% can be obtained by further deacetylation steps with the compensation of partial degradation of the polymer chains and an increase in the possibility of reacetylation (Islam et al. 2020). Hence, the source and extraction method of the chitosan are two crucial factors that determine the molecular weight and DD, which ultimately influence the physicochemical properties of chitosan.

Chitosan is extracted from the exoskeleton of crustaceans such as crabs, lobsters, and shrimps; shells of tortoise and insects; and fungal cell walls (Ahsan et al. 2018; Islam et al. 2020). It is obtained by partial deacetylation of chitin using a chemical, biological, or a combination of both methods (Rahman and Rashid 2013). These methods are sometimes coupled with several irradiation processes such as microwave irradiation, γ-irradiation, and ultrasound treatment to facilitate the deacetylation process (Rahman et al. 2013; Sebastian et al. 2019; Zhu et al. 2019). Currently, most commercial chitosan is extracted from the exoskeletal of crustacean by employing three main steps: deproteinization, demineralization, and deacetylation (Rashid et al. 2012; Croisier and Jérôme 2013; Islam et al. 2020). In the deproteinization step, the washed and dried exoskeletal is treated with an alkali solution, e.g., NaOH, to remove protein. Then, the deproteinized shells are treated with a mineral acid, e.g., HCl, to remove the minerals (mostly $CaCO_3$). This step is known as a demineralization process. The insoluble part is separated with centrifugation and subsequent washing. The dried sample is termed as chitin. The chitin is further treated with a concentrated solution of an alkali (mostly NaOH) at boiling conditions for several hours. In this step, chitin is converted to chitosan by the deacetylation process. The obtained product is repeatedly washed, dried in an oven, and stored at room temperature. For some pharmaceutical and biomedical applications, chitosan is decolorized by using different oxidizing agents, such as potassium permanganate, hydrogen peroxide, ozone, etc. Sometimes, chitosan is further purified by dissolving in aqueous acetic acid and then separating the insoluble impurities using a filtration process. After re-precipitation from the clear solution by neutralizing with an alkali solution, purified chitosan is obtained.

Chitosan exhibits remarkable intrinsic properties due to the presence of amino and hydroxyl groups in its structure (Fig. 19.1). These groups of the chitosan are responsible for (1) dissolution in the diluted aqueous acidic solutions ($pH < 6$) by the formation of polyelectrolyte through protonation of $-NH_2$ groups; (2) complexation with various species, such as metal ions and dyes; (3) formation of hydrogels through self-cross-linking or cross-linking with other cross-linkers; (4) high-ordered crystallinity which confronts the solubility of chitosan in conventional solvents like water; (5) preparation of blends with other polymers; and (6) ionic interaction between chitosan and many anionic moieties of structural molecules of the human

body (Croisier and Jérôme 2013). Moreover, due to the polycationic nature, chitosan exhibits antibacterial activity, along with antifungal, mucoadhesive, and hemostatic properties, and biocompatibility with the physiological medium. All these features make chitosan a superior candidate for biomedical applications.

19.2.2 Applications in Tissue Engineering

Chitosan has been widely used in food (Tian and Liu 2020), pharmaceuticals (Rashid et al. 2014; Shariatinia 2019), cosmetics (Aranaz et al. 2018), agriculture (Bandara et al. 2020), textiles (Pan et al. 2020), pulp and paper (Rahmaninia et al. 2018), energy (Hasan et al. 2020), biotechnology (Verma et al. 2020), and environmental chemistry (Dey et al. 2016; Biswas et al. 2017, 2020; Rashid et al. 2017; Morin-Crini et al. 2019). However, owing to its negligible toxicity and excellent biodegradability and biocompatibility, chitosan has garnered considerable attention in the biomedical application (Zaman et al. 2015; Islam et al. 2017, 2020; Nasrin et al. 2017). Among various biomedical applications, tissue engineering is an emerging field in the area of regenerative medicine which involves the generation of biomaterials to enhance cell adherence, proliferation, and differentiation eventually leading to the regeneration of a new tissue (Ahsan et al. 2018). Chitosan is effectively used in tissue engineering having some fundamental properties, such as (1) surface properties that promote cell attachment, differentiation, and proliferation; (2) biocompatibility with tissues; (3) a biodegradability rate corresponding to the rate of new tissue formation; (4) nontoxicity and non-immunogenicity; (5) no acute or chronic response; (6) optimal mechanical strength for handling and to mimic the damaged tissue; and (7) adequate porosity and morphology for transporting cells, gases, metabolites, nutrients, and signal molecules both within and across materials and host environment (Kim et al. 2008; Croisier and Jérôme 2013; Islam et al. 2020). The important fields of tissue engineering application of chitosan include cell regeneration, bone tissue engineering, cartilage tissue engineering intervertebral disk tissue engineering blood vessel tissue engineering, corneal regeneration, skin tissue engineering, periodontal tissue engineering, and tissue fixation (Badhe et al. 2017; Venkatesan et al. 2017; Doench et al. 2019; Shamekhi et al. 2019; Islam et al. 2020).

19.3 Hydrogels

19.3.1 General Aspects

Hydrogels are exceptional polymeric networks arranged in three-dimensional structures (Kabir et al. 2018; Rahman et al. 2019; Rusu et al. 2019). Having a high number of hydrophilic groups or domains, these networks show a high affinity for water. But, the chemical or physical bonds formed between the polymer chains prevent them from dissolving. The hydrogels are able to swell either in water or in

biological fluids allowing diffusion of nutrients which offers them the advantage of biocompatibility with living tissues and physicochemical similarity to the extracellular matrix (ECM) in biological conditions, allowing its biomedical applications (Pellá et al. 2018).

Polymeric hydrogels are classified into two types depending on their mode of cross-linking: (1) physical hydrogels and (2) chemical hydrogels. In physical hydrogels, the polymer molecules are held together by chain entanglements and/or reversible noncovalent interactions, including ionic cross-links, dipole-dipole interaction, hydrogen bonds, and hydrophobic interactions. Conversely, chemical hydrogels are formed by irreversible covalent bonds. Hydrogels with hydrophilic functional groups, such as hydroxyl (–OH), carboxyl (–COOH), amine (–NH_2), and amide (–CONH–$CONH_2$), can absorb a high amount of water leading to hydrogel swelling. However, hydrogels with hydrophobic chains (such as polylactic acid, polypropylene oxide) have lower water swelling capacity than hydrophilic lattices (Hamedi et al. 2018).

Hydrogels are prepared from both synthetic polymers (poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA), poly(propylene fumarate) (PPF)), and natural polymers (cellulose, chitosan, gelatin, etc.), involving a wide range of chemicals including cross-linkers and with different mechanical, physical, and chemical properties. These polymeric materials found applications in a range of fields such as agriculture (Abdel-Raouf et al. 2018), wastewater treatment (Rashid et al. 2019), food industry (Zhang et al. 2020), cosmetics (Mitura et al. 2020), tissue engineering (Lee and Kim 2018), wound healing (Hamedi et al. 2018), drug delivery (Yegappan et al. 2018), cell growth and transplantation, and regenerative medicine (Du et al. 2019).

19.3.2 Chitosan-Based Hydrogels

Chitosan has hydrophilic functional groups, such as hydroxyl (–OH), amine (–NH_2), and acetyl amine (–$NHCOCH_3$), that enable them to form both physical cross-linking (electrostatic interaction) and chemical cross-linking (covalent interaction using cross-linker). Depending on the materials used and modes of fabrication, different types of interactions are observed in chitosan-based hydrogels, such as (1) small anionic molecules-chitosan (electrostatic interaction), (2) polyanions-chitosan (electrostatic interaction), (3) polymer-chitosan (H-bond or hydrophobic interaction), (4) self-assembly, (5) thermally induced entanglement of polymer chains, (6) coordination complex cross-linking, and (7) covalent cross-linking (Zhang and Khademhosseini 2017). Figure 19.2 illustrates different types of interactions between chitosan chains and other polymers or small molecules. All these interactions lead to the formation of the cross-linked structure of the hydrogels that prevents complete destruction of the hydrogel cross-links and dissolution while swelling. Chitosan-based hydrogels can be designed in a variety of physical shapes, including spherical, cylindrical, bead, blocks, microparticles, nanoparticles, coatings, and films.

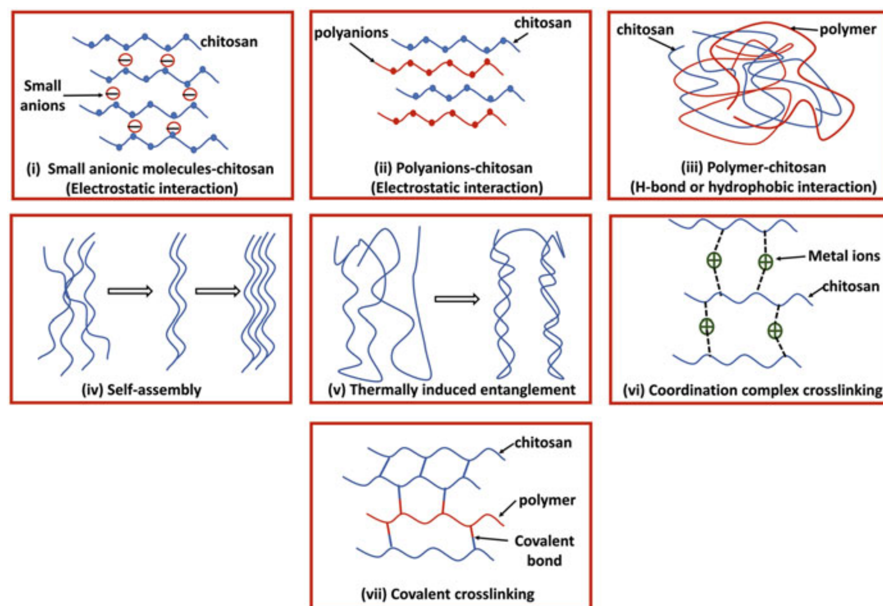

Fig. 19.2 Illustration of interactions in chitosan-based hydrogels in different systems-physical cross-linking (i–vi) and chemical cross-linking (vii). (i) electrostatic interaction between the small anionic molecule and polycationic chitosan chain; (ii) electrostatic interaction between opposite charges of polyanion molecule and chitosan chain; (iii) H-bond or hydrophobic interaction between polymer molecule and chitosan chain; (iv) self-assembly of chitosan molecules to fold into scaffolds by weak noncovalent bonding mechanisms—including hydrogen bonds, van der Waals forces, and hydrophobic interactions; (v) thermally induced physical entanglement of chitosan chains in response to a temperature change, typically caused by an alteration in their solubility and the formation of packed polymer backbones that are physically rigid; (vi) coordination complex cross-linking between multivalent metal ions and chitosan chain; and (vii) covalent cross-linking among functional moieties of chitosan chains and/or polymer chains, sometimes with the help of cross-linkers

Like other hydrogels, chitosan-based hydrogels find their applications in numerous fields. However, their notable properties regarding high water content, nontoxicity, facile control of solute transport, cell-guided degradability, and inherent cellular interaction, as well as tissue biocompatibility, make them a suitable candidate for tissue engineering application that includes cell regeneration, bone tissue engineering, cartilage tissue engineering, intervertebral disk tissue engineering, blood vessel tissue engineering, corneal regeneration, skin tissue engineering, periodontal tissue engineering, and bone fixation (as shown in Fig. 19.3) (Pellá et al. 2018). Many synthetic and natural polymers as well as many small molecules are incorporated with chitosan to fabricate modern hydrogels with desired physicochemical properties. However, these materials should be biocompatible, safe, nontoxic, with no or very limited immunological response as they may continuously interact with the body during hydrogel degradation and cellular regeneration.

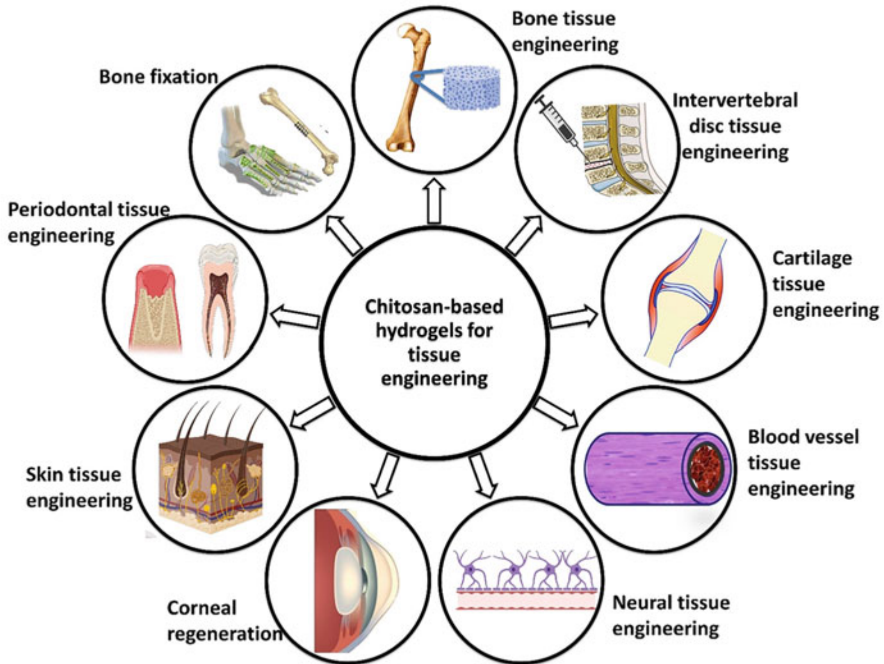

Fig. 19.3 Schematic diagram of applications of chitosan-based hydrogels in tissue engineering

19.4 Chitosan-Based Hydrogels for Tissue Engineering

19.4.1 Bone Tissue Engineering

Bone tissue engineering (BET) is a multidisciplinary field of tissue engineering that deals with the repairment of critical bone defects resulted from fracture, trauma, tumor resection, and infection, which cannot heal spontaneously (Awad et al. 2014). While traditional methods of treatments which include grafting of bones, autografts, allografts, xenogafts, and exploitation of metal prostheses are associated with numerous severe disadvantages, such as expensiveness, limited availability, risk of infections, the requirement of additional surgeries, immune rejections, inflammation, and chronic pains, BTE provides better solution avoiding various postoperative complexities (Tang et al. 2020a; Yue et al. 2020). Various inorganic, organic materials, such as glycerophosphate, tricalcium phosphate, chitosan, hydroxyapatite, and polylactic acid are generally utilized to fabricate various scaffold materials for BTE applications due to their ability to facilitate osteoblast adhesion, proliferation, and mesenchymal stem cell differentiation (Dessì et al. 2013; Rogina et al. 2016; Yu et al. 2017; Nie et al. 2020). However, in many cases, especially when the defect is irregular in shape and hard to reach, insertion requires surgical procedures (Dessì

et al. 2013). Therefore, in order to mitigate the surgical requirements and make the tissue engineering approaches more effective, injectable in situ formed hydrogel can be an effective treatment strategy. Although pure chitosan can provide favorable conditions for osteoblast attachment, proliferation, and mineralization, the required level of mechanical properties cannot be obtained (Levengood and Zhang 2014). Therefore, several chitosan-based hydrogels have been developed by exploiting polymers, nanomaterials, and encapsulation of bioactive molecules with chitosan (in Table 19.1) that have exhibited structural and biological similarities with components of the extracellular matrix (ECM) in the biomineralization process of bones (Hu et al. 2020; Lavanya et al. 2020). Besides, these hydrogels can also offer polyelectrolyte action, low immunogenicity, low cost of production, biodegradability, and biocompatibility.

In an investigation, a chitosan-based hydrogel has been developed utilizing β-glycerophosphate (β-GP) for cross-linking and β-tricalcium phosphate (β-TCP) as a reinforcing agent (Dessì et al. 2013). The incorporation of β-GP (a diol) is predicted to neutralize the ammonium group presented in the chitosan backbone that enhances hydrophobic interactions among chains and facilitates sol-gel transition. On the other hand, the addition of β-TCP facilitates attachment, growth, and proliferation of osteogenic cells by providing a suitable microenvironment. As nanoparticles are quite well-known for their ability to improve various properties of the fabricated polymer, many previous studies have revealed that Zn has a significant effect on bone formation and mineralization by exciting cellular protein synthesis with the help of tRNA synthetase (Yamaguchi 1998; Nagata and Lönnerdal 2011). Therefore, Zn, together with β-glycerophosphate, has been incorporated in a chitosan-based hydrogel which has exhibited high antibacterial activity and osteoblast differentiation. Antibacterial property is observed due to the ability of Zn nanoparticles to create a bond with bacterial membrane and causing lysis. Besides, it also delays bacterial cell division (Niranjan et al. 2013). In another investigation, pectin and genipin have been utilized for fabricating chitosan/pectin/genipin hydrogel containing interconnected pore structures. This type of structure facilitates the delivery of nutrients and the exchange of waste materials which are important for the effective application of these scaffolds. Moreover, chitosan/pectin/genipin hydrogel has provided higher strength and enhanced proliferation of the osteoblast cells incorporated inside it (Liu et al. 2015).

Hydroxyapatite (HA) is one of the main components of bone; hence, many investigations have been conducted for the utilization of this material for BTE. But low mechanical strength is the major barrier for successful implications (Bonfield et al. 1981; Suchanek and Yoshimura 1998; Depan et al. 2014). In order to impart higher mechanical properties, graphene oxide (GO) has been incorporated in an investigation to fabricate chitosan/HA/GO hydrogel (Depan et al. 2014). Graphene oxide has various peripheral functional groups, increasing interfacial interaction with chitosan and imparting higher mechanical strength. Besides, the carbon-based material also has demonstrated the ability to promote osteoblast adhesion and proliferation (Agarwal et al. 2010). Despite having these improved combined characteristics in the fabricated hydrogel, it is not suitable for practical applications

Table 19.1 Application of chitosan-based hydrogels in bone tissue engineering

Materials	Types of investigation	Remarks	References
Chitosan, Zn, β-glycerophosphate	In vitro	• Demonstrates enhanced antibacterial activity • Increases osteoblast attachment, differentiation, and proliferation • Turns into gel at body temperature	Niranjan et al. (2013)
Chitosan, Zn, nanohydroxyapatite, β-glycerophosphate	In vivo and In vitro	• Enhancement in swelling, protein adsorption, and biomineralization • Accelerates bone regeneration	Dhivya et al. (2015)
Chitosan, genipin, pectin	In vitro	• Production of type I collagen with alkaline phosphate activity • Interconnected porous structure • Osteoblast proliferation	Liu et al. (2015)
Chitosan, graphene oxide, hydroxyapatite, genipin, sodium ascorbate	In vitro	• Self-assembled into an oriented microstructure • High porosity, large pore size with high mechanical properties and elastic modulus • Ability to osteoblast adhesion and proliferation	Yu et al. (2017)
Chitosan, graphene oxide, glycerol phosphate	In vitro	• Improvement in protein absorption and swelling ability • Enhancement in osteoblast differentiation and proliferation • Advanced physicochemical properties	Saravanan et al. (2018)
Carboxymethyl chitosan, α-cyclodextrin, PEG_{1000}	In vitro	• Less invasive with ability to fill an irregular shape • Porous interconnected structure suitable for extracellular matrix diffusion • Enhances cell growth and proliferation	Saekhor et al. (2019)
Chitosan, silk fibroin, crude water extract of longan seed	In vitro	• Sol-gel transition at 4–10 min at 37 °C • Antimicrobial activity against *E. coli* and *S. aureus*	Pankongadisak and Suwantong (2019)

(continued)

Table 19.1 (continued)

Materials	Types of investigation	Remarks	References
		• Enhances differentiation and mineralization	
Carboxymethyl chitosan, calcium phosphate, glucono lactone	In vivo and In vitro	• Provides a large amount of Ca^{2+}, PO_4^{3-} for bone formation	Zhao et al. (2019)
		• Excellent attachment and proliferation of mesenchymal stem cells	
Collagen, aldehyde modified nanocrystalline cellulose, chitosan, Au nanoparticles	In vitro	• Uptakes large volume of water	Nezhad-Mokhtari et al. (2020)
		• Easily controllable morphology, swelling degree, gelation time, and degradation rate of hydrogels by changing ratio of elements	
		• Enhancement of cell growth and proliferation by Au NPs	
Hydroxyethyl chitosan, polyvinyl alcohol, biphasic calcium phosphate nanoparticle	In vitro	• High compressive strength (5–7 MPa)	Nie et al. (2020)
		• Porous structure with thicker pore wall	
Chitosan, glycerol phosphate, hydroxyapatite	In vitro	• Application of chitosan hydrogel as bio-ink for 3D printing	Demirtaş et al. (2017)
		• Strong gel network with high elasticity	
		• Enhanced cell viability, proliferation, and osteogenic differentiation.	
Chitosan, hydroxyapatite, PLA	In vitro	• Mechanical support provided by PLA	Rogina et al. (2016)
		• Very good interconnected porous structure with enhanced biocompatibility	
		• Enhanced osteogenesis of mesenchymal stem cell	

due to a lack of the required level of mechanical strength (Yu et al. 2017). Further, the strength of chitosan significantly reduces after uptaking water. Therefore, in another investigation, a cross-linking agent, genipin, together with reducing agent sodium ascorbate (SA) has been used in chitosan/HA/GO hydrogel (Yu et al. 2017). The 3D self-assembled hydrogel enhances HA fixing ability by strong H-bond with HA on the surface of GO, and the strong π-π interactions of GO overlap with one another leading to an oriented morphology. Further, a combination of both genipin and SA causes the formation of uniform hydrogel mitigating delamination, which

has been resulted from uneven shrinking among various parts of hydrogel while the only cross-linking agent was used. Other than HA, in another study, glycerophosphate (GP) has been exploited with GO to manufacture an injectable chitosan/GO/GP hydrogel (Saravanan et al. 2018). An incorporated mesenchymal stem cell has demonstrated an increase in osteogenic differentiation, which has ensured the ability to provide a suitable environment for cell growth and proliferation of chitosan/GO/GP hydrogel.

Apart from the abovementioned systems, some other chitosan-based hydrogels have been introduced in recent times to impart higher qualities. Such as chitosan derivatives, carboxymethyl chitosan has been exploited to avoid a lower solubility of chitosan at lower pH (6.4). The incorporation of PEG with α-cyclodextrin has facilitated gel formation by forming an inclusion complex. The leophylized hydrogel has exhibited an interconnected porous structure, which is suitable for higher diffusion of the extracellular matrix that ensures supply of all required materials and removal of undesired products offering cells a higher chance to survive and proliferate (Saekhor et al. 2019). In order to promote bone regeneration, a mesenchymal stem cell has been incorporated in a different type of hydrogel based on carboxymethyl chitosan and amorphous calcium phosphate (Zhao et al. 2019). The addition of amorphous calcium phosphate not only has provided a large amount of Ca^{2+}, PO_4^{3-} for the formation of bones but also has demonstrated outstanding cell adhesion and proliferation. In another investigation, nanocrystalline cellulose and Au nanoparticles (NPs) have been incorporated in a chitosan/collagen-based hydrogel which successfully has imparted higher mechanical properties and degradation resistance of the fabricated hydrogel with easy control of various properties, including morphology, swelling degree, gelation time, and degradation rate of the hydrogel by changing ratio of components (Nezhad-Mokhtari et al. 2020).

Currently, bioprinting has evolved as a promising and innovative technology in the field of tissue engineering where bio-ink, containing required biomaterials (e.g., living cells, extracellular matrix), has been utilized for layer-by-layer construction of tissue or organ (Matai et al. 2020). In order to exploit this technology in BTE, a chitosan hydrogel has been manufactured as bio-ink for 3D printing by incorporating glycerol phosphate disodium salt and hydroxyapatite, which has exhibited better performance than alginate, the material currently using for bioprinting. For fabricating bioprintable hydrogel, chitosan and HA mixture has been added with GP and preosteoblast. Later, bioprinting has been conducted using this hydrogel bio-ink (Fig. 19.4). Incorporated preosteoblast cells have demonstrated well osteogenic mineralization and differentiation where the application of hydroxyapatite has enhanced cell viability, proliferation, and differentiation (Demirtaş et al. 2017). Although the chitosan/hydroxyapatite system has very significant applications in BTE, it suffers from lower mechanical strength. Therefore, 3D printed macroporous poly(lactic acid) (PLA) has been incorporated to produce a hydrogel with better mechanical strength (Rogina et al. 2016). Apart from satisfying the limitation, this hydrogel also has imparted higher osteoinductivity and bioresorbability. Besides, the porous macrostructure has offered an efficient supply of nutrients together with better cell adhesion and migration.

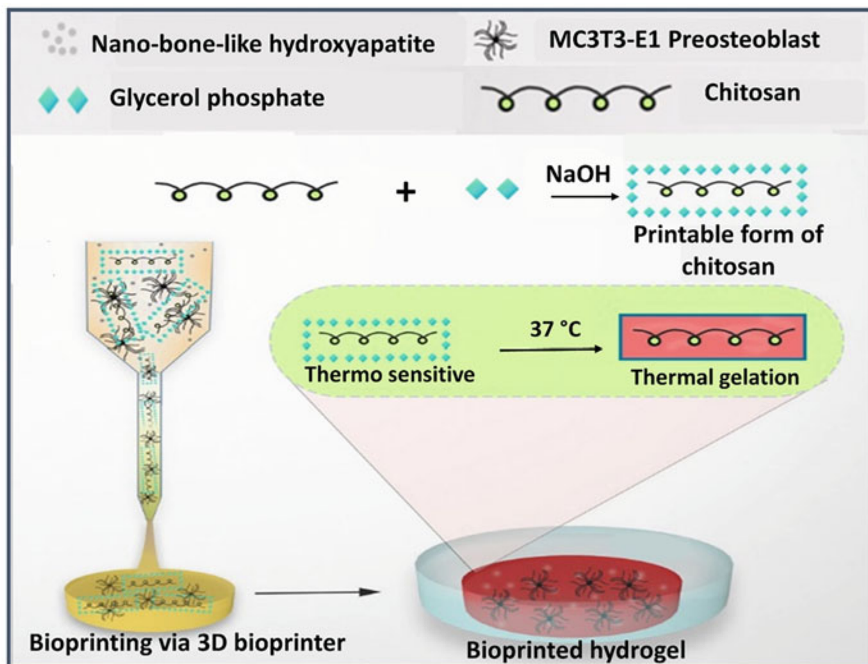

Fig. 19.4 Schematic illustration of chitosan/hydroxyapatite/glycerol phosphate hydrogel production by biopriniting. M3C3T3-E1 preosteoblast cells are incorporated in hydrogel solution to print a disk-shaped hydrogel by 3D bioprinter. (Reprinted with copyright permission from reference Demirtaş et al. 2017. Copyright © 2017 IOP Publishing Ltd.)

Chitosan-based hydrogel systems have exhibited auspicious results in in vitro investigations; however, still several lacking hinder their applications for practical purposes. Administration of injectable hydrogels requires in situ gel formation, and there is no chance to remove undesired materials. Therefore, the investigation should be conducted to find effective and biocompatible materials for gelation with the ability to enhance bone regeneration. Further, many hydrogels of improved quality have been fabricated using GO and nanoparticles (Nagata and Lönnerdal 2011; Yu et al. 2017; Nezhad-Mokhtari et al. 2020). Interaction of these materials in the human body and their long time exposure should be critically evaluated.

19.4.2 Cartilage Tissue Engineering

Cartilage tissue, formed with chondrocytes, demonstrates significant biomechanical operations, like shock absorption, wear resistance, and forming bone joints (Dash et al. 2011). As a consequence of the complex structure, avascular property, intensive heterogeneity, and meager cell population, treatment of cartilage tissue injuries is quite troublesome (Balasundaram et al. 2014). Articular cartilage exhibits lacking

property due to trauma, osteoarthritis, or other illness, which results in tremendous joint pain, as well as joint affliction. Cartilage tissue engineering (CTE) applies a summation of biocompatible scaffold cells and growth factors for fabricating a cartilage-like tissue with almost identical biomechanical characteristics as a native cartilage tissue (Kotecha 2017). At present, tissue engineering technology has garnered tremendous attention as an alternative treatment for articular cartilage repair, because current treatments, such as microfracture, mosaicplasty, and autologous chondrocytes injection, do not provide the same structural similarity with native cartilage (Islam et al. 2020). In recent times, various types of injectable hydrogels (in situ produced) are gaining attention in tissue engineering as implantation surgery can be substituted by an easy injection procedure that is not invasive. In addition, the combination of growth factors and the cells can operate straight away, and prior to the gel structure, the hydrogel can be easily modified to give the desired shape and better adjustment with the surrounding tissue (Gutowska et al. 2001). In contemplation of fabricating hydrogels, chitosan has been extensively investigated for cartilage tissue engineering as a result of the structural similarities with cartilage ECM (extracellular matrix) proteins. Some in vitro studies have demonstrated that chitosan-based hydrogels facilitate chondrogenic activity and accommodate the development of cartilage ECM proteins by chondrocytes (Sechriest et al. 2000). Moreover, being a polycationic polysaccharide, chitosan can enzymatically deteriorate in vivo via lysozyme, a polycationic protein existing in the ECM of human cartilage (Moss et al. 1997; Vårum et al. 1997). For this reason, chitosan may be an effective candidate for the modulation of chondrocyte (cartilage + cell) morphology, differentiation, and stimulating chondrogenesis.

In an early study, chitosan-grafted glycolic acid (GA) and phloretic acid (PA) based hydrogel (CH-GA/PA) have been fabricated by enzymatic cross-linking with horseradish peroxidase (HRP) and H_2O_2 (Jin et al. 2009). The hydrogel has exhibited an easily degradable property via lysozyme because of the hydrolysis of the glycosidic bonds which appear in the chitosan backbone (Pangburn et al. 1982; Vårum et al. 1997). Moreover, chondrocytes in this hydrogel have maintained their orbicular shape and possess great durability after 2 weeks of the study. However, fast gelation is necessary to preserve the cells and bioactive materials at the injection site. By applying vial titling method with (1 weight%) CH-GA/PA solution, the gelation time was observed to be approximately 4 min which can be reduced to 10 s by increasing concentration. From the rheological analysis, a lower value of a damping factor (ratio of loss modulus to storage modulus) was enumerated between 10^{-3} and 2×10^{-3}, pointing out the strong elastic property (Jin et al. 2009). Later, new hydrogels were developed via poly(ethylene oxide-co-glycidol)-CHO and glycol chitosan precursors by in situ fabrication using cross-linking among the amino groups of glycol chitosan and aldehyde groups of the polymer precursor. Superior physical characteristics like optimum gelation time, high gel content (up to 90%), decreased water uptake, and higher stiffness have been achieved by increasing the concentration. The deterioration of hydrogels could be the reason for an alteration in the 3D microenvironment, resulting in a negative impact on the activity and proliferation of encapsulated cells (McKinnon et al. 2014). Chondrocytes encapsulated in

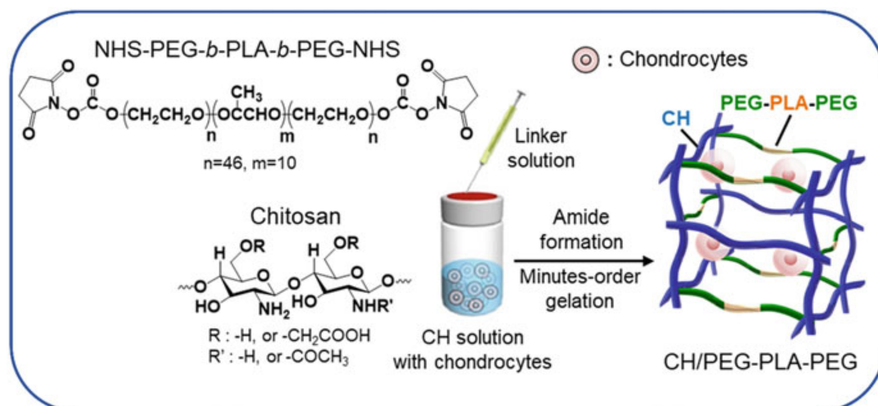

Fig. 19.5 Schematic illustration of a chitosan-based hydrogel (CH/PEG-PLA-PEG) synthesized with a low gelation time (within minutes) by condensation reaction among amine group of chitosan (backbone) and the succinimide group of NHS-PEG-b-PLA-b-PEG-NHS (cross-linker). (Reprinted with permission from ref. Ishikawa et al. 2020. Copyright © 2019 Wiley Periodicals, Inc.)

sustained elevated viability and a spherical morphology after 2 weeks of three-dimensional culture was obtained (Cao et al. 2015).

The merger between chitosan and gelatin biopolymers has garnered tremendous attraction in cartilage tissue engineering due to the nontoxicity and biocompatibility of the biopolymers (Xia et al. 2004). Gelatin, an essential element of cartilage ECM, is derived from collagen and beneficial for cell connection. In addition, it can sufficiently absorb a large amount of water that helps in nutrient transportation and possess low antigenicity (Elzoghby 2013). Several imitative types of protein-based hydrogels were fabricated from chitosan and collagen for cartilage tissue engineering applications as the combination of collagen and chitosan has demonstrated enhanced mechanical properties, lessen biodegradation rate, and ameliorated cell proliferation (Yan et al. 2010). Shen et al. developed durable and firm chitosan-gelatin hydrogels using the in situ precipitation method that has exhibited a prominent compressive strength of 2.15 MPa and Young's modulus of 3.25 MPa, which is greater than human cartilage (Shen et al. 2015).

In the latest study, chitosan-based hydrogels were fabricated to use as scaffolds for culturing chondrocytes accompanied by hydrolyzable poly(DL-lactide) (PLA) segments. The combination of chitosan solution together with a bi-functionalized decomposable triblock cross-linker, NHS-PEG-b-PLA-b-PEG-NHS, has formed CH/PEG-PLA-PEG (chitosan/poly(ethylene glycol)-poly(DL-lactide)-poly(ethylene glycol)) hydrogels within minutes with the help of the reaction among the amine group of chitosan and the succinimide group of the cross-linkers (Fig. 19.5) (Ishikawa et al. 2020). To evaluate the hydrogel's degradation properties as a cell culture scaffold, the reduction of weight of the hydrogels with encapsulating chondrocytes was calculated to be 35% at 40 days with embedded chondrocytes. On the other hand, in the absence of embedded chondrocytes, the calculated weight

Table 19.2 Application of chitosan-based hydrogels in cartilage tissue engineering

Materials	Types of investigation	Remarks	References
Chitosan/poly(ethylene glycol)-poly(DL-lactide)-poly(ethylene glycol)	In vitro	• Increases the production of the sulfated glycosaminoglycan	Ishikawa et al. (2020)
		• Accelerated degradability	
		• Drastically enhances the properties of collagen from the encapsulated chondrocytes	
Glycol chitosan (GC) and poly(ethylene oxide-*co*-glycidol)-CHO	In vitro and in vivo	• Maintains a high viability and a round cell morphology for a long time. Preserves cell proliferation and conserved the phenotype of the chondrocytes	Cao et al. (2015)
		• Provides a versatile platform for constructing injectable in situ hydrogels	
Chitosan-gelatin (CG) hydrogel	In vitro	• Higher compressive strength and large Young's modulus	Shen et al. (2015)
		• Supreme adhesion and proliferation of human thyroid cartilage cells on these hydrogels	
		• Exhibits well biodegradability, cytocompatibility, microporous structures	
Chitosan-graft-glycolic acid (GA) and phloretic acid (PA)	In vitro and in vivo	• Long-term viability and retention to the round shapes	Jin et al. (2009)
		• Short gelation time	
		• Demonstrates a good solubility at up to pH 10	
Silk fibroin (SF) and carboxymethyl chitosan (CMCS) composite hydrogel	In vitro and in vivo	• High material properties and degradation rate, good biocompatibility	Li et al. (2019)
		• Remarkable tissue compatibility in vivo	
		• Sustains and even promotes chondrogenic phenotype	

reduction was 25% (Ishikawa et al. 2019). Moreover, with the addition of the chondrocytes, a swift degradation was noticed, and these hydrogels produce developed sGAG (glycosaminoglycan) and collagen.

Table 19.2 represents some recent works that attempted to use chitosan-based hydrogels in the treatment of cartilage injury by cartilage tissue regeneration through cell implantation. Further studies in this field are still necessary, as most of the researches are at their primitive stage. In addition, most of the results submitted are build on in vitro tests. For implementation in human bodies, comprehensive and thorough in vivo experimental evaluation is required.

19.4.3 Intervertebral Disks Tissue Engineering

Intervertebral disks (IVDs) are mainly made up of fibrocartilages lying in between two vertebrae, responsible for the normal function of the spine and absorbing stress and shock imposed on it (Choi et al. 2019). IVD can be damaged in various ways, which may contribute to lower back pain and cause a lot of suffering to affected people. In order to mitigate sufferings caused by IVD degeneration and improve conditions of damaged IVD, surgical excision, vertebral bone fusion, case, or prosthesis insertion are the available treatment strategies which hinder the natural biological function of the spine and sometimes lead to further deterioration of adjoining sections and contribute spinal complications (Guyer and Ohnmeiss 2003; Hughes et al. 2012). Although some advanced cell-based, ECM-based, and molecular therapies have been introduced, these methods also suffer from several disadvantages, including the inability to obtain complete functionality, lamination, and precipitation while directly inserted and failure in maintaining catabolism and anabolism (Ganey and Meisel 2002; Anderson et al. 2005). Therefore, various chitosan-based hydrogels are currently being investigated for repairing damaged IVDs due to their ability to impart higher mechanical properties and structural similarities with ECM of native IVD (Cheng et al. 2010; Tang et al. 2020b).

IVDs are formed of three major parts: (1) inner nucleus pulposus (NP), (2) outer annulus fibrosus (AF), and (3) cartilage endplates (CEP) (Humzah and Soames 1988). NP is the gelatinous portion situated at the core and mainly consists of a large amount of water and proteoglycans, enabling it to withstand the compressive load. On the other hand, the laminated AF protects and holds NP in position (Alonso and Hart 2014; Tang et al. 2020b). IVD degeneration, caused by aging, modification of matrix proteins, and accumulation of cell waste products, results in loss of proteoglycans, one of the major components of NP. All these phenomena lead to the failure of integrity among different parts of IVDs. Besides, the inability to receive the proper amount of nutrients due to avascular nature and a lower rate of cell proliferation, NPs cannot regenerate to the desired level (Buckwalter 1995; Urban et al. 2004). Therefore, a chitosan-based hydrogel is developed by utilizing gelatin, glycerol phosphate, and disodium salts as a vehicle for NP restoration (Cheng et al. 2010). The addition of gelatin has imparted higher strength and significantly mitigated the time required for gelation. The hydrogel has provided a favorable microenvironment where incorporated NP cells have demonstrated desired extracellular matrix (ECM) production with high mRNA gene expression for type II collagen and aggrecan (a proteoglycan), prohibiting the production of undesired ECM (e.g., type I collagen).

Another chitosan-based hydrogel has been fabricated using a gelling agent made by combining sodium hydrogen carbonate and β-glycerophosphate and phosphate buffer (Alinejad et al. 2019). At specific compositions, it mimics mechanical properties similar to human NP and can provide a favorable microenvironment for the growth and proliferation of NP cells. In order to impart higher mechanical strength, cellulose nanofiber has been integrated into fabricating chitosan-based hydrogel in another recent investigation (Doench et al. 2018). This reinforced

hydrogel has shown the ability to improve the height of degenerated IVD together with the reduction in back pain by mitigating compression of nerve roots. Apart from these approaches, some other studies have been conducted on chitosan-based hydrogel (listed in Table 19.3), where gelatin has been used for enhancing hydrophilicity, fibroin silk for increasing stability and durability, and ZrO_2 to impart radiopacity that helps in noninvasive evaluation after in vivo implantation (Ghorbani et al. 2017; Gullbrand et al. 2017).

The application of decellularized ECM (dECM) is another effective approach to treat IVD degeneration. In the decellularization method, the natural cells are removed from tissue or organ which are mainly responsible for immunogenicity. Therefore, fabricated scaffold materials can serve their purposes without inflammatory and immune responses together with avoiding the risk of tissue rejection (Kabirian and Mozafari 2020). In order to exploit this strategy in IVD degeneration, a chitosan-based hydrogel has been developed by using dECM of AF, genipin, and basic fibroblast growth factor (bFGF) where stem cells derived from AF have exhibited adhesion and proliferation (Liu et al. 2019a). Further, the hydrogel has ensured the sustained and continuous release of bFGF, which has facilitated higher gene expression for collagen I, collagen II, aggrecan, and glycosaminoglycans.

Recently, a new treatment method has been introduced where a relatively complete structure of IVD has been produced by tissue engineering (Yuan et al. 2019). In such an investigation, a highly hydrated chitosan hydrogel has been utilized to form NP, whereas poly(butylene succinate-co-terephthalate) (PBST) has been used to fabricate AF (Yuan et al. 2019). The hydrogel has exhibited excellent viscoelasticity and rheological characteristics with suitable mechanical properties required for satisfying its purpose. In vivo investigation on this fabricated IVD has suggested adhesion, growth, and expression of ECM by NP cells. Another tissue-engineered IVD has been introduced recently in which a similar-type chitosan hydrogel has been used to excite NP, while poly(ether ether ketone) (PEEK) and PBST have been utilized for fabricating outer AF and inner AF, respectively. PBST film produced by electrospinning was rolled over chitosan-based hydrogel, and together these were inserted on molded PEEK scaffold to fabricate whole tissue-engineered IVD. After emerging into a culture medium containing IVD cells separated from porcine lumber, it was implanted into the porcine spine (Illustrated in Fig. 19.6) (Yang et al. 2018). Most of the previous investigations have been carried out on small animal models where the actual environment and stress loaded on fabricated IVD are much higher. Therefore, this hydrogel-based tissue-engineered IVD has been incorporated in the damaged spine of pigs. Various in vitro and in vivo investigations have revealed the potentiality of this for future application.

Despite many fruitful investigations, chitosan-based hydrogels are still lagging behind from clinical applications. Lower mechanical strength of the fabricated scaffolds and inability to provide proper lead-bearing capacity are seemed to be the main reasons which should be overcome to make these hydrogel-based systems suitable for practical applications. The hydrogel of improved characteristics with appropriate growth factors, developed by exploiting advanced technologies, can be a possible solution for overcoming these challenges.

Table 19.3 Application of chitosan-based hydrogels in intervertebral disk tissue engineering

Materials	Types of investigation	Remarks	References
Chitosan, gelatin, glycerol phosphate, disodium salts	In vitro	• The higher strength of gel with the reduction in gelation time • Suitable microenvironment for desired ECM production prohibiting undesired ECM • High mRNA gene expression for type II collagen and aggrecan	Cheng et al. (2010)
N-carboxyethyl chitosan, oxidized dextran, teleostean, ZrO_2 nanoparticles	–	• Imparts radiopacity by adding ZrO_2 nanoparticles • Recover mechanical strength of damaged IVD • Allows survival and differentiation of mesenchymal stem cells and NP cells • In vivo investigation	Gullbrand et al. (2017)
Chitosan, β-glycerophosphate, hyaluronic acid, chondroitin-6-sulfate, collagen, gelatin, fibroin silk	In vitro	• Rise in hydrophilicity by adding gelatin • Highly porous and structurally similar to native IVD • Thermosensitive hydrogel	Ghorbani et al. (2017)
N-hexanoyl glycol chitosan	In vivo and in vitro	• Longer gel stability (28 days) • Interconnected macroporous structure • Facilitates cell growth and migration • Thermosensitive hydrogel	Li et al. (2018)
Chitosan, sodium hydrogen carbonate, β-glycerophosphate, phosphate buffer	In vivo	• Mechanical properties similar to human NP at certain composition • Facilitates better production of proteoglycans • Thermosensitive hydrogel	Alinejad et al. (2019)
Chitosan, cellulose nanofibers	In vivo	• Recovers viscoelastic properties of the disks • Enhancement in the disc height, • Reduction in back pain by mitigating compression of nerve root	Doench et al. (2018)

(continued)

Table 19.3 (continued)

Materials	Types of investigation	Remarks	References
Chitosan, 1,6-diisocyanatohexan, polyethylene glycol,	In vivo and in vitro	• Excellent viscoelasticity and rheology and mechanical properties • Demonstrates growth and ECM expression by NP cells	Chen et al. (2014); Yuan et al. (2019)
Chitosan, decellularized matrix of annulus fibrosus, genipin, and basic fibroblast growth factor (bFGF)	–	• Porous 3D network structure • Sustained and gradual bFGF release • Higher level of gene expression for collagen I, collagen II, aggrecan • In vitro investigation	Liu et al. (2019a)
N-carboxyethyl chitosan, oxidized dextran, teleostean, ZrO_2 nanoparticles	–	• Imparts radiopacity by adding ZrO_2 nanoparticles • Recovers mechanical strength of damaged IVD • Allows survival and differentiation of mesenchymal stem cells and NP cells • In vivo investigation	Gullbrand et al. (2017)

19.4.4 Blood Vessel Tissue Engineering

Several cardiovascular diseases, such as coronary heart disease, peripheral arterial disease, and cerebrovascular diseases, are the major causes of death worldwide. These diseases occur mainly due to the growth of plagues in arteries that end up blocking the flow of blood to essential organs (Frâncu and Veștemean 2013). Blood vessels provide oxygen and necessary nutrients throughout the body. These are also enormously involved in angiogenesis and the onslaught of cancer cells (Carmeliet and Jain 2000; Zheng et al. 2012). Angiogenesis means the construction of new blood vessels that is necessary for ordinary wound healing by granting cells and growth factors to reach the wounded tissues (Klagsbrun and D'Amore 1996). Nowadays, in blood vessel tissue engineering, artificial grafts are being applied for regenerating tissues that suffer from low feasibility due to host cell infiltration, calcification, sluggish remodeling, structural inconsistency, deficiency of supply, or preexisting vascular diseases (Fukunishi et al. 2016). The applied grafts also must exhibit some specific properties, like biocompatibility and controlled degradation for avoiding inflammation and calcification (Islam et al. 2020). Moreover, regulable degradation is essential, as too fast degradation of the graft leads to shape failure, while slow degradation causes obstruction in cell proliferation and integration. Chitosan demonstrates the required properties for blood vessel tissue engineering

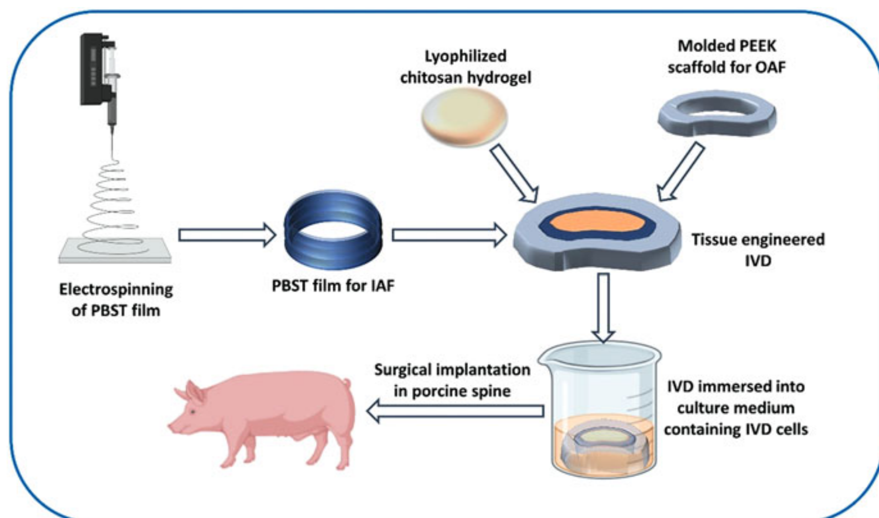

Fig. 19.6 Schematic illustration of whole tissue-engineered IVD. Chitosan hydrogel is used to fabricate nucleus pulposus, polyetheretherketone (PEEK), and poly(butylene succinate-co-terephthalate) (PBST) is used to fabricate outer AF (OAF) and inner AF (IAF), respectively. PBST film produced by electrospinning is rolled over chitosan-based hydrogel, and together these are inserted on molded PEEK scaffold to fabricate tissue-engineered IVD. After emerging into a culture medium containing IVD cells separated from porcine lumber, it was implanted into the porcine spine. (Figure is redrawn based on information of ref. Yang et al. 2018)

as a result of its highly porous and gel-forming nature. The extracellular matrix of blood vessel tissue is assembled with glycosaminoglycans that possess a structural resemblance with chitosan. So, fabricating chitosan grafts may facilitate cell infiltration and cell proliferation and experience swift remodeling (Chupa et al. 2000; Zhang et al. 2006; Huang et al. 2011). However, the cytocompatibility of chitosan with endothelial cells and vascular smooth muscle cells doesn't show noticeable results. For getting better cytocompatibility, researchers attempt to develop various types of chitosan-based hydrogels and scaffolds, which exhibit prominent results and tremendous improvements in mechanical strength, and cell adhesion and proliferation have also been noticed.

In an early study, a tubular sandwich scaffold has been developed using a chitosan tube immersing into chitosan solution with shrouding the internal and external surface via a layer of chitosan-gelatin complex solution followed by freeze-dehydration (Zhang et al. 2006). The scaffold demonstrated appropriate swelling property, the burst strength of almost 4000 mmHg, considerable joint retention strength, and long sustaining mechanical strength (mass loss of 18.7% after degradation for 2 months). Most importantly, vascular smooth and balanced muscle cells can generate and propagate fantastically on that scaffold. Apart from this study, 3D printing synthetic blood vessel has been developed by applying poly (ε-caprolactone) (PCL), chitosan, and hydrogels joined biocomposite (Ulag et al.

2019). 3D printing cannot only adjust instrumentation readily but also able to mimic biomedical processes for synthesizing tissues without any vascularization problem. These biocomposites have exhibited very high elastic modulus (ca. 174 MPa). The degradation time was found to be higher than the swelling time, as a result of cross-linking among the hydrogel networks. This further has displayed resistance to dissolution and accelerates the degradation time of PCL. As a result of the addition of hydrogel, cell life and attachments enhance, confirming that the biocomposite can be used as a biomaterial for vascular tissue engineering. Moreover, 3D printed vessels may be effective for generating functional blood vessels when integrated with the sufferer's endothelial cells.

Over the past few years, vascular layered patches have also been extensively pursued, because the deposition of polymer in the layers enhances cell attachments and proliferation (Zhang et al. 2019). In addition, they can be a potential alternative to the polycomponent biomaterials, which are frequently unstable and fabricated by complex procedure. In a study, a chitosan and gelatin-based hydrogel scaffold was developed that possesses a bilayered tubular structure synthesized by solvent casting-co-particulate leaching method (Badhe et al. 2017). The prepared scaffold contained an internal macroporous layer covered up by a nonporous external layer resembling the 3D matrix of blood vessels with cellular attachments and proliferation. The inner layer delineates remarkable channeling of the pores that facilitates nutrient flow and material transfer rate and grants a high surface area for cell attachment and proliferation. On top of that, these scaffolds exhibit a commendable tensile strength, elongation, and porosity (given in Table 19.4).

The current researches in this field are mainly based on a single component fabricated via a conventional method for treating an original material which possesses various types of demerits, like the inconvenience of fabrication as well as the unreliability of numerous components (Li et al. 2017; Zhu et al. 2017; Gan et al. 2018). In spite of demonstrating enhanced biocompatibility and exacerbated endothelialization, they are incapable of handling some complications of the patients, like blood clotting before surgery and occurrence of infection at the place of surgery (Tang et al. 2017; Zhang et al. 2018). As a result, the fabrication of vascular patches via a facile and expedient system is an imperious requirement that can ensure the stability of multiple components. With this consideration, the fabricated layer-by-layer (LbL) self-assembly method grants remarkable possibilities. In a study, polyelectrolyte multilayered vascular patches have been developed via self-assembly technique by excessive deposition of heparin and chitosan on polyurethane-coated decellularized scaffold (PU/DCS) (Fig. 19.7) (Zhang et al. 2019). These patches have demonstrated exalted biocompatibility, reduced hemolysis rate, augmented in vitro coagulation time, and higher resistance to platelet adhesion. Furthermore, they have ameliorated attachment and proliferation of endothelial progenitor cells and the PEM vascular patches can sustain indelible patency of the surgical arteries (Zhang et al. 2019). For all these reasons, this approach provides an eminent potential in compensating vascular defects, as well as a feasible strategy for constructing vascular patches. Therefore, novel

Table 19.4 Application of chitosan-based hydrogels in blood vessel tissue engineering

Materials	Types of investigation	Remarks	References
Chitosan, gelatin	In vitro	• Appropriate swelling property • Large joint retention strength • Vascular smooth muscle cells can grow perfectly on the scaffold	Zhang et al. (2006)
Chitosan, gelatin	In vitro	• A structure resembling a 3D matrix of blood vessels with cellular adhesion and proliferation • The scaffold exhibits a high tensile strength • Imparts controlled swelling and biodegradation	Badhe et al. (2017)
1,2-propanediol, chitosan acetate	In vitro	• Demonstrates necessary mechanical strength for resisting physiological stresses • Higher compliance for eliminating adverse hemodynamic environments	Aussel et al. (2017)
Poly (ε-caprolactone), chitosan	In vitro	• Effective for the fabrication of functional blood vessels when combined with the patient's own endothelial cells • Exhibits high elastic modulus • Possesses high cell vitality and attachment	Ulag et al. (2019)
Heparin, chitosan	In vitro and in vivo	• Sustains the long-term patency of the surgical arteries. • Enhances attachment and proliferation of endothelial progenitor cells. • Demonstrates enhanced biocompatibility, reduced hemolysis rate, and prolonged in vitro coagulation time.	Zhang et al. (2019)

scaffolds fabricated by chitosan-based hydrogels provide a better solution in vascular tissue engineering.

19.4.5 Neural Tissue Engineering

At present, the fundamental interest in the central nervous system (CNS) has been concentrated on cell replacement techniques for recuperating injured neurons, as maximum neurons in a mammalian cannot self-proliferate. The greater portion of CNS diseases is frequently ascribable to the progressive loss of functions and death of neurons. The accrual of inserted cells should be controlled to demonstrate differentiation and neurite outgrowth. As a result, the proper selection and study of compatible scaffold materials for facilitating cells on implantation are mandatory (Crompton et al. 2007). Therefore, the scaffolds ought to grant favorable chemical and spatial microenvironment for cell proliferation, differentiation, and axon extension. To be used as cellular scaffolds, hydrogels have many amenities due to

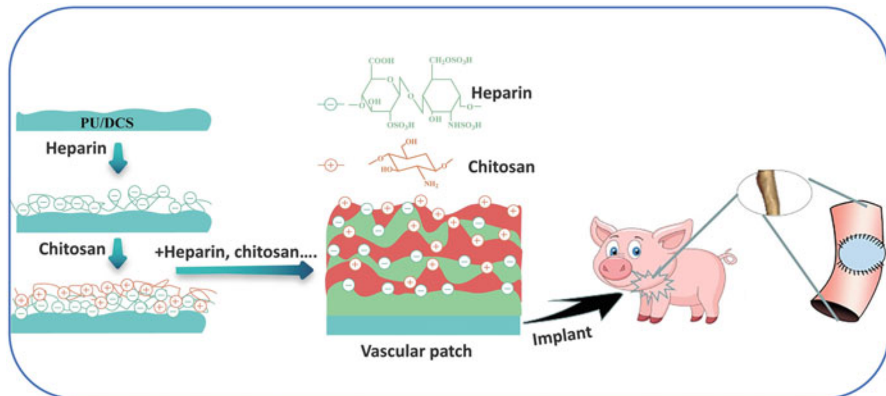

Fig. 19.7 Schematic illustration of the fabrication of a multi-structured vascular patch through the alternately layer-by-layer deposition of heparin and chitosan onto the substrate polyurethane-coated decellularized scaffold (PU/DCS). The in vivo investigation was performed in a porcine model with B ultrasound, Doppler spectrum, and computed tomography angiography (CTA). (Reprinted with the permission of Zhang et al. 2019. Copyright © 2019 Elsevier B.V.)

analogous mechanical characteristics with soft tissue and low interfacial tension that grant cells to move across the tissue-implant boundary (Andrade 1976). Although chitosan facilitates cell line attachment and growth, extensive usage of chitosan has been restricted in neural tissue engineering due to low solubility at physiological pH, higher gelation time, and incapability to tune the mechanical properties to assist three-dimensional (3D) neurite propagation. The application of cross-linkable chitosan hydrogels can be promising in neural tissue engineering attributable to the minimum usage of cytotoxic chemicals in the synthesis method, the potential comfort of encapsulating cells or bioactive agents beneath the gel, and efficiency to compensate complex 3D voids created on account of a wound (Leipzig and Shoichet 2009; Leipzig et al. 2011). Apart from that, peripheral nerve injuries (PNIs) may generate on account of complicated surgical processes, trauma, and intrinsic defects that result in impermanent or permanent perplexities (Jones et al. 2016). In order to deal with PNIs, the plan should be based on the intensity of the primary injury, and currently autologous nerve grafts are used widely for the treatment (Isaacs and Browne 2014; Xie et al. 2014; Muheremu and Ao 2015). However, they demonstrate several inconveniences like patient site morbidity, restricted supply of tissue, vulnerability to infections, and inconsistency among size and shape (Robinson 2000; Gu et al. 2014). The aforementioned drawbacks related to longer nerve gap led to the evolution of novel neural scaffolds that exhibit prominent results in dealing with peripheral nerves defect (Bagher et al. 2016). Hybrid chitosan hydrogel has been extensively investigated as scaffolds in neural tissue engineering because of their suitable mechanical properties as well as cell interactions (Peppas et al. 2006).

Two auspicious seed cells named olfactory ensheathing cells (OECs) and neural stem cells (NSCs) are extensively used in neural tissue replacement. OECs being an identical kind of glial cells are generated from the olfactory placode and travels

through the glial scar, hence facilitating axonal regeneration. In addition, the in vivo studies have demonstrated that transplantation of OECs elevates functional healing of rats following spinal cord injury (SCI) (Wang et al. 2010). On the other hand, NSCs possess the capacity for regenerating cells as well as the ability to distinguish among the central nervous cells like neurons, astrocytes, and oligodendrocytes. Although the mentioned characteristics establish NSCs in cell transplant treatment, many of the cells are separated into astrocytes that impairs the outcome of implantation (Cao et al. 2001; Han et al. 2002).

In an early study, a novel chitosan-based photo-cross-linkable hydrogel scaffold has been fabricated for neural tissue engineering, which has demonstrated desired physiochemical and mechanical characteristics in order to assist neurite propagation and cell transplantation (Valmikinathan et al. 2012). Contemptible cytotoxic properties were audited when the human mesenchymal stem cells have been cultivated with the scaffolds. Moreover, the hydrogels not only assisted in forming developed neurite that is superior to primary cortical neurons but also facilitated segregate tubulin positive neurons and astrocytes. Further investigations led to the development of a natural polymer-based complex chitosan-alginate hydrogel scaffold synthesized by the powerful ionic bonding among the amino groups of chitosan and the carboxyl groups of alginates (Wang et al. 2017). This scaffold has been applied most extensively in neural tissue engineering. The hydrogel contains a highly inner porous structure, as well as elevated hydrophilic property, and from the cell proliferation study, it has been observed that both OECs and NSCs proliferated successfully on the hydrogel.

In the latest study, the alginate-chitosan hydrogel has been applied for peripheral nerve reconstruction with varying concentrations of hesperidin (Bagher et al. 2020). The hydrogel has shown excellent blood compatibility and superior antibacterial characteristics. In addition, the hydrogel also has exhibited no toxicity effect and capable of enhancing the proliferation rate of olfactory ecto-mesenchymal stem cells. Furthermore, biodegradability was verified by applying the weight loss measurement. The loss percentage was approximately 80% after 14 days. In vivo experiments, the 1% concentration of hesperidin has exhibited better sciatic nerve regeneration than 0.1% and 10% concentration. As a result, hesperidin added alginate-chitosan hydrogel might be deliberated as an effective substance for peripheral nerve regeneration in the future. Apart from that, there are some other investigations on alginate-chitosan hydrogel in sciatic nerve tissue engineering, which has been highlighted in Table 19.5. Apart from these studies, electroactive hydrogel, built upon chitosan-aniline oligomers/agarose with self-gelling features, has garnered much attention due to the interdisciplinary imitation of the mechanical, electrical, and electrochemical characteristics of neural tissues (Bagheri et al. 2019). For sustaining the characteristics of hydrogel, aniline oligomer performed a leading role by directing the glass transition temperature and thermal properties. Moreover, slower swelling and degradation rates were observed owing to the hydrophobic nature of the aniline oligomer. Some other prominent investigations have been listed in Table 19.5 (Crompton et al. 2007; Alhosseini et al. 2012).

Table 19.5 Application of chitosan-based hydrogels in neural tissue engineering

Materials	Types of investigation	Remarks	References
Chitosan, glycerophosphate salt, and poly-D-lysine	In vitro	• Exhibits proper cell adhesion properties • Good neuron compatibility at low concentrations • Neuron survival enhances	Crompton et al. (2007)
Carboxymethyl chitosan and methacrylated chitosan	In vitro	• Exhibits enhanced neurite separation from cortical neurons • Robust and longer neurite lengths • Applicable for peripheral and central nervous system repair after injuries simultaneously	Valmikinathan et al. (2012)
Polyvinyl alcohol and chitosan	In vitro	• Exhibits balanced characteristics to meet the basic required specifications for nerve cells • Increases viability and proliferation of nerve cells	Alhosseini et al. (2012)
Chitosan-alginate	In vitro	• OECs and NSCs proliferated well on the chitosan-alginate hydrogel • Widely applied in nerve tissue engineering to repair peripheral nervous system (PNS) injuries and CNS injuries	Wang et al. (2017)
Alginate-chitosan	In vitro and in vivo	• Exhibits promising blood compatibility • Maintains a suitable substrate for cell survival.	Salehi et al. (2019)
Chitosan, aniline oligomers, and agarose	In vitro	• Appropriate conductivity for applications in neural tissue engineering • Decreases swelling and degradation rates • Maintains the hydrogel properties by regulating the glass transition temperature and thermal properties	Bagheri et al. (2019)
Alginate-chitosan and hesperidin	In vitro and in vivo	• Demonstrates good blood compatibility and antibacterial properties • Does not display toxicity effect • Capable of increasing the proliferation rate	Bagher et al. (2020)

Over the last decade, diverse efforts have been given for the combination of tissue engineering fields into strategies for treating various parts of the human body. However, neuronal treatment remains one of the most challenging and difficult areas because of the perplexity of the structure, function of the nervous system,

and the less effectiveness of the conventional repair methods. So, further investigations should be carried out in order to increase the effectiveness of these systems.

19.4.6 Corneal Regeneration

The cornea is a transparent, avascular tissue on the ocular surface responsible for normal vision as it exhibits the 2/3 of the total refraction taking place in the human eye. This outermost lens of the eye is composed of five layers: (1) uppermost epithelium layer, (2) fibrous Bowman's layer, (3) collagen-rich central stroma layer (90% of the total thickness of the cornea), (4) Descemet's membrane, and (5) endothelium layer (Dua et al. 2013; Meek et al. 2015). All layers must be transparent and stay intact to ensure perfect geometric shape for normal vision. In order to maintain the transparency of the cornea, corneal endothelial cells (CECs), monolayer, flat, and polygonal cells have a very significant role, as these cells are mainly responsible for actively pumping fluid across the cornea. The active pumping of fluid keeps the stroma hydrated and maintains proper thickness, which is important for the transparency of the cornea (Engelmann et al. 2004; Bonanno et al. 2012). These CECs layers can be deteriorated due to various reasons, including trauma, disease, and aging. As CECs cause 0.5% reduction every year, which cannot be compensated naturally, deterioration of CECs under critical value leads to corneal dysfunction with stromal edema, which subsequently results in blindness. Replacement of cornea is the currently available treatment that suffers from several disadvantages, such as scarcity of donor, allograft rejection, and failure in grafting in some cases (Gao et al. 2008; Liang et al. 2011a, b). Therefore, the culture and transplantation of these CECs are a better method that needs suitable matrix materials where the cells can survive, grow, and proliferate.

As previously mentioned, hydrogel based on chitosan is a suitable matrix due to mimic similarities in many characteristics with extracellular matrix (ECM) and having the ability of cell proliferation and differentiation (Liang et al. 2011a, b). Therefore, with a view to regenerating the damaged endothelium, various chitosan-based hydrogels have been investigated. As pure chitosan cannot often impart the desired properties, various other biocompatible materials such as poly(ethylene glycol) (PEG), gelatin, glycerol phosphate, and numerous growth factors are incorporated for promoting corneal reconstruction. PEG is a common biocompatible polymer that is frequently used to fabricate chitosan-based hydrogels for corneal regeneration (Liu et al. 2010). An ultrathin chitosan-PEG hydrogel film has been developed by cross-linking chitosan with diepoxy-PEG and cystamine. In vitro study has revealed that this hydrogel has exhibited attachment and proliferation of CECs. Isolated CECs for culture in this hydrogel have increased 9 times in 7 days with slightly higher cell density. Besides, this hydrogel film has also exhibited transparency above 95% in the visible spectrum with desired mechanical properties that enabled the film to insert easily without any instrumental (sutures or Busin's glide) aid in ex vivo surgery (Ozcelik et al. 2013). However, poor solubility of

chitosan in the physiological solvent is a major drawback for direct utilization. Hence, various chitosan derivatives demonstrating good solubility in physiological solvent are utilized in various investigations. In a recent study, hydroxypropyl chitosan (HPCS) has been exploited with oxidized sodium alginate dialdehyde (SAD), which has led to the rapid formation of the hydrogel by self-cross-linking between them. CECs encapsulated by this hydrogel to grow on Descemet's membranes have exhibited successful reconstruction of the damaged endothelium. Self-cross-linking has eliminated the requirement of cross-linking agents and reduced the risk of toxicity which could result from the cross-linking agent. Besides, the easy replacement procedure has caused minimal damage to the cornea, and therefore, postoperative inflammatory responses have significantly reduced (Liang et al. 2011a, b).

The corneal epithelium is another important layer of the cornea. This is the uppermost nonkeratinized, stratified layer, which has a significant role in refracting the light and focusing into the retina. In order to maintain normal vision, the integrity of epithelium is very important which can be obtained by the continuous renewal of these cells through proliferation and differentiation of basal layer and limbal stem cells (Liu and Kao 2015). But, the corneal epithelium can be damaged due to various reasons, including exposure to chemicals and UV radiations, surgical injury, Steve-Johnson syndrome, and some other diseases (Puangsricharern and Tseng 1995; Tang et al. 2017). In an attempt to reconstruct damaged epithelium cells, induced pluripotent stem cells (iPSCs) has been reprogrammed from human corneal keratocyte (Chien et al. 2012). iPSCs with carboxymethyl-hexanoyl chitosan (iPSCs/CHS) hydrogel has demonstrated better wound-healing properties which can restore severely damaged cornea due to alkali burn, surgery abrasion injury by mitigating oxidative stress and promoting endogenous epithelium cell for reconstruction. Chitosan hydrogel works as a suitable medium for a rapid and effective delivery of these bioactive materials. In another study, stromal cell-derived factor-1 alpha (SDF-1) has been incorporated in hydrogel developed by using chitosan and gelatin. SDF-1 is widely known for its ability to tissue repair (Askari et al. 2003). Exogeneous SDF-1 alpha excited local limbal epithelial stem cells and mesenchymal stem cells in the cornea to migrate to injured sites and proliferate. This facilitates the synthesis of growth cytokines that help restore epithelium (illustrated in Fig. 19.1) (Tang et al. 2017). However, when oxidative damage of the cornea results from chemicals or exposure to ultraviolet radiations, this leads to the generation of various reactive oxidative species (ROS) (Youn et al. 2011). Although numerous endogenous antioxidants (e.g., vitamins, glutathione, dismutase, superoxides) can maintain normal redox homeostasis;, in acute cases, it fails to mitigate oxidative stress and consequently cell death. Therefore, exogenous administration of natural antioxidants is required which could be a potential treatment procedure (Chen et al. 2009). Ferulic acid, a phenolic compound-based natural antioxidant, has gained the attention of scientific communities due to its ability to mitigate the impact of ROS by the formation of phenoxy radical stabilized by resonance in a phenolic nucleus and side chains (Graf 1992). Besides, FA facilitates cell adhesion and proliferation (San Miguel et al. 2011; Wang et al. 2011a). But, the lower bioavailability and short

residence time limit the effectivity of that antioxidant. Therefore, a thermosensitive hydrogel developed by utilizing chitosan, gelatin, and glycerol phosphate has been loaded with FA to ensure proper concentration and sustained released (Tsai et al. 2016). In another investigation, a limbal stem cell has been incorporated in carboxymethyl chitosan hydrogel where SAD has been utilized for cross-linking purpose to form the hydrogel (Xu et al. 2019). This system also has exhibited rapid formation in alkali burn and significantly enhance epithelium restoration.

Increasing demand for corneal replacement, limited availability of donors, and associated postoperative complications have suggested the need for better treatment procedures. In current studies, it is found that, for corneal epithelium and endothelium regeneration, chitosan-based hydrogels (Table 19.6) can be an effective treatment method, together with providing a suitable environment for cell adherence, growth, proliferation, and differentiation, and ability to carry growth factors and other important bioactive materials. These hydrogels also provide flexibility of modification and good mechanical properties (Ahmadi et al. 2015). However, as these investigations are still in the exploration stages, there are many limitations. In some cases, the obtained result is not up to the marks. Sometimes, the time required for healing is very long (Liang et al. 2011a, b). Besides, very few bio-ingredients are being incorporated in chitosan hydrogel. Further investigation incorporating various growth factors and formulations is needed to develop advanced formulation having the ability to work with much effectiveness and a significant reduction in time required to regenerate corneal cells.

19.4.7 Skin Tissue Engineering

Skin, being the largest organ of the human physique, is critically liable for keeping the body to function by not only acting as a barrier to hinder pathogens perforation in the direction of internal organs but also restrains excess water evaporation from the body (Zhao et al. 2015; Albanna and Holmes IV 2016). On account of the unending contact with the extraneous environment, skin injuries are an inevitable issue that requires to be considered specially. As a result, functional healing after injury stands as the fundamental objective of skin tissue engineering research. After acquiring either acute or chronic wounds, the body commences a process that consists of four sections—hemostasis, inflammation, proliferation, and reconstruction at the injured site for healing wounded tissues, as well as regenerating the barrier property (Reinke and Sorg 2012; Eming et al. 2014). Although, in the case of perfunctory wounds, intervention is not required, but in cases of severe injuries like second or third-degree burns owing to the long-term treatment, the ordinary system of wound healing is flawed. Consequently, atomical and psychological scars may occur and cause chronic dysfunction or even a serious cause of aberration and fatality (Wisniewski et al. 2000; Dabiri et al. 2016). Chitosan-based hydrogels having a complex 3D structure of hydrophilic cross-linked polymers provide dynamic equilibrium among the superfluous exudates and impart moisture to the wound surface (No et al. 2002; Fürst and Banerjee 2005; Chen et al. 2013). Therefore, these collaborate autolytic

Table 19.6 Application of chitosan-based hydrogels in corneal regeneration

Materials	Types of investigation	Remarks	References
Chitosan-poly (ethylene glycol), cystamine	In vitro	• Transparency above 95% in visible spectrum with desired mechanical properties • Attachment and proliferation of CECs • Readily permeable to glucose and albumin	Ozcelik et al. (2013)
Sodium alginate dialdehyde, hydroxypropyl chitosan	In vivo	• Survival and growth of transplanted CECs on hydrogel • Reduction of toxicity due to self-cross-linking with quick biodegradation • Minimal damage in the cornea during transplantation and low postoperative inflammation	Liang et al. (2011a, b)
iPSC/CHC hydrogel	In vivo	• Mitigates oxidative stress and promotes endogenous epithelium cell for restoration • Utilizes corneal surgical residue • No incident of tumorigenesis	Chien et al. (2012)
Chitosan, gelatin, stromal cell-derived factor-1 alpha	In vivo and in vitro	• Facilitates local expression of growth factors • Enhances reconstruction of epithelium with a more native structure • Thermosensitive hydrogel	Tang et al. (2017)
Chitosan, gelatin, glycerol phosphate, ferulic acid	In vivo and in vitro	• Accelerates corneal wound healing resulted from alkali burn • Decreases inflammation level • Can be an alternative of traditional eye drop	Tsai et al. (2016)
Carboxymethyl chitosan, sodium alginated dialdehyde	In vivo and in vitro	• Provides a good microenvironment for cell differentiation and proliferation	Xu et al. (2019)
Limbal stem cell		• Rapid restoration of corneal integrity with reduced scar formation	

acts and adjust pH, temperature, and moisture. In addition, the hydrogels also grant for gaseous exchange that gives rise to the higher oxygen tensions promoting the favorable environment for cellular metabolism, thus enhancing healing (Tozzi et al. 2016; Zhu and Marchant 2011). Furthermore, they promote accelerated wound termination with enhanced angiogenesis (Boateng and Catanzano 2020).

Chitosan-based hydrogels can be used in skin tissue regeneration as well as in wound healing. Various blended polymers like collagen, gelatin, and fibrinogen with chitosan repeatedly offer the necessary properties for making scaffolds for skin tissue engineering (Table 19.7). Chitosan and collagen combined scaffolds are

Table 19.7 Application of chitosan-based hydrogels in skin tissue engineering

Materials	Types of investigation	Remarks	References
Chitosan/nanofibrin composite	In vitro and in vivo	• Improved blood clotting and platelet activity	Kumar et al. (2013)
		• Able to absorbing fluid	
		• Cells are properly attached and proliferated	
		• Microporous, flexible, biodegradable, and nontoxic	
Dimethyl 3-3, dithio bis' propionimidate cross-linked chitosan	In vitro	• Exhibits increased the tensile strength	Adekogbe and Ghanem (2005)
		• Less toxic	
Chitosan, gelatin, and polycaprolactone	In vitro	• Possesses great elastic modulus and good cell adhesion	Gomes et al. (2017)
Nanocurcumin chitosan-g-pluronic	In vitro and in vivo	• Prevents the prolonged presence of oxygen free radicals	Dang et al. (2018)
		• Contains great stability	
		• Demonstrates well fibroblast proliferation and ability in anti-microbacteria	
		• Higher regenerated collagen density and thicker epidermis layer formation	
Chitosan/maleic terminated polyethylene glycol	In vitro and in vivo	• Contains a porous structure with swelling ratio	Jafari et al. (2019)
		• Can support human fibroblast cell proliferation	
		• Improves the wound contraction process with improved vascularization	

applied for skin regeneration and burnt skin treatment (Ma et al. 2003). Similarly, chitosan- and fibrin-blended scaffolds are considered superior quality scaffolds as they can facilitate angiogenesis, repair tissue, and ennobles cell binding and growth (Pocaterra et al. 2016). In a study, a composite was developed by using chitosan hydrogel and nanofibrin composite bandages (CFBs) that have demonstrated enhanced properties, like microporous, high flexibility and biodegradability, improved blood clotting and platelet activity in comparison with chitosan hydrogels only. Moreover, CFBs have the ability to absorb fluid and contained more attached and proliferated cells. As a result of collagen deposition, the nontoxicity property has enhanced (Kumar et al. 2013). Apart from this, nanofibrous scaffolds were developed by applying blends of chitosan, gelatin, and polycaprolactone for skin tissue engineering (Gomes et al. 2017). At first, the gel was produced from electrospinning solutions of polymeric blends (chitosan, gelatin, and polycaprolactone) followed by cross-linking with glutaraldehyde (GTA). The resultant nanofibrous scaffolds demonstrated hydrophilicity and the general porosity of nonwoven fiber mats. The

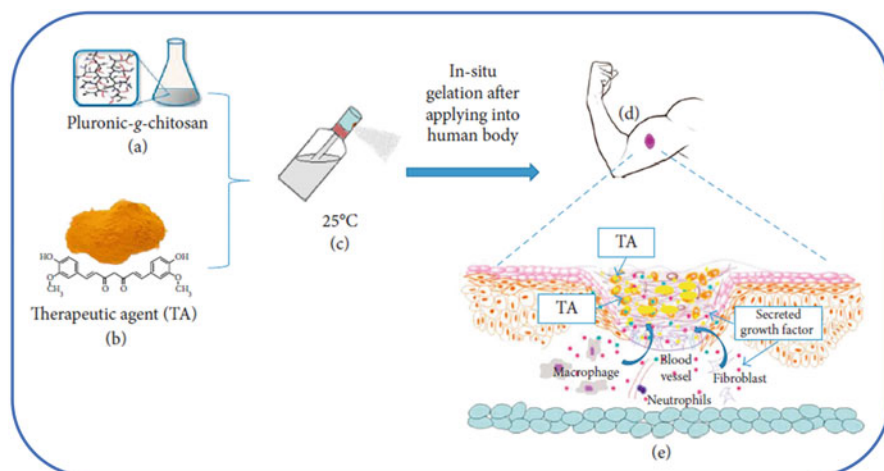

Fig. 19.8 Schematic illustration of the fabrication process of nCur-CP nanocomposite hydrogel by (**a**) CP is made by the combination of pluronic F127 and amide groups of chitosan at the appointed ratio of 15 to 1, respectively, (**b**) conversion of curcumin into a nanoform by the assistance of CP for the formation of hydrogel, (**c**) the sol-gel alteration near the temperature of 25 °C, (**d**) conversion of the solution in situ gelation after being applied into the human wound, and (**e**) nCur-CP nanocomposite hydrogel may be a hopeful therapeutic treatment for the management of burn wound. (Reprinted with the permission of Dang et al. 2018. Copyright © 2018 Le Hang Dang et al.)

scaffolds also exhibited a maximum elastic modulus of 48 MPa as well as superior dimensional stability. In a recent study, a chitosan-based hydrogel has been developed through thermal-cross-linking among chitosan (backbone) and polyethylene glycol terminated with maleic anhydride (PEG-MA) (cross-linking agent) (Jafari et al. 2019). Enhanced properties were observed after the addition of TiO_2 nanoparticles to the matrix. The experimental outcome has implied that the hydrogels contain a porous structure with a swelling ratio in the range of 240–280%. In vivo estimation showed that the hydrogel has exhibited completely biocompatible property and facilitated human fibroblast cell proliferation during testing time resulting in accelerated wound termination with improved vascularization. Apart from this study, another promising hydrogel (chitosan-g-pluronic hydrogel) formulated with nano curcumin has been fabricated that has demonstrated an extreme potential for burn treatment (Dang et al. 2018). Burn treatment is a complicated multiphase process which confides on coordinated signaling molecules to overcome. Curcumin, being a strong antioxidant and anti-inflammatory agent, restrains the prolonged appearance of oxygen free radicals, enhancing the optimum healing process. For fabricating the hydrogel (Fig. 19.8a, b), first chitosan-g-pluronic (CP) has been made from the coupling of pluronic F127 and amide groups on chitosan. After that, curcumin, the therapeutic agent has been converted into nanostructure with the help of CP resulting in the fabrication of nCur-CP nanocomposite hydrogel that has converted to sol-gel form at the by the temperature

of the human body. Thus, gelation has started after this solution coming in contact with the burnt surface (Fig. 19.8c, d). From the storage study, the superior stability of the hydrogel has been observed for a long time using UV-Vis and (dynamic light scattering) DLS. From in vitro experiments, nCur-CP has demonstrated significant fibroblast proliferation and anti-microbacterial property. Moreover, from the in vivo wound healing experiment, the activity of the nanocomposite hydrogel group has displayed an exceptional regeneration of collagen and denser epidermis layer formation, together with more excellent collagen content, enhanced granulation, and better wound maturity.

19.4.8 Periodontal Tissue Regeneration

Periodontitis is an inflammatory disease due to bacterial infection affecting the tooth-supporting tissues gingiva, periodontal ligament, cementum, and alveolar bone, leading to tooth loss in severe cases (Iwata et al. 2014; Kinane et al. 2017). Besides, patients suffering from periodontitis have a higher risk of developing some other diseases, including diabetes, Alzheimer's disease, and atherosclerosis (Ide et al. 2011; Stewart and West 2016; Dominy et al. 2019). Therefore, effective treatment procedures to get rid of this disease, as well as increase the living standard of affected people, have become crucial. The removal of subgingival bacterial biofilms by scaling and root planning is the current treatment strategy for this disease. Although this restricts further deterioration of the affected tooth, regeneration of the damaged tissue is still a gigantic challenge. Moreover, going through repetitive treatment sometimes makes the condition worse (Shaddox et al. 2010; Shen et al. 2020). Therefore, tissue engineering for restoring the structure and function of the damaged periodontium has become a considerable area of interest for the researchers where chitosan has been utilized to develop various hydrogel scaffolds for tissue restoration and active delivery of various bioactive materials to stimulate that process.

A novel chitosan-based thermoresponsive hydrogel has been prepared by using chitosan, quaternized chitosan, and α,β-glycerophosphate (Ji et al. 2009). Quaternized chitosan addition increases the solubility, whereas incorporation of glycerophosphate enhances the ionic interaction among ions that leads to the formation of densely packed hydrogel structure which retards drug release rate. This hydrogel has been utilized for local drug delivery purpose where ornidazole (ORN) has been used as a drug due to demonstrating better effectivity in treating chronic and acute periodontitis (Pradeep et al. 2012). Additionally, the quick phase transition from its liquid state at 37 °C (3 min) and longer duration of drug release (3 h) have been observed, which provides further benefit for its utilization. Although this hydrogel has exhibited mainly antibacterial activity with noticeable tissue regeneration, incorporating bioactive materials can further improve the effectivity by promoting tissue restoration (Ji et al. 2009, 2010). Bone morphogenetic protein-7 (BMP-7), also known as osteogenic protein-1, is well-known for its ability to enhance osteocementoblast differentiation and regeneration of damaged periodontal tissue (Gothard et al. 2014). Therefore, (BMP-7) and ORN together have been

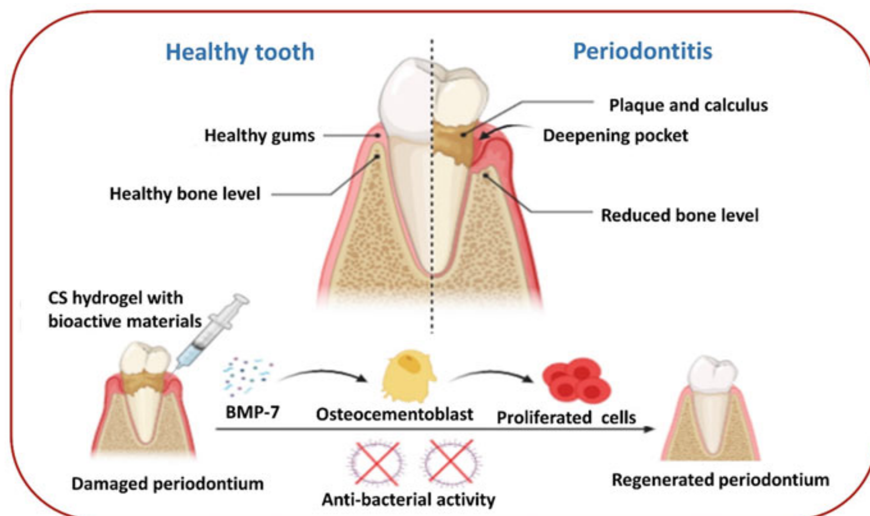

Fig. 19.9 Schematic illustration of periodontitis treatment utilizing chitosan-based hydrogel. Bioactive materials (e.g., bone morphological protein-7 (BMP-7)) and antibacterials (ornidazole) are incorporated in the chitosan/β-glycerophosphate hydrogel. BMP-7 excites osteocementoblast to proliferate and induces periodontium regeneration, whereas antibacterial destroys bacteria in plaque and calculus. (Redrawn from the work of Zang et al. 2019)

incorporated in chitosan/β-glycerophosphate (CS/β-GP) hydrogel in an investigation (illustrated in Fig. 19.9) (Zang et al. 2019). Good antibacterial properties and periodontal tissue regeneration with no synergistic effect between these two incorporated materials have been observed. Moreover, the exploitation of CS/β-GP hydrogel has ensured a sustained release of these incorporated bioactive materials and assisted in adherence, proliferation, and differentiation of cells in its 3D structure.

Hyaluronic acid (HA), one of the major components of the extracellular matrix plays a significant role in the tissue restoration process as well as wound healing (Litwiniuk et al. 2016). Besides, it is also widely known for its ability to mitigate gingival inflammation and alveolar bone regeneration; as a result, HA has gained significant attention in periodontal tissue engineering applications (Pilloni et al. 2011; Dahiya and Kamal 2013; Jimbo et al. 2014). Therefore, in an investigation, chitosan has been incorporated with HA to produce hydrogel for periodontal tissue regeneration purposes (Miranda et al. 2016). The hydrogel facilitates cell migration and tissue restoration with the help of CD44. Moreover, it can also prohibit matrix metalloproteinases (MMPs) induction in chondrocytes which is responsible for periodontal tissue deterioration and sustained inflammation (Julovi et al. 2004; Ji et al. 2010). In another study, triclosan and flurbiprofen, an antimicrobial and an anti-inflammatory drug, respectively, together has been incorporated in chitosan to develop a nano-hydrogel for localized sustained delivery of these drugs which can avoid further deterioration of damaged periodontium (Aminu et al. 2019). The

hydrogel has exhibited combined anti-inflammatory and antibacterial activity with good bio-adhesiveness. However, treatment of periodontitis for the diabetic affected patient is a big challenge as the sudden rising level of glucose may cause a reduction of the required dose available at particular sites for antibacterial activity (Jepsen and Jepsen 2016). Moreover, wound healing gets slower due to the restricted function of the immune system (Chapple et al. 2013). Therefore, a glucose-sensitive photo-cross-linked chitosan hydrogel film has been developed incorporating metronidazole. This hydrogel can monitor glucose levels and adjust the inner pore structure accordingly to control drug release, which enhances the antibacterial activity despite the higher level of glucose (Liu et al. 2019b).

Before inserting these hydrogels into our body, sterilization is a basic requirement to avoid further infection. This is generally done by using radiation (UV, gamma), ethylene oxidation, aqueous alcohol solution, which include various problems, such as deterioration of physicochemical, biological, and mechanical properties of the hydrogel, the release of toxic chemicals, degradation of chitosan structure, and so on (Holy et al. 2000; Yue et al. 2009; Zang et al. 2014). Therefore, steam sterilization has become a simple but effective method for sterilization. But autoclaved chitosan solution suffers from several problems (e.g., reduction of molecular weight, increased gelling time, and viscosity). Therefore, in an investigation, the hydrogel has been prepared to utilize autoclaved chitosan powder with glycerophosphate (Zang et al. 2014). This hydrogel has demonstrated faster gelation, higher viscosity, higher water absorption, together with enhancing periodontal ligament cell proliferation compared to autoclaved chitosan solution.

In a recent study, it has been found that although bacteria initiate periodontitis, but further degradation of the condition continues due to the release of pro-inflammatory macrophage (PIM) in response to bacteria (Darveau 2010; Hajishengallis 2014). On the other hand, anti-inflammatory macrophage (AIM), another type of macrophage, mitigates inflammation and facilitates tissue restoration (Shapouri-Moghaddam et al. 2018). Therefore, transforming PIM to AIM can be an effective treatment procedure for periodontitis. Exosomes derived from stem cells are known for their immunomodulatory and anti-inflammatory properties. Therefore, dental pulp stem cell-derived exosomes (DPSC-Exo) have been incorporated in chitosan hydrogel for periodontitis treatment in another investigation. The system can facilitate the reconstruction of alveolar bone and periodontal epithelium (Shen et al. 2020). Besides, this hydrogel can transform PIM to AIM what can mitigate inflammation. In vivo study has revealed its possibility as a suitable treatment procedure for periodontitis.

In the recent decade, investigations over chitosan-based hydrogels for periodontal tissue engineering have achieved significant progresses. Many systems have been developed (Table 19.8) that have exhibited combined antibacterial as well as tissue regeneration properties and the findings are getting improved by further researches and investigations. A combination of various bioactive materials with suitable dose and formulation may enhance the effectivity of these fabricated systems to the desired level for practical application. Besides, attention should be given in finding

Table 19.8 Application of chitosan-based hydrogels in periodontium regeneration

Materials	Types of investigation	Remarks	References
Chitosan, quaternized chitosan, (α,β-glycerophosphate), ornidazole (ORN)	In vitro and in vivo	• Sol-gel transition above 25 °C. Faster at 37 °C (3 min)	Ji et al. (2009, 2010)
		• Strong antibacterial activity with sustained release of drug	
		• Facilitates proliferation and alkaline phosphate activity in human periodontal ligament cells	
Chitosan, β-glycerophosphate, ornidazole, bone morphogenetic protein-7 (BMP-7)	In vitro and in vivo	• Enhance osteocementoblast and periodontal tissue regeneration in class III furcation defect	Zang et al. (2019)
		• Good antibacterial activity	
		• No synergistic effect between ORN and BPM-7	
Chitosan, hyaluronic acid	In vitro	• Exhibits higher phosphate buffer solution uptake	Miranda et al. (2016)
		• increased cellular viability and higher migration of cells on hydrogel	
		• Mitigates matrix metalloproteinases induction responsible for periodontal tissue deterioration and inflammation	
Chitosan, triclosan, flurbiprofen,	In vitro and in vivo	• Demonstrates strong bio-adhesiveness	Aminu et al. (2019)
		• Activity of antimicrobial against both gram-positive and gram-negative bacteria	
		• Anti-inflammatory action and mitigation of plaque	
Photo-cross-linked chitosan	In vitro	• Glucose-sensitive drug delivery system suitable for diabetic periodontitis treatment	Liu et al. (2019b)
		• Good mechanical property and enzymatic activity	
		• Better antibacterial ability against *Porphyromonas gingivalis*	
Autoclaved chitosan powder/β-glycerophosphate hydrogel	In vitro	• Faster gelation, higher viscosity, higher water absorption	Zang et al. (2014)
		• Enhances periodontal ligament cell proliferation	
DPSC-Exo/chitosan hydrogel	In vivo and in vitro	• Converts macrophages to anti-inflammatory phenotype and mitigates inflammation	Shen et al. (2020)
		• Facilitates healing of alveolar bone and the periodontal epithelium	

the most effective growth factors that can enhance tissue regeneration more effectively than the currently available one.

19.4.9 Bone Tissue Fixation

Bone fracture is one of the most common health-related complications occurring millions every year all over the world due to aging, accidents, and natural disasters. Currently, for clinical purposes, various fixation devices based on metal (e.g., stainless steel, titanium, cobalt) have been exploited, which is associated with numerous problems, including repetitive surgeries, allergic and toxic reactions, stress-shielding phenomena, and high cost (Van der Elst et al. 1995; Wang and Hu 2010). Therefore, in the last decade, various chitosan-based systems have been developed as a suitable replacement for metal fixation devices in order to promote bone fracture fixation.

Hydroxyapatite, a major component of human bone, has a very significant role in bone repair and regeneration (Kokubo 2008). As a result, a hydroxyapatite/chitosan nanocomposite gel rod has been developed by covalent cross-linking using aqueous glutaraldehyde solution as a reinforcing agent in an investigation (Wang and Hu 2010). Cross-linking has imparted very high mechanical properties with bending strength and modulus 178 MPa and 5.2 GPa, respectively. Further, glutaraldehyde can mitigate the inflammatory response that was previously observed in another study on chitosan/collagen scaffold (Lie et al. 2003). One more chitosan/hydroxyapatite hydrogel rod has been fabricated using sodium tripolyphosphate as cross-linking agent by Pu et al. (2012). Apart from higher mechanical properties, this system has also demonstrated the ability to facilitate cell viability and proliferation, avoiding any cytotoxic effect. In another investigation, chitosan gel rods of higher mechanical properties (bending strength and modulus 186.3 MPa and 5.17 GPa, respectively) have been developed by cross-linking with glutaraldehyde (Wang et al. 2011b). Multi-walled carbon nanotubes (MWCNT) are well-known for their extraordinary mechanical strength (Shokrieh et al. 2013). Besides, several studies have revealed the ability of MWCNT to promote bone formation by prohibiting osteoclastic differentiation and mitigating transcription factors responsible for osteoclastogenesis (Narita et al. 2009; Lobo et al. 2010; Huang et al. 2019). Therefore, MWCNT has also been used as a reinforcing agent in fabricating chitosan rods (Choi and Wang 2011). Effective dispersion of MWCNT in aqueous media was a major challenge that has been overcome by utilizing poly(phenylacetylene) due to its ability of uniform dispersion by wrapping its chain with MWCNT surface. Further, noncovalent interaction has contributed to the mechanical strength of fabricated chitosan gel rods. As previously mentioned, various nanoparticles have demonstrated remarkable biocompatibility, cell attachment, growth, and proliferation. TiO_2 nanoparticles due to these properties have been utilized to fabricate a chitosan-based hydrogel (Guo et al. 2020). Fabricated hydrogel has imparted good thermal stability, cell attachment, growth, and differentiation. Besides, the

incorporation of TiO_2 nanoparticles has facilitated alkaline phosphate activity, which is important for mineralization, as well as bone formation.

Application of bioabsorbable materials in fixation of hard and soft tissue have been available for treatment more than 20 years. These bioabsorbable implants have been reported to suffer from brittleness, lower mechanical strength, development of sinus at insertion place, early material failure, and accumulation of sterile fluid (Givissis et al. 2010; Mukherjee and Pietrzak 2011). In order to remove these limitations, a chitosan-based implant has been fabricated using hydroxyapatite (HA), tricalcium phosphate (TCP), and plasticizer. Glycerol and sorbitol have been used as plasticizers separately in two different systems (Figueiredo et al. 2020). These systems have demonstrated higher mechanical properties. Further addition of plasticizers (glycerol/sorbitol) has enhanced the ductility and strength of that materials.

In order to fabricate an effective material for bone tissue fixation, a combination of various properties, including stiffness, strength, hardness, and flexibility, are required with safe insertion in the human body (Figueiredo et al. 2020). But imparting all required properties combining various materials in a single hydrogel is still a gigantic challenge. The incorporation of MWCNT, HA, nanoparticles in various investigations has demonstrated optimistic improvements in several characteristics. However, the availability of very few papers on this topic (as listed in Table 19.9) indicates the necessity of further researches and investigations. Proper attention should be given to the biocompatibility and toxicity study of these investigated materials for effective utilization.

19.4.10 Miscellaneous

Apart from the above mentioned applications, the chitosan-based hydrogels have gained much attention in some other promising fields. One such application is to act as a barrier for obstructing postoperative peritoneal adhesions (Wei et al. 2009). Postoperative peritoneal adhesion is a common problem in surgery and occurs after most of the abdominal and pelvic surgery, which may result in severe abdominal/ pelvic pain and infertility (Arung et al. 2011). Currently, although various pharmacological and barrier-based methods are exploited effectively, these suffer from difficulties in fixation and handling. In order to avoid these problems, a new thermoresponsive hydroxybutyl chitosan hydrogel system has been developed to prevent adhesion after the operation due to its ability to mitigate the formation of scar tissue. The hydrogel has exhibited high effectiveness and quickly gave sol-gel transition and formed durable hydrogel, which has survived adequately long until the healing process has been finished. Therefore, this system has come up with undeniable amenities over existing barrier methods.

A heat-sensitive cisplatin-loaded chitosan hydrogel has been fabricated in combination with radiotherapy, which has been applied in order to treat patients with locoregionally severe nasopharyngeal carcinoma (NPC) (Peng et al. 2019). Although cisplatin-based chemoradiotherapy is still recognized as the maximum

Table 19.9 Application of chitosan-based hydrogels in bone tissue fixation

Materials	Types of investigation	Remarks	References
Chitosan, hydroxyapatite (HA) nanocomposite	In vitro	• Potential application in internal bone fracture fixation	Hu et al. (2004)
		• Well dispersion of HA in chitosan matrix	
		• Reduction in water absorption	
Chitosan, hydroxyapatite, glutaraldehyde	In vitro	• Much tight layer-by-layer structure	Wang and Hu (2010)
		• Very high mechanical properties	
		• mitigates inflammatory response	
Chitosan, hydroxyapatite, sodium tripolyphosphate	In vitro	• High mechanical strength	Pu et al. (2012)
		• Ability of cell viability and proliferation	
Chitosan, glutaraldehyde	In vitro	• Much tight layer-by-layer structure	Wang et al. (2011b)
		• Mechanical strength similar to commercially available biodegradable devices	
Chitosan, multi-walled carbon nanotubes, poly (phenylacetylene), Fe_3O_4 nanoparticles	In vitro	• Higher mechanical strength with excellent bending modulus	Choi and Wang (2011)
		• One-dimensional nanostructure	
		• Enhances cell growth and proliferation	
Chitosan, gelatin, TiO_2 nanoparticles	In vitro	• Significant effect on cell attachment and viability	Guo et al. (2020)
		• High alkaline phosphate activity and higher rate of mineralization	
Chitosan, hydroxyapatite, tricalcium phosphate, glycerol/ sorbitol	In vitro	• Increases in ductility and reduction in brittleness by adding plasticizer	Figueiredo et al. (2020)
		• Ability to machining into a shape of any geometry	

feasible treatment for locoregionally developed NPC, as a result of enhanced treatment cost in comparison with chemotherapy and radiotherapy, it desponds to demonstrate the optimum therapeutic dose (Lee et al. 2011; Tang et al. 2018; Lv et al. 2019). In addition, it also exhibits sluggish retention in tumor tissues resulting in evasive interactions, leading to intense side effects on sound tissues (Cavaletti et al. 2011; Qi et al. 2016). So, the fabricated cisplatin-loaded chitosan hydrogel has

exhibited an extraordinarily enhanced survival time of 81 days. The fundamental mechanism not only raises the death rate of cancer cells but also has averted cell proliferation by blocking (Ki-67). The aforementioned outputs display the therapeutic potential of chitosan hydrogel and radiotherapy summation for localized NPC treatment.

In recent times, primary attention to wound healing researches have also been given for evaluating the synergistic effects of multi-polymeric methods in the form of hydrogels, as they provide fast reepithelialization (Gyles et al. 2017). A chitosan-based hydrogel has been fabricated that exhibited desired swellability, flexibility, gelling, and surface morphological characteristics along with compatible micropores, which are essential for speedy wound recovery. So, this hydrogel may be potential wound dressing materials with fantastic forming and improved wound healing.

19.5 Challenges and Future Prospects

Limitations of currently available treatment procedures have inspired researchers to exploit tissue engineering strategies for various purposes. With some other biomaterials, chitosan also has been utilized for numerous applications in tissue engineering. Among various chitosan-based scaffolds, hydrogels have been regarded as one of the most promising materials because of their mimicking similarities with extracellular matrix and ability to provide a microenvironment for cell adhesion, growth, and proliferation (Du et al. 2013; Debnath et al. 2015). Therefore, over the years of researchers have introduced a myriad of chitosan hydrogel-based systems for various tissue engineering applications which has been summarized in this chapter. Although, some investigations have demonstrated promising results, still several drawbacks are keeping these systems far from clinical applications. To impart the desired level of progress and make them suitable for clinical applications, the currently existing problems and possible future research directions as solutions are outlined in the following points:

- One major problem regarding chitosan-based hydrogel for tissue engineering application is the low mechanical strength of fabricated hydrogel. Higher mechanical strength can be imparted either by combining with other materials or the addition of suitable cross-linkers or combining both approaches. Therefore, various materials including graphene oxide, nanocellulose, nanoparticles of TiO_2, ZrO_2, Au, and Zn have been incorporated to fabricate hydrogels with higher mechanical strength (Zazakowny et al. 2016; Gullbrand et al. 2017; Saravanan et al. 2018; Nezhad-Mokhtari et al. 2020). Although studies have reported these materials as biocompatible, some other studies also have suggested various complications regarding the application of metal nanoparticles and carbon-based materials, as these have the ability to catalyze metabolism and act differently based on the physiological environment (Choi and Wang 2011; Reina et al. 2017). Such as TiO_2 has been reported to create ions that can affect our

metabolism and cause inflammation (Souza et al. 2019). Moreover, the impact of long time exposure of these materials in body fluid is another important aspect to be critically considered. Therefore, while investigating with graphene-like carbon materials and metal or metal oxide nanoparticles, a proper toxicity study should be conducted concerning their size, layer, dimensions, and other required parameters. Another way of imparting higher mechanical strength is effective cross-linking. Therefore, various hydrogels have been developed by utilizing several cross-linking agents. As the currently using cross-linking agents are unable to provide the desired level of strength, the investigation should be conducted in search of novel materials which can be obtained either by exploiting derivatives of the existing one or making a suitable combination among two or more to enhance synergistic action of them.

- In order to obtain superior properties, integration of various growth factors and drugs has been proved to be an effective method, where chitosan-based hydrogel plays the role as a carrier that ensures sustained and continuous release of incorporated components and provides the environment of cell adhesion and proliferation. In many investigations, growth factors and drug incorporation has exhibited promising results (Ji et al. 2010; Aminu et al. 2019; Liu et al. 2019a; Zang et al. 2019). A combination of both growth factors and required drugs for satisfying various purposes can be the approaches for effective tissue engineering applications. For example, the application of ornidazole and bone morphogenetic protein-7 simultaneously has offered hydrogel with higher effectiveness in periodontitis treatment (Zang et al. 2019). Therefore, a combination of various elements and their synergistic effects should be rigorously studied.
- Many of the reports published recently have circumvented their evaluation within in vitro, which lags in a proper understanding of the performance of fabricated hydrogels. Because the original condition encountered by these hydrogels is different from the in vitro studies, therefore, in vivo investigation should be conducted maintaining similar environmental factors and conditions at which hydrogel will be practically exposed. Again, for in vivo investigations, small lab animal (e.g., mouse, rabbit) may not give the best possible results, as the load imposed on these implanted scaffolds are far different than human body in terms of load as well as the physiological environment (Yang et al. 2018). Therefore, the selection of larger animals (e.g., porcine, sheep) may provide the opportunity for precise evaluation.
- Over the years, studies and researches have introduced various advanced technologies and procedures. 3D bioprinting and electrospinning are two such technologies for fabricating high-quality scaffold for biomedical application. Electrospinning provides the opportunity to fabricate nanofibers suitable for use as a scaffold in tissue regeneration purposes, while bioprinting offers forming a precise layer-by-layer structure of cells, growth factors, and scaffold and required materials (Liu et al. 2013; Bishop et al. 2017).
- Decellularized ECM, a promising approach for tissue engineering, has been found to be applied in numerous fields, including IVD, cartilage tissue regeneration, and skin tissue, and in various other tissue engineering applications (Hanai

et al. 2020; Khoshnood and Zamanian 2020; Kim et al. 2020; Rothrauff and Tuan 2020). However, studies over these promising technologies and approaches are very limited. The exploitation of these technologies and strategies is believed to impart superior quality hydrogels for which further rigorous studies should be conducted.

19.6 Concluding Remarks

After initiating the journey as an effective and easy way of tissue repair, regeneration, or artificial production, tissue engineering has seen a tremendous growth where chitosan-based hydrogel systems have held considerable attention in various field, including bone, cartilage, IVD, blood vessel, neural, skin, periodontal tissue engineering, as well as bone fixation and corneal regeneration with some other applications which have been discussed in this chapter. Most of the investigations are in the developing stage, and still, these need to go a long way for materializing into clinical applications. However, the results obtained from many of the investigations are quite promising, which have persuaded the researchers to investigate deeper inside the possibilities. The currently existing challenges and their possible research direction are also outlined in the previous sections. The analysis of current development in this field suggests a combination of suitable materials, and advanced technologies will be able to eradicate existing problems and equipped humankind with tissue engineering as an advanced treatment strategy.

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20 Bioceramics-Based Biomaterials for Bone Tissue Engineering

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Abstract

Finding a novel bioactive bone graft substitute is an emerging area of research for all the time to overcome the traditional bone graft procedures. Bone graft substitute, which induces osteogenesis, is considered suitable graft material in orthopedic clinical practice. Different kinds of biomaterials are used to develop bone grafts using polymers, ceramics, carbon, and metal-based materials. Hydroxyapatite and bioactive glasses are widely used bioceramics. In this chapter, different bioceramics with the combination of alginate, graphene oxide, polylactic glycolic acid, and chitosan are discussed. The preparative procedure, in vitro cell interaction, and in vivo study of bioceramics are also presented. Moreover, the osteogenic properties of the hydroxyapatite and bioactive glasses and their composite biomaterials are discussed in detail. The in vitro and in vivo studies have proven that HA and bioactive glass can induce osteogenesis, a vital parameter for bone tissue regeneration. Hence, this chapter emphasizes that a

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scaffold system containing hydroxyapatite and bioactive glass composites are ideal for a bone graft substitute.

Keywords

Alginate · Bioactive glass · Bone tissue engineering · Chitosan · Hydroxyapatite

20.1 Introduction

Bone is a hierarchically organized dynamic tissue in the body that has regeneration/degeneration capacity. Bone loss or defects can occur due to several causes, including diseases, trauma, aging, osteoarthritis, tumor resection, motor accidents, bone fracture, and congenital disabilities. Bone loss or damage significantly affects the patient's life. Autograft and allograft are widely used grafting techniques in orthopedic surgery to treat defective/diseased bone. Autograft is considered the gold standard for bone graft substitutes. The success rate of autograft substitute is due to osteoconductive, osteoinductive, and osteogenic properties (Venkatesan et al. 2014; Amini et al. 2012). These bone grafting procedures have some disadvantages. The drawbacks of the autograft technique are insufficient donor sites, secondary surgery, chronic pain, and donor site morbidity. Allograft has disadvantages: contracting diseases and rejection to the host bone tissue (Amini et al. 2012; Shegarfi and Reikeras 2009). The synthetic bone graft can be an alternative substitute to overcome the weaknesses of autograft/allograft techniques.

Bone tissue engineering approaches are being used to construct the artificial synthetic bone graft substitute using materials, cells, and growth factors. Bone formation (osteogenesis) happens due to the cellular activity of osteoblasts, osteocytes, and osteoclasts (Van Heest and Swiontkowski 1999; Dadhich et al. 2016; Shegarfi and Reikeras 2009). The bioactive scaffolding materials can easily integrate with the native tissue (Karageorgiou and Kaplan 2005). Titanium-based scaffolding systems have been extensively used for treating a long bone fracture. Metal implant techniques (titanium alloys and stainless steel) have shortcomings, including non-degradability, high stiffness, and lack of integration into the host tissue (Rezwan et al. 2006; Roseti et al. 2017). To overcome all these problems in the grafting material, researchers are developing alternative synthetic bone graft substitutes. Significant research has been carried out to create artificial bone graft substitutes using bioceramics, polymers, co-polymers, and growth factors.

Poor vascularization, insufficient mechanical strength, lesser osteointegration, leaching materials from the implanted site, bacterial infection, and poor osteoinductive effects are still existing problems in the synthetic bone graft substitute. Porosity, biocompatibility, osteoinductive, osteoconductive, and mechanical strength of the bone grafts substitute play an essential role in proper bone regeneration in the defective bone sites. Scaffold having porosity provides cell attachment and cell growth and is sufficient for better bone tissue regeneration. Hence, developing an orthopedic bone graft substitute with mechanically robust, sufficient porosity prevents microbial infection, and it is challenging to create an artificial

synthetic bone graft substitute (Yang et al. 2018; Ikada 2006; Amini et al. 2012; Novosel et al. 2011; Winkler et al. 2018).

20.2 Bioceramics (Hydroxyapatite and Bioactive Glasses)

Bioceramics, including calcium phosphate, hydroxyapatite and bioactive glass, are extensively studied for bone tissue engineering fields. These bioceramics are biocompatible with human tissues and also help in improving the mechanical strength of the graft substitute. Cytotoxicity, degradation assessment, hemocompatibility, sensitization, and the genotoxicity of the developed bone graft substitute can be evaluated with International Organization for Standardization procedures (Reeve and Baldrick 2017).

20.2.1 Hydroxyapatite

Hydroxyapatite (HA) is one of the calcium phosphate materials, and it is biocompatible to human bone tissue due to similar biomimicking properties to the native bone. HA is less immunogenic and can be integrated with native tissue with required metabolic functions (Demirkiran 2012; Lobo and Livingston Arinzeh 2010). HA has several important properties, including biocompatible, osteoconductive, mechanical strength, and biodegradable and noninflammatory. Bioactive glass is a kind of glass-ceramics that consists of calcium sodium phosphosilicate. Bioactive glass integrates with bone tissues (Chen et al. 2006; Gerhardt and Boccaccini 2010; Fu et al. 2011; Jones et al. 2006). To improve the physical, chemical, and biological properties of bone graft substitute, it is often combined with polymers, metal nanoparticles, growth factors, and stem cells (Leukers et al. 2005; Wei and Ma 2004; Lin and Yeh 2004; Saini et al. 2019; Saravanan et al. 2017; Swetha et al. 2010; Venkatesan et al. 2012; Zhou and Lee 2011). In the current book chapter, we have discussed the effectiveness of the HA and bioglass and their composite biomaterials toward bone tissue engineering applications. The preparatory procedure of composite biomaterials, biomaterials-cell interaction, gene expression, and mineralization results was discussed in detail (Habraken et al. 2007; Lakhkar et al. 2012; Samavedi et al. 2013; Sethu et al. 2017; Shaheen et al. 2019; Sowjanya et al. 2013).

20.2.2 HA-Polymer Biocomposites

HA combines natural and synthetic polymers to improve its biological properties for better bone tissue regeneration. Alginate is a polyionic natural polysaccharide, which contains β-(1→4)-linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronic acid (G) structures. Alginate is commonly isolated from brown seaweed. Alginate interacts with the divalent cations and forms hydrogels, fibers, foams, and microspheres (Lin and Yeh 2004; Venkatesan et al. 2015). HA with alginate is one of the best-studied biomaterials composite for bone tissue engineering

applications. Biomimetic fibrous scaffold containing alginate and HA can be used as a bone implant (Chae et al. 2013), and in vivo bone mineralization with alginate and HA-containing biocomposites is well-proven (De Paula et al. 2009). Barros et al. have reported optimized composition of the alginate and nano-HA-containing hydrogel system for bone regeneration applications (Barros et al. 2019). Maji et al. developed nano-HA with alginate paste using the photo-cross-linking method to incorporate bone morphogenic protein-2 (BMP-2). In vitro cell interaction study was done with mesenchymal stem cells and reveals that developed paste has osteogenic properties. The MTT assay revealed that the prepared paste material is favorable for cell attachment and proliferation.

Further, an immunocytochemistry study reveals that the addition of nano HA and BMP-2 to the paste is responsible for enhancing the osteogenic properties (Maji et al. 2020). Lin et al. have developed scaffolds of porous alginate and HA by using the phase separation technique. Further, biological assays were carried out with rat osteosarcoma UMR 106 cells. Cell attachment was observed by scanning electron microscopy (SEM) images, whereas histology analysis proves the osteogenic properties of the developed scaffold (Lin and Yeh 2004). Brun et al. have studied micro-computational (μ-CT) image analysis of the alginate and HA composition. It has been observed that the final framework of the scaffold can be defined by usingμ-CT images (Brun et al. 2011).

Tricomponent systems with HA, both synthetic and natural polymers, provide better biological properties. A microporous scaffold containing polylactic acid, sodium alginate, and HA was developed using 3D bioprinting technology. In vitro biomineralization test in simulated body fluid (SBF) showing scaffold can induce mineralization. SBF-treated composite scaffold showed compressive strength of 13.61 MPa (Fernández-Cervantes et al. 2019). Li et al. have developed hydrogels containing graphene oxide, alginate, and HA and checked the osteogenic potentials in cells. Alizarin red S assay shows hydrogels can induce mineralization. Simultaneously, ALP assay shows enhancement in the alkaline phosphatase activity and its increases with implantation time (Li et al. 2020). Bernhardt et al. has reported the osteogenic differentiation capacity of the scaffolding system containing alginate, gelatin, and HA composition. In vitro osteogenic properties of the fabricated scaffold were checked with human bone marrow stromal cells. Further, osteogenic differentiation of the scaffold was confirmed by osteogenic gene expression analysis, DNA content and alkaline phosphatase (ALP) activity, and lactate dehydrogenase (LDH) activity (Bernhardt et al. 2009). Besides this, several biocomposites, including collagen-alginate-HA, silk fibroin-alginate-HA, gelatin-alginate-HA, cellulose-polycaprolactone-alginate-HA, and alginate-hydroxymethyl cellulose-HA, were developed and checked bone tissue regeneration applications (Zheng et al. 2014; Tohamy et al. 2018; Nabavinia et al. 2019; Iqbal et al. 2018; Hokmabad et al. 2019; Jo et al. 2017).

20.2.3 HA-Metal Biocomposites

Metals have been extensively studied biomaterials for orthopedic treatment. Several research reports have proven that, metal nanoparticles can provide significant properties for bone graft substitute, including biocompatible, antimicrobial, and improved mechanical properties. Besides this, metals also induce osteogenic cell differentiation and regulate cell growth pathways. Metals including titanium oxide, molybdenum disulfide, silver nanoparticles, strontium, and cobalt were explored for bone tissue engineering applications (Eivazzadeh-Keihan et al. 2020; Dhivya et al. 2015; Xue et al. 2020; Yang et al. 2020).

Molybdenum disulfide (MoS_2) is an inorganic transition metal dichalcogenide and gained much attention in the biosensor, tissue engineering, and drug delivery applications. Recently, Wang et al. and Zhang et al. have studied the osteogenic differentiation of MoS_2 (Wang et al. 2017; Zhang et al. 2018). Yadav et al. have fabricated nanocomposite containing MoS_2 nanosheets and HA and studied the in vitro cell studies on MG-63. The developed nanocomposites are nontoxic, increased alkaline phosphatase activity, and enhanced osteogenic differentiation. Furthermore, in vivo studies were conducted on the rat distal femoral defects. In vivo radiological examinations, ALP activity in blood samples, and osteogenic gene expression assays showing enhancement in the osteogenic gene expressions and bone healing have increased within 12 weeks of post-implantation time (Yadav et al. 2019).

Ignjatovic et al. have conducted a study on the composition of nano cobalt (Co) and HA for bone tissue engineering applications. In vivo studies were conducted on the artificially created osteoporotic defects, and results were observed after 12 and 24 weeks. In vivo biomineralization, scanning electron microscopy (SEM) images, and histological analysis show that HA containing 12% Co has higher osteogenesis potential than HA containing less than 12% Co (Ignjatovic et al. 2015). Lin et al. have studied the scaffolding system containing Co with polycaprolactone and HA for bone regeneration applications. In vitro biodegradation studies in PBS show long-term controlled degradation behavior. MTT assay and staining assays with human osteosarcoma cell lines (MG-63) prove the biocompatibility and osteogenic properties of the scaffold (Lin et al. 2019). Strontium (Sr) incorporation with HA shows better bone tissue regeneration when compared to HA alone. Sr reinforced nano-HA was coated on a titanium surface and studied biocompatibility with BMSCs. Alamar blue assay proves that HA and Sr-HA materials are nontoxic and biocompatible. Further, ALP assay and gene expression study showed enhancement in the ALP activity and osteocalcin secretion. The developed biocomposites can induce mineralization, and Sr-HA shows superior results than bare HA (Yang et al. 2015).

Bacterial infection associated at the implanted site is a significant complication in orthopedic treatment and leads to failure of the bone implants. The substitution of the antimicrobial agent in the scaffolds can be a better alternative to solve microbial infection at the implanted site. Silver is known to have antimicrobial potential (Eivazzadeh-Keihan et al. 2020; Arjunan et al. 2020; Marsich et al. 2013). Stanic

et al. have studied monophasic silver with HA and investigated the efficacy of bone tissue regeneration. Antibacterial potential of the developed biocomposition was checked against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Finding results show that the developed biocomposition possesses excellent antimicrobial potential (Stanić et al. 2011). Sol-gel technique was used to develop silver-containing HA coating material. In vitro cell interaction study was carried out on human embryonic palatal mesenchymal cells to check cell proliferation and osteogenic differentiation. Cell proliferation and osteogenic differentiation were confirmed by double-stranded DNA assay and ALP assay (Chen et al. 2007). Also, silver-HA composite developed by ultrasonic spray pyrolysis method can be used as a bone implant. Colony count method, inhibition zone assay, in vitro analysis of biofilm formation, and in vivo imaging prove the antibacterial efficiency of the developed biocomposition. Besides, the developed biocomposite is biocompatible with MC3T3-E1 cells (Honda et al. 2013). Biosynthesized chitooligosaccharide-coated silver nanoparticles were developed by using a facile and straightforward microwave-assisted method. Next, microspheres were fabricated by using silver nanoparticles, alginate, and HA. Biocompatibility and cell proliferation of the microspheres with MG-63 cell lines were proven by MTT, Hoechst staining, and acridine orange/ethidium bromide (AO/EB) staining assay. In addition, microspheres show inhibition against *Staphylococcus aureus* and *Escherichia coli* (Dalavi et al. 2020). Khan et al. have developed scaffolds of nano-HA containing β-glucan and acrylic acid, and silver was coated on this scaffold using the dip-coating method. The developed scaffold shows excellent antibacterial activity against DH5 alpha *Escherichia coli*. In vitro cell interaction with MC3T3-E1 preosteoblast cell lines was done to check the biocompatibility of the scaffold. Neutral red assay proves that cells are viable and nontoxic with the developed composite scaffold (Khan et al. 2020). Besides, silver doped in HA and polyvinyl alcohol (PVA) nanofibers can be used as a bone implant for osteogenesis application (Anjaneyulu et al. 2017).

20.2.4 HA-Carbon-Based Biocomposites

Carbon-based materials have been studied for bone tissue engineering applications due to their exceptional mechanical strength. Lee et al. have developed nanocomposite-containing graphene oxide-HA as an orthopedic graft. In vitro cell interaction of graphene oxide-HA with MC3T3-E1 cells was carried out to check osteogenic differentiation. Alizarin red S (ARS) staining and Von Kossa staining assay prove that nanocomposite can induce mineralization. In vivo studies, calvarial defects, histopathology, and histomorphometry assay show new bone formation without agitating response (Lee et al. 2015). Sumathra et al. have checked osteogenic properties of the cisplatin loaded in graphene oxide, HA, and chitosan biocomposite material. ALP activity and osteogenic gene expression study reveal that developed biocomposites have osteogenic properties (Sumathra et al. 2018).

20.3 Bioactive Glass

Since the decades, bioactive glasses have been used to heal bone defects. Larry Hench discovered 45S5 bioglass (calcium sodium phosphosilicate composed of 45 wt% SiO_2, 24.5 wt% CaO, 24.5 wt% Na_2O, and 6.0 wt% P_2O_5), and it can mimic the bone healing process by integrating with physiological body fluid and apatite formation. The creation of porosity in bioactive glass and a lower degradation rate is the biggest challenge while using bioactive for bone regeneration applications. Researchers are adopting several synthetic routes to tailor the biological properties of bioactive glass (Gerhardt and Boccaccini 2010; Jones 2013). To achieve higher osteoinductive properties, researchers are using bioactive glass containing metals including zinc (Zn), strontium (Sr), manganese (Mn), and boron (B). Besides this, bioactive glasses and bioactive polymers containing biocomposites are attractive materials for bone regeneration applications (Gentile et al. 2014).

20.3.1 Bioactive Glass-Polymer Biocomposites

Polymers with bioactive glass composites are studied for bone tissue engineering application (Gentile et al. 2012). Both natural and synthetic polymers were used to combine with bioactive glass for better bone tissue regeneration. Poly(lactic-co-glycolic acid) (PLGA) is a nontoxic, biocompatible, and biodegradable Food and Drug Administration-approved biopolymer. PLGA is widely used in bone tissue engineering because of its biodegradable properties, and it can be tailored to regulate cell interaction for better bone regeneration capacity (Gentile et al. 2014; Danhier et al. 2012). The utilization of PLGA in bone tissue engineering has some shortcomings, including lack of mechanical strength and poor osteoconductive properties. Hence, composite biomimetic scaffolds containing bioactive glass and PLGA are a suitable alternative for bone regeneration applications (Gentile et al. 2014). The pressure-activated microsyringe was used to fabricate a scaffold containing bioactive glass and PLGA. Further, in vitro cell interaction study was carried out with MG-63 osteoblast-like cells. MTT assay and osteogenic gene expression study proved that scaffold has osteogenic properties (Mattioli-Belmonte et al. 2017).

Chen et al. developed PLGA and bioactive glass biocomposite scaffold by the sol-gel method. In vitro biomineralization test of the scaffold in SBF proves that PLGA/bioactive glass-containing scaffold has a more extraordinary ability to induce apatite formation than bare PLGA (Chen et al. 2010). Incorporating PLGA in bioactive glass can increase the mechanical strength of the composite material (Filipowska et al. 2014). Bioactive glass and PLGA biocomposite was fabricated and effect of photobiomodulation strategy were applied to develop bone graft. In vivo studies on the rat calvarial bone defect model were studied, and histomorphometric analysis shows that new bone tissue forms at an implanted site (Magri et al. 2019b).

Magri et al. have developed biocomposite containing bioactive glass, collagen, and PLGA. In vitro cell interaction studies with MC3T3-E1 cell lines demonstrate that biocomposite can proliferate cells and enhance ALP activity and cell adhesion. Further, in vivo studies were done on the calvarial bone defect model in rats. Next, histology and immunochemistry assays confirm that a significant volume of new bone has formed (Magri et al. 2019a). A composite scaffold containing HA, PLGA, and bioactive glass was used. In vitro cell interaction study was done with the MC3T3-E1 preosteoblast cells. Cell viability of the cells on the developed scaffold was proven by live/dead assay. Next, Alizarin red S assay showing the scaffold can induce mineralization. Further, an immunocytochemistry study shows enhancement in the osteogenic gene markers (Ryu et al. 2019). Cheng and his co-workers have reported the utility of vancomycin loaded into the bioactive glass and PLGA composite scaffold for bone regeneration and to prevent bacterial infection associated with bone grafts (Cheng et al. 2018).

20.3.2 Bioactive Glass-Chitosan Biocomposites

Chitosan is a cationic polysaccharide that is commonly isolated from the shells of the crustacean's exoskeleton. Chitosan has been studied extensively for bone tissue engineering applications due to its properties, including biocompatibility, biodegradability, and nontoxic nature. Chitosan can promote and proliferate osteoblast cells (Levengood and Zhang 2014). Biodegradable composite of polymers like chitosan and ceramic-like bioactive glass is one among the best choice to use as a bone implant (Venkatesan and Kim 2010). Several research reports are available on nano-bioactive glass with chitosan hybrid composite for osteogenic applications (Oudadesse et al. 2020). Remineralization of the chitosan and bioglass composite under different dynamic mechanical conditions was reported (Caridade et al. 2013b). Chitosan membrane containing micro-bioactive glass and nano-bioactive glass was developed by using the solvent casting method. In vitro bioactivity tests show that the chitosan membrane with nano-bioactive glass shows better biomineralization than the chitosan membrane with micro-bioactive glass composites (Caridade et al. 2013a). An injectable hydrogel system containing chitosan and bioactive glass nanoparticles has osteogenic properties and can be used as a bone implant (Couto et al. 2009).

Covarrubias et al. have fabricated chitosan and gelatin reinforced bioactive glass for bone regeneration applications. Five percent bioactive glass nanoparticles were used in the biocomposite. MTT assay reveals that fabricated nanocomposite has exceptional cytocompatibility, whereas in vitro biomineralization in SBF shows scaffold can induce apatite formation. It is found that the APL assay shows enhancement in the ALP activity. Furthermore, in vivo study was performed on the femoral defect model in rats. After 8 weeks of implantation, it has been observed that a significant volume of fresh bone has formed at the defective site (Covarrubias et al. 2018). Chitosan and niobium (Nb)-doped glass containing scaffolding system was developed by the electrophoretic method. It was found that the release of Nb from

the scaffold was nontoxic to the MG-63 cells. Besides, the antibacterial property of the scaffold was reported (Bonetti et al. 2020). Ding et al. have fabricated polyhydroxybutyrate, polycaprolactone, and bioglass containing hybrid biocomposite, to achieve superior osteogenic properties. Biogenic assays prove that the developed scaffold is nontoxic and biocompatible with MG-63 osteoblast-like cells. Besides, it is observed that scaffold can induce remineralization (Ding et al. 2016).

45S5 bioactive glass-chitosan-poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Li et al. 2015), calcium phosphate-bioactive glass-chitosan-collagen (Mooyen et al. 2017), bioactive glass-chitosan-collagen (Moreira et al. 2016), bioglass-poly(vinyl alcohol)-chitosan-collagen (Pon-On et al. 2014), *N*-(2-carboxybenzyl) chitosan-nano TiO_2-bioglass (Nerantzaki et al. 2017), cerium-doped bioactive glass-chitosan-polyethylene oxide (Saatchi et al. 2020), bioactive glass-HA-PCL-chitosan (Shalumon et al. 2013), bioglass-chitosan-PCL (Yao et al. 2014), and chitosan-polyvinyl pyrrolidone-45S5 bioglass (Yao et al. 2015) composites were studied for bone tissue engineering applications. Around 31 clinical trials are ongoing with the hydroxyapatite with the combination of chitosan nanoparticles, platelet membrane, antibacterial drugs, and natural collagen for bone tissue regeneration (ctri.nic.in n.d.). Seventy-one studies are completed/ongoing related to hydroxyapatite for dental and bone tissue regeneration (clinicaltrials.gov n.d.).

20.4 Conclusion

Bioceramics and their composite biomaterials are widely utilized for bone tissue engineering applications due to their biocompatible, osteoconductive, and biodegradable nature. Improved properties (biocompatible, biodegradable, mechanical strength, alkaline phosphatase activity, osteocalcin, and enhanced mineralization) were observed with the combination of bioceramics with polymer and metal nanocomposites. The combination of hydroxyapatite and bioactive glass with PLGA, alginate, chitosan, graphene oxide, and metal nanoparticles are extensively studied biomaterials for bone tissue engineering. In vitro and in vivo studies of hydroxyapatite-alginate, hydroxyapatite-metal nanoparticles, bioactive glass-PLGA, and bioactive glass-chitosan composites show improved physical, chemical, and biological properties for better bone tissue regeneration. Stem cells play an essential role in bone tissue regeneration and remodeling for a better regenerative effect with bioceramics and polymers. However, large animal models need to be performed to transfer the composite biomaterials to clinical trials.

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21 Recent Advances in Hydrogels and Stem Cells

Ailar Nakhlband, Laleh Saleh-Ghadimi, Marziyeh Fathi, Mohammad Samiei, Jaleh Barar, and Yadollah Omidi

Abstract

Regenerative medicine is a field of science that has shown enormous applications for efficient therapy and has been advanced by recent sophisticated achievements in bioengineering. Modern techniques deployed in manufacturing tissues and

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designing structures, which are functional in preserving, restoring, and regenerating damaged tissues and organs, have enhanced medicine and health care. Methods of merging biomimetic materials, cells, and bioactive molecules are critical in advancing the revival of wounded tissues or therapeutic systems. In recent decades, one of the most popular materials used for engineering tissues is the hydrogel-based scaffolds. They represent the ability to configure a distinct 3D structure that provides the cells within the tissues with elegant mechanical support for the cells in the engineered tissues and replicate the native extracellular matrix. In this chapter, as an important class of stem cell vehicles, hydrogels are articulated, and their fabrications and applications for tissue regeneration are discussed. We focus on the application of the hydrogels for 3D cultures in regenerating different tissues, such as skin, bone cartilage, vascular, cardiac, and neural tissue regeneration.

Keywords

Biomaterials · Stem cells · Tissue engineering · Injectable hydrogel · Smart scaffolds · Regenerative medicine

21.1 Introduction

Stem cells can differentiate into several specific lineages and form new tissues to treat several diseases or repair damages. The stem cells differ in their capability of differentiation and regeneration; therefore, their applications vary accordingly (Zakrzewski et al. 2019). Stem cells are able to differentiate and regenerate designated functional tissue under specific condition and determining factors (Juul et al. 2020). Moreover, stem cells can produce specific biomolecules that are required for the cell survival and tissue regeneration. It has widely been accepted that the secretion of such biomolecules from stem cells has several impacts on the surrounding tissue, including (1) the suppression of fibrosis and inflammation and (2) the induction of proliferation and differentiation. As a result, stem cells not only can serve for tissue regeneration but also offer a great promise as therapeutic. Besides, the paracrine effect of stem cells has provided significant therapeutic potentials for cell therapy by increasing angiogenesis and stimulating tissue regeneration (Choe et al. 2018). The diseased/damaged tissues can be regenerated through (1) direct replacement of the diseased cells with stem cells (e.g., bone marrow transplantation) or (2) stem cell-based stimulation of the patient's cells to assist the repair/regeneration process. In the latter situation, stem cells are employed to support the diseased cells to be self-repaired by secreting various factors necessary for the regulation of their behaviors and functions—a phenomenon so-called paracrine effect.

Although stem cell therapy has provided new insights into regenerative medicine, still, some challenges are required to be addressed to enhance their therapeutic efficacy. For instance, the post-transplantation viability of stem cells is very low, and the fate of cells after entering into a designated biological microenvironment is not certain, which might profoundly reduce the success of transplantation (Kröger 2015). Several investigations have been performed toward overcoming these challenges through the usage of carriers with the capacity to (1) expand the survival of stem cells, (2) improve their growth conditions, (3) modulate biological activity, (4) increase cell residence at the target sites, and (5) exhibit well-mimicking potential of the extracellular matrix (ECM) (Amiryaghoubi et al. 2020a; Regmi et al. 2019). Moreover, a promising carrier should protect stem cells from possible mechanical insults or biochemical injuries while providing the cellular niche essentials. Ideally, an efficient vehicle must offer required growth factors, supply nutrients and oxygen, and facilitate metabolic waste removal. An impeccable vehicle for the delivery and transplantation of stem cells should be nontoxic or have trivial toxicity for stem cell and their target tissue. Furthermore, depending on the therapeutic purpose, stem cell carriers should display optimal biodegradability, adhesion, flexibility, and non-immunogenic properties (Luan et al. 2017).

The success of stem cell therapy modality seems to be largely dependent on the biocompatibility, handling simplicity and the application of the scaffolds, which need to be considered. In this regard, it is well established that stem cells are sensitive to the mechanical and biochemical signals from their microenvironment (Ma et al. 2018; Chacón-Martínez et al. 2018). The memory of the previous mechanical signals also affects the stem cells' fate in the long term, even after transferring into the body (Hinge et al. 2020; Carty et al. 2018). It should also be pointed out that the migration of stem cells from the target site to distant tissues is one of the major obstacles, because the presence of sufficient cells in the target tissue site is of critical importance after the transplantation (Kumar et al. 2017). Moreover, damage to the stem cell membrane during the transplantation is another problem with the injectable stem cell carriers. These concerns mostly can be prevailed using the appropriate scaffolds with a protective structure. Such a biomimicking setting can also increase the survival and proliferation of stem cells consequently. Collectively, regarding the viability of stem cells, hydrogel-based scaffolds seem to be the most favorable platform (Luan et al. 2017). Many studies have asserted that the hydrogel-based carriers can be employed as promising delivery vehicles for a variety of stem cell therapy applications (Ma and Huang 2020). There is a wide range of hydrogel-based carriers according to their ingredients, configuration, and function. However, it should be noted that most of them have advantages such as biocompatibility, low toxicity, optimal and convenient simultaneous encapsulation of stem cells, and directing bioactive factors. The hydrogel-based scaffolds can be formulated using (1) natural polymers such as chitosan (CS) (Pereira et al. 2013), alginate (Venkatesan et al. 2015), cellulose (Dugan et al. 2013), hyaluronic acid (Collins and Birkinshaw 2013), and proteins (Yue et al. 2015); (2) synthetic polymers like poly(lactide-co-glycolide) (PLGA) (Gentile et al. 2014), 2-hydroxyethyl methacrylate (HEMA) (Rogero et al. 2003), poly(ethylene glycol)

(PEG) (Bakaic et al. 2015), and polycaprolactone (PCL) (Bhavsar and Amiji 2008); or (3) their hybrid systems. Depending on the composition of hydrogels, they offer some advantages and disadvantages. Given the delicacy of stem cells and the sensitivity of their applications in the human body, hydrogels based on natural materials are preferred. On the other hand, the mechanical properties of synthetic-based hydrogels with a good degree of biocompatibility are better than natural polymers, which make these materials suitable for specific cell therapy applications, including muscle and bone tissue regeneration. Besides, the composites of synthetic and natural polymers, such as proteins and polysaccharides, can introduce new hydrogels for tissue engineering purposes (Kaczmarek et al. 2020).

21.2 Hydrogels as Stem Cell Carriers

Hydrogel-based scaffolds provide structural support for stem cells by providing a permissive milieu. Thereafter, they potentially mimic the three-dimensional (3D) biochemical and biophysical environment of the cells. The large surface area of hydrogel-based constructs promotes cell-matrix interactions and exchange of nutrients and wastes as well as oxygen and carbon dioxide. The improved viability and proliferation of stem cells have made the hydrogels the main scaffolds for recreating stem cell microenvironment (Farhat et al. 2018). Additionally, the emergence of novel technologies such as biochips, 3D bioprinting, and microfluidics provides simple approaches for the design and fabrication of monodisperse and uniform hydrogels (An et al. 2019). In this line, bioink 3D bioprinting can straightaway result in the development of scaffolds with greater precision and control in terms of composition and architecture (Diaz-Gomez et al. 2020).

There is an intricate relationship between hydrogels and stem cells. Specifically designed hydrogels play a vital role in the fate of stem cells by regulating their differentiation (Zhang et al. 2017). Hydrogels can be complemented with various nanomaterials to have an adjustable behavior (Zhang et al. 2019). They can also include various substances (e.g., growth factors) to modulat cell differentiation toward specific path (Chan et al. 2019). Of note, the physical characteristics and the chemical properties of ingredients should be considered as influencing factors on the stem cells' fate, including morphology, geometry, stiffness, porosity, physical properties and nature of ingredients, injectability, the relaxation time of hydrogels, and their byproducts (Tsou et al. 2016). For instance, the faster relaxation characteristic of hydrogel leads to more efficient cell spreading, differentiation, and proliferation compared to the slow relaxation counterparts (Cacopardo et al. 2019). Given that the ECM naturally displays viscoelastic behavior with stress-relaxation potential to accommodate cells, an ideal hydrogel should resemble such features (Eddhahak and Zidi 2015; Li et al. 2020).

One of the most important issues to consider in hydrogel engineering is the biodegradation of the materials (Peng et al. 2018). As a stem cells' carrier, hydrogels need to be a safe and biocompatible milieu with a good degree of biodegradation at the desired time. If the hydrogel remains permanently in the

body, it may cause physiological and psychological damages. On the other hand, the necessary space for cell stretching and proliferation is of paramount necessity through the biodegradation of hydrogels, particularly in the case of hydrogels with smaller pores. Furthermore, the stiffness of hydrogel critically affects the adhesion, migration, and differentiation of stem cells (Leipzig and Shoichet 2009). It is well established that the aim of the application and type of the laden stem cells must determine the hydrogel types and features such as stiffness. As an example, stiff scaffolds direct the cells to the myogenic lineage, while softer ones yield neurogenic origin. A study investigated the correlation of photopolymerizable methacrylamide CS-based hydrogel stiffness with the differentiation of neural stem/progenitor cells (NSPCs). It was presented that the type of specific cells derived from the stem cell differentiation was largely related to the stiffness of the hydrogel. The softest hydrogel ($<$1 kPa) led to differentiation of stem cells into neuronal cells. NSPCs were shown to differentiate into oligodendrocytes within the stiffer scaffolds ($>$7 kPa) (Alvarado-Velez et al. 2014). Of note, the embryonic stem cells' (ESCs) amplification and stemness properties seem to be better preserved in stiff scaffolds like glycosaminoglycan-binding hydrogels, while softer hydrogels were not able to efficiently retain their pluripotency potential (Tsou et al. 2016; Hunt et al. 2014). The porosity of biological materials plays a key role in revascularization, since porous structures allow the penetration of vessels without degradation of the compact matrix. The porosity of the system provides the proper conditions for post-transplant angiogenesis, and the space required for stem cells to reside and interact with the essential bioactive factors. The size and distribution of the pores of the hydrogels accounted as decisive features in the performance and effectiveness of the hydrogel. The optimum pore size of hydrogels differs based on the purpose of tissue regeneration. For example, microporous hydrogels with pores of 5 μm are ideal for neovascularization, while those with 20–125 μm pores are suitable for regeneration of adult mammalian skin or 20 μm for the ingrowth of hepatocytes. Larger pores (100–350 μm) are required for bone regeneration, and macroporous scaffold with pores bigger than 40 μm is appropriate for the of central nervous system (CNS) tissue engineering (Tsou et al. 2016).

In addition to the factors mentioned above, the dynamic nature of the hydrogels' constituents and their binding characteristics such as hydrogen, hydrophobic/or electrostatic molecular interactions, matrix, and cell ligand bindings can also affect the differentiation and final fate of the stem cells. A body of evidence has shown that the concentration and type of ligands and hydrogel dimensions have different effects on the differentiation of hematopoietic stem cells (Yan et al. 2020).

The diameter and dimensions of the hydrogels should also be taken into account in the design of the hydrogel, depending on the number of loaded stem cells. In the case of injectable hydrogels, the viscosity of the hydrogel must be appropriate in terms of syringeability. Hydrogels must also be flexible enough to have sufficient mechanical strength to protect stem cells from pressure during or after the injection. Considering all these conditions, proper and efficient preparation methods with the capacity for large-scale fabrication, while being simple and inexpensive, need to be employed. Furthermore, it is conceivable that purposed engineering and

modifications of hydrogels can yield novel types of scaffolds with much more likeliness to the microenvironment of the loaded stem cells and even specific to the desired stem cell. For instance, the use of ECM-like materials such as gelatin methacryloyl (GelMA) has been shown to reduce the probability of an immune reaction and improve the adhesion of stem cells (Kim et al. 2020). It has also been reported that efficient proliferation can be maintained by incorporating various peptide moieties such as peptide-modified methacrylated glycol CS-based hydrogels (Dhillon et al. 2019) and cell-adhesive peptide-functionalized hydrogels (Zhang et al. 2021).

21.2.1 Hydrogel Fabrication Methods

So far, various techniques have been employed for engineering hydrogel-based structural matrices, which have been lately influenced by the evolution of other state-of-the-art techniques (e.g., bioink 3D bioprinting) and the inclusion of advanced biomimetic materials. Some of the fundamental techniques for manufacturing porous hydrogels with application in tissue engineering are discussed in the following sections.

21.2.1.1 Emulsification

This is a basic method for effectively producing gel-based formulations. This method is a common practice for developing micro-structured gels with applications in tissue engineering. The procedure of this method starts with a multi-phased solution mechanically mixed with a hydrophobic phase like oil to generate hydrogel droplets. It is possible to produce droplets with different sizes just by altering the hydrogel precursor's viscosity, regulating the usage of surface acting groups and the intensity of mixing. Such modulation is mandatory for restricting the hydrogel's surface tension and preventing their agglomeration. This method introduces an outstanding capacity to design cell-laden hydrogel structures. This can be simply done by inserting cell types into a phase mixture that will be eventually encapsulated within the gels. However, this method faces a limitation when it comes to biomedical applications because it is only capable of generating spherical gel structures (El-Sherbiny and Yacoub 2013).

21.2.1.2 Freeze-Drying (Lyophilization)

This method begins with forming an emulsion by adding a polymeric material and a designated solvent to water. Then, the resultant solution should go through a fast-cooling procedure ranging from −70 to −80 °C, which can introduce thermal instability to the structure. By applying partial vacuum, the frozen material should undergo sublimation that lets the solvent evaporate. This will finally result in spongy voids in the freeze-dried construction, resulting in the formation of the scaffold (Bencherif et al. 2013). The freeze-dried process facilitates a control on the pore size of the structure which in turn provides the permeability of these matrices. Such an approach is increasingly exploited to manufacture 3D interconnected porous

hydrogel scaffolds, which can be in favor of biomolecular and cellular transports (Rich et al. 2015; Grenier et al. 2019). There has been a great amount of work carried out to examine this method further for the ability to keep a porous network. For instance, Autissier et al. have worked on a favorably porous structure, whose key polymer was a polysaccharide. The system was produced by undergoing fast cooling and sublimation. As a result, it was proposed that this method can maintain both pore diameter and porosity within the structures (Autissier et al. 2010).

21.2.1.3 Porogen Leaching

Porogen leaching, which is often used for the preparation of large 3D porous scaffolds, can improve the structure of the electrospun scaffold. It is one of the most utilized featured easy techniques, in which porogens of various sizes can be included in an aqueous polymer. As a result, the polymeric structure is created with the embodied porogen (salt crystals, or bubbles of gas), the subsequent removal of which can give rise to the formation of open spaces within the structure. Thus, 3D hydrogel-based scaffolds are formed. Practically, salt particles (e.g., NaCl) are selected based on the desired pore size with proper crushing and polymer used. These particles are transferred to a solvent matrix, and the solvent is obtained from biocompatible organic polymers. After forming the preferred shape through shattering and solid-state extrusion, the solvent of mold evaporates via freeze-drying, and the trapped salt particles are leached within the matrix, resulting in the formation of proper porosity—an important feature in tissue engineering applications (Bencherif et al. 2013; Ma 2004). The only problem with this method is the presence of some residual salt within the network, which might be the cause of the defective pore structures and undesired biological effects (El-Sherbiny and Yacoub 2013).

21.2.1.4 Gas Foaming

Gas foaming designs interconnected scaffold networks with high porosity using gaseous effervescence. This technique utilizes a solvent-free method via applying ammonium and sodium bicarbonate salts as gas-forming mediators. In this method, first, the condensation of molded biodegradable polymer is carried out with the salts, and then, gas bubbles are produced in the mold utilizing chemical or physical mediators, which consequently decreases the solubility when the polymer is in the saturated form. After building up the pressure, the gas nucleation begins, which can result in the formation of the interconnecting pores (Dehghani and Annabi 2011). This method develops macroporous scaffolds and promotes the creation of evenly sized pores ranging from 100 to 200 μm (Nam et al. 2000). The effect of citric acid and other acidic salts on the enhancement of gas nucleation has also been investigated (Yoon and Park 2001). This method can be simply recruited for the production of microarchitecture hydrogels. While this approach has the capacity to eliminate the need for an organic solvent and improve cell adhesion, it might suffer from some shortcomings such as the paucity in the pore interconnectivity with limited pore sizes (Bedell et al. 2020).

21.2.1.5 Electrospinning

As one of the oldest methods, the electrospinning technique is used to fabricate the submicron fibers possessing proper mesh formation (Hutmacher et al. 2008). In this approach, having employed a direct high-voltage current, a syringe pump and an earthed rotating collector are used to produce porous polymeric matrices. In the first step, an electric repulsion occurs within the polymer solution in the syringe tube. This repulsion jets the polymer out of the nozzle tip as slim filamentous strands, which can finally accumulate to the rotating target collector based on the final properties required for the application of scaffold (Sequeira et al. 2012; Parrag et al. 2012). The main pros of the electrospinning technique are to create fibers from difficult to use materials (e.g., the immiscible blend of polymers) and form hollow fibers. Nevertheless it might entail some cons such as the lack of control over fiber formation and morphology, as well as the difficulties in manufacturing scale-up (Chandra et al. 2020).

21.2.1.6 3D Printing

To date, the 3D printing technology for the fabrication of the hydrogels has been considered and used to produce structurally precise multipurpose scaffolds with the desired size, shapes, and features. The 3D printing approach can precisely control the geometrical parameters, the assembling of biological constructions, and cell distribution within the scaffold to simulate the transplanted organ with their 3D design (Huang et al. 2011). To be transplanted and subjected to rapid prototyping, the construction of the tissue is managed via computer, and a layer-by-layer scaffold network is formed by the flow of polymeric materials toward a multilayer system (El-Sherbiny and Yacoub 2013). However, the bio-inkjet/3D printing strategy has some drawbacks such as low resolution, limited choice of materials, and relatively high cost of fabrication (Ma 2004). Thus, more broad studies are needed to develop biological inks for tissue engineering.

21.2.1.7 Photolithography

The photolithography technique is a selective-illumination top-down engineering procedure that has extensively been used to engineer polymeric 3D scaffolds. It can be done by a two-step approach. First, the photoresponsive polymer kept over the substrate is covered with preferred shapes and sizes and subjected to the UV light, in which the photopolymerization can occur within the uncovered areas. Second, a solvent removes the unexposed polymeric substrate to produce the patterned 3D scaffolds (Khan et al. 2015). Photolithography, as a computer-aided precise fabrication technique, can create a 3D pattern with higher surface areas and excellent architecture. Some other methods following this approach are introduced to boost the development of better hydrogel-based scaffolds. For example, blue light was used to synthesize a crosslinkable photopolymer for tissue engineering applications (Bryant et al. 2000).

21.2.1.8 Sol-Gel Technique

The sol-gel approach is a chemical fabrication method, in which a solution or a colloidal solution is created. It consists of a liquid and solid phase inside them which divides them into two phases. The morphology of the formed gels is based on their variances in the two-phase system (solid and liquid phases) applied in the production of the hydrogel. Superior purity and uniform nanostructure are important advantages of this method, which is achieved at low temperatures. To design scaffolds with specific features, they can be modified. Accordingly, under several cycles of hydrolysis and polymerization reactions, scaffolds are usually made by dissolving organic or inorganic metal compounds in a solvent to form a colloidal suspension or a tuber structure. Additionally, nanoparticle-based hydrogels are obtained by molding this structure after subsequent drying and heat application. Nowadays, the sol-gel method has been mostly used to adjust the particle size and network morphology for tissue replacement, in large part due to its improved homogeneity and potential to undergo conversions at relatively low temperatures. In the sol-gel transition method, to modulate specific properties of scaffolds (e.g., conductivity), it is possible to add doping agents in the matrix of the polymer without any chemical unbalancing and physical deformation. These advantages make the doped polymers suitable materials even for the development of different types of biosensors used in biomedical tissue engineering and also in controlled drug delivery systems (DDSs) (Garg et al. 2012).

21.2.2 Injectable Hydrogels as Carriers for Stem Cell Transplantation

The injectable hydrogel is technically a gelling system that requires specific processing steps. They can be designed to result in more sophisticated control of gelation kinetics. Of note, where drug delivery and tissue engineering are deemed, injectable hydrogels under mild conditions are of particular interest since they facilitate a unique carrier quality in 3D, biocompatible, low-invasive, and adaptable shape for administration (Chao et al. 2020; Ayoubi-Joshaghani et al. 2020). In recent years, these materials have gained more attention than ordinary gels because they require a lower-invasive surgical procedure as well as a more adaptable shape in real time that is formed locally (Sun et al. 2020; Liu et al. 2017a). Due to this capability, the injectable hydrogels might require fewer carriers, which turn them into the most favorable means for the delivery of cells and drugs/genes. Despite the aforementioned advantage, an injectable hydrogel needs to remain in the sol state during injection into the targeted location—a feature so-called impeccable syringeability by the injection equipment with no/trivial safety issue. Thus, injectable hydrogels should be distinguished from in situ-forming gels.

Certain criteria must be met if an injectable hydrogel is required to be used for biomedical applications. First, the transition of sol-gel ought to undergo mild conditions. Second, the gelation must be done steadily in good time, so that the outflow of the material to the surrounding tissues should be avoided throughout the process. Third, the creation and degradation of the hydrogel are supposed to impose no damaging/toxicity effects on the target site or the delivered cells. It should be

noted that the injectable hydrogels are developed by either physical (Lee 2018) or chemical crosslinking of polymers (Lee 2018; Wang et al. 2018). The former provides safer hydrogels as a result of the exclusion of tiny compounds for the beginning of hydrogel formation. On the other hand, the latter produces stronger hydrogels by strengthening their mechanical properties (Dimatteo et al. 2018; Wang and Han 2017). One of the fundamentals of preparing ionic crosslinked hydrogels is to combine ionizable polymers with counter-ions. If the pH, temperature, or concentration of ions is modified, then the dynamics of sol-gel transition might affect the injectability of the hydrogel (Park et al. 2013; Zhang et al. 2018).

To have superior control on the mechanical characteristics of the hydrogels, two parameters should be taken into consideration, including the molecular weight of the polymers, and the crosslinking mass of the hydrogel (Bidarra et al. 2014). Such measures can be attained by adjusting the concentration of the polymer or the counter ions. One of the classical and natural macromolecules in this field is alginate. It features nontoxic and non-thrombogenic characteristics, upon which it has widely been applied in the development of cosmetic, biomedical, pharmaceutical, and food products (Etter and Oldinski 2018; Zhao et al. 2018). Also, it contains a lot of carboxyl groups that can be crosslinked by introducing cationic donors like calcium chloride ($CaCl_2$) to create an egg-box-like arrangement. By adding more Ca^{2+} into the matrix, the mechanical properties of the alginate hydrogel can be promoted (Lee and Mooney 2012). Nevertheless, this should be done cautiously because increasing the volume of cations might intrinsically harm the viability of the incorporated cells and hence impose negative impacts on the curative feature of the hydrogel. As a result, an optimal condition should be sought to ensure the efficiency and safety of the bioactive hydrogel.

Dynamic covalent bonding has lately become another candidate for the crosslinking of polymers. This method offers a great possibility to obtain safer injectable hydrogels with improved performance (Deng et al. 2012; Qu and Yang 2016). Hydrogen bonding (H-bonding) displays a dynamic nature together with the ability to be broken at high temperatures. Therefore, these two properties permit the H-bonding to stand out as a suitable crosslinking process for developing injectable hydrogel. Moreover, it can facilitate the hydrogels with thermoplasticity, self-healing property, and re-processability at the same time (Dai et al. 2015; Ruel-Gariépy and Leroux 2004). However, hydrogels crosslinked via H-bonding may suffer from weaker resistance in the water. This is because the hydration process might dissociate the H-bonding between polymer units. Liu et al. have worked on several projects related to the fabrication of the hydrogels crosslinked via H-bonding (Wang et al. 2015; Tang et al. 2010). The main purpose of their research has focused on proposing a methodology for enhancing a hydrogel's performance in the water medium. One proposal was to synthesize poly(*N*-acryloyl glycinamide) (PNAGA), whose NAGA monomer possesses two amides. This process might provide a much more stable structure through a paired dual hydrogen bonding, and hence, the mechanical property of the PNAGA hydrogel has proven to be more consistent in water (Dai et al. 2015). In another study, it is proposed to employ nanoclay so that the viscosity of the NAGA monomer solution could be tuned. As is illustrated in

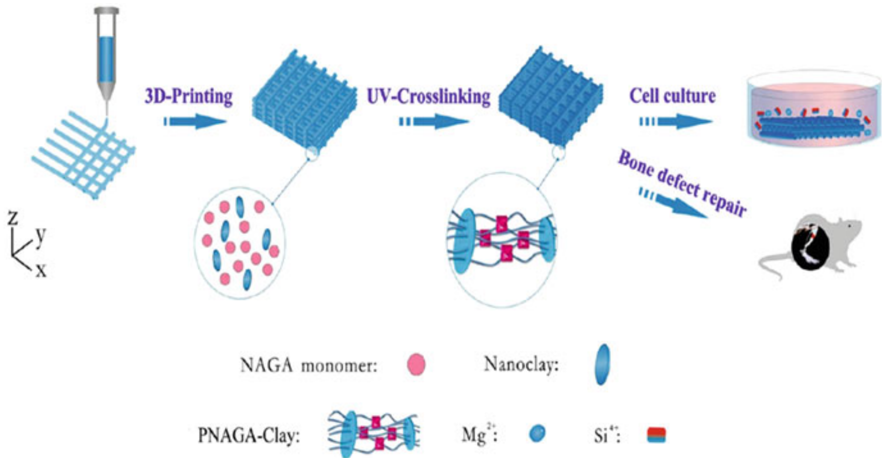

Fig. 21.1 Hydrogel comprised of *N*-acryloyl glycinamide (NAGA) monomer and nanoclay was prepared by 3D printing technique for the manufacture of load-bearing tissue engineering scaffolds for bone defect repair. (Adapted with permission from Zhai et al. 2017)

Fig. 21.1, the PNAGA-clay structure should be manufactured via the 3D printing technology where the doping solution is used as a bio-ink. Finally, the crosslinking is created with the aid of UV rays (Zhai et al. 2017). The other feature of the PNAGA-based hydrogel is the flexibility of sol-gel and gel-sol conversion upon appropriate cooling-heating cycles. As a result, the processed gel can reveal thermoplasticity if being injected at 90 °C, while the de-molded hydrogel products even have high-level modulus at room temperature (Dai et al. 2015). It has also proposed that by introducing acrylamide (AAm) to PNAGA as a comonomer, the injection temperature of the copolymer can be reduced to 47 °C which falls into the range of human body temperature (Shi et al. 2016).

The type of stem cells also plays a key role in determining the destiny of the cells and the outcome of the cell therapy (Scarfe et al. 2018; Amer et al. 2018). The direct injection seems more effective, healthier, and faster. However, there are still several challenges such as the possibility of secondary infection, which need to be fully addressed (Amer et al. 2018). Of injectable hydrogels, much attention has been paid to designing advanced in situ-/ex situ-forming hydrogels. Briefly, in situ-forming hydrogel carriers are in liquid form after the incorporation of stem cells pre-injection, while they undergo a sol-gel transition via crosslinking mechanism under a stimulus and anchor in the target site of the injection. Although in situ-forming structures have many advantages such as minimal invasive delivery, they may also associate with some challenges such as undesired interactions. There are not ample stimuli for the physical or chemical sol-gel transformation, which might raise some safety concerns. The failure to provide proper encapsulation time by the physical or chemical crosslinking may lead to inadvertent cell leakage from the target tissue to distant tissues and cause subsequent problems. Due to the

pre-administration fluidity of the hydrogel in this approach, the carrier has less potency to protect the stem cells against mechanical damages during an injection (Young et al. 2019). However, several in vivo studies have shown that some hydrogels fabricated via in situ polymerization with host-guest interaction might provide adequate protection for the stem cells against mechanical stress (Yan et al. 2020). It should be noted that the hydrogels per se might also induce undesired impacts on the incorporated cells by depriving stem cells to access sufficient nutrients and oxygen, elicit nonspecific interactions, or induce toxic byproducts.

In the ex situ approach conversely, hydrogels are polymerized in the external environment and turn into a gel. This approach is suitable for tissues with insufficient stimuli for the sol-gel transition of the carrier. This method might be suitable for reducing mechanical insult during the injection considerably. Since the crosslinking occur ex vivo, the components of these hydrogels are also simple. A body of evidence has shown that the extensional flow force through the syringe needle can be accounted as one of the main causes of stem cell death, and hence, the ex situ forming hydrogel may protect stem cells much more efficiently (Luan et al. 2017). Injectable hydrogels have proven their benefits for manufacturing cardiac tissues with the loading of functional cells and molecules (Peña et al. 2018). A study on 27 patients suffering from myocardial infarction (MI) was conducted by using an injectable IK-5001 hydrogel (a solution of 1% sodium alginate plus 0.3% calcium gluconate) to treat left ventricular remodeling (Frey et al. 2014). Based on a 6-month follow-up, it was found that the intervention in patients was without any significant adverse reactions, while echocardiographic assessments confirmed the desired clinical impacts. Further, the outcomes have acknowledged both the safety measures and the effectiveness of the approach in high-risk patients. Nonetheless, the capacity of the carrier, which is used to convoy injectable hydrogels, needs to be improved significantly. It is known that injectable hydrogels embedded in hard tissue (like bone and cartilage) are only used for filling and delivery aims though they might not be capable of enduring the damaged tissue (Wang et al. 2017; Amiryaghoubi et al. 2021). In contrast, it has already been concluded that the soft injectable hydrogels can enhance the quality of cartilage repair, in large part by providing an accommodating setting. For example, Lu et al. have developed an injectable hydrogel based on collagen (Lu et al. 2019). This hydrogel was conjugated with carbon dots (CDs) and genipin was used as the crosslinker. The CDs were found to strengthen the rigidity of the gel and produce reactive oxygen species (ROS), which in turn could boost the chondrogenic differentiation of bone marrow-derived stem cells toward cartilage regeneration. Since they are featured as an in situ gelling modality, they might benefit from their carrier proficiency and flexibility in handling. Moreover, the administration involves less invasion and allows to fill irregular-shaped cavities. Nevertheless, it obligates a sol-gel transition under moderate conditions and well-controlled kinetics to succeed in a medical injection. Collectively, there is no doubt that the injectable hydrogels have demonstrated their desired properties for biomedical applications. However, despite offering numerous advantages, the process of developing injectable hydrogels, which are ought to meet clinical requirements, may

encounter with some obstacles that need to be fully addressed to have an optimized entity.

21.2.3 Smart Hydrogels as Stem Cell Carriers

"Smart" or "intelligent" hydrogels can respond to external stimuli like pH, temperature, light, magnetic, electric fields, and ionic strength (Fig. 21.2) (Mantha et al. 2019; Barar et al. 2016a; Fathi et al. 2015). Smart hydrogels have revealed their ability in noninvasive and remote-controlled therapies such as targeted DDSs (Panyam and Labhasetwar 2003; Hamidi et al. 2008; Saleh-Ghadimi et al. 2014; Fathi et al. 2019), regenerative medicine (Bettinger et al. 2014; Kim et al. 2015), tissue engineering (Khademhosseini and Langer 2016; Kasiński et al. 2020), and implanting artificial organs (Pei et al. 2020; Barthes et al. 2020; Jalili et al. 2016). These hydrogels are a type of polymers that are capable of an immediate reaction to the changes in the pH, ionic strength, or temperature by rapidly restructuring their swelling behavior, network structure, sol-gel transition, penetrability, or mechanical strength. However, ordinary hydrogels can only perform the swelling-deswelling process if they are surrounded by a suitable environment where enough water is guaranteed (Karchoubi and Pahlevani 2019).

Bioactive and stimuli-responsive hydrogels consist of biological instructive functionalities/moieties that are suitable for interacting with the biological environment. These hydrogels are capable of being applied to a whole living organism. This feature makes them particularly unique and persuasive because they provide the opportunity of using biomolecules such as peptides that are cleavable under the

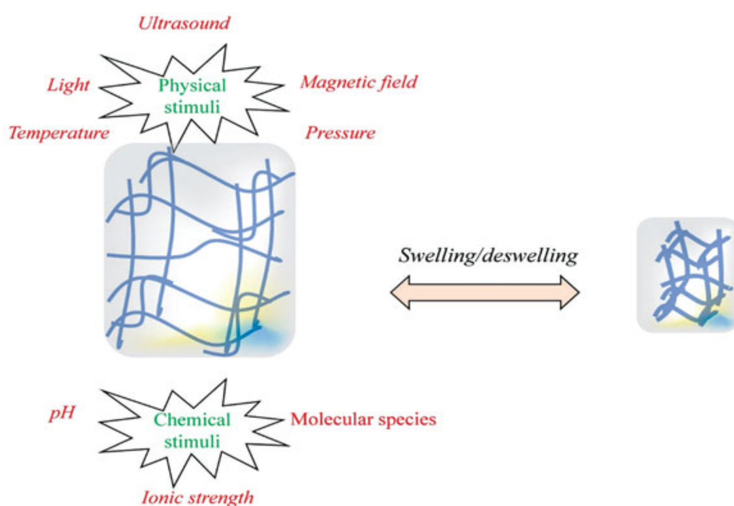

Fig. 21.2 Alteration of hydrogel behavior in response to physical or chemical stimuli. (Reproduced with permission from Fathi et al. 2015)

performance of particular enzymes (Sood et al. 2016). As a result, the catalytic functioning of the enzyme on the substrate can change the swelling properties of the gel. Enzyme matrix metalloproteinases (MMPs) can be considered as an example (Mano 2008; Li et al. 2019). This family highlights the ability of decomposing ECM molecules, which also highly associates with tissue development and remodeling. As enzymes are extremely valuable in their reactivity as well as crucial elements in different biological applications, it is essential to design materials so that they can respond to a certain enzyme associated with the particular substrate. Upon the occurrence of tissue damaged, a change might emerge in local physiological electricity, which can result in the change(s) in the transferring of electrical signals between cells. If the exogenous electrical signals are introduced, the variation of electrical signals between cells can be reverted. This phenomenon can then guarantee the migration, adherence, proliferation, and separation of stem cells that altogether promote tissues to be recovered (Genchi et al. 2016). Thus, it is a promising and sound strategy to choose electrically active materials to develop conductive scaffolds when it is necessary for cell therapy and tissue regeneration (Feiner et al. 2016). Such advanced materials enable the successful delivery of electrical signals to cells since they provide the proper foundation(s) to establish a biomimetic-electro microenvironment, which can in turn efficiently induce the differentiation of stem cells. To develop injectable electroactive hydrogels, Cui et al. have proposed a multi-interaction approach by synthesizing tetra aniline capped polylactide-poly(ethylene glycol)-polylactide (CTA-PLA-PEG-PLA-CTA) copolymer (Cui et al. 2013). This approach includes the stereo-complexation of PLA units and the hydrogen bonding between the CTA and PEG parts as well as the π-π stacking of the CTA groups. Moreover, Liu et al. have been successful in the synthesis of a self-healing, shear-thinning, and injectable metallo-folate hydrogel. In this study, first, through hydrogen bonding, tetramers were created by the pterin group in the folic acid, and then, the tetramers generate nanofibers by π-π stacking. These nanofibers are finally employed to make a hydrogel via a crosslinking by Zn^{2+}. This is a reversible non-covalent complexation that allows the hydrogel to be printable with a syringe needle as schematically shown in Fig. 21.3 (Liu et al. 2018).

Amphiphilic polymers can form a hydrogel with the corporation of the hydrophobic moieties as physical crosslink points (Ruel-Gariépy and Leroux 2004; Le et al. 2018). The synthesis of these polymers can be performed either via block copolymerization, random copolymerization, or using hydrophobic pendent groups to modify the hydrophilic macromolecules (Gyles et al. 2017). For the injection, the polymeric amphiphiles might state either lower critical solution temperature (LCST) or upper critical solution temperature (UCST). This behavior warrants a sol-gel transition by a change in the temperature of the surrounding environment. The samples are made up of the derivatives of natural polymers, including chitosan, cellulose, xyloglucan, and synthetic polymers involving blocks like poly(*N*-isopropylacrylamide) (PNIPAAm), poly(*N*,*N*-diethylacrylamide-co-acrylic acid) (PDEAAm), and poly(2-carboxyisopropyl acrylamide) (PCIPAAm) (Qu et al. 2019; Jommanee et al. 2018; Saekhor et al. 2019). Thus, the gelation process of these macromolecules is highly contingent on temperature. Due to this fact, the

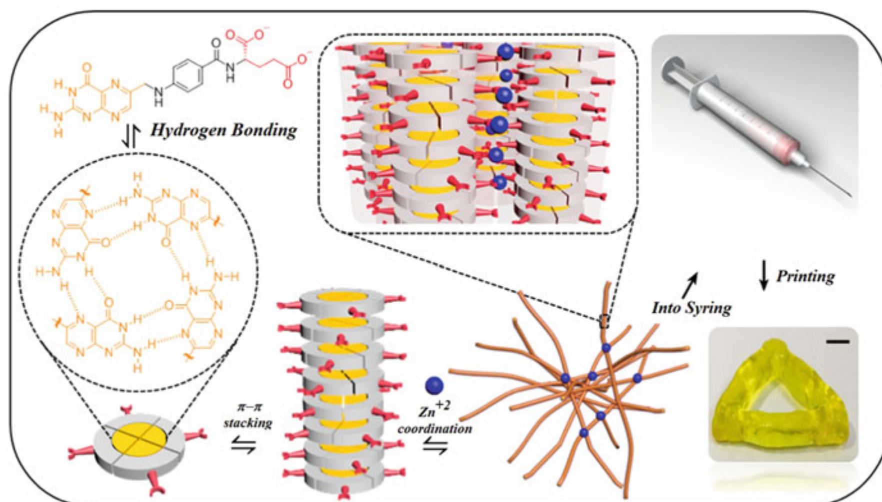

Fig. 21.3 The injectable metallo-folate hydrogel synthesized via multiple π-π interactions among the nanofibers through stacking the assembled folic acid tetramers. (Adapted with permission from Liu et al. 2018)

central challenge is to reach a critical temperature suitable for the human body when such materials are applied to a living organism (Saekhor et al. 2019; Jones et al. 2012). One of the well-known candidates to establish biomedical needs for a thermoresponsive system is PNIPAAm homopolymer, whose LCST of 32–34 °C lets it standing out among others. It is noteworthy to state that the soft tissues (e.g., skin, tendons, fat, ligaments, muscles, and blood vessels) are mainly composed of water, collagen, elastin, and ground substance that represents viscoelastic possessions similar to a hydrogel. Such remarkable similarities nominate the hydrogels as one of the materials of choice, which can efficiently adapt to the applied microenvironment after the implantation (Latifi et al. 2018). For example, injectable hydrogels were shown to offer remarkable usefulness in repairing damaged skin (Sun et al. 2020), cardiac (Hasan et al. 2015), and eye tissues (Wang and Han 2017), as well as segmental organ substitution (Sun et al. 2020). It should also be noted that the skin not only serves as the protecting shield of the body but also plays key roles in the homeostasis of the whole body. Further, the human skin is armed with a high healing capacity. Given all these capacities, any profound damages to the skin tissue may turn into a critical problem, while the skin tissue regeneration using smart hydrogel-based stem cell therapy has created great hopes (Sivashanmugam et al. 2015; Unal and West 2020).

21.3 Hydrogels for 2D Culture Versus the 3D Culture of Stem Cells

The two-dimensional (2D) cell culture approach is a robust method that is used to cultivate cells since the early 1900s. This method is of the utmost importance in the discovery of biological procedures and diseases. The 2D cell culture, despite all the limitations, is one of the most common methods of cell culture due to its simplicity and versatility. Several studies on stem cells have also been performed on the 2D cell culture system, which was cultured on plates coated with various biological materials such as smooth, hard polystyrene plate with a collagen or laminin coating (Li and Wang 2020). The 2D stem cell culture is also beneficial in that it provides a simple environment for controlled analysis and evaluation of the impacts of single molecules on stem cell behavior. Hydrogels can partially simulate the natural environment of the cell in the 2D culture. Most of the stem cells cultured on the 2D cell cultivation systems are subjected to various molecular biology analyses using DNA, RNA, and protein entities. On the other hand, the preparation of hydrogel-embedded cell samples faces more technical challenges in terms of the required analyses. Several strategies including mechanical or enzymatic degradation can be useful for releasing embedded cells. Such techniques need to be implanted with extra care and skills to maintain the integrity of intracellular components besides sufficient efficiency (Caliari and Burdick 2016). It is well established that culturing stem cells in a 2D culture medium provides a completely different environment from that of the in vivo physiological conditions. As a result, it may alter the cell pluripotency properties and lead to some sort of dysfunctional and heterogeneous differentiation. The 2D cell culture creates cellular polarity, which leads to the interaction of some parts of the cell membrane with the ECM and adjacent cells, while the rest of the cells is exposed to the empty culture medium. Subsequently, the abnormal and polarized connections to some entities such as integrin might affect the intracellular signaling and phenotypic fate of cells. Moreover, cells are afforded by the homogenous concentration of nutrients, growth factors, and cytokines in contrast to the presence of dynamic spatial gradients in vivo. Therefore, cells should be constructed in 3D model cultivation environments to provide better and more accurate cell physiology, mechanical transfer, and tissue morphogenesis studies in vitro. Of note, the 3D cell cultivation systems are believed to collect important mechanical and biochemical markers from native ECM while facilitating classified processes such as migration and tissue organization (Jensen and Teng 2020). The emergence of the 3D systems in cell culture can enhance the heterogeneity in the environment around the cells compared to the 2D cell culture, which can also provide an environment that is closer to the physiological conditions of the tissue. The 3D cell models may be considered as a desirable platform for performing various biological studies using stem cells. Hydrogels can also facilitate the imitation of the natural tissue microenvironment and enable the reconstruction and deeper understanding of complex tissue processes. The hydrogel networks with specific porosity facilitate cell growth and provide stem cells with sufficient nutrients and substances necessary for proliferation and differentiation. However, special

considerations must be taken into account in the production of hydrogels, some of which have already been mentioned. In the 3D cultivation media, nutrient concentration gradients can occur, while the diffusion of some substances, especially proteins, can be limited and may happen as a heterogeneous distribution. Altogether, it seems that the imaging and analysis of functionalities of cells in the 3D cell culture system are much closer to that of the reality in vivo (Tibbitt and Anseth 2009).

21.4 Hydrogels for the Regeneration of Different Types of Tissues

In recent years, the advent of regenerative medicine using stem cells promises innovative therapeutic options. To date, many advances in stem cell-based therapies have aimed at replacing complete tissue or organ, or repairing of damaged tissues and organs. This approach has provided new opportunities in cell-based therapeutics for the treatment of several diseases, including leukemia, hereditary blood diseases, spinal cord lesions, skin diseases, bone, and cartilage tissues. Given the need for advancing regenerative medicine and overcoming the current limitations, extensive studies have been conducted through the designing and production of smart scaffolds for the delivery of stem cells and the controlled release of growth factors. For instance, agarose-carbomer-based hydrogel grafted with RGD peptide was shown to improve the viability and density of human mesenchymal stem cells (hMSCs), overcome pro-the inflammatory conditions, and maintain the immunomodulatory properties (Caron et al. 2016). One of the remarkable improvements in regenerative medicine is to use patients' endogenous adult stem cells for the repair of damaged tissue—an approach so-called personalized regenerative medicine (Harris 2014; Pushp et al. 2021). For this purpose, hydrogels are employed as a carrier system for the targeted delivery and release of regulatory molecules (e.g., cytokines, chemokines, growth factors, and proteins) in the target tissue to modulate the desired physiological functions and behaviors. As follows, they can direct the endogenous adult stem cells from their population site (generally bone marrow) to the target tissue and induce tissue-specific cell lineage differentiation (Lim et al. 2013). Having capitalized on this strategy, Lim et al. aimed to enhance the infiltration of the endogenous neural progenitor cells (NPCs). To this end, they employed injectable gelatin hydroxyphenylpropionic acid-based hydrogels encapsulating polyelectrolyte complex nanoparticles (PCNs) loaded with stromal cell-derived factor-1α (SDF-1α) for its controlled delivery to cavitary lesions in the brain. They reported that the SDF-1α and its receptor C-X-C chemokine receptor 4 (CXCR4) can function as the chief regulatory molecules of cell homing to the bone marrow. These molecules have key roles in enhancing the migration of bone marrow cells into the peripheral circulation and local grafting into the target tissues. As a result, they showed the successful infiltration of NPCs to cavitary lesions in the brain. In addition to neural regeneration, myocardial tissue repair using SDF-1α carrying hydrogels scaffolds has also been reported (MacArthur Jr et al. 2013). In the following sections, some examples of different tissue regeneration with the aid of hydrogels typify the

importance of hydrogel in tissue engineering even though several issues require to be addressed and further works are necessary to prevail some limitations associated with the bench-to-bedside translation of the cell medicines.

21.4.1 Hydrogels Serve as a New Therapeutic Tool for Skin Stem Cell Therapy

To date, wound healing has been facilitated through several interventions, including surgical correction, skin grafting, tissue engineering, and other procedures. However, complete wound healing without a scar and full function gaining of damaged tissue is one of the main goals of regenerative medical skincare. Wound dressing with a sterile pad, gauze, or hydrogel has been one of the most widely applied methods. Recently, much attention has been paid to the dressing of the wound with the 3D hydrogels carrying stem cells to induce the desired tissue repair. In this approach, stem cells differentiate and secrete the factors necessary for the wound healing process. Moreover, chronic wound healing through employing multifunctional hydrogels with the controlled release capacity of bioactive factors has presented new avenues to the wound healing methodologies. In this regard, an injectable, self-healing, and antibacterial polypeptide-based F127/OHA-EPL (FHE) hydrogel was developed as a stimuli-responsive system for the delivery of adipose-derived mesenchymal stem cell exosomes (AMSCs-exo) to improve chronic wound healing and skin regeneration. As shown in Fig. 21.4, satisfactory results obtained from in vivo studies demonstrated improved angiogenesis and diabetic wound healing (Wang et al. 2019). In another study, graphene oxide/hyaluronic acid (GO/HA) composite hydrogels were designed for skin wound covering. The hydrogels displayed high swelling, degradability, tunable rheological property with close mechanical properties to human skin. Moreover, owing to the presence of polydopamine, the hydrogel displayed unique features, including self-healing capacity, antioxidant activity, tissue adhesiveness, and hemostatic ability (Zhao et al. 2020). In brief, injectable in situ-forming carriers are favored, while microgels or microspheres have opened up unique opportunities for the skin therapeutic modalities.

21.4.2 Hydrogel Applications in Bone and Cartilage Regeneration

Stimulation of osteogenic lineage stem cells can lead to bone regeneration. Stem cell-containing hydrogel scaffolds provide a therapeutic approach to overcome the issues of the standard treatment for defective bone. Given that the hydrogel porosity plays a key role in terms of stem cell fate, such capacity needs to be considered. Concerning bone tissue regeneration, the loose and porous structure of the scaffold is of utmost importance, in large part due to providing favorable conditions for cell-matrix interactions and oxygen and nutrient exchange. To develop implantable systems for osteogenic differentiation and bone repair, Ahn et al. capitalized on a

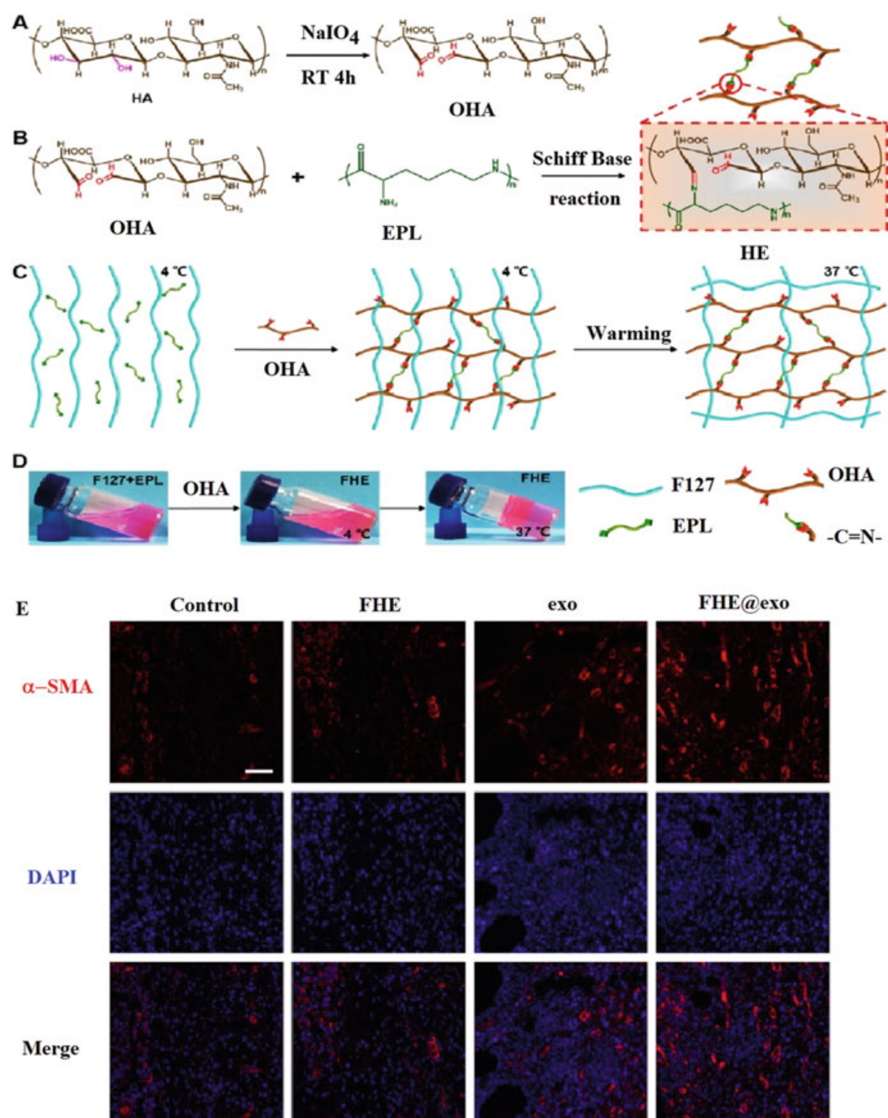

Fig. 21.4 The injectable multifunctional FHE hydrogel. (**a**) Oxidized hyaluronic acid (HA) synthesis. (**b**) Schiff base reaction between oxidized HA and polypeptide (ε-poly-L-lysine, EPL). (**c**) The thermoresponsive sol-gel transition of the hydrogel (composed of F127-EPL and oxidized HA). (**d**) Optical pictures of the sol-gel transition. (**e**) The impact of FHE@exo hydrogel on neovascularization of wounds. Alpha-smooth muscle actin (α-SMA) (red) and DAPI (blue) staining of blood vessels 7 days after the operation. (Adapted with permission from Wang et al. 2019)

thermosensitive diblock copolymer-based gel that forms a gel in body temperature. These permeable scaffolds were composed of PEG and PCL/acrylic acid (PCL/AA) and encapsulated human adipose tissue-derived stem cells (hADSCs) together with the osteogenic factors. Upon the usage of the system, substantial hADSCs' attachment, growth, and differentiation into an osteogenic linage were reported, which are of paramount importance for bone formation in vivo (Paul et al. 2016; Amiryaghoubi et al. 2020b). To stimulate osteogenesis without the involvement of osteoinductive growth factors, Paul et al. developed a high-porous light crosslinkable collagen-based hydrogel loaded with the optimal concentration of silicate nanoparticles (nSi) (Paul et al. 2016). In vitro studies have shown that these hydrogels can successfully direct stem cells toward osteogenesis without stimulating apoptosis or inflammatory cytokines. Engineered hydrogels also increased the migration and proliferation of cultured cells. These results were also confirmed with in vivo experiments in male Wistar rats. Moreover, they indicated that the incorporation of nSi in the manufactured hydrogels has a significant role in improving osteogenic differentiation of hMSCs. This study provides substantial evidence for designing a biomimetic scaffold for in situ bone regeneration and therapy independent of osteoinductive growth factors.

Cartilage is one of the functional and vital tissues of the human and animal body. The main function of cartilage is to provide a smooth surface to move and facilitate the transfer of mechanical loads. Most cartilage tissues in the body tolerate large mechanical stresses and usually carry heavy loads; and hence, they can easily be damaged. Given that the damaged cartilages are hardly able to heal and repair, restoring structure and returning normal function to damaged cartilage is one of the challenges of orthopedics and sports medicine. Cartilage regeneration therapy offers a possible alternative strategy by implanting chondrogenic cells in or on biocompatible scaffolds to yield engineered cartilage to repair damaged cartilage. In addition to providing a 3D growth environment for cells, hydrogel structures protect living cells and cushions from shear damage and mechanical stresses in hard knee environments. The scaffolds maintain the cells attached to the target tissue and prevent the cells from being washed away by the joint fluid. Some hydrogels have properties such as self-healing or being injectable that can cover the damaged area and integrate with the surrounding cartilage tissue, thus providing many benefits. Injectable hydrogel based on oligo-(poly(ethylene glycol) fumarate) (OPF) carrying rabbit marrow MSCs and gelatin microparticles (MPs) loaded with TGF-β1 has been applied for cartilage tissue engineering. An increase in the chondrocyte cell markers in MSCs demonstrated the potential of OPF hydrogel for cartilage tissue engineering (Park et al. 2007). Moreover, some studies have clarified that the encapsulated TGF-β1 and other efficient growth factors in hydrogel can enhance the expression of chondrocyte cell markers in stem cells (Chen et al. 2020). Consequently, appropriate hydrogel loading with TGF-β1 might provide a proper microenvironment for the differentiation of stem cells. An in situ photo-crosslinkable platelet-rich plasma (PRP) complexed hydrogel glue (HNPRP) with controlled release ability growth factor was shown to significantly improve the repairment of the defected cartilage tissues. Several growth factors existing in this structure are competent wound

healers. Besides, light can accelerate the gel-forming in situ and covalent binding between HNPRP hydrogel, and the tissue surface provided a strong tissue adhesiveness. In addition to enhancing the proliferation process, HNPRP was reported to enhance the migration of chondrocytes and bone marrow stem cells (Liu et al. 2017b). Glycopeptide nanofiber has been replaced by hyaluronic acid in some hydrogel systems. In vitro studies demonstrated that the self-assembly characteristic of glycopeptide amphiphilic molecules allows the hydrogel to interact with the CD44 receptor of MSCs and stimulate chondrogenic differentiation in MSCs. In vivo investigations showed that hydrogel enhances the migration of the endogenous MSCs and holds a remarkable amount of them at the injured site. Conclusively, glycopeptide nanofibers are promising materials for the regeneration of cartilage tissue without any requirement to the exogenous growth factors (Ustun Yaylaci et al. 2016)

21.4.3 Vascular and Cardiac Regeneration Improvements by Hydrogel-Based Structures

Regenerating the vascular network serves as a new tool to combat many diseases such as ventricular myocardial microstructure (Lichtenauer et al. 2011; Schipke et al. 2017; Hughes et al. 2020). Attributed to the fact that vascular engineering is one of the ways for overcoming cardiovascular disease and stroke, regenerating blood vessels using biofunctional hydrogels is one of the attractive fields of tissue engineering (Brewster et al. 2020). Several synthetic and natural hydrogels have been employed for vascular engineering strategies. In a study, the blood vessels were constructed from human pluripotent stem cells (PSCs) and endothelial cells cultivated in the Matrigel and collagen I substrate. These miniature constructions have demonstrated a firm and broad vascular tree setting with arteries, arterioles, and venules in immunodeficient NSG mice. Based on both in vitro and in vivo findings, the artificially engineered organoids were proposed as an efficient tool for several purposes from transplantation to disease models such as diabetic vasculopathy (Wimmer et al. 2019).

Undoubtedly, hydrogels made of natural materials (biomimicking polymers) are more efficient than other types since they have similar properties to the ECM of the human tissues. Of the hydrogels used for engineering arteries, fibrin is one of the most suitable constituents because it plays a key role in the blood clotting process and further contributes to the growth of new blood vessels. Nevertheless, it should be stressed that the low stability of fibrin hydrogels is one of the main drawbacks of these systems, which can be overcome by the addition of other natural components such as collagen fiber. Further, the use of vascular models can benefit the high-throughput platforms for the discovery and development of vascular drugs. With advances in this field, vessels made from human cells might serve as a noble alternative to animal models in preclinical trials. In short, the development of human vascular structures seems to be largely dependent on the use of appropriate hydrogel materials and structures (Sisak et al. 2020), which should display full

biocompatibility with the associated cells necessary for the regeneration of damaged heart tissue after a heart attack. Several stem cells and carriers have been employed to improve the efficiency of stem cell-based myocardial infarction therapeutics. However, hydrogel-based carriers have promised innovative therapeutic options, but the main issue of hydrogels as a stem cell carrier for cardiac tissue is blocking blood vessels after solidification. In this respect, in situ-forming hydrogels might pave the way for overcoming the issue, owing to the possibility of administration via three routes: intracoronary, epicardially, or trans-endocardially. On the other hand, minimally invasive delivery is possible by employing catheter technology. Collectively, hydrogels might serve for regeneration purposes in the cardiac tissues in three ways, including (1) bulk hydrogels without any therapeutic biomolecules or cells just to provide mechanical support to the infarcted heart; (2) hydrogels carrying various biomolecules including growth factors, cytokines, and small molecules; and (3) hydrogels that are applied as a vehicle for stem cell delivery to the desired site. Several studies have been performed to demonstrate the most efficient supportive structure for cardiac tissue regeneration. It seems that the integration of both natural and synthetic polymers-based hydrogels is the most appropriate strategy. In this line, the hybrid hydrogels offer innovative prospects to improve the regeneration of a defected myocardium after myocardial infarction (Ahadian et al. 2016).

21.4.4 Hydrogel-Based Neural Regeneration

It is well established that the human nervous system has limited capacity to repair damaged parts after any injury or disease. Therefore, many efforts and studies have been performed in the field of nerve cell regeneration. There is no effective clinical treatment to restore central nervous system function after injury. Regarding stem cell therapy, the short half-life and systemic effects of injectable growth factors, and the poor survival, differentiation, and migration of transplanted stem cells lead to the failure of these new therapeutic strategies. In general, the brain is not considered to be a favorable environment for stem cells localization and function. Damaged brain lesions host various proteases and inflammatory molecules that create an invasive environment. Additionally, the blood-brain barrier, which restricts the passage of cells or molecules to the brain, is a major obstacle (Barar et al. 2016b; Omidi et al. 2021). To address the abovementioned issues, multifunctional scaffolds capable of carrying therapeutic cells and effective molecules can be the promising approach (Marchini et al. 2019). Several studies have emphasized the key role of growth factors in determining the differentiation of neural progenitor cells (NSPCs). Kornev et al. (2018) categorized hydrogels employed for neural regeneration into three main classes, including (1) hydrogels that are appropriate for brain injury therapy (e.g., HA-, collagen type I-, alginate-, CS-, methylcellulose-, fibrin-, and self-assembling peptide-based hydrogels) where fibrin-based hydrogels offer great achievements, (2) hydrogels that do not provide the basic conditions desirable to treat human brain injury mainly because of being inflexible and non-degradable, and (3) multifunctional potent hydrogels that require further studies.

Moreover, polymeric hydrogels are employed as bio-inks for the development of the 3D bio-printed platforms for the regeneration of neural cells/tissue (Maiti and Diaz Diaz 2018). Considering the performed investigations and the existing gaps, the future focus on hydrogels design for neurodegenerative diseases must be on the complex hybrid systems based on neuroprosthetic and bioprinting technologies. Taken all, it is also necessary to conduct the in-depth investigation for the discovery of much more biocompatible biopolymers and develop novel in vitro and ex vivo models of neuroregeneration using microfluidic-based multistage and multilayer systems.

21.5 Conclusions and Final Remarks

The improved viability of incorporated cells within the biomimicking hydrogels makes these formulations the main scaffolds for the proliferation and differentiation of stem cells. If designed carefully with the right composition, the hydrogels can mimic the 3D biochemical and biophysical environment as a permissive milieu to accommodate stem cells. They have been developed using a variety of fabrication methods, while the emergence of new technologies (e.g., 3D bioprinting) appears to substantially improve the design and fabrication of multifunctional hydrogels. Notably, a wide range of polymers (e.g., natural, synthetic, or hybrid) offer a great plausibility for the formulation of hydrogel with desired properties for various tissue engineering purposes. Hydrogels with smart and injectability characteristics can be used in a variety of biomedical applications, depending on the final endpoint of the cell therapy. Injectable smart hydrogels have shown promising potential for drug delivery and tissue engineering since they facilitate a unique carrier quality as a porous 3D context for drug loading with a great deal of biocompatibility and adaptability yet with low invasiveness and no/trivial toxicity. The stimuli-responsiveness characteristics of the smart hydrogels have provided compelling data on their benefits in tissue engineering. They can show an immediate reaction to the changes in pH, ionic strength, enzyme, or temperature by rapidly restructuring their swelling behavior, network structure, sol-gel transition, penetrability, or mechanical strength. To fabricate safe scaffolds, some critical issues should be considered in terms of the optimization of the injectable hydrogel, including the cytocompatibility and cell safety with no toxic degraded materials, the sol-gel transition at mild conditions, and the steady gelation with good timing to avoid the outflow of material to the surrounding tissues. Taken all together, the success of hydrogel-based tissue regeneration is largely dependent upon the type of the cells and their interactions with and responses to the desired differentiation pattern. As a result, it is critically crucial to develop the most desirable hydrogel for stem cell therapy based on the target microenvironment to which the cells are applied considering all biological factors that may influence the proliferation and differentiation of the cells.

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Chitosan-Based Biomaterials: Their Interaction with Natural and Synthetic Materials for Cartilage, Bone, Cardiac, Vascular, and Neural Tissue Engineering

22

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Abstract

Chitosan is a special material because of its nature as a natural polymer, which has been used for the preparation of safe and biocompatible scaffolds for tissue engineering and regenerative medicine. The development of advanced bioscaffolds using bioploymers such as chitosan in tissue engineering and regenerative medicine is a fast-growing multidisciplinary field of science and technology to restore defective and damaged organs. A large number of investigations have been carried out, employing chitosan and/or its derivatives, to develop advanced biomaterials to serve as suitable biological platforms for cell therapy and tissue regeneration. This chapter emphasizes various techniques used to produce chitosan-based biomaterials. The main focus is to provide insights into the blend of chitosan with other natural and synthetic polymers and figuring out their applications. Various chitosan-based biomaterials are discussed in terms of their applications for regeneration of multiple types of tissues such as bone, cartilage, cardiac, vascular, and neural tissues.

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22.1 Introduction

As a natural carbohydrate, chitosan (CS) is derived from chitin and considered an exceptional biopolymer with unique characteristics such as viability and biodegradability. Most of these extraordinary traits are related to the existence of the primary amines of the CS backbone, which makes this polysaccharide an attractive biomaterial for tissue regeneration. CS exhibits hydrophilic characteristics, by which it can improve the cell attachment, growth, and differentiation and also induce a minor external body response to implantation (Croisier and Jérôme 2013; Li et al. 2005). CS is a nontoxic biodegradable natural polymer with trivial immunogenicity. The application of CS has widely been investigated in drug/gene delivery and cell delivery and tissue regeneration. As a linear biopolymer, CS shows some structural resemblance to glycosaminoglycans of the extracellular matrix (ECM), which makes it very suitable for the development of scaffolds. It can also favor cell-cell adhesion and interaction.

To date, significant advances in the field of biomaterials have substantially improved tissue engineering and regenerative medicine. The main feature of such biomaterials is to show the highest biocompatibility with the biological settings. As one of the amplest biopolymers, CS has been employed in tissue engineering, in large part due to its unique characteristics, including biodegradability, low toxicity, no/trivial immunogenicity, and antimicrobial functions. To improve the mechanical strength of CS, this biopolymer has been further enhanced through covalent, non-covalent, or coordination complex crosslinking with other organic/inorganic materials. As a proof of application, CS-based hemostatic bandages have been developed for emergency applications by HemCon Medical Technologies Inc. Of note, it is estimated to have a marked expansion in the market size for the pharmaceutical and biomedical applications of CS globally.

The biocompatible nature of CS seems to promote the adhesion and proliferation/differentiation of various cell types (Amiryaghoubi et al. 2020a). CS can immobilize negatively charged entities such as enzymes, proteins, and nucleic acids, being polycationic polymer. CS can be blended with other advanced materials such as graphene to meet the required properties (Choudhary et al. 2020). To serve tissue engineering and regenerative medicine, CS can be engineered as fiber, hydrogel, film, and sponges. Accordingly, various types of CS-based formulations can be carried out to meet patient-specific needs. To this end, CS-based biomaterials can be developed as 3D soft gel, thin film, electrospun microfiber, 3D-printing scaffold (Rajabi et al. 2021; Gao et al. 2021), which make it a great candidate for many biomedical applications such as cartilage (Yamane et al. 2005), bone (Balagangadharan et al. 2017), cardiac (Martins et al. 2014), vascular (Zhang et al.

2006), and neural tissues (Crompton et al. 2007). This chapter aims to provide comprehensive information on the CS-based biomaterials and their extensive applications in tissue engineering.

22.2 Chitosan Structure and Properties as a Biomaterial

Structurally, CS comprises β(1→4)-linked glucosamine components, which make it a polycationic polymer with great potential for functionalization with other entities. CS is typically attained through the alkaline deacetylation of chitin. In this regard, the degree of deacetylation can be considered an indication of the number of amino groups based on the quantity of D-glucosamine entities. CS offers unique properties such as nontoxicity, biocompatibility/biodegradability, hemostatic properties, high swelling ratio, and antibacterial potentials. All these traits make CS a polymer of choice for cell therapy and tissue regeneration (Fakhri et al. 2020; Rinaudo 2006). CS derivatives can be produced by modification of the –OH and $–NH_2$ active functional groups on CS (Razmi et al. 2019; Ren et al. 2019). Chemically, CS resembles the glycosaminoglycans (GAGs), which are the component of cartilage tissue and hence have many potential properties for the application as a scaffold in tissue regeneration (Dutta et al. 2011). CS has extensively been utilized in bone tissue regeneration, in large part because of its potential in enhancing the calcium-rich matrix formation by osteoblasts (Seol et al. 2004). It has been shown that CS can degrade through enzymatic hydrolysis in the human body. The degradation behavior of CS is a key part of tissue engineering and host response (Kim et al. 2008). To improve the mechanical strengths and structural arrangement of the CS for bone tissue engineering, the incorporation of various natural/synthetic polymers has been evaluated, some of which have been utilized as the 3D-lyophilized, hydrogels, and fibrous scaffolds (Deepthi et al. 2016; Kavya et al. 2013). Structurally, protonated amino groups of CS make it a polycationic entity that can form ionic complexation with different types of anionic materials. By the same token, the degradation rate of CS is largely dependent on (1) the molecular mass and (2) the degree of deacetylation of the polymer (Croisier and Jérôme 2013). Based on its structural capacity, CS can be crosslinked with other organic or even inorganic entities through covalent, non-covalent, or coordination complex reactions. It can be used for the engineering of multistage biomaterials as the layer-by-layer 3D structures (Silva et al. 2017; Ma et al. 2021), which are important scaffolds for tissue engineering.

22.3 Chitosan-Based Hydrogels for Tissue Regeneration

Hydrogels provide a 3D organization of chemically/physically crosslinked polymeric networks, which are a proper platform for tissue regeneration. They present structural arrangement, outstanding biodegradation, more penetrability, viability, superior mechanical properties, and injectability (Amiryaghoubi et al. 2020b). Hydrogel generates a biocompatible and flexible analog of the extracellular cellular

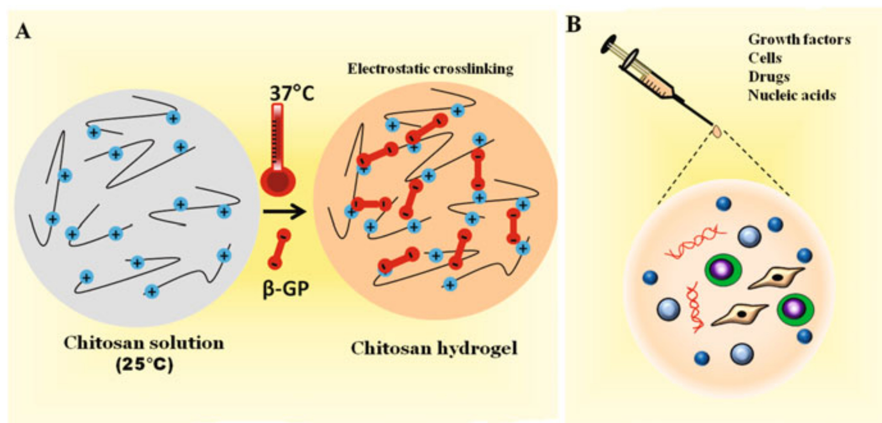
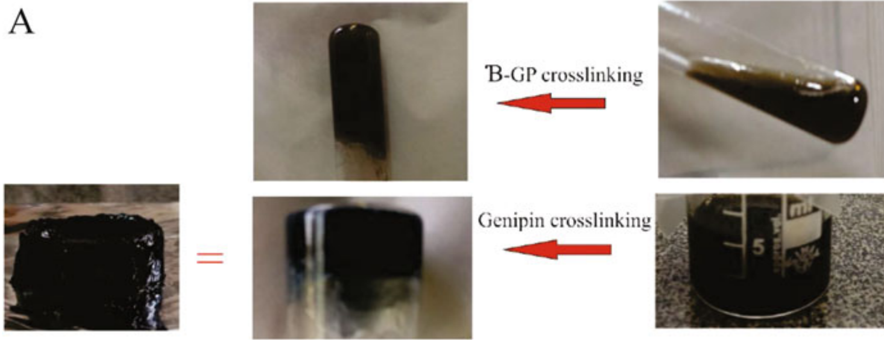


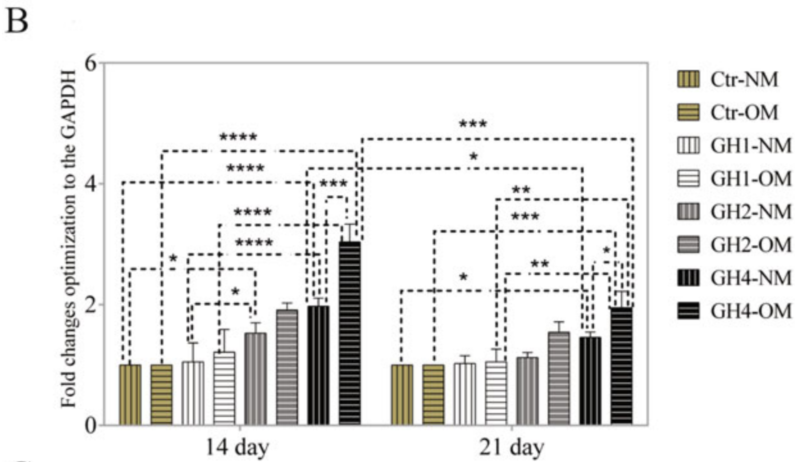
Fig. 22.1 (**a**) Chitosan crosslinked with β-glycerophosphate (β-GP) for tissue regeneration. (**b**) Injectable hydrogel incorporated with cells and other necessary substances and drugs. (Reproduced with permission from Saravanan et al. 2019, Copyright 2019 Elsevier)

matrix (ECM), which offers an appropriate microenvironment for cell attachment and growth (Nieto-Suárez et al. 2016). Among various crosslinking techniques, physical and chemical crosslinking are two primary techniques to prepare hydrogels (Hu et al. 2019). Hydrogels with physical crosslinking are normally produced by secondary forces such as non-covalent interactions, including hydrophobic bonds, hydrogen interaction, and electrostatic bonds between polymeric network chains (Korah et al. 2018; Bordi et al. 2002). These hydrogels are usually reversible with insignificant mechanical properties (Zhang et al. 2013). Chemically crosslinked hydrogels are fabricated via covalent bonding through free radical polymerization, condensation polymerization, and Michael addition (Kolb et al. 2001; Censi et al. 2010). The chemically crosslinked hydrogels are irreversible and offer outstanding mechanical stability (Kuo et al. 2015). Among various hydrogels, CS-based hydrogels offer substantial benefits as a scaffold in tissue engineering, mainly due to their biodegradability and biocompatibility (Zheng et al. 2019). For instance, an injectable CS hydrogel loaded with proteins and mesenchymal stem cells (MSCs) was shown to offer appropriate cell adhesion, mechanical strength, cell viability, and biodegradability (Molinaro et al. 2002). Further, CS hydrogels can act as a stimuli-responsive vehicle in response to the variations in pH and temperature (Turabee et al. 2019). This behavior of CS hydrogels, which results from the protonation/deprotonation of the basic amine groups (NH_2), can change with the exogenous pH (Xu and Matysiak 2017). Due to the deprotonation of its NH_2 groups, CS is less soluble in water at pHs above its pK_a (Rizwan et al. 2017). Among different crosslinkers, β-glycerophosphate (β-GP) provides uniform deposition of CS gel thermosensitive potential (Fig. 22.1) (Saravanan et al. 2019). It has been shown that a CS/β-GP injectable hydrogel is an important rich matrix for the regeneration of bone tissue (Zhou et al. 2015) In a study, composite hydrogels of poly(*N*-

isopropylacrylamide) (PNIPAm) with graphene oxide (GO) engineered using physical and chemical crosslinking have been evaluated for bone tissue engineering (Amiryaghoubi et al. 2020c). The chemically crosslinked hydrogels (GH1-GH4) were formed through the amide bonds between the CS and the genipin (GN) as chemical crosslinker, whereas physically crosslinked hydrogel (GH5) was formed via electrostatic interaction between the amine groups of CS and the phosphate groups of β-GP. The hydrogel was sensitive to the pH and body temperature (Fig. 22.2a). The hydrogel enhanced the formation of calcium and the activity of alkaline phosphatase (ALP), because of the oxygen and amine groups of GO and CS. The fabricated hydrogels promoted the expression of Runt-related transcription factor 2 (*Runx2*) and osteocalcin (*OCN*) of human dental pulp stem cells (hDPSCs) (Fig. 22.2b, c) (Amiryaghoubi et al. 2020c).

Recently, researchers formulated CS-based hydrogels using *Aloe vera* for biomedical applications (Drabczyk et al. 2020). The hydrogels were prepared by photopolymerization of a mixture of CS 1% (w/v) solution and desirable amounts of crosslinking agent, poly(ethylene glycol diacrylate) (PEGDA 575) or PEGDA 700, as well as a sufficient amount of *Aloe vera* juice. As a result, the hydrogels modified with *Aloe vera* exhibited a higher swelling ratio. On the other hand, the hydrogels prepared with higher amounts of crosslinking agent displayed a lower swelling ratio due to higher crosslink density. At the same crosslinker concentration, the hydrogels modified with *Aloe vera* were more flexible compared with the unmodified ones. *Aloe vera* enhanced the elongation of hydrogels up to 23%. Besides, the hydrogels prepared using 10 mL of *Aloe vera* and 10 and 12 mL PEGDA 700 demonstrated the highest mechanical strength. The addition of *Aloe vera* as an adjusting factor was found to result in the smoothing of the hydrogel surface. Thus, the unmodified hydrogels possessed a relatively rougher surface as compared to the hydrogels modified with *Aloe vera*. The L929 murine fibroblasts cells loaded in these hydrogels showed noticeably high viability over 70% after incubation for 24 h (Drabczyk et al. 2020). In 2020, Nezhad-Mokhtari et al. reported a study, in which injectable hydrogels were fabricated using collagen (COL)/cellulose nanocrystals (CNCs)/chitosan loaded with AuNPs (COL/CNCs/CS-Au) for tissue engineering (Nezhad-Mokhtari et al. 2020). The incorporation of CNCs and CS-Au enhanced the mechanical strengths and slowed down the degradation rate. The cytotoxicity assay in the 3T3 fibroblasts revealed no toxicity for the prepared hydrogels (Nezhad-Mokhtari et al. 2020). CS-based hydrogels have been prepared through crosslinking with β-GP, which were further intensified via physical bonds with β-tricalcium phosphate (β-TCP). The hydrogels showed a gel-phase transition at 37 °C, forming a 3D system with a suitable rheological and elastic profile. As a robust system, the hydrogel enhanced the attachment and proliferation of the incorporated cells. The addition of β-TCP improved the structural features of the hydrogel providing appropriate physicochemical characteristics based on the natural condition of the bone tissue (Dessì et al. 2013).





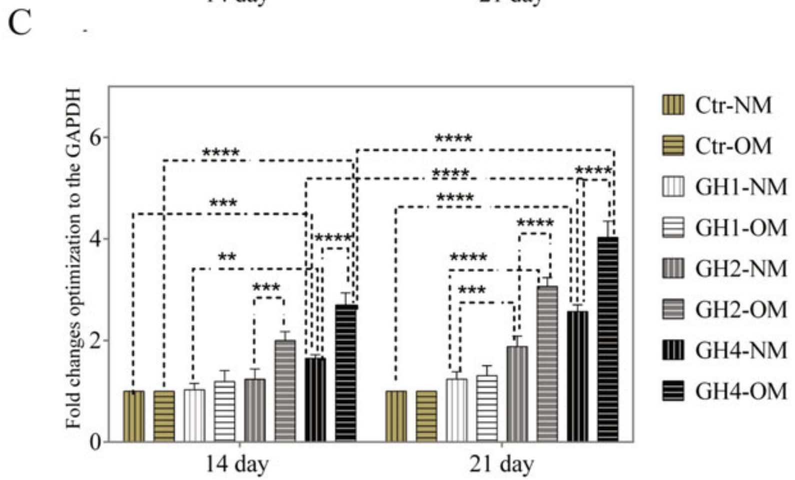


Fig. 22.2 (**a**) The image of GH4 and GH5 hydrogel formation. (**b**) The *Runx2* expression. (**c**) The *OCN* expression. The expression was analyzed on the prepared hydrogels in the growth media and osteogenic media and after 14 days and 21 days of cell culture, respectively. (Reproduced with permission from Amiryaghoubi et al. 2020c, Copyright 2020 Elsevier)

22.4 Chitosan for the Restoration of Different Types of Tissues

22.4.1 Chitosan for the Regeneration of Cartilage Tissue

Aggrecan, a chondroitin (CHN)/keratan sulfate-comprising proteoglycan, is the main element of the cartilage tissues. Cartilage contains a relatively low number of chondrocytes spread within the extracellular matrix (ECM) (Sundar Raj et al. 1995). Aggrecan is an abundant CHN sulfate proteoglycan in articular cartilage tissue, which can cause water retention and also maintain the osmotic resistance necessary for cartilage resistance against compression (Chandran and Horkay 2012). Early osteoarthritis (OA) is generally associated with the loss of aggrecan, in which the chondrocytes play a key role as the distinctive cell type for cartilage restoration even though they have a shortage of resources and slight differentiation capacity (Barbero et al. 2004). In recent years MSCs have been considered as the alternative cell source for the regeneration of cartilage tissue (Pittenger et al. 1999), which is believed to further advanced using biomimetic scaffolds and cells to revitalize the function of damaged tissues (Zhang et al. 2009; Ansari and Eshghanmalek 2019). Different natural and synthetic polymers mixture have accordingly been utilized as biocompatible and biodegradable scaffolds to promote cell growth, ECM deposition, and structural strengthening (Liao et al. 2014).

22.4.1.1 Chitosan-Based Natural Polymers for the Restoration of Cartilage Tissue

CS-alginate (Alg) scaffold has been developed via an interconnected 3D porous construction by thermally induced phase separation and freeze-drying, which was shown to enhance cell growth and phenotype expression of HTB-94 chondrocytes (Liao et al. 2014). An injectable hydrogel containing methacrylate glycol chitosan (MeGC) and hyaluronic acid (HA) was produced by a riboflavin photoinitiator with visible radiation. The irradiation time from 40 to 600 s meaningfully enhanced the compressive modulus of the platforms (Fig. 22.3a). The viability of chondrocytes embedded in the MeGC platform, after a 300 s radiation, increased to ~80–87% during 21 days (Fig. 22.3b–d). The addition of HA in MeGC hydrogels promoted the growth and formation of cartilaginous ECM (Park et al. 2013). Silk fibroin (SF)/CS mixed scaffolds were investigated for the regeneration of the cartilage tissue. The SF/CS (1:1) blended platform indicated the most accumulation of GAG and COL. The cell-seeded settings showed more dynamic stiffness at high frequencies compared to the control (Bhardwaj et al. 2011). The improved ratio of the polymer solutions of CS, agarose, and gelatin (CAG) was manufactured via cryogelation and crosslinking by incubating the mixture at sub-zero temperature (−12 °C). The CAG cryogels were found to offer suitable cell attachment of primary goat chondrocytes as analyzed by scanning electron microscopy (SEM). The implantation of the scaffolds in the animal models confirmed the in vivo viability of the prepared scaffolds (Bhat et al. 2011). In a recent study, the photocrosslinking maleilated chitosan (MCS) and methacrylate-grafted SF (MSF) micro-/nanoparticles (NPs) were prepared using Darocur 2959, a photoinitiator. When the MSF amount was

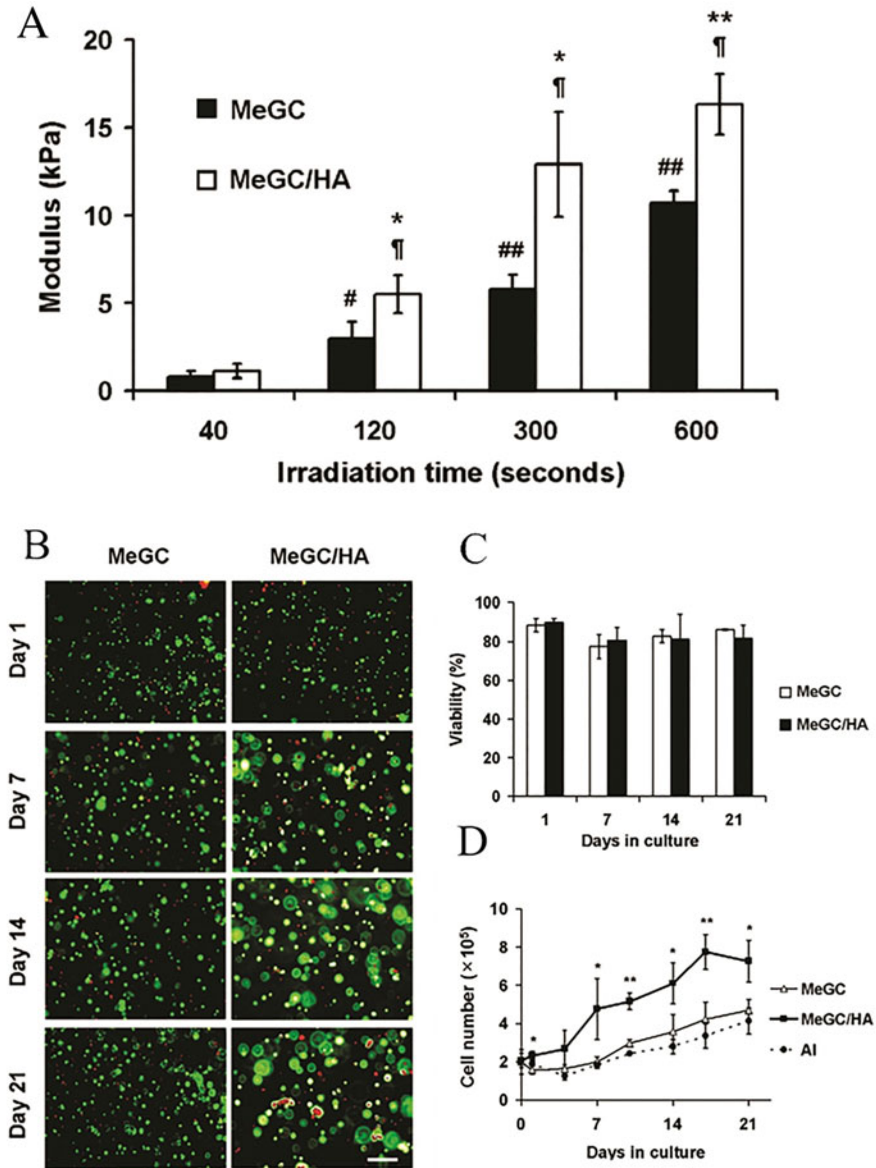


Fig. 22.3 (**a**) Mechanical strengths of prepared platforms polymerized with different irradiation times. (**b**) Live/dead assay. (**c**) Viability of encapsulated chondrocytes in prepared hydrogels, and (**d**) Alamar blue test indicating growth of chondrocytes seeded in hydrogels for cartilage regeneration. *MeGC* methacrylate glycol chitosan, *HA* hyaluronic acid. (Adapted with permission from Park et al. 2013, Copyright 2012 Elsevier)

enhanced to 0.1%, the hydrogel showed the compressive modulus in the range of articular cartilage tissue value. The micro-/nanocomposite hydrogels with transforming growth factor-β (TGF-β1) were found to be a nontoxic setting for chondrocytes and hence regeneration of cartilage tissue (Zhou et al. 2018). In a study, COL/CS/HA polymer blend was prepared by the freeze-drying technique (Yan et al. 2006). The mechanical properties and water binding capacity were elevated by the incorporation of CS and hyaluronan (HYA). DNA and GAG amounts were meaningfully enhanced using COL/CS/HA platform compared to the COL scaffold. As a result, the prepared scaffold was proposed as a potential candidate for the bioengineering of cartilage tissue (Yan et al. 2006). The integration of CS with COL type I (CS/COL-I) by freeze-drying and crosslinked by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) resulted in the production of a sound scaffold. It was shown that synovium-derived stromal cells (SDSCs) cultured using the CS/COL-I scaffold promoted the functional expression of aggrecan and COL type II (Gong et al. 2010). Rabbit articular chondrocytes were seeded on porous polymer blend scaffolds, which were prepared using COL, CS, and CHN (COL-CS-CHN), and provided homogeneous cartilaginous tissue after the implantation for 12 weeks. Hematoxylin and eosin staining indicated the rounded morphology of the chondrocytes on the prepared scaffolds (Yan et al. 2007).

22.4.1.2 Chitosan-Based Synthetic Polymers for the Restoration of Cartilage Tissue

CS-coated poly(L-lactide) (PLA) microspheres were designed for the regeneration of the cartilage tissue. The PLA microspheres were prepared via an oil/water emulsion solvent evaporation technique and then hydrolyzed in an alkaline solution to create a greater number of carboxyl groups. CS was covalently attached to the microspheres by water-soluble EDC as a crosslinking agent. The more CS on the PLA microspheres promoted the attachment and proliferation of cells after 24 h and 7 days culture (Lao et al. 2008). Hydrophilic fibrous scaffolds were fabricated by a mixture of poly(3-hydroxybutyrate) (PHB) and CS for the cartilage tissue regeneration. The electrospun of PHB (9 wt%) solutions was mixed with CS in trifluoroacetic acid. The incorporation of CS reduced the water contact angle and mechanical strength. Chondrocyte adhesion was promoted on the surfaces of these scaffolds (Sadeghi et al. 2016). In a study, CS/poly(vinyl alcohol) (PVA)/GO nanofibers were produced by the electrospinning method (Cao et al. 2017). The tensile strength of the scaffold was enhanced by the incorporation of GO in the nanofiber structure. The CS/PVA/GO (6 wt%) revealed a greater growth of ATDC5 cells as compared to the CS/PVA/GO (4 wt%), which can be used as a possible scaffold for generating the artificial cartilage (Cao et al. 2017). The electrospun CS/PVA has been inspired by the natural addition of $CaCO_3$ inside a chitin complex. With the addition of $CaCO_3$ NPs from 1 to 5 wt%, the diameter of fibers was increased (Fig. 22.4a–f). Young's modulus of the scaffold with 4 wt% $CaCO_3$ was enhanced up to 912.5 ± 60.6 MPa (Fig. 22.4g). The $CaCO_3$-loaded fibers revealed the formation of apatite minerals in the simulated body fluid (SBF) after 14 days and enhanced growth of the ATDC5

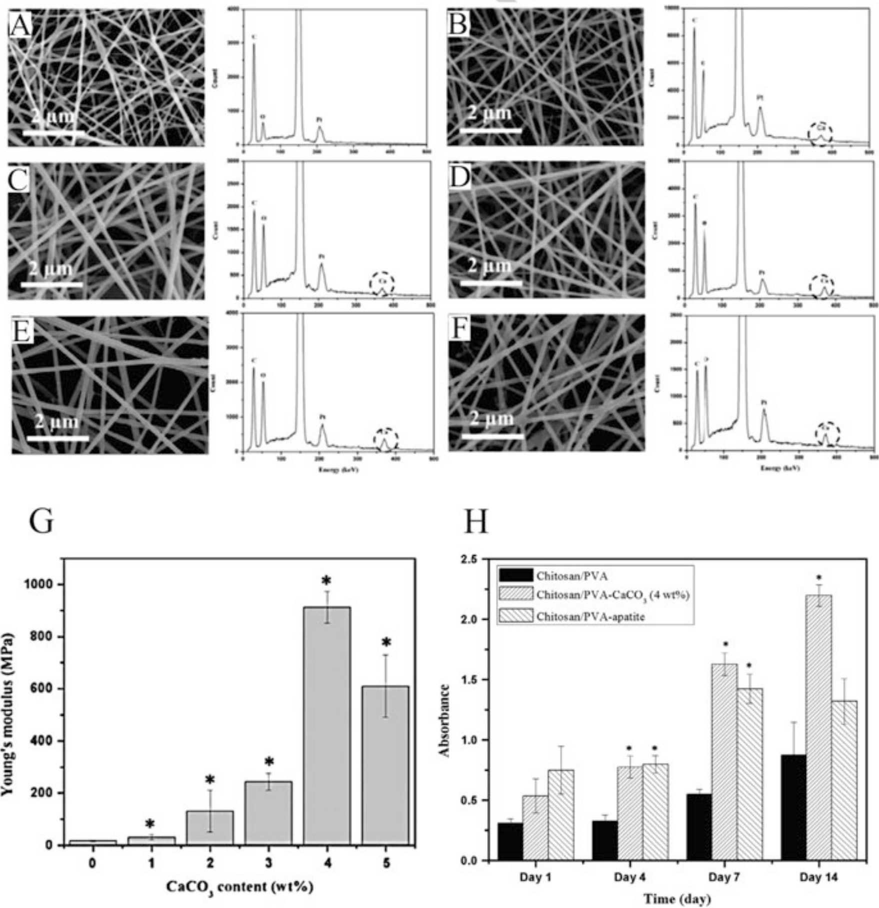


Fig. 22.4 The SEM micrographs and EDS analysis of CS/PVA fibers with various amounts of $CaCO_3$. Panels (**a**)–(**f**) represent 0, 1, 2, 3, 4, and 5 (wt%), respectively. (**g**) Young's modulus meaningful difference among groups shown by * $\sim p < 0.05$ as compared to CS/PVA. (**h**) Proliferation of ATDC5 cells on prepared scaffolds. The difference among samples is shown by * $\sim p < 0.05$ compared with CS/PVA. *SEM* scanning electron microscopy, *EDS* energy-dispersive X-ray spectroscopy, *CS* chitosan, *PVA* poly(vinyl alcohol). (Reproduced with permission from Sambudi et al. 2015, Copyright 2015 Elsevier)

cells (Fig. 22.4h). The $CaCO_3$-reinforced CS/PVA could be a potential scaffold for artificial cartilage engineering (Sambudi et al. 2015).

In 2017, Karbasi and Alizadeh reported about three different amounts (0.5, 0.75, and 1 wt%) of multiwall carbon nanotubes (MWNTs), which were added to improve the PHB/CS electrospun scaffold (Karbasi and Alizadeh 2017). The water contact angle (WCA) of the prepared platforms was decreased by the addition of MWNTs. With the initial strength of 3 MPa, the mechanical strength of the PHB/CS scaffold

was enhanced using various amounts of MWNTs. Therefore, PHB-CS/MWNTs scaffold exhibited high enough mechanical strength, which can be utilized in the regeneration of the cartilage tissue (Karbasi and Alizadeh 2017). The CS/γ-poly (glutamic acid) (γ-PGA) platforms with diverse mass fractions were fabricated utilizing GP as a crosslinker, and their surfaces were enriched using elastin, human serum albumin (HSA), and poly-L-lysine (PLL). The fabricated CS/γ-PGA platforms (at a ratio of 3:1) enhanced the porosity, mechanical strength, and biodegradation up to 90%, 4 MPa, and 30–60%, respectively. As a result, the CS/γ-PGA scaffold can serve as a desirable platform for articular cartilage tissue regeneration. The surface change of the platforms resulted in suitable cell adhesion, growth, and GAG, COL II synthesis of bovine knee chondrocytes, and recovery of the cartilaginous tissue regeneration (Kuo et al. 2017). A porous poly(L-lactide-co-ε-caprolactone) (PLCL) was fabricated and crosslinked at the surface to CS to enhance its hydrophilicity. The Young's modulus of the cartilage tissues on the CS improved the PLCL platform as compared to the unmodified PLCL. The inclusion of CS in the PLCL platform promoted the cell viability of the scaffold (Yang et al. 2012). Novel CS/polybutylene succinate fiber-based platform (CS-PBS) was utilized to evaluate their usage for the cartilage tissue regeneration, in which the non-woven polyglycolic acid (PGA) felts were utilized as reference materials. An increasing GAG formation was detected in both the CS-PBS and PGA platforms, while the latter indicated higher contents of synthesized GAGs (Oliveira et al. 2011). The CS, PLA, and pectin (PC) compositions were tailored to produce polyelectrolyte complex-based platforms. The platform was crosslinked with EDC and *N*-hydroxysuccinimide (NHS) solution incorporating CHN sulfate to provide the structure with a similar arrangement of the ECM of the cartilage tissue. The pore size and porosity percentage of the sample were in the range of 49–170μm and 79–84%, respectively, which might allow the diffusion of nutrients and cell growth. The addition of CHN sulfate in the porous platform was shown to promote the formation of GAGs within the scaffold (Mallick et al. 2018).

22.4.2 Chitosan for the Restoration of Bone Tissue

Bone is a vigorous and dynamic organ that owns excessive repair and renewing capacity. This complex tissue indicates an individual biological setting with mechanical properties and aligned structure toward different biological and physiological functions. The bone ECM (bECM) contains COL-I, mineral, OCN, and osteopontin (OPN) (Amiryaghoubi et al. 2020a; Robey and Boskey 2009; Ingram et al. 1993). The bone inorganic structure is nanocrystalline, which largely entails hydroxylapatite [$Ca_{10}(PO_4)_6(OH)_2$] (HAp) and plays a key role in the reinforcement of the tissue (Grynpas and Omelon 2007). As a late osteogenic marker, OCN is a calcium-binding protein that is largely involved in the development of bone tissue (Dowd et al. 2003; Stewart et al. 2002). As the most common biochemical gene for osteogenic differentiation, *ALP* can promote the calcification of bECM via the hydrolysis of phosphate groups (Golub and Boesze-Battaglia 2007; Meng et al. 2013). Large bone

impairment is a serious problem, which can mostly be induced by extreme trauma, osteomyelitis, tumor, infection, osteolysis, and rheumatoid arthritis (Gong et al. 2015; Ardeshirylajimi et al. 2015; Liao et al. 2017; Li et al. 2015). Up to now, the conventional autografting and allografting techniques have been known as the medical gold standard method for the repair of damages in the bone tissue (Bauer and Muschler 2000). However, several problems limited the usage of the aforementioned grafting method in clinical applications, including the lack of donors, infectious diseases, and immune responses (Banwart et al. 1995; Friedlaender 1983). Bone tissue regeneration has been considered a potential approach for the restoration of the damaged bone tissue by utilizing a mixture of scaffolds and cells (Liu et al. 2017a). As for scaffolds, various synthetic and natural polymers have been introduced based on their features (e.g., mechanical strength, wettability, surface functionalization, interconnected porosity, and osteoconductivity). Of these, nanocellulose/poly(lactide-co-glycolide) (PLGA) nanocomposite has been successfully used for the cultivation of the NIH 3T3 cells (Tang et al. 2017).

22.4.2.1 Chitosan-Based Natural Polymers for the Restoration of Bone Tissue

Composite platforms of CS-gelatin (Gel) have been fabricated with nano-bioactive glass-ceramic (nBGC) (CG/nBGC) by mixing CS and Gel with nBGC. The pore size range in the prepared scaffold was from 150 to 300μm. With the incorporation of nBGC, the degradation and swelling ratio of the nanocomposite scaffolds were diminished, while the protein adsorption was increased. The CS/Gel/nBGC nanocomposite provided more mineral deposition with improved cell adhesion and promoted spreading as compared to the CSGel scaffolds. The advanced nanocomposite might be considered as a selection for alveolar bone tissue engineering (Peter et al. 2010). The researchers fabricated CS-Alg and fucoidan incorporated CS-Alg (CS-Alg-fucoidan) by a freeze-drying technique for bone tissue engineering. The pore sizes of the CS-Alg and CS-Alg-fucoidan platforms were 62–490μm and 56–437μm, respectively. The ALP activity and proliferation of the MG-63 cells incorporated in the CS-Alg-fucoidan scaffold were enhanced as compared to the CS-Alg sample (Venkatesan et al. 2014). The CS/Alg and CS/Alg/nano-silica ($nSiO_2$) samples were prepared by the freeze-drying method. The incorporation of $nSiO_2$ enhanced the formation of apatite and the adsorption of protein on these scaffolds (Sowjanya et al. 2013). The CS-Gel/β-TCP hybrid polymer network combined with glutaraldehyde (GTA) was produced by the freeze-drying technique. With the incorporation of β-TCP, the compressive modulus of the platforms was significantly increased (3.9–10.9 MPa) (Yin et al. 2003). The nanocomposite HAp (nHAp) and blend of CS and carboxymethyl cellulose (nHAp/CS/CMC) were produced by the freeze-drying technique. The composite scaffold was formed mainly through electrostatic interactions between the CS and the CMC, by which the pore size and porosity were increased up to 500μm (from 100μm) and 77.8%, respectively. Besides, the MG63 and MSCs cells attached and flourished on the platform in vitro. The implantation of the scaffold in vivo indicated that numerous blood vessels proliferated into the porous organization, while the platform was

steadily biodegraded (Yin et al. 2003). The extremely porous 3D β-TCP platforms with two internal perpendicular canals were produced utilizing the foam-casting method. The platforms were covered with a gel layer (Bastami et al. 2017). The recombinant human bone morphogenetic protein-2 (rhBMP2)-loaded CS NPs were then distributed into the COL hydrogel and filled into the platform canals. The results indicated that Gel-coated TCP scaffold encompassing rhBMP2 NPs provided a mechanically and biologically compatible framework with sustained delivery of rhBMP2 for the differentiation of human buccal fat pad-derived stem cells (hBFPSCs) toward the osteoblast lineage (Bastami et al. 2017). The porous 3D platforms based on CS, CS/SF, and CS/SF/HA scaffolds were prepared for bone tissue engineering and regeneration/repair. The porosity of the CS/SF/HA and CS were $89.7 \pm 2.6\%$ and $94.2 \pm 0.9\%$, respectively. The CS/SF/HA scaffold showed more SaOs-2 cell adhesion, growth, and ALP activity as compared to other tested scaffolds on Days 7 and 21 (Lima et al. 2013). In a study, the clinoptilolite (CLN)-nHA/CS-Gel scaffold was fabricated for bone tissue regeneration (Sadeghinia et al. 2020). The addition of the CLN and nHA enhanced surface area and biomineralization and decreased the rate of degradation in SBF while enhancing the mechanical strength. The scaffold promoted the proliferation of the h-DPSCs. The adult human bone marrow-derived stem cells (hBMSC) encapsulated in hydrogels of CS/COL at different ratios (i.e., 100/0, 65/35, 25/75, and 0/100 wt%) displayed high biocompatibility on Day 1. The DNA content increased in the scaffold containing collagen but decreased about half in pure CS after 12 days. CS also enhanced the ALP activity and mineralization in the osteogenic media.

22.4.2.2 Chitosan-Based Synthetic Polymers for the Restoration of Bone Tissue

The 3D CS/PLGA composites were prepared by sintering into CS/PLGA microspheres to serve bone tissue regeneration. The presence of CS upregulated the gene expression of *ALP* and bone sialoprotein. The growth of the MC3T3-E1 osteoblast-like cells on the CS/PLGA microspheres was found to be much higher than other platforms (Jiang et al. 2006). In a study, an injectable thermosensitive CS hydrogel (CSG) was added to the 3D-printed PCL platform to produce a hybrid platform for bone tissue engineering (Dong et al. 2017). Rabbit bone marrow mesenchymal stem cells (BMMSCs) and BMP-2 were encapsulated in CSG. The stronger osteogenesis and bone-matrix deposition was observed for the BMMSCs using the hybrid scaffold after 2 weeks in osteogenic culture (Dong et al. 2017). The HA NPs, in a distinct rodlike form, were uniformly dispersed inside the CS-PLA matrix. The results indicated that the incorporation of PLA led to the formation of uniform composites that could meaningfully resist higher stresses. It also enhanced the elastic modulus, which makes these platforms suitable candidates for surgical applications (Cai et al. 2009). The researchers fabricated the photopolymerized injectable hydrogels from the CS derivative/poly(ethylene glycol) dimethacrylate-dimethylacrylamide (EGAMA-CS/PEGDA/DMMA). The prepared scaffold promoted the proliferation and attachment of the human bone sarcoma SW1353 cells. The photopolymerized hydrogels displayed high water absorption,

excellent mechanical properties, and good thermal stability (Ma et al. 2010). The nanofibrous scaffolds based on PCL/CS and PCL/CMC mixtures were developed for bone tissue engineering via electrospinning. The PCL/CMC platforms significantly increased surface water absorption as compared with the PCL/CS ones. The fiber diameter of prepared scaffolds was decreased at higher concentrations of CMC. The PCL/CMC nanofibers exhibited more proliferation of the human osteoblast MG63 cells compared to the PCL and PCL/CS samples, particularly at the extreme concentrations of CMC (Sharifi et al. 2018). The PCL/PVA/CS hybrid was fabricated by multi-jet electrospinning for bone regeneration. The expression of an early and late phenotypic osteogenic marker of rat MSCs was found to be upregulated in the scaffold cultured using the osteogenic media. The PCL/CS/COL groups loaded with different amounts of GO (0, 0.5, 3, and 6 wt%) were considered for bone tissue regeneration. The addition of GO enhanced hydrophilicity, bioactivity, MG-63 cell attachment, and proliferation (Aidun et al. 2019). The *n*-HA/CS/PEG was prepared via a coprecipitation approach at 25 °C. The MTT assay indicated the greater growth of the murine fibroblast L929 cells on the *n*-HA/CS/PEG setting as compared to the *n*-HA/CS scaffold. A faster swelling rate and higher mechanical strength were observed for the *n*-HA/CS/PEG scaffold (Shakir et al. 2015). The 3D composite platforms of nBG/$nTiO_2$ were produced by the foam replication method. PHB and PHB/CS were coated on platforms by a dip-coating technique. The polymer coating enhanced the mechanical properties but did not change the interconnectivity of the pore construction. The presence of CS reduced the contact angle and increased the bioactivity and antibacterial properties (Parvizifard et al. 2020). In a study, CS/poly(lactide-co-glycolide) (PLAGA) sintered microsphere platforms were functionalized via heparin immobilization for bone tissue engineering (Jiang et al. 2010). The heparinized CS/PLAGA possessed an interconnected porous structure with 172.33 ± 5.89μm pore size. The heparinized CS/PLAGA platforms with less heparin loading (1.7μg/scaffold) displayed more MC3T3-E1 cell growth and *OCN* expression as compared to CS/PLAGA scaffold and CS/PLAGA platform with more heparin load (14.1μg/scaffold) (Fig. 22.5a). The compressive modulus and compressive strength of heparinized CS/PLAGA scaffolds were 403.98 ± 19.53 and 9.83 ± 0.94 MPa for native trabecular bone (Fig. 22.5b, c) (Jiang et al. 2010). The CS/PCL/Zn scaffold was compatible with the MSCs and promoted the differentiation of osteoblast cells for bone regeneration. The prepared scaffold promoted calcium deposition and ALP activity. The expression of Runx2, type 1 COL, OCN, and osteocalcin (OC) of MSCs which were isolated from various sources were upregulated by CS/PCL/Zn scaffold (Chandramohan et al. 2020). The researchers indicated that electrospun PLA/poly(ε-caprolactone)-poly (ethylene glycol)-poly(ε-caprolactone) (PCEC) fibrous platforms could promote the adhesion and growth of the human placenta-derived mesenchymal stem cells (hPMSCs) due to an interconnected and porous arrangement as well as structural similarity to bECM. The SEM micrograms showed that fiber diameter and the WCA reduced as PCEC concentration increased from 0% to 50% (Wang et al. 2014). The porous samples of PCL loaded with BG NPs (0–15 wt%) and 7 wt% strontium (Sr) were produced for bone regeneration. The platform was then covered with CS

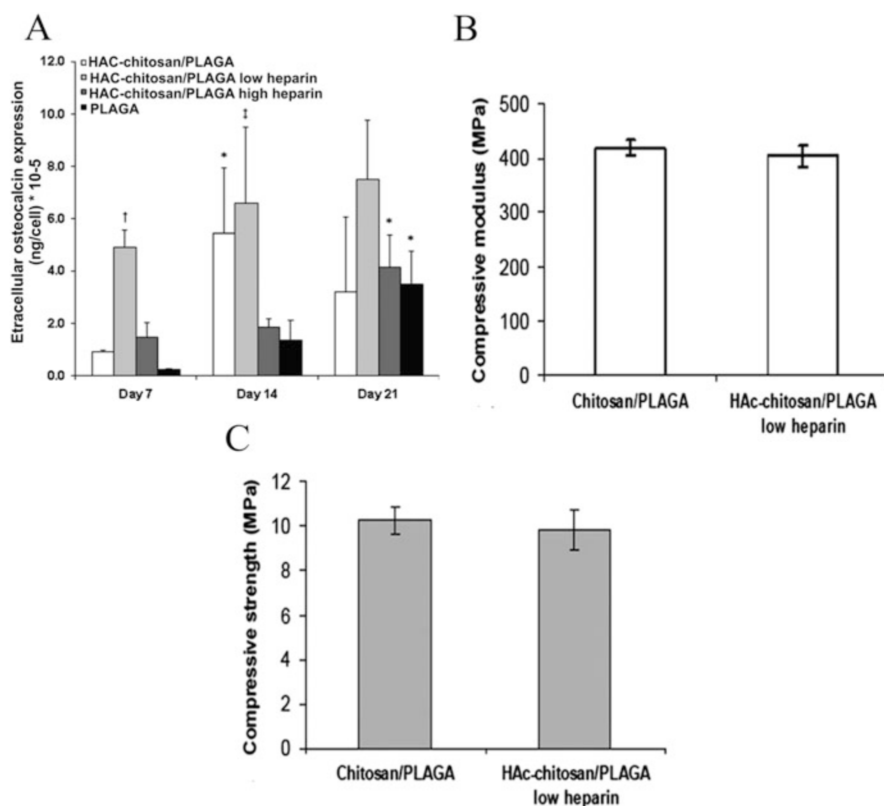


Fig. 22.5 (**a**) The *OCN* expression of MC3T3 cultured on the prepared scaffold after 7, 14, and 21 days. (**b**) Compressive modulus. (**c**) Compressive strength of PLAGA scaffolds and HAc-CS/PLAGA low heparin platforms. *CS* chitosan, *PLAGA* ploy(lactic acid-co-glycolic acid), *HAc* acetic acid. (Adapted with permission from Jiang et al. 2010, Copyright 2010 Wiley)

containing 15 wt% Sr-replaced BG NPs. The porous PCL-P and BG platform coated with CS containing 15 wt% BG NPs promoted both the ALP activity and the cell adhesion (Shaltooki et al. 2019). Further, alumina nanowires have been incorporated to PHB-CS alloy composite to engineer a platform through electrospinning technique. The tensile strength of the PHB-CS structure enhanced with the addition of 3 wt% alumina. The growth of MG-63 cells and the ALP activity were meaningfully higher on platforms containing alumina than that of the PHB or PHB-CS scaffolds (Toloue et al. 2019).

22.4.3 Chitosan for the Regeneration of Cardiac Tissue

The cardiac tissue includes a complex internal microvasculature consisting of endothelial cells (ECs), fibroblasts, and pericytes (Pinto et al. 2016). As an organ

with a nominal capacity to regenerate, the cardiac tissue plays an important role in pumping blood and oxygen to other tissues, supplying nutrients, and keeping blood circulation homeostasis (Zhang et al. 2015). The contractile behavior and synchronized beating of cardiomyocytes (CMs) are entirely dependent on cellular orientation, elongation, and anisotropic microarchitecture of the cardiac tissue. Myocardial infarction (MI) might occur because of blood clotting in the coronary artery, which reduces the delivery of nutrients and oxygen to the heart muscle, and, consequently, cardiomyocytes start to die because of ischemia that ultimately results in necrotic or apoptotic processes, heart failure, and death. In this process, cardiac muscles are unable to contract rhythmically, inducing abnormalities in the electrical function of the cardiovascular system such as arrhythmias and left ventricle (LV) enlargement. Moreover, during myocardial infarction (MI), the left ventricle becomes stiffer compared to the normal left ventricle, in large part because of COL I majority over COL III in damaged tissue (Nian et al. 2004; Boateng et al. 2005). For cardiac tissue engineering, stem cells can be differentiated into cardiac cells using natural or synthetic polymer-based scaffolds such as multilayered structures of CS and gelatin (Pok et al. 2013), electroconductive CS/carbon scaffolds (Martins et al. 2014), and nanofibrous scaffold of CS/PLA (Liu et al. 2017b). CS has also been exploited for heart-on-a-chip systems (Zhang et al. 2015). The main principles of cardiac tissue engineering are to efficiently improve the contractile and electrophysiological role of the heart muscles and to appropriately reorganize cells into tissues (Boateng et al. 2005).

22.4.3.1 Chitosan-Based Natural Polymers for the Regeneration of Cardiac Tissue

In a study, a platform from the solubilized cardiac ECM was supplemented with Alg and CS for cardiac tissue engineering (Tamimi et al. 2020). The combination of ECM with CS and Alg considerably enhanced the mechanical strength of ECM. The immunofluorescence staining indicated more upregulation of cardiac marker in the blended ECM with CS and Alg ternary scaffold as compared to ECM alone (Tamimi et al. 2020). The COL/CS scaffolds were fabricated using various crosslinking agents such as GTA, GP, and tripolyphosphate (TPP) for cardiac tissue engineering (Fang et al. 2020). All the samples exhibited extraordinary porosity (>65%) and a good swelling ratio suitable for myocardial regeneration. TPP crosslinked platforms displayed exceptional mechanical strength and elastic modulus in the biological range of the natural myocardium (20–100 kPa). GP and TPP crosslinked platforms showed more viability than GTA crosslinked platforms, as confirmed by the live/dead and growth test. More expression of cardiac protein and the contractile property was observed for cardiomyocytes cultured on TPP crosslinked scaffolds. TPP was considered the most appropriate crosslinking agent for COL/CS platform in myocardial tissue regeneration (Fang et al. 2020). In a study, the healing potential of CS-HYA/SF (CHS) cardiac patches in rats with MI was evaluated for 8 weeks (Chi et al. 2013). The CHS platform profoundly promoted the secretion of paracrine elements such as vascular endothelial growth factor (VEGF) in the MI sections of LV ($p < 0.05$, $n = 4$) as compared to that of the MI group. The expression of

paracrine factors (e.g., VEGF) in the MI regions of LV was enhanced in the CHS sample, suggesting CS-HYA/SF as a potential platform for cardiac healing in MI conditions (Chi et al. 2013).

22.4.3.2 Chitosan-Based Synthetic Polymers for the Regeneration of Cardiac Tissue

A 3D platform, encompassing self-accumulated PCL sandwiched in the Gel-CS hydrogel, has been prepared for the surgical regeneration of the damaged heart. The PCL core provided surgical usage and great initial tensile strength, whereas the Gel-CS part with the desired pore size and mechanical strength supported the adhesion of cells for the cardiomyocyte function. The compressive modulus of the composite tissue resembled natural tissue (15 kPa for 50% Gel and 50% CS). The Gel-CS feature of the gel as a 3D porous construction with a diameter of 80μm provided a great possibility for the migration and proliferation of the neonatal rat ventricular myocytes after 7 days, which resulted in spontaneously beating system (Pok et al. 2013). The electrospun fibrous scaffolds have recently been formulated for cardiac tissue regeneration using different ratios of PLA and CS. The fiber diameter was reduced with CS concentration, whereas the mechanical strength and the water absorption were enhanced. The platforms with the aligned fibers showed a higher mechanical strength as compared to the random fibers. The platforms with the aligned fibers and PLA:CS ratio of 7:1 were shown to promote cardiomyocyte proliferation and elicit cell elongation and synthesis of troponin I and reconstruction of myocardia (Liu et al. 2017b). Conductive platforms can be proper candidates for cardiovascular tissue regeneration due to their structural similarity to native ECM of cardiac tissue. Nanofiber platforms based on PVA, CS, and various concentrations of carbon nanotubes (CNT) were prepared by electrospinning (Fig. 22.6a). The nanofiber scaffold loaded with 1% of CNT displayed a good elastic modulus (130 ± 3.605 MPa), water absorption, cell attachment, and cell proliferation up to 80% (Fig. 22.6b). The results indicated that the PVA-CS-CNT1 scaffold with 1% of CNT could be ideal for cardiac differentiation. Likewise, the real-time polymeric chain reaction (real-time qPCR) revealed a more expression of Nkx2.5, troponin I, and β-MHC of rat MSCs cultured on PVA-CS-CNT1 as compared to the control group after 10 and 20 days. The incorporation of CNTs meaningfully enhanced the conductivity and elastic modulus of scaffolds and the attachment of MSCs to platforms (Mombini et al. 2019). A biologically active macromolecular (e.g., PEG/CS) hydrogel was fabricated in the presence of spherical TiO_2 NPs to be used for the regeneration of cardiac tissue. The TiO_2 NPs were distributed in the hydrogel construction and markedly enhanced the mechanical strength, swelling ratio, and cell adhesion. The TiO_2-PEG/CS composite hydrogel presented a better synchronous function compared to the PEG/CS hydrogel (Liu et al. 2018). The researchers have made a copolymer containing PCL attached to a chemically improved CS-backbone (CS-g-PCL) in order to study the response of Wharton's jelly (WJ) MSCs. The results indicated a high cell attachment on the CS-g-PCL scaffold in the first periods in cultivation, while the growth was promoted after 3 and 7 days (Chatzinikolaidou et al. 2014).

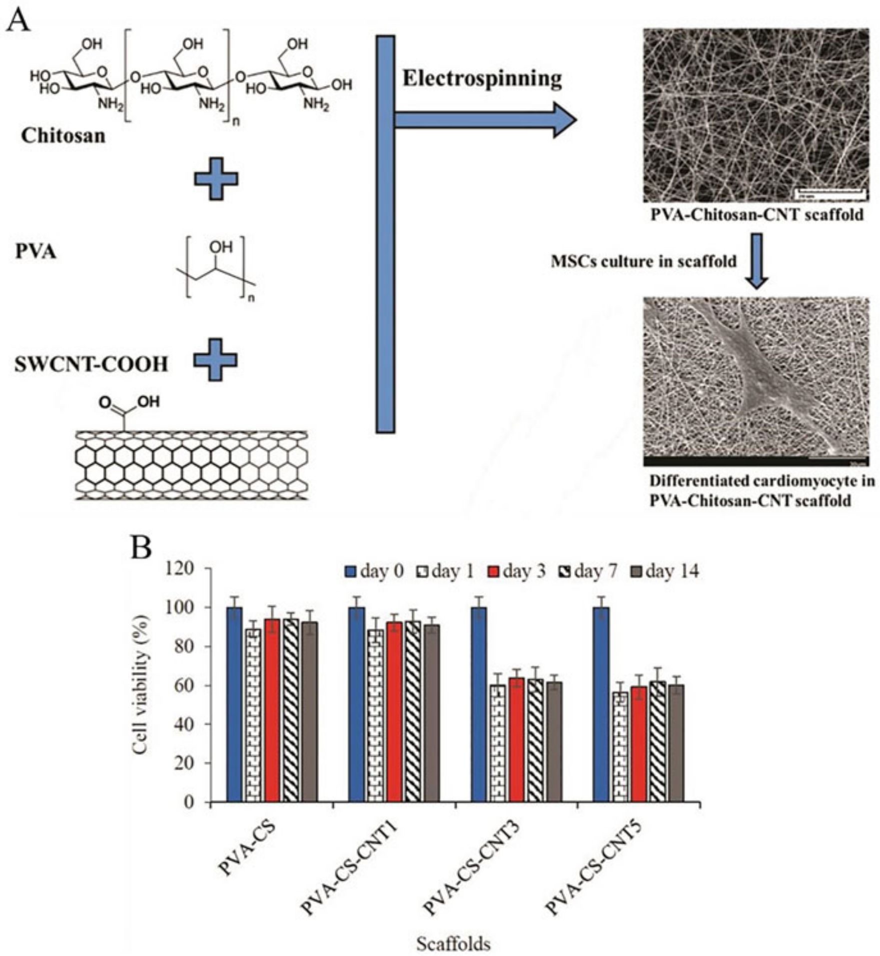


Fig. 22.6 (**a**) The schematic representation of nanofiber formulated used poly(vinyl alcohol) (PVA), chitosan (CS), and single-wall carbon nanotube (CNT). (**b**) Cell proliferation of MSC after treatment with various scaffolds after 1, 3, 7, and 14 days. (Reproduced with permission from Mombini et al. 2019, Copyright 2019 Elsevier)

22.4.4 Chitosan for the Regeneration of Vascular Tissue

The vascular system helps to transport vital nutrients and oxygen throughout the body and expunge carbon dioxide and metabolic waste. They also help to support the body and control the body temperature. Defects in the vascular system can impose a detrimental environment for the cell (Malda et al. 2007), which may be resolved by the vascular tissue engineering approaches to regenerate the blood vessels (Edelman 1999). The artificial vascular network includes proangiogenic inducer such as a VEGF and scaffold. Vascular tissue regeneration can provide important solutions in

a variety of medical complications (Chang and Niklason 2017). The combination of CS with some other synthetic and natural polymers was utilized as a platform for vascular tissue regeneration (Mombini et al. 2019; Goonoo et al. 2013; Rez et al. 2017; Zhu et al. 2014; Liu et al. 2013).

22.4.4.1 Chitosan-Based Natural Polymers for the Restoration of Vascular Tissue

Recently, novel human-like collagen (HLC)/CS hybrid platforms were prepared using various mixture ratios by crosslinking and examined for vascular tissue engineering. The SEM micrographs showed that when the mixture ratio was 0.02%, the porous morphology was uniform with 46 ± 9 mm in size. The HLC/CS (0.02%) scaffold promoted more human venous fibroblasts cell attachment and growth as compared to the pure CS, and 0.2% CS/HLC platforms (Zhu et al. 2009). An elastic composition of electrospun collagen, CS, and poly(L-lactic acid-*co*-ε-caprolactone) (P(LLA-CL)) at a ratio of 20:5:75 was shown to have a potential in vascular graft applications. The prepared scaffold showed the highest tensile strength, elongation at break, and elastic modulus (Fig. 22.7a). The burst pressure strength of the platform remained more than 3365 mmHg, and the compliance amount was 0.7%/100 mmHg (Fig. 22.7b, c). The endothelial cell growth was meaningfully promoted on the combined platforms as compared to the P(LLA-CL) sample (Yin et al. 2013).

22.4.4.2 Chitosan-Based Synthetic Polymers for the Regeneration of Vascular Tissue

Natural polymers can commonly be combined with synthetic polymers either by physical or chemical modification to fabricate improved tissue-engineered grafts with desired biological function, strength, and firmness. The vertically aligned CS/PCL nanofibrous platforms with CS and PCL by serial quantity arranging co-electrospinning approach. The heparinization and immobilization of VEGF in the gradient CS/PCL were utilized to create a blood vessel microenvironment. The results indicated that more heparin reacted with the CS nanofiber in gradient CS/PCL as compared to the homogenous CS/PCL nanofibrous scaffolds. The adhesion and growth of human umbilical vein endothelial cells (HUVEC) were promoted on the top side of the gradient CS/PCL platform. Therefore, the application of vertical gradient heparinized CS/PCL nanofibrous platforms with the cells could offer blood vessels with the native characteristics of anticoagulation property (Du et al. 2012). The PVA/CS hydrogels were prepared by freeze-thawing cycles for vascular tissue engineering. The surface of the samples was more improved with COL I adsorption and cultured with bovine aortic vascular smooth muscle and endothelial cells. The COL coating induced an enhancement in initial cell adhesion and growth (Vrana et al. 2008). The PLA fiber films were coated with CS and heparin by the electrostatic self-gathering (ESA) technique and used as platforms for vascular regeneration. The growth of vascular smooth muscle cells (VSMCs) on the pure PLA films was more than that of the covered fiber films. On the other hand, the growth of endothelial vascular cells (EVCs) on the covered films was more than that of the pure

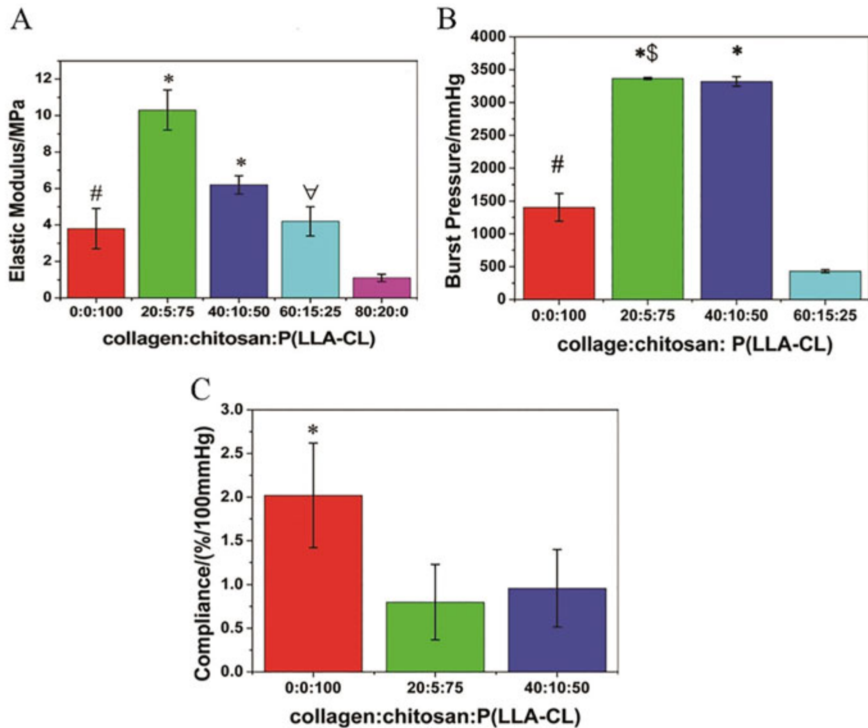


Fig. 22.7 (**a**) Elastic modulus of P(LLA-CL) in a different ratio. (**b**) Burst pressure of scaffolds with various COL:CS: P(LLA-CL) ratios. *shows meaningful difference from 0:0:100 to 60:15:25 ($p < 0.01$), # shows significant difference from 60:15:25 ($p < 0.01$). (**c**) Compliance values for the various COL:CS:P(LLA-CL) platforms. (Adapted with permission from Yin et al. 2013, Copyright 2019 Wiley)

PLA samples (Vrana et al. 2008). In 2017, Rez et al. reported on the production of tubular nanofibrous PCL/CS scaffold through the electrospinning method. The prepared scaffold promoted the adhesion and proliferation of HUVECs (Rez et al. 2017). The co-electrospinning human-like COL (HLC) with the CS/PLA was performed at 25 °C and normal pressure. The HLC/CS/PLA scaffold indicated more biomimetic construction than PLA, and the fiber diameters resembled the native ECM of vascular tissue. Besides, the HLC/CS/PLA scaffolds showed more hemocompatibility as compared to other ones (Zhu et al. 2014).

22.4.5 Chitosan for the Neuron Tissue Engineering

The nervous system is composed of neural tissue, also identified as nerve cells, which receive and transfer signals. Further, neuroglia, similarly recognized as glial cells or glia, support the propagation of the nerve impulse and support the neurons

(Stewart and Wiley 1981). Nerve tissue regeneration is one of the greatest potential approaches to revitalize the central nervous system (CNS) in tissue engineering and regeneration (Navaei-Nigjeh et al. 2014). Neural tissue regeneration emphasizes the advancement of biomaterials that could favor the restoration of neurons after trauma and the damages induced by the degenerative disorders (Matyash et al. 2012). In general, natural polymers such as CS provide viability and bioactivity, whereas synthetic polymers offer mechanical strength and organizational firmness. Therefore, a blend of the two types of polymers might improve the polymeric conduits to mimic the natural biological condition of the normal neural tissues and enhance the restoration of injured nervous tissues (Boni et al. 2018). A CS-based photocrosslinkable hydrogel system was successfully fabricated for neural tissue engineering (Valmikinathan et al. 2012). Neural stem cells (NSCs) cultivated within the photocrosslinkable CS hydrogels were found to promote the differentiation into the tubulin-positive neurons and astrocytes. As a result, significant enrichment was observed in the expression of the glial marker and glial fibrillar acidic protein (GFAP) in the cells cultivated using the CS hydrogels after 14 days.

22.4.5.1 Chitosan-Based Natural Polymers for the Regeneration of Neural Tissue

In a study, CS-Alg hydrogel was prepared via robust electrostatic interactions between the CS and Alg molecules. The SEM analysis and WCA test indicated the inner porous arrangement and hydrophilic behavior of the CS-Alg hydrogel. The cell growth was found to be fine on the CS-Alg hydrogel that might provide a suitable platform for neural tissue regeneration (Wang et al. 2017). The CS/Gel porous platforms comprising HA and heparan sulfate (HS) were produced by the freeze-drying method. The CS/Gel/HA/HS composite platforms displayed a uniform and interconnected porous structure with porosity above 96%. The addition of HA and HS into the platform considerably enhanced the cellular attachment potential and facilitated the proliferation of cells in 3D conditions (Guan et al. 2013). In addition, researchers prepared the blend of agarose with CS gel composite to enhance the proliferation of neural cells in a 3D manner (Cao et al. 2009). An ideal range of CS concentration was around 0.66–1.5 (wt%), which was found to promote neural cell adhesion and differentiation (Cao et al. 2009). Icariin, a prenylated flavonol glycoside, has been extracted from *Epimedium brevicornum Maxim* and utilized to relieve atherosclerosis (Xu et al. 2009). As a result, the CS/COL/icariin composite was fabricated by mixing and crosslinking CS with COL and icariin to serve for nerve regeneration. After the cultivation, cells presented orders of spiral-resembling and aligned analogously with the channel construction on the surface of the composite. Collectively, the CS/COL/icariin composite scaffold showed the highest growth of Schwann cells and PC12 cells and substantial elongation in the average neurite length (Yang and Di Chen 2013).

22.4.5.2 Chitosan-Incorporated Synthetic Polymers for the Regeneration of Neural Tissue

Recently, a hydrogel system comprising CS-g-oligo(L,L-lactide) copolymer, PEGDA, and Irgacure 2959 photosensitizer was fabricated by a molding method. The results indicated that the prepared hydrogel could improve the efficient metabolic function of neonatal rat cortical cells. The hydrogels did not interrupt intracellular calcium signaling and metabolic function of the cultivated neuronal cells (Grebenik et al. 2020). The PHB/CS electrospun aligned, and random fibrous scaffold was prepared for nerve tissue regeneration. It was shown that CS increased surface porosity and the WCA was reduced for both structures. The MTT results revealed a higher growth of the B65 cells on the electrospun-aligned PHB/CS platforms as compared to the random PHB/CS platforms. Besides, the SEM images indicated that the nerve cells were aligned in the direction of the fiber arrangement. Also, the aligned PHB/CS fibrous platforms exhibited a milder speed of degradation than the random PHB/CS fibrous platforms (Karimi Tar et al. 2020). In addition, fibrous conductive PVA/poly(3,4-ethylenedioxythiophene) (PEDOT) platforms were prepared in various structures. PVA scaffolds comprising 1 wt% of PEDOT was efficient in improving the conductivity of the non-conductive polymers. Moreover, neural differentiation of MSCs was promoted due to the electrical stimulation on the PEDOT platform and the presence of differentiation factors, including retinoic acid (RA), 3-isobutyl-1-methylxanthine, and Forskolin. The cell spreading was improved for the scaffold containing PEDOT (Fig. 22.8a–f). Furthermore, the expression of nestin gene in the platforms with conductivity was higher than the other scaffolds (Fig. 22.8g) (Babaie et al. 2020). Notably, 2% CS together with Gel and PLL were found to be the best blend to serve neural tissue regeneration. All blends formed polyelectrolyte complexes due to the electrostatic interactions. Unlike the CS-Gel-PLL scaffold, the CS-Gel system presented a meaningfully minor pore size. Astrocytes and olfactory ensheathing cells cultivated on the Gel and CS-Gel films displayed compacted and spindle cell morphologies. Neurites from the dorsal root ganglia prolonged the most on the CS-Gel films. Collectively, the CS-Gel blend was found to have a great potential for neural regeneration (Martín-López et al. 2012).

22.5 Final Remarks and Future Perspective

The paramount necessity in tissue regeneration is to ensure the safety of the cell therapy modality. The success of a cell therapy and tissue engineering strategy is largely dependent on several parameters, including the quality of cells, the efficiency of scaffolds in accommodating cells, the compatibility of the scaffolds with the microenvironment of the body, and the desired functions of bioactive substances incorporated in scaffolds. The advent of advanced biomimicking materials and artificial scaffolds has revolutionized tissue engineering approaches. Many fascinating advancements have been developed and employed to regenerate damaged tissues and recover their functions. Of these advanced biomaterials, chitosan-

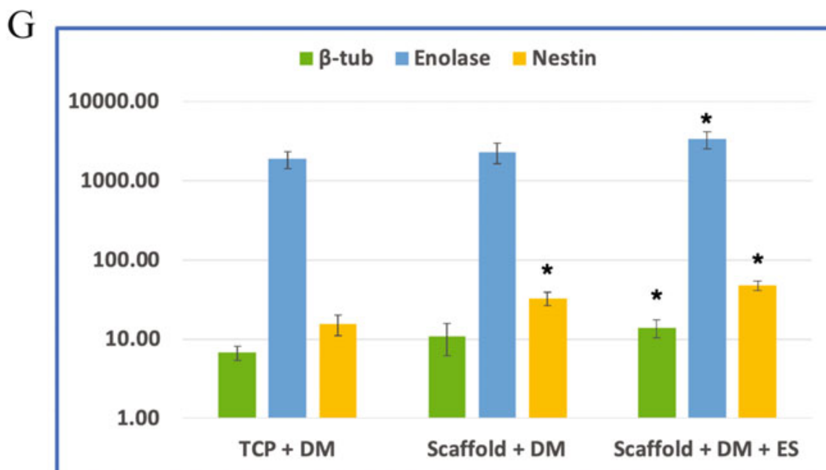


Fig. 22.8 The SEM micrographs of the cell adhesion after 7 days, (**a**) the pure PVA, (**b**) PVA/PEDOT (0.1), (**c**) PVA/PEDOT (0.3), (**d**) PVA/PEDOT (0.6), (**e**) PVA/PEDOT (1), and (**f**) PVA/PEDOT (3). (**g**) The expression of neural marker genes in various treatments. * represents

based scaffolds have been developed with the incorporation of other natural and synthetic polymers to serve as a platform for the regeneration of bone, cartilage, cardiac, vascular, and neuron tissues. Of various biopolymers, CS is a distinct natural polymer that has been widely utilized not only in tissue regeneration but also in the delivery of small and macromolecules to cells/tissues. The application of CS for developing bioactive scaffolds for the restoration of different tissues can be improved via the incorporation of other polymers depending on the purpose of tissue engineering. As a mucoadhesive biopolymer, CS can interact with the negatively charged cells/tissues through electrostatic interactions that can be in favor of cellular adaptation in the CS-based scaffolds. This biopolymer has a unique potential for biomedical usages, especially in tissue regeneration. Based on its potential for functionalization and blending with other entities, CS-based scaffolds appear to be a promising context to serve the defected tissue. Despite all the signs of progress made during the past several years, still little is known about the impacts of the physicochemical structure of scaffolds on the physiological behaviors of the embodied cells in the implanted microenvironment. Such a dilemma might be due to the complexity of the biological settings and the diversity of factors that can influence cell therapy and tissue engineering. It is envisioned that all these questions will be addressed very soon, while the clinical applications of tissue engineering and regeneration should be ethically and technically approved.

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Fig. 22.8 (continued) statistical significance. *DM* differentiation media, *ES* electrical stimulation, *SEM* scanning electron microscope, *PVA* polyvinyl alcohol, *PEDOT* poly (3,4-ethylenedioxythiophene). (Adapted with permission from Babaie et al. 2020, Copyright 2020 Elsevier)

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23 Applications of Decellularized Extracellular Matrix for Regenerative Medicine

Zihan Chai and Zongjin Li

Abstract

With a three-dimensional connective fiber network, the decellularized extracellular matrix (dECM), which is composed of various proteins, glycosaminoglycans, proteoglycans, collagens, and elastin, has been shown to play critical roles in supporting cells as well as provide the metabolic site for cells. However, the application for tissue repair of dECM is limited owing to the source of dECM and the lack of bioactive molecules. The combination of stem cells and decellularized materials will affect the differentiation of stem cells and regulate the function of stem cells by microenvironmental factors. In this chapter, we will focus on the current understanding of the characteristics and therapeutic mechanisms of dECM and discuss various decellularized methods of multiple organs. Finally, we will provide a detailed introduction of existing processes in the implementation of dECM with stem cells to alleviate skin, cartilage, heart, kidney, liver, lung, and dental pulp injuries.

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Keywords

Stem cell · Regenerative medicine · Extracellular matrix · Decellularization · Hydrogel

23.1 Introduction

Tissues and organs are both composed of cellular structures and noncellular structures. The noncellular structure is called the extracellular matrix (ECM) that contains some cell components secreted on the surface or intercellular substances, which constitute the three-dimensional network structure. ECM is a heterogeneous connective fiber network composed of collagen, elastin, proteoglycan, glycosaminoglycan, etc. It has the function of connecting and supporting cells and the basic framework and metabolic site for cell attachment. The morphology and function of the constructed tissues are always influenced by the ECM of them. As a result, different ECMs provide good living conditions for different types of cells, which is significant to maintaining the homeostasis and balance of the organism (Mouw et al. 2014). More than that, it can regulate the processes of cell differentiation, cell proliferation, cell growth, and cell migration through mediating the signal transmission and information exchange of cells. In these processes, ECM carries many small molecules that are involved in vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF). ECM plays an irreplaceable role in tissue regeneration and repair (Paolillo and Schinelli 2019).

Decellularized extracellular matrix (dECM) removed the antigens and retained biological activity ingredients via a physical or chemical method of acellular treatment for allograft tissue. The tissue-specific dECM material is a complicated structure composed of various protein components, growth factors, and small molecules. The component structure is always in dynamic change and plays an essential role in cell physiological functions. The hypothesis of the acellular tissue matrix was first put forward by Peerut in 1955, whereas it failed to digest cartilage with trypsin. In 1975, Meezan made the acellular basement membrane of some tissues using chemical detergents; thus, the development of decellularized materials was started. After more than 10 years, a decellularized dermal matrix called AlloDerm was first approved for clinical in the United States (Wainwright 1995). In the late 1990s, with the rise of the tissue engineering field on a global scale, dECM has aroused the interest of many researchers, and then it gradually becomes one of the hotspots in tissue engineering (Fig. 23.1).

Theoretically, the ideal dECM should be removed from all cellular components through several specific methods and retain all ECM components. Wilson observed the decellularized canine common carotid artery with both optical and electron microscopes, both of which result in a relatively complete structure holding collagen and elastin, etc. It can be found that the soluble proteins and cell components have been successfully removed after chemical treatment, leaving some insoluble matrix

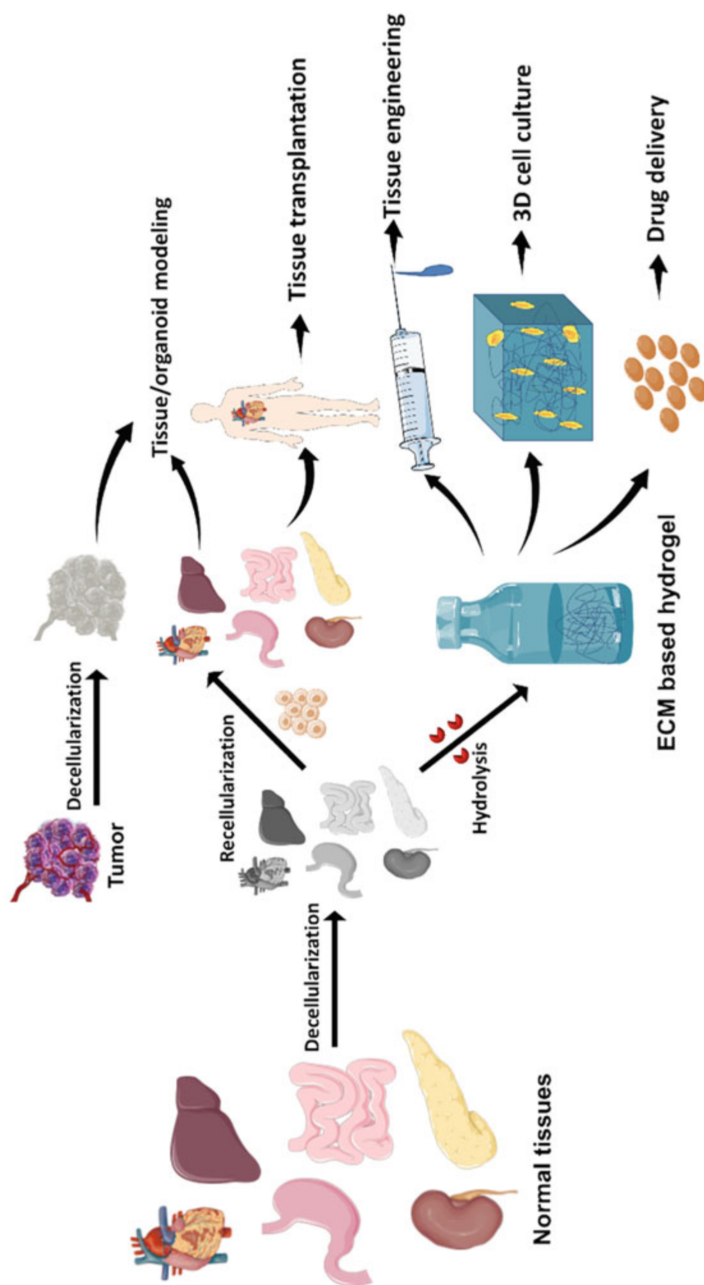

Fig. 23.1 The application of the decellularized materials. The decellularized tissues can be acquired from normal tissues by a suitable decellularization method. It has a recellularization effect for tissue reconstruction and transplantation and a gelation effect forming ECM hydrogels for tissue engineering, 3D cell culture, and drug delivery. (Reproduced with copyrights permission from Yao et al. 2019)

components that have a relatively complete appearance of the original tissue. The bioactivity and biocompatibility of the injectable dECM material indicate that it can be used as the best candidate material for cell delivery. By detecting the capillary density, it can be found that the injectable dECM material can be a good material for vascular reconstruction (Fu et al. 2019). ECM not only plays a role as a structured scaffold but also regulates cell phenotype and function. The dECM materials are considered ideal scaffolds and have been widely used in the research of constructing biomimetic cardiac tissue. In the heart-related research, it has been found that the extracellular matrix activated fibroblasts and promoted the proliferation and differentiation of vascular endothelial cells by regulating cytokines and growth factors (Chen et al. 2014a). In the study of myocardial injury, there will be significant changes in the ECM network after myocardial infarction. ECM can repair part of the infarcted heart when the subsequent inflammation and proliferation stages are coming (Frangogiannis 2017). Because of low immunogenicity and high biological activity, it has been used in many applications of tissue repair and regeneration, including vascular repair, artificial heart valve, urinary system repair, skin wound healing, and so on (Liu et al. 2013).

In recent years, with the widespread development of tissue engineering, many decellularized biomaterials using stem cells have been emerging. Generally, the dECM plays the role of a 3D natural scaffold for various cells, showing satisfying regeneration and repair capability. ECM derived from stem cells serves as a candidate culture substrate for chondrocyte and cartilage regeneration of the candidate bioactive material (Yang et al. 2018). It can be seen that the ECM of stem cells can be used as a culture substrate for the damaged tissue cells to promote their implantation and proliferation, as well as be used as a culture scaffold to provide a suitable microenvironment (Mazini et al. 2020). More than that, the combination of stem cells and decellularized materials can influence the behavior of stem cells, including promoting the differentiation of stem cells to various tissues and regulating the function of stem cells by microenvironmental factors. Thus, we will first introduce dECM extracted from stem cells and then depict the various functions of dECM combined stem cells containing the therapeutic effect for tissue injury, supportive effect as scaffold materials, and substitution effect of implantation fillings in this chapter. Moreover, we will describe recent strategies to decellularize cells via physical, chemical, and biological methods and several problems during the decellularization process. Finally, we will summarize the current data on the applications of dECM combined stem cells employed in tissue engineering for diverse organs.

23.2 Different Functions of dECM

With the development of more than 40 years since 1975, there have been some dECM products related to the skin mucosal system, cardiovascular system (pericardium, heart valve), and urinary system (bladder, ureter). Although different dECMs from different tissues have tissue specificity and the ECM components and growth

factors contained after decellularization are not the same, the ultimate goal of all tissue engineering studies using ECM is to repair or replace the impaired tissue (Marinkovic et al. 2021). At present, there are three main functions of the dECM: (1) the characteristics of low immunogenicity and high bioactivity to directly repair or replace damaged tissue (2) as a scaffold material for tissue engineering, supporting stem cells repairing the damaged tissues through the recellularization of biological scaffolds; and (3) used in plastic surgery as natural biomaterials implanted and filled. Among them, the most in-depth research is the second one. Combined with stem cells and dECM, this composite biomaterial has the cell growth microenvironment of natural ECM, which can induce the differentiation of stem cells (Rao Pattabhi et al. 2014). Therefore, it has been widely studied, which can be used for the repair and reconstruction of various damaged tissues and organs.

23.2.1 Application of dECM for Tissue Repair

For skin burn wounds, a product called AlloDerm was developed by LifeCell in the United States in the early 1990s. With the strategy of transplant a composite material of dermal matrix and autologous mesh blade thick skin, AlloDerm can provide a microenvironment rich in nutrients and a suitable concentration of oxygen for cells in the injured area to promote local angiogenesis and granulation tissue regeneration, which has been used in clinical deep burn wounds (Wainwright and Bury 2011). Compared with the expanded polytetrafluoroethylene patch used to repair the abdominal wall defect of rats, the result shows that the allogeneic decellularized dermal matrix had more advantages for this repair. The extracellular matrix is often used as a method of direct replacement transplantation to repair the bladder, ureter, and urethra (Eitan et al. 2010b). Removing 40–50% of the bladder in animal experiments and taking the dECM of the allogeneic bladder for repair, the regenerated bladder is difficult to distinguish from the original bladder in the 12 weeks after the operation. Subjected to left pulmonary valve replacement surgery for the dog, the ultrasound results showed that the valve was in good shape in 1 month after the operation.

Due to the unique structure of the dECM, most of them are studied as scaffolds to support tissue repair, making it easier for the corresponding cells to migrate into it. In subsequent studies, there are only a few reports on the application of bioactive small molecules of dECM for repair directly. It is worth noting that the perinatal tissues are recognized as a natural treasure house of stem cells, due to the rich stem cells and abundant growth factors of dECM in perinatal tissues (Rao Pattabhi et al. 2014). Three different perinatal tissues including the placenta, umbilical cord, and amniotic membrane are, respectively, subjected to decellularization treatment. After testing the decellularization effect, they were made into dECM hydrogels by enzymatic method. The efficacy has been proved in the mouse full-thickness wound model, which is a typical example of stem cell-derived dECM directly applied to tissue damage repair. It is believed that the dECM biomaterials of perinatal tissues will be expected to be used in the repair of other tissue injuries such as acute kidney injury, liver injury, lower limb ischemia, and so on shortly.

23.2.2 dECM Serves as Scaffolds

Tissue engineering, also known as "regenerative medicine," was proposed by the National Science Foundation of the United States in 1987. Scaffold materials with three-dimensional structures are one of the core contents of tissue engineering (Cossu et al. 2018). Existing tissue engineering scaffolds can be divided into three types: natural biomaterials, synthetic organic materials, and inorganic materials. Compared with the latter two types of materials, multiple methods such as physics, chemistry, and enzymatic hydrolysis are used to treat whole organs (heart, liver, lung, kidney, etc.) to obtain a three-dimensional biological scaffold material. This material, called dECM scaffold, is one of the best choices for preparing biomimetic scaffolds because of no cell retention but almost the whole natural extracellular matrix. Allogeneic organ transplantation has the weakness of donor shortage, immunological rejection, and lifelong immunosuppression (Startseva et al. 2019). This natural biological dECM scaffold has better histocompatibility that can provide cells with a microenvironment similar to that in the body, which is conducive to cell proliferation and differentiation and hopefully forms the same function as the original organ. As technology continues to mature, it is expected to become an important source of tissue repair and reconstruction or organ replacement in the future.

The decellularized tissue was obtained from natural ECM. After being implanted in the body, it can be gradually replaced by the patient's tissue over time, or even it will grow with the growth of the patient's tissue when in good condition. In choosing stem cells from different sources to embed in dECM biological scaffolds, the different matrix components will induce stem cells to exhibit different functions, differentiate, and proliferate in different directions, thereby effectively repairing and even forming regeneration. At present, a variety of allogeneic or xenogeneic dECM scaffolds have been successfully prepared for the skin, blood vessels, cartilage, heart, liver, kidney, lung, and other organ tissues making significant progress in tissue engineering (Kc et al. 2019). According to the report, donor organs such as the heart, liver, lung, and kidney provide acellular ECM-based scaffolds through the decellularization process, which have been shown the potential for recellularization of selective cell populations (especially stem cells). The dECM of stem cells has also become an effective biological scaffold for controlling the fate and function of stem cells in the tissue. The interaction between cells and ECM is considered an essential process that determines cell growth and triggers the transformation of cells from proliferation to tissue structure formation (Song and Ott 2011). To understand the underlying mechanisms and matrix characteristics that can guide stem cells to a specific developmental lineage, it is important to study the interaction between stem cells and the matrix.

The ECM has provided an environment and intermediary for the interaction between cells and matrix for tissue regeneration. The spleen of the rat was taken into a decellularized scaffold, the shape of the spleen was preserved. And then, the scaffold was recellularized with BMSCs, which result showed that BMSCs could locate and survive in the ECM scaffold of the spleen. This study suggests that the

three-dimensional decellularized spleen scaffold can support the replantation and differentiation of BMSCs (Xiang et al. 2015). It can reconstruct a new tissue by co-cultivating multiple types of tissue cells in a reasonable and orderly manner, which is also one of the most effective purposes of tissue engineering. Decellularize the understructure of the amniotic membrane, that is, the outermost layer of the umbilical cord with a certain tough structure, and then composite MSCS to treat skin injuries. Stem cells can also be used as auxiliary cells to promote tissue recovery, metabolism, growth, and repair, emphasizing their importance to organ regeneration (Paschos et al. 2015). If the stem cells can proliferate and differentiate into the corresponding damaged tissue cells in the dECM of different tissues, it will provide more imagination space for tissue engineering. Although this approach seems to be a long way from clinical, there are still researchers who have successively carried out experiments in the clinic to lay the foundation for the treatment of patients in the future.

23.2.3 dECM for Implantation Fillings

The dECM began to be used for burn wound coverage in the 1980s. Since then, it has been widely used as the implantation filling for a variety of plastic repair operations, including breast reconstruction, face and neck plastic surgery, abdominal wall reconstruction, and scar treatment, which is crucial in the field of plastic surgery. Because bio-scaffolds made of dECM can quickly integrate into living tissues as well as have high ductility and low-induced inflammation, they are very popular in various surgical operations, especially in the mammaplasty field (Gravina et al. 2019). Breast plastic surgery includes breast cosmetic surgery, breast repair surgery, prosthetic reconstruction surgery, and so on. There are many related complications, such as capsular contracture, bilateral asymmetry, pain and infection, exposed prosthesis, etc. Among them, capsular contracture can significantly affect the softness and beauty of the breast. Studies have suggested that the underlying cause may be the fibrosis process caused by various factors, but the exact mechanism is still unknown (Henderson et al. 2020). At present, the application of ACTM is expected to become a new method for treating capsular contracture. In breast repair surgery, the reoperation rate of traditional surgery is 20–40%, while dECM has the characteristics of low recurrence rate, good aesthetic effect, and high overall patient satisfaction. Various studies have shown that this kind of natural biological material has practical significance for breast repair and reconstruction.

The dECM is mainly used to fill the skin and soft tissue defects that cannot be solved by flap technology in ophthalmology and plastic surgery. In the treatment of dECM for lacrimal duct reconstruction, the method selected five patients (five eyes) with partial or complete lack of lacrimal duct. Four cases (four eyes) had lacrimal duct trauma, and one case (one eye) had a lack of congenital lacrimal drainage system. The flush test confirmed that five tear ducts were effective, and five patients all successfully reconstructed the lacrimal duct, which proved the reliability and effectiveness of dECM (Chen et al. 2014b). In the other fields, rhinoplasty, one of the

most common plastic surgery operations in the Asian Youth Injection Center, is a surgical operation to reshape the nose. It can correct the natural defects or injuries of the nose and even help to relieve some breathing problems. However, the contour of the allograft becomes evident over time in rhinoplasty patients, which is an important reason for revision. dECM has been shown that using it to cover the silicone prosthesis is an alternative method to reduce the visibility of the prosthesis contour (Suh et al. 2017).

Malignant tumors of the neck include laryngeal cancer, hypopharyngeal cancer, maxillary sinus cancer, and tracheal tumors causing soft tissue damage after resection (van Oldenrijk et al. 2010). For this, dECM can solve the problem of insufficient soft tissue in the area that needs to be repaired. In 40 cases of hypothalamic hypopharyngeal reconstruction surgery from February 2011 to October 2012, the application of dECM in hypopharyngeal reconstruction led to stable wound healing and low complication rate (Li et al. 2016). The surgery of dECM is easy to operate with many advantages involving the shortened operation time, low risk of postoperative infection, reduced fistula, and stenosis. In addition to the mentioned above, the dECM is also used as an implantation filling, such as the reconstruction of gingival recession, cleft palate repair, vagina reconstruction, etc. All these play a role in repair and aesthetics effect by filling tissue defect.

23.3 Strategies in Decellularization

ECM materials have been widely used in clinical treatment and basic research in vivo and in vitro. The quality of decellularized technology is essential. The incomplete decellularization effect will cause a series of immunological rejections after the material is implanted in the body, which ultimately leads to the failure of treatment. The decellularization process is the core of the removal of immunogenicity as well as the key point of the dECM material preparation process. Natural ECM materials can be obtained and prepared from isolated tissues by decellularization methods, including physical methods, chemical methods, and biological enzymatic methods.

23.3.1 Physical Methods for Decellularization

Physical methods include mechanical shock, mechanical scraping, repeated freezing and thawing, electroporation, high pressure, ultrasound, superfluid, etc. The mechanism is mostly destructing the cell membranes directly so that the tissue can release cell contents and remove the cells (Table 23.1). The basic principle of the repeated freezing and thawing method is to produce ice crystals in the cells through repeated freezing, thereby destroying the cell membrane and causing cell lysis (Nonaka et al. 2014). Studies have pointed out that using -80 °C low temperature or liquid nitrogen storage will produce larger ice crystals in the cells during the rapid freezing process, leading to destroy the cell membrane structure and promote to the removal

Table 23.1 Physical methods for tissue decellularization

Method	Principle	Advantages	Disadvantage	Applicative tissues
Snap-freezing method	Procreant ice crystals destroy the cell membrane	Remove cells efficiently	Require the perfusion to take cells out of the matrix	Tendons, ligaments, and nerves tissues
Mechanical force (scraping/oscillation)	Mechanical external force to rupture the cells	Prevent the tissue from contacting with chemical reagents	No uniform standard for mechanical force (easy to destroy the ECM components in the decellularized process)	Mechanical scraping: small intestine Mechanical oscillation: high toughness tissues (heart, liver, blood vessels)
High-pressure method	A series of gradient pressures help cell lysis	Remove cells efficiently	The equipment is expensive	Bladder tissue
Ultrasound method	Ultrasonication	Remove cells efficiently	Easy to destroy the submicroscopic structure of the ECM	Umbilical cord tissue
Supercritical fluids method	The low viscosity and high flux characteristics of supercritical fluids	Both sterilization and virus inactivation by supercritical CO_2 adopting mild conditions	Limited applicability	Aortic tissue
Electroporation method	Form micropores on the cell membrane	Slowly release cell components from the cells	The structure and function of small molecules of ECM will be destroyed because of the electric field	Carotid artery tissue
Rinsing and shaking method	Combine the chemical reagents	Remove cells efficiently	A longer rinsing time and higher conditions for tissue thickness	Blood vessels, muscles, peripheral nerves, skin, bladder tissue

of the tissue cells. However, this method also requires perfusion to take cells out of the matrix so that they achieve the goal of decellularization. This method is mainly suitable for the decellularization of tendons, ligaments, and nerves.

The mechanical scraping method removes the main cell components and retains the submucosa as an ECM material by scraping the mucosal layer, serosal layer, and muscle layer, which is mainly used for the decellularization of the small intestine.

Similar to the mechanical scraping method, the mechanical oscillation method also needs a mechanical external force to rupture the cells on the surface to remove the cells. It is mainly suitable for the heart, liver, blood vessels, and other tissues with high toughness. This method can prevent the tissue from contacting with chemical reagents to cause the degradation of the ECM components. However, the disadvantage is that there is no uniform standard for mechanical force, which is very likely to destroy the ECM components in the decellularized process (Crapo et al. 2011). In later research, the combination of mechanical oscillation and chemical methods can speed up the decellularization speed and get a better decellularization effect. For example, combined with mechanical oscillation and a chemical detergent, the process of decellularization of the cornea can have full contact with this chemical detergent, getting a more fully decellularized corneal tissue (Hopkinson et al. 2008).

The high-pressure method can apply a series of gradient pressures to the tissue during the decellularization process to increase cell lysis efficiency and detach cell debris from the tissue. Yoshihide used high static pressure technology to effectively remove cells while maintaining the arrangement of collagen fibers. The decellularized cornea obtained by this method can be used as a scaffold for corneal tissue regeneration (Hashimoto et al. 2010). This method does not require detergents and has no cytotoxicity, but the equipment is expensive so that the preparation cost is too high. Applying the ultrasound method to remove cells, the shortage is that this method is easier to destroy the submicroscopic structure of the ECM although it can remove cells efficiently. This method is mainly suitable for tissues such as the umbilical cord. The low viscosity and high flux characteristics of supercritical fluids can be used as a simple decellularization method (Guler et al. 2017). With no residue solvent and dissolved grease, supercritical CO_2 adopts mild critical coordinates, such as a pressure of 7.38 MPa and a temperature of 31 °C, so it has the advantages of both sterilization and virus inactivation (Chou et al. 2020). Due to the high cost of technical equipment, the applicability of supercritical fluids in the decellularization remains to be determined.

The electroporation method causes autolysis of cells by forming micropores on the cell membrane. This method can slowly release cell components from the cells (Brun et al. 2019). Nevertheless, under the disturbance of the electric field, the structure and function of small molecules of ECM could be destroyed, so only relatively small tissues such as carotid artery can be applied. The rinsing and shaking method can efficiently remove cells in thinner tissues, such as blood vessels, muscles, peripheral nerves, skin, bladder, etc. It requires a longer rinsing time and has higher conditions for tissue thickness, so it is not widely used right now. Physical methods are generally unable to completely remove cell components while retaining the ECM components due to the excessively violent decellularization process and other reasons, so further chemical or biological methods are required.

23.3.2 Chemical Methods for Decellularization

Common chemical reagents include acid-base reagents, ionic or nonionic detergents (SDS and Triton X-100), cross-linking agents, chelating agents, hypertonic or hypotonic solutions, and other inorganic or organic reagents (Gilbert et al. 2006). The mechanism includes the destruction of connections between cells or between cells and ECM, enabling cell lysis to dissolve cytoplasmic components and destroy the connections between nucleic acids, proteins, and lipids in the cells (Table 23.2). In the process of decellularization, alkali treatment and acid treatment can dissolve cell membranes and organelles. The main acid reagents include peroxyacetic acid, acetic acid, hydrochloric acid, sulfuric acid, etc. The advantage of this method is that some reagents with disinfection function can enter the tissue to make it clean. By decellularizing four kinds of horse synovial tissues, it was found that incubating with 0.1% peroxyacetic acid twice is the best way to produce horse synovial ECM, which can remove most of the DNA and retain the villa-like structure of the synovium (Reisbig et al. 2016). The disadvantage is that it may damage some of the more important components of the ECM such as collagen, GAGs, growth factors, etc. This will cause their structural change and content to decrease, failing the ECM function. For denaturing cell chromosomes and plasmids, alkaline reagents include ammonium hydroxide, sodium hydroxide, and calcium hydroxide, which are mainly used to remove hair in the dermal tissue (Kawecki et al. 2018). Similar to acid treatment, the wrong concentration of the alkali reagent will cause a lot of ECM material to degrade.

Ionic detergent is a kind of strong detergent, which has effects on cytoplasm, cell membrane, and the cell nucleus. It can destroy cells and make the decellularization effect stronger. However, having damage to the remaining tissues, it is extremely easy to denature extracellular proteins. Common ionic detergents involved sodium dodecyl sulfate (SDS), sodium deoxycholate, and so on (Mendoza-Novelo et al. 2011). For the first time, Uygun used gradient perfusion SDS to intubate the hepatic portal vein and then prepared a decellularized scaffold for rat liver (Uygun et al. 2010). The decellularization effect of SDS has been proven effective in a large number of studies. Since SDS is often playing a role in denaturing and decomposing proteins in polyacrylamide gel electrophoresis, it will destroy the covalent bonds between proteins. In the process of decellularization, to reduce the negative impact of SDS on the ECM, it is necessary to reduce its concentration as much as possible, reducing the soaking time of the tissue in it. Some evidence has shown that SDS, as a powerful detergent, is difficult to completely remove from the tissue soaked in it. Such residual SDS will reduce the structure of ECM, reduce the retention of important components of the ECM, and even affect the growth and reproduction process of stem cells in it, thereby reducing its role in tissue damage repair (Woods and Gratzer 2005).

Nonionic detergents having relatively mild effects on the structure of ECM are widely used in the decellularization of various tissues and organs. It will only leave protein interactions and destroy lipid interactions, lipid-DNA interactions, and lipid-protein interactions, so that protein molecules and enzymes in the ECM will have

Table 23.2 Chemical methods for tissue decellularization

Method	Principle	Reagents	Advantages	Disadvantages
Alkali treatment	Dissolve cell membranes and organelles	$NH_3 \cdot H_2O$, NaOH, $Ca(OH)_2$	Remove cells efficiently	The wrong concentration (too high) will cause a lot of extracellular material to degrade
Acid treatment	Dissolve cell membranes and organelles	CH_3COOOH, CH_3COOH, HCl, H_2SO_4	Some reagents with disinfection function can enter the tissue to make it clean	May damage some of the more important components of the ECM
Ionic detergent	Destroy protein-protein interactions, lipid interactions, lipid-DNA interactions, and lipid-protein interactions	Sodium dodecyl sulfate (SDS), sodium deoxycholate	A kind of strong detergent, which has effects on cytoplasm, cell membrane, and cell nucleus	Destroy protein-protein interactions and difficult to completely remove from the tissue
Nonionic detergents	Destroy lipid-lipid interactions, lipid-DNA interactions and lipid-protein interactions	Triton-100	Have relatively mild effects on the remaining protein-protein interactions	Make lower laminins
Zwitterionic detergents	Both properties of ionic detergent and nonionic detergents	CHAPS, sulfobetaine	Protect natural ECM contents	The impact of ECM is smaller than ionic detergent but bigger than nonionic detergents
Hypertonic and hypotonic solutions	Osmotic shock to dehydrate or swell cells	–	–	The residual DNA is difficult to remove from tissues
Chelating agent	Bind divalent cations to cell adhesion sites, making it difficult for cells to attach to the ECM and fall off	EDTA, EGTA	Comparatively moderate condition than other chemical reagents	The residual chelating agent can destroy the ECM

their functional conformations. Triton-100 has less damage to extracellular protein molecules, glycosaminoglycans, and growth factors than SDS (Ren et al. 2013). For different tissues, there is no uniform standard for the suitable concentration of Triton-100. It is necessary to select the most appropriate decellularization concentration according to the thickness of the tissue structure. Octyl glucoside, a new

nonionic surfactant with low surface tension, has characteristics including good water solubility, delicate and stable, nontoxic, and quick explanation. Studies have shown that compared to SDS, the application of this reagent for decellularization can remove cells better and has higher biological activity (Dong et al. 2013).

With a net charge of zero, zwitterionic detergents can protect the natural structure of the protein during decellularization. The most commonly used are 3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS), sulfobetaine, etc. Compared with ionic detergents and nonionic detergents, zwitterionic detergents can remove cells but retain more ECM components, removing 95% of the cells in the tissue (O'Neill et al. 2013a). The principle of hypertonic and hypotonic solutions is an osmotic shock to dehydrate or swell cells and break away from the surrounding tissue (Woods and Gratzer 2005). The hypertonic solution can increase the solubility of DNA, which can remove the residual nucleic acid components in the later stage of decellularization. More conducive to cell lysis, the hypotonic solution destroys the cell membrane through the osmotic effect by combining the use of detergents. This method usually requires a combination of biological, enzymatic, or chemical methods to remove the cell debris. Binding firmly to the central metal ion, the chelating agent always forms a cyclic complex. EDTA and EGTA are the most common chelating agents, which bind divalent cations to cell adhesion sites, making it difficult for cells to attach to the ECM and fall off (He and Callanan 2013). Since ethylenediaminetetraacetic acid and ethylene glycol tetraacetic acid are often mixed with other decellularization reagents, their direct effects on the ECM have not been reported before.

23.3.3 Biological Methods for Decellularization

Common enzymes for decellularization include various nucleases (DNase and RNase), protease (trypsin), and esterase (phospholipase A). The mechanism of them is to hydrolyze nucleic acids and proteins, so that they remove DNA and destroy the connection between cells or cells and ECM. Trypsin is the most commonly used enzyme in the decellularization protocol. It can selectively cleave the carboxyl end of arginine and lysine of adhesion proteins, thereby detaching cells from the tissue. However, if trypsin acts for a too long time, it will destroy collagen and other matrix components (He and Callanan 2013). Treated with 0.02% trypsin for 1 h to porcine adipose tissue, no significant changes were found in the internal structure of the tissue; immersed in 0.5% trypsin for 48 h to sartorial tissue, the cytolysis was removed, while the ECM ingredients had been destroyed (Meyer et al. 2006). Providing the proper concentration of the enzyme and appropriate treatment time, it can be more effective and have lower biological toxicity. Prasertsung et al. suggested that the application of 1% trypsin in the process of removing cells in porcine dermal tissue should not exceed 24 h to remove most of the cells and protect the internal collagen from damage (Prasertsung et al. 2008).

DNase and RNase are endonucleases that hydrolyze deoxynucleotide chains and nucleotide chains respectively. Nucleases (deoxyribonuclease and ribonuclease) can

destroy nucleic acid sequences and help remove nucleic acid components. When one reagent does not achieve a good decellularization effect, these enzymes are often used to further remove the remaining DNA components, but the ECM components and surface structure can also be destroyed by the nuclease (Mangold et al. 2015). After 24 h of treatment of the aortic valve tissue with SDS, it was found that there were still cell nuclei remaining in the tissue that had not been eluted, adding DNase and RNase to obtain a completely decellularized material (Grauss et al. 2005).

Similarly, adding to decellularization reagents, phospholipase A2 can hydrolyze the phospholipids in the tissues while retaining the ultrastructure of collagen, which reduces the content of glycans to a certain extent. Moreover, proteolytic enzymes are commonly used in the first step of decellularization of many tissues including dermal tissue and corneal tissue and then cooperating with other physical methods or chemical reagents to better complete the decellularization work (Prasertsung et al. 2008). For example, porcine corneal tissue was soaked in 4 g/L proteolytic enzyme II for 45 min and then soaked in 1.5 mol/L hypertonic saline for 12 h. A more effective method is to soak in 0.1% SDS for 12 h (Gonzalez-Andrades et al. 2011). Special consideration in the method of decellularization using enzymatic reagents is that natural protease inhibitors released from lysed cells will inhibit the activity of enzymes. Experiments have shown that the cell removal rate will increase significantly with the renewal of the enzyme solution, for example, trypsin activity will decrease to 60% after 12 h (Prasertsung et al. 2008). Adding a small amount of protease inhibitors may improve the limitations of this problem. Enzymatic decellularization is a common method in current research, but it is insufficient for the effect of enzyme decellularization alone. Moreover, the enzymes remaining in the scaffold will stimulate an immune response and affect the proliferation and differentiation of re-implanted stem cells. Now the most common method is a chemical combined enzymatic method. The mixed use of multiple methods will make the decellularization effect better and lower the cytotoxicity (Gilpin and Yang 2017). Depending on the tissue to be decellularized, the decellularization method always requires a combination of physical, chemical, and enzymatic methods. It is usually desirable to use some gentle methods to produce ECM materials without harming the tissue structure and functional protein components of it.

23.3.4 Sterilization Method for the Derivation of dECM

At present, many decellularized biological products have to wait for approval, including the decellularized dermis, decellularized bladder, decellularized heart valve, and so on. Some of them have been used to repair cartilage, diabetic ulcers, breast reconstruction, oral problems, and other tissue damage (Boccafoschi et al. 2017). It is always utilized for ionizing radiation, ethylene oxide sterilization, etc., because the material transplantation requires relatively high cleanliness. Firstly, researchers believed that peroxyacetic acid can serve both as a decellularization and a sterilization reagent. However, it could not effectively sterilize thicker tissues by immersing in it alone. Afterward, the ionizing radiation method was used for

sterilization. The principle is to change the DNA/RNA in the microorganisms which makes them lose their activity to achieve sterilization. Studies have found that low-dose gamma rays can improve the mechanical properties of the ECM. When the dose exceeds 15 kGy, the structural strength of the decellularized material will gradually decrease as the dose increases (Uriarte et al. 2014).

Ethylene oxide is the most typically used gas sterilization compound, causing protein inactivation by interacting with macromolecular proteins in cells. Studies have reported that something about the adhesion of ECM and growth factors secreted by fibroblasts will be altered or partially destroyed due to ethylene oxide sterilization (Hodde et al. 2007). Some scholars claim that it will produce harmful substances or mutations during the process of ethylene oxide sterilization. Besides, collagen and ethylene oxide will form a covalent bond after the reaction; if it remains, it is difficult to remove and will affect the immune response of the host. For the above reasons, the method of ethylene oxide sterilization still needs further research.

23.3.5 Evaluation of Decellularization Efficiency

The advantage of decellularized material is that it has a natural tissue cell microenvironment and extremely low immunogenicity. Especially for heterologous tissues, if the residual cells cause severe immune rejection in the body, the transplantation will be failed. Regardless of residual DNA, cell membrane proteins, cytoskeletal proteins, etc., they will have a greater impact on the implantation of decellularized materials (Saldin et al. 2017). Quantitative detection of residual cellular components is required because most decellularization methods cannot completely remove all cells. With the increasing application of ECM in clinical, researchers have gradually begun to establish evaluation criteria for the effect of ECM.

Earlier suggestions were based on the amount of remaining nucleic acid as a reference, and the standard was divided into three points: (1) The decellularized tissue after hematoxylin-eosin staining was red in the visual field, and no obvious nuclei should be observed; (2) residual DNA should not be greater than 200 bp; and (3) calculated by dry weight, the residual amount of double-stranded DNA should be less than 50μg/g (Crapo et al. 2011). Later, the team pointed out that the above three points are not enough to evaluate the effect of decellularization. The addition includes the removal of biofilm components (including cell membranes and intracellular membranes), the retention of the structure and components of the ECM (such as collagen, glycosaminoglycans, etc.), and the decellularization of products without cytotoxicity. Scanning electron microscopic observation of the prepared decellularized material shows that there are many filamentous collagen fibers and no cell debris, which can also prove the good effect of decellularization.

Due to the wide application of decellularization technology, there are many kinds of decellularization tissues. The abovementioned standards are not necessarily suitable for all tissues of origin because the host immune response brought by different tissues is also different. Some pointed out that not only nucleic acid content

needs to be tested, but the remaining cell components can also be used as testing standards, such as mitochondria. From the evolutionary perspective of cell biology, mitochondria evolved from bacteria to endosymbionts and finally became organelles. Cells will produce a series of stress responses after tissue damage involving the release of mitochondrial DNA will also cause a systemic immune response (Zhang et al. 2010). With the continuous improvement of decellularization technology, the mechanism between the decellularized material and the host will continue to be discovered, and the criteria for evaluating the decellularization effect will also continue to evolve.

23.3.6 Adverse Effects of Residual Reagent

Since chemical reagents have irreplaceable advantages in destroying the structure of cells, the abovementioned decellularization methods, whether physical, chemical, or biological enzymatic methods, all include the application of some chemical reagents. However, if the decellularization is not done properly and there are still higher concentrations of chemical reagents, it will have a greater impact on the transplanted host cells. Studies have shown that residual SDS in decellularized materials has strong toxicity for cell growth (Startseva et al. 2019). To detect the residual amount of SDS, it always uses a microplate reader which principle is that a blue compound will be produced when it interacts with methylene blue in an acidic environment. This colorimetric assay can be a quantitative analysis of the residual amount of sodium lauryl sulfate in the matrix (Keane et al. 2015). The residual amount of SDS can effectively be reduced by applying BAS or PBS after decellularization or using Triton X-100.

It is worth considering that the difference is more due to the different structure and composition of the decellularized material obtained by different decellularized methods rather than the different dosages of residual sodium, which means that the decellularization method will more clearly affect the response of the ECM material to the host. It was found that no matter what the rinsing method and the residual dosage of SDS are used, there is no significant effect on cell growth and activity. These data indicate that the composition and structural changes in tissue ECM are one of the main factors affecting cell growth. However, more studies are still needed to confirm whether other decellularized tissues are also following this phenomenon. Moreover, many decellularization processes are applied to bovine-derived enzyme reagents (such as DNase, RNase, and trypsin). These enzymes can also produce adverse immune responses to the host (Startseva et al. 2019). Washing with PBS repeatedly is the simplest and most effective way to remove the chemicals in the decellularization process and wash the cell debris. The decellularized materials can be processed in combination with an appropriate degree of ultrasound. Do not take too long, otherwise, the tissue structure will be destroyed.

23.4 Application of dECM for Regenerative Therapy

Necrosis or functional failure of vital organs seriously threatens human health. According to statistics, there are close to 230 million patients with cardiovascular disease in China, and three million people die from cardiovascular disease every year, ranking first among various causes of death (Nemet et al. 2020). There are approximately 79 million patients with liver disease and eight million patients with end-stage liver failure. For patients with end-stage vital organ failure, organ transplantation is the most fundamental and effective treatment. With the rise of tissue engineering, the important role of ECM in tissue regeneration has been gradually discovered. Its destruction or excessive production will affect tissue damage and repair. Stem cell-related decellularized materials have this prospective development in the fields of skin tissue engineering, bone and cartilage tissue engineering, heart tissue engineering, oral cavity, and dental pulp regeneration engineering, and some have entered the preliminary clinical stage.

23.4.1 dECM for Skin Tissue Engineering

As the largest organ of the human body, the skin is composed of epidermis, dermis, and subcutaneous tissue. Skin defects caused by trauma, burns, and other reasons are relatively common clinical diseases. Large-scale skin defects will seriously affect the health and survival of patients (Zhang et al. 2018). According to the depth of the burn, it can be divided into first-degree burn, second-degree burn, and third-degree burn, which involve the epidermis, dermis, and subcutaneous layer, respectively. The more frequent method is to remove the area of degeneration and necrosis and then perform skin transplantation. Among them, the thin-part-thickness grafting has won universal recognition. However, the poor results of skin transplantation are mainly due to insufficient skin source in the donor area and adverse reactions, such as the insufficient area of the donor area and pain, which limit its wide application. Xenografts and allografts have problems such as immune rejection, risk of disease transmission, and ethics problems (Zhang et al. 2014). As a result, with the continuous rise of tissue engineering in recent years, increasingly skin tissue substitutes have been produced, bringing new hope to patients with large area skin defects.

In skin wound healing, fibroblasts play an important role in the wound healing process, providing some remodeling enzymes such as protease and collagenase. The natural structure of ECM is easier to support the growth of stem cells. Comparing the two ECMs of human lung fibroblast-derived matrix (hFDM) and umbilical cord blood mesenchymal stem cell-derived matrix (UMDM), the result was that the total proteins of UMDM and angiogenesis-related cytokines are more abundant than hFDM, showing that the UMDM significantly promotes wound healing within 24 h (Savitri et al. 2020). Researchers implanted stem cells into the dECM to interact with each other, thereby promoting stem cell adhesion, proliferation, and differentiation. Using stem cell self-renewal and multidirectional differentiation potential regulates the release of inflammatory factors and promotes the formation of

granulation tissue. In addition, it regulates the deposition of ECM and promotes the migration of fibroblasts and keratinocytes, thereby accelerating the wound healing process.

The activated BMSCs' composite three-dimensional dECM scaffold can promote the survival and function of stem cells in the body, increasing their therapeutic applications (Varkey et al. 2015). Similarly, the ECM scaffolds of decellularized adipose-derived stem cells (ADSCs) can support the adhesion and proliferation of primary human fibroblasts and dermal microvascular endothelial cells, indicating their advantages in the field of wound healing (Riis et al. 2020). The amniotic membrane can be used as a tissue engineering scaffold after decellularization, which can be combined with BMSCs to repair skin damage. ECM can not only be applied as a natural biological scaffold material but also be used alone. After decellularization of ADSCs, forming a gel at 37 °C, these factors released from the ECM can promote the proliferation of fibroblasts and angiogenesis (van Dongen et al. 2019).

Perinatal tissue is an important organ for material exchange between the fetus and the mother. Among them, the placenta is a joint organ between mother and child that grows from the embryonic membrane and maternal endometrium during human pregnancy. The umbilical cord is a tubular structure connecting the fetus and placenta. Perinatal tissues such as the placenta and umbilical cord are rich in ECM and basement membrane. This ECM contains more abundant growth factors than ordinary human tissues, so it can be used to promote organ damage and repair. For example, the human decellularized amniotic membrane can promote the adhesion and proliferation of amniotic MSCs. Others, the umbilical artery vascular, have good mechanical properties and histocompatibility after decellularization. ECM hydrogel materials derived from perinatal tissues have significant tissue damage repair and medical regeneration functions (Spang and Christman 2018). Involving collagens, glycosaminoglycans, and bioactive factors secreted by a variety of stem cells, these dECM materials derived from perinatal tissues are the key to organ injury healing. With good biocompatibility and good therapeutic effect, it has been widely used. Glycosaminoglycans (GAG), a heteropolysaccharide with the repeated disaccharide structure, has negatively charged carboxyl or sulfate groups, so the anionic polysaccharide chain is acidic. Recent studies have shown that GAGs in dECM play a key role in tissue regeneration (Dutta and Dutta 2010). However, the glycomics and GAG regulatory functions of organ-specific dECM in perinatal tissues have been poorly studied. Therefore, a perinatal tissue-derived dECM hydrogel was prepared by the chemical combined enzymatic method, and glycomics analysis was used to identify the mechanism that promotes tissue repair and regeneration in the mouse skin wound healing model.

High-sensitivity LC-MS/MS was used for glycosaminoglycanomics analysis to explore the main components of the repair (Li et al. 2015). This result showed that the placenta-derived dECM (PL-dECM) hydrogel has a high content of chondroitin sulfate (CS) and heparan sulfate (HS). By bioluminescence imaging (BLI), the anti-inflammatory and pro-angiogenic responses were monitored in wound healing after dECM hydrogel treatment. BLI showed that PL-dECM hydrogel exerts the best

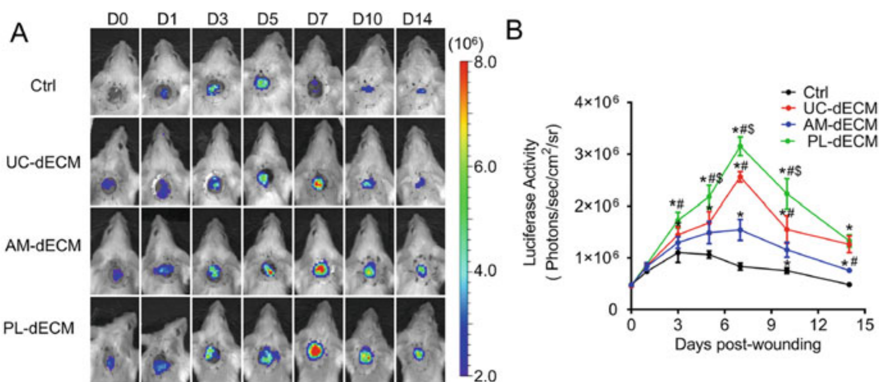

Fig. 23.2 ECM hydrogel enhanced the repair of wound healing by enhancing early angiogenesis at the injured sites. (**a**) The expression of VEGF-R2 was tracked by BLI following ECM treatment. (**b**) Quantitative analysis of BLI signals in each group during Day 1 to Day 14. Data are expressed as mean ± SD, $n = 3$. *$p < 0.05$ vs. Nor, $^{\#}p < 0.05$ vs. Ctrl, $p < 0.05$ vs. UC-ECM, $p < 0.05$ vs. AM-ECM. (Reproduced with copyrights permission from Wang et al. 2021)

anti-inflammatory and pro-angiogenesis effects in the skin wound healing model. Further analysis in vitro showed that CS with 6-O-sulfo group (CS-6S) has anti-inflammatory effects, while HS with 6-O-sulfo group (HS-6S) plays a crucial role in angiogenesis.

These findings reveal that the glycosaminoglycan of dECM modulates chondroitin sulfate (CS-6S) with 6-O-sulfo group and heparan sulfate (HS-6S) with 6-O-sulfo group during tissue repair, which plays an important role in inflammation and angiogenesis (Fig. 23.2). This study shows that it is an attractive candidate for drug delivery and tissue engineering for the use of glycosaminoglycan-containing placental dECM hydrogels. It can be seen that it is feasible to apply stem cell-related dECM to repair skin injuries. It can promote tissue anti-inflammatory, angiogenesis, etc., thereby effectively promoting wound healing and reducing scar formation.

23.4.2 dECM for Bone and Cartilage Tissue Engineering

Cartilage tissue is characterized by lack of blood vessels, low nutrients, low supply of progenitor cells, low cell content, lack of neurons, and lymphatic drainage (Steinert et al. 2007). Due to the specificity of cartilage tissue causing its ability to regenerate is very limited, there is no particularly effective treatment for cartilage damage in clinical now. In the clinical repair process, the unstable metabolism of the synovial joint after injury could produce poor repair, which often leads to the occurrence of osteoarthritis and brings greater pain to the patient.

For tissue engineering, the unique three-dimensional structure of ECM is required to provide mechanical support for cells. After implanting cells on it, they will help the proliferation of cartilage cells as they grow, thereby helping the damaged tissues

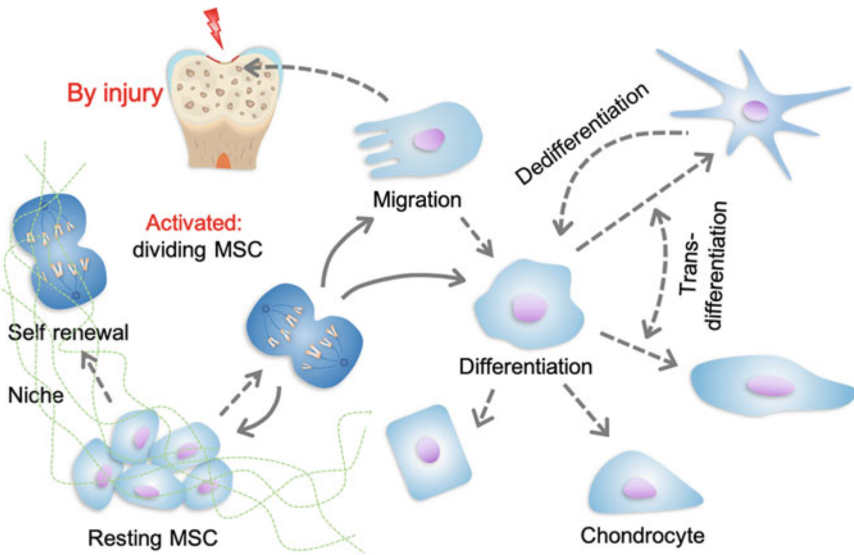

Fig. 23.3 Simplified scheme summarizing the mechanisms by which MSCs could influence cartilage repair. In physiological status, MSCs are always localized in a niche with renewing in an asymmetric manner. Once in external stimulation, they start a division in an asymmetric manner producing two different daughter cells. Attracted by chemokines, one of them can migrate to the tissue injury site. The other can stay in the niche with the function of stem cells, meaning that it can dedifferentiate, transdifferentiate, differentiate terminally (hypertrophy), or differentiate into various directions (chondrocytes, osteocytes, adipocytes, fibroblasts, tenocytes, myocytes) because of the various microenvironments

to gradually restore structure and function (Tang et al. 2013). The decellularized material has a unique natural structure that has significant advantages in terms of biocompatibility, mechanical properties, and biodegradability. The small extracellular molecules among them can regulate the growth state of cells, thus becoming the hotspot of cartilage repair. Different types of cell-derived ECM combined with articular chondrocytes have been studied, such as ADSCs, bone marrow stromal cells, synovial stem cells, etc. In the repair of cartilage defects, the most common method is to embed bone marrow-derived MSCs and adipose-derived mesenchymal stem cells into the dECM of cartilage tissue and then transplant them to the damaged bone. In the tissue or cartilage tissue, the combined scaffold can promote the proliferation and differentiation of cells and accelerate the repair of damaged parts (Murphy et al. 2013). The specific mechanism by which MSC affects cartilage repair is as follows (Fig. 23.3). Under physiological conditions, MSC is located in an ecological niche. Once a certain stimulus is received, the activated MSC will renew itself in two ways. The first is the symmetrical division, which can maintain the characteristics of the stem cell and make it stay in the original niche. The two daughter cells produced by division are similar. In the second asymmetric way, one of them stays in the stem cell pool and the other can migrate. After cartilage

tissue is stimulated by damage, cells that can migrate are attracted by chemokines. Under the induction of different factors, they can differentiate into different directions such as bone cells, chondrocytes, muscle cells, and fibroblasts.

Bone marrow-derived MSC has high proliferation activity and is suitable for composite decellularized scaffolds for cartilage tissue repair. After treated with 2.5% SDS, the BM-MSCs were implanted into the bovine articular cartilage tissue. Compared with the control group, the bovine articular cartilage scaffold provided a more suitable growth environment for BM-MSCs, which the adhesion and proliferation effects were higher than those of the control group (Tavassoli et al. 2015). With good structure and ideal biological properties, a new type of cartilage matrix-derived porous scaffold retaining most of the ECM after decellularization becomes one of the perfect candidates for cartilage tissue engineering cell carriers. Using natural cartilage cell ECM scaffolds, isolated canine bone BMSCs were inoculated onto the scaffold. Scanning electron microscopy revealed that there were BMSCs distributed on the surface and inside of the scaffold accompanied by collagen and proteoglycans (Zhang et al. 2019). Compared with the use of a single scaffold to treat rabbit cartilage defect repair, the group using cartilage ECM-ADSCs composite scaffolds for the treatment was shown a good defect tissue filling and satisfactory repair effect (Kang et al. 2014). In the same way, we put MSCs derived from the synovium on the ECM scaffold formed by the decellularization of the equine synovium and proved that the implantation of cell composite biological scaffolds can significantly improve cartilage damage in vivo (Reisbig et al. 2019). BM-MSCs adhered, proliferated, and differentiated into chondrocytes after 21 days of culture on the decellularization of human cartilage tissue, forming cartilage-like tissue after 4 weeks (Yang et al. 2008).

Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) decellularized ECM has obvious advantages in the formation and differentiation of cartilage, which can retain the differentiation ability of chondrocytes as well as promote the proliferation of chondrocytes (Zhang et al. 2019). Observe the microstructure of ECM material formed by inducing BMSCs to form cell sheets in vitro. To evaluate the repair effect, the ECM scaffold was implanted into the rabbit osteochondral defect model. The interesting finding was that the ECM promoted the potential of Bmscs to differentiate into osteoblasts under osteogenic conditions and its potential to differentiate into chondrocytes under cartilage conditions (Wang et al. 2020).

Moreover, the researchers extracted newly designated cartilage ECM-derived particles with a diameter of 215–313μm from the ECM of natural cartilage tissue after crushing, screening, and decellularization. Cultivated under suitable conditions, BMSCs can quickly colonize and proliferate on their surface, and the cell viability is high (Yin et al. 2016). The use of stem cell-related dECM is providing a prospective method for transplantation to reconstruct articular cartilage and subchondral bone. However, there is still a certain distance from the clinical. Many questions are all required in cartilage tissue engineering, such as the quality of dECM, the integration of scaffolds and surrounding tissues, the colonization and growth of stem cells, the matching of tissue regeneration speed, and the reconstruction of tissue heterogeneity.

23.4.3 dECM for Dental Tissue Engineering

Periodontitis is an inflammatory disease characterized by the destruction of tissues that support the teeth, including the alveolar bone, periodontal ligament, and cementum, which the main cause is the host's immune response to periodontal pathogens (Kajiya et al. 2010). If left untreated, periodontitis can cause irreversible tissue damage, and in severe cases, it may cause tooth loss. Therefore, to achieve periodontal regeneration, methods including various growth factors, solving inflammatory mediators, and using bioactive agents have a certain degree of effect (Kim et al. 2015). However, for this complex process, needing to be balanced with each other, it is necessary to predict possible reconstruction methods from three aspects: cementum, cementum, and periodontal ligament. New cell therapies need to provide some stem cells and tap their potential to differentiate in the desired direction.

The ECM can not only simulate the natural biological environment but also has a 3D structure composed of a collagen network so that it is considered one of the most suitable scaffold materials for dental tissue engineering (Ravindran et al. 2014a). Although MSC-ECM is still in the basic research stage, it has broad application prospects in the dental clinic, especially in the fields of oral implantation and periodontal field. BM-MSCs have the characteristics of self-renewal and pluripotency, which are considered an effective choice for periodontal tissue regeneration by transplantation into periodontal defects. Due to concerns about insufficient biomechanical properties of dECM scaffolds, chemical cross-linking methods are often used to form composite scaffolds. After decellularizing the bovine pulp, the effects of chemical cross-linking and nonchemical cross-linking were tested. After that, implanting hBMSCs into the scaffold, the proliferation effect of the cells in the cross-linked scaffold was better and the angiogenesis effect was increased (Fig. 23.4). Cross-linking improves biocompatibility but reduces biodegradability (Bakhtiar et al. 2020). This method does not integrate well into the oral cavity due to the addition of chemical cross-linking agents or organic reagents. The results show that dECM from different tissues has different degrees of mechanical properties.

In choosing the homogeneous or heterogeneous dECM as the scaffold, implant some stem cells such as BMSCs to study the ability of the cell-matrix composite scaffold to promote the repair of periodontal tissue. Then, the MSC-ECM was prepared to verify its ability to promote periodontal tissue regeneration in vivo (Takewaki et al. 2017). The dECM of human umbilical vein endothelial cells (HUVECs) was extracted for decellularization, and stem cells from the exfoliated deciduous teeth were implanted in it. It was found that compared to chemically synthesized scaffold materials, it was arranged more neatly by dECM (Gong et al. 2017). These proved that biological scaffolds with dECM alone can be used as an effective means for periodontal tissue regeneration. More than that, different decellularization methods have different effects on the ECM of the bovine dental pulp:

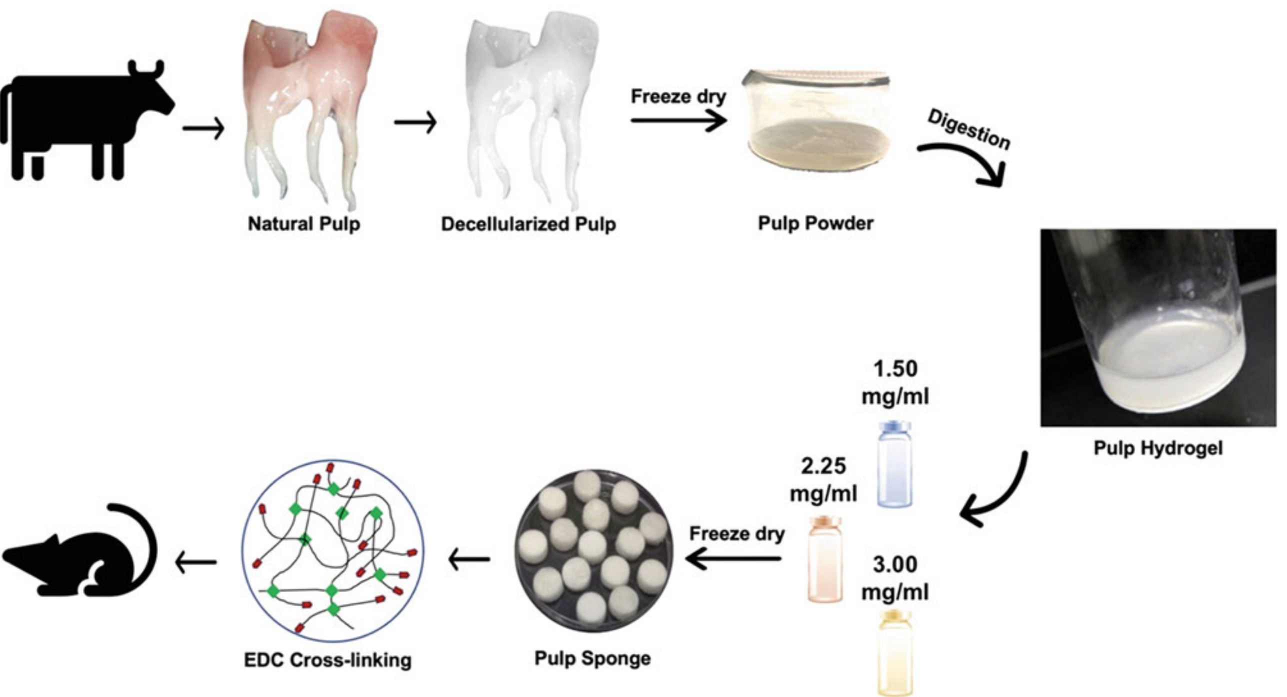

Fig. 23.4 Schematic representation of the bovine pulp sample preparation and decellularization. Pulp tissue was decellularized by trypsin 0.02%, ethylenediaminetetraacetic acid (EDTA) 0.05%, 0.1% w/v sodium dodecyl sulfate (SDS) extracted from bovine teeth. The pulp lyophilized powder was digested to form a pulp hydrogel and freeze-dried to get pulp sponge. (Reproduced with copyrights permission from Bakhtiar et al. 2020)

Scheme 1: After 1 h of Triton-100 treatment and 12 h of EDTA or trypsin treatment, without the addition of SDS, this program has the highest GAG content retention rate (Laudani et al. 2020).

Scheme 2: After 1 h of EDTA or trypsin treatment and 48 h of SDS treatment, without Triton-100 added, this program has the most remaining DNA content (Laudani et al. 2020).

The preparation of dental pulp stem cell-derived ECM by decellularization method can promote the proliferation and calcification of dental pulp stem cells. Some factors in ECM can regulate the process of odontogenic differentiation (Zhang et al. 2017). Porcine decellularized dental pulp tissue was implanted into a dog root canal model for 8 weeks. This dECM supports the expression of dentin markers and cell infiltration, indicating that ECM therapy of dental pulp tissue is expected to replace the traditional root canal therapy method (Alqahtani et al. 2018). Human dental pulp from healthy extracted teeth can be successfully decellularized by different methods, and the resulting scaffold could support the proliferation and differentiation of apical papillary stem cells (Song et al. 2017). The results show that the new calcified tissue has a good regeneration effect after transplantation. The decellularized porcine dental pulp ECM contains components that are conducive to the proliferation and differentiation of stem cells and mediates dental pulp, which is one of the potential treatments for regeneration (Hu et al. 2017).

Besides, the dECM of bone marrow-derived MSCs can promote the neuronal differentiation of dental pulp MSCs. Since dental pulp stem cells are derived from the embryonic premise of neuroectoderm, they are considered to have a greater tendency to differentiate into nerves. Experiments show that ECM enhances the role of dental pulp stem cells in neuronal induction. The presence of ECM increases their differentiation tendency, which can be used to support the growth and differentiation of MSCs (Laudani et al. 2020). The dECM scaffolds can promote the odontogenic differentiation of human periodontal ligament stem cells and human dental pulp stem cells and finally form the dental pulp-like tissue, which provides a new direction for the natural biomimetic scaffold to treat dental caries (Ravindran et al. 2014b). Without the cell components, ECM is easy to store and has relatively small ethical restrictions. If the problem of adhesion and fixation can be solved, and the surface osteogenic component can be improved, it can be gradually put into commercial production.

23.4.4 dECM for Heart Tissue Engineering

Cardiovascular disease is still the disease with the highest mortality rate. Since 1990, the global cardiovascular disease and mortality rate have been on the rise. After myocardial infarction (MI), the normal healing response begins, during which the damaged myocardium is replaced by fibrotic scar tissue. However, this leads to poor ventricular activity (decreased ejection fraction), which ultimately leads to heart failure and death (Segers and Lee 2010). The cardiac acellular matrix material is a

new biomaterial in recent years. After the natural heart tissue is decellularized, its ECM shows ideal mechanical properties in terms of elasticity and toughness with extremely low sensitivity and high biological activity. Therefore, it has become a hotspot in cardiac tissue engineering research. Since the cardiac dECM was successfully prepared in 2008, related decellularization methods have been continuously improved. The dECM of the heart can be used as a myocardial patch alone to repair the damaged myocardium, or it can work together with the cells colonized in it, or it can also be made into an injectable hydrogel for the treatment of MI.

The current cell therapy for MI is also subject to many restrictions in the process of entering the clinic, such as low cell retention rate and so on. Researchers hope to strengthen cell adhesion and improve the clinical effects of cell therapy. Physically pressing the dECM onto the polyvinyl alcohol hydrogel will produce a stretchable ECM. This is not only preserving the microenvironment provided by the ECM but also enhancing its mechanical properties. It has been proven that the ECM of this composite hydrogel can increase MSC retention and improve cardiac remodeling (Kim et al. 2019). However, the disadvantage is that the biocompatibility of polyvinyl alcohol is not good enough, in which low toxicity of the material will affect the recovery of functions such as cardiomyocytes.

A better way is to colonize stem cells into the dECM of the biological heart to jointly help the damaged heart restore function. In recellularizing the ECM of the porcine heart, human mesenchymal stem cells (hMSCs) were implanted into it. The authors evaluated that from multiple angles, during the process of ECM recellularization, it will increase the protein density and increase the plasticizing effect between fibers (Au-Yeung et al. 2017). After decellularization of the ECM produced by cardiac fibroblasts, MSCs derived from human embryonic stem cells are seeded on it, which can be used for mouse MI models (Schmuck et al. 2014). The ECM of bovine cardiomyocytes successfully removed most of the cells after hypotonic washing and enzymatic hydrolysis (Fig. 23.5). The histological evaluation confirmed that it does not only simulate the natural ECM environment but also induce the phenotypic transformation of stem cells into cardiomyocytes, which suggests that it has large application potential (Arslan et al. 2018). Three types of cells (cardiomyocytes, fibroblasts, and mesenchymal stem cells) were cultured and then seeded on the cardiac ECM scaffold. MSC cells were able to maintain cell viability after 24 cultured on the cardiac ECM (Eitan et al. 2010a).

Different methods of decellularization of heart tissue can lead to different effects. Animal ECM was prepared by whole organ perfusion or immersion, and the effect of the decellularization process on the ECM of the myocardium was analyzed. For example, 2 h for lysis, 6 h SDS treated, and fetal calf serum were incubated for 3 h to remove the remaining DNA. This scheme can decellularize well and retain the complete structure of ECM, which will make cells better adhere and grow (Kim et al. 2019). If the SDS is not cleaned after treatment, the toxicity will have an adverse effect on the regeneration of hMSC. The ECM scaffolds formed by different decellularization methods will have a great impact on the preservation of the ECM niche and the regeneration of cells (Liu et al. 2016).

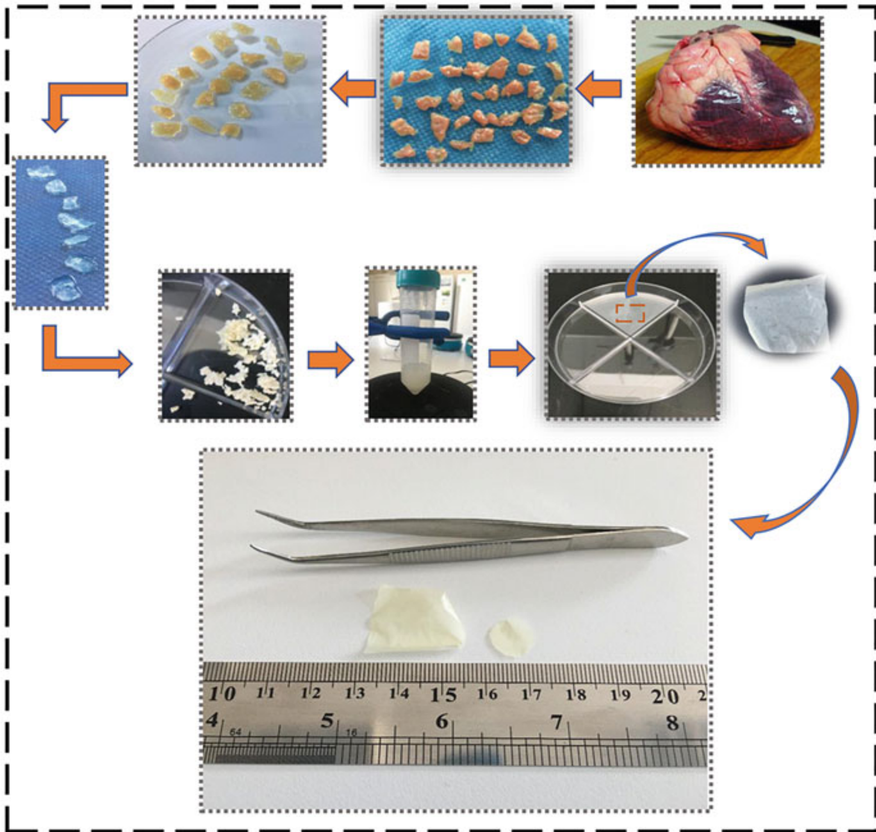

Fig. 23.5 Schematic illustration of the decellularization stages for obtaining films from decellularized bovine heart tissues. Obtaining a bovine heart, the dECM was lyophilized after treating with the hypotonic solution for 48 h and with a hypertonic solution including 1% SDS for 48 h. Digest this by pepsin to get the soluble solution; prepare the film with the dECM soluble solution. (Reproduced with copyrights permission from Arslan et al. 2018)

In previous studies, the myocardium after pathological changes was considered an irreversible process and difficult to repair itself. BMSC transplantation has become a potential treatment for ischemic heart disease because of its effect on improving myocardial function (Schachinger et al. 2006). Further studies have found that comparing human heart tissue and umbilical cord tissue dECM, BMSCs have a better proliferation effect on the umbilical cord ECM and can promote cell migration (Robert et al. 2017). Moreover, cell therapy with cardiac c-kit cells can restore the function of the damaged heart. After using Triton-100 for decellularization, c-kit cells were cultured on the cardio-derived MSC-ECM for 21 days; the expression of cardiac troponin increased by 1.7 times, indicating that MSC-ECM can promote the growth and proliferation of c-kit cells (Ng et al. 2019). Current research on cardiac tissue engineering has shown that dECM hydrogels can promote angiogenesis;

dECM compositing cells can promote the differentiation of stem cells into cardiomyocytes, which means dECM materials can effectively repair damaged heart tissue. Nevertheless, the preparation of the heart acellular matrix scaffold can only be seen as the first step in building a biological heart. Due to the multicellular nature of heart tissue, many questions need to be answered. The future directions are how to stably recellularize this scaffold, whether it can induce stem cells to differentiate into pacemaker cells, and how to make them beat regularly.

23.4.5 dECM for Other Tissue Regeneration

The lung is a complex organ with a complicated three-dimensional structure and composed of more than 40 kinds of cells. In clinical treatment, lung transplantation is the fundamental treatment method for a variety of terminal lung diseases (Wagner et al. 2013). Lung transplantation mainly includes autologous transplantation, allogeneic transplantation, and synthetic tissue substitutes. Here, autologous transplantation cannot be implemented for many patients with lung diseases. The immune rejection of allogeneic transplantation has always been the biggest problem for all organ transplants. Artificially synthesized tissue substitutes have restricted their application due to long-term chemical instability, poor biodegradability, and poor biocompatibility.

In recent years, tissue-engineered lung transplantation has attracted the interest of lung transplant researchers. After seeding stem cells cultured in vitro on a suitable cell scaffold, the cell-matrix complex is transplanted into the human body to achieve the effect of tissue repair or replacement. The use of decellularized lung tissue as a scaffold material maintains the microenvironment of the original lung tissue and has the biological characteristics of human lung tissue such as elasticity, plasticity, adsorption, porosity, and absorbability (Tebyanian et al. 2019). Recellularization of decellularized lung scaffolds has always been a hot issue in tissue-engineered lung transplantation.

Experiments have shown that the 0.1% SDS solution perfusion as the decellularization method was used to decellularize rat and human lungs. After decellularization, human-induced pluripotent stem cells (iPSCs) were inoculated. The cells showed proliferation after 5 days as well as the upregulation of Nkx2.1 expression was detected after several days of culture. The decellularized lung matrix supports the culture of human iPSC-derived cells and can even promote their differentiation into a variety of lung cells (Gilpin et al. 2014). Using SDS to decellularize mouse lung tissue, lung tissue-related markers can be detected after transplanting ES cell-derived embryoid bodies and culturing for 14 days, which proves that lung tissue decellularized matrix can have the potential of ESCs to differentiate into lung cells (Kawai et al. 2018). However, due to the high complexity of the lung structure, a key challenge in the lung tissue engineering of stem cell-related dECM is to determine how to replicate the three-dimensional matrix structure in the lung tissue and promote the function between ECM and cells for gas exchange and physiological interactions. The glomerulus, the basic functional unit of the

kidney, can cause various kidney diseases via glomerular damage caused by various reasons. At present, the most effective method for terminal-stage renal disease is kidney transplantation, but the shortage of donor organs is the biggest problem for organ transplantation. As a result, kidney tissue engineering continues to rise. The application of dECM kidney scaffold in kidney bioengineering has become one of the popular investigations, which has a natural microstructure and ECM. The renal progenitor cells and endothelial cells derived from human-induced pluripotent stem cells are planted in the kidney dECM. After proper cultivation, the assembly of glomeruli can be achieved, indicating that this is a feasible method to repair kidney damage (Du et al. 2016). After the kidney tissue is treated with a modified decellularization method, ADSCs are combined with it (Fig. 23.6). Bioluminescence imaging shows that kidney dECM can significantly increase the retention rate of ADSCs in damaged kidneys and reduce the chance of escape, so it is considered to have the potential to treat renal ischemia-reperfusion injury (Ross et al. 2009). Mouse ESCs can proliferate after intraarterial injection into the decellularized whole kidney scaffolds of the rat, showing the differentiation evidence of morphology, immunohistochemistry, and gene expression (Ross et al. 2012).

Due to the high regional diversity of the kidney tissue matrix, the three typical areas of the kidney (cortex, medulla, and papilla) were prepared for dECM. Compared with the former two, kidney stem cells cultured on the papillary outer matrix have lower proliferation ability, higher metabolic activity, and also differences in cell morphology, arrangement and structure formation (O'Neill et al. 2013b). This shows that tissue and region-specific ECM can provide different development directions for ECM combined with stem cell therapy. Stem cell-based therapies are considered to be one of the most potent methods for the treatment of kidney damage. However, there are still many details facing challenges in the efficiency of the delivery of cells. There are still some challenging problems in the construction of functional and engineered kidneys. A large number of experiments and clinical studies are needed to further evaluate its effectiveness and safety.

Recent studies have shown that it is possible to use detergent-treated whole decellularized liver as a scaffold, which retains the entire vascular network and complete ECM. In addition to decellularized whole-organ scaffolds, it is critical to the function of the bioengineered liver that how to select the seed cells for reconstructing decellularized liver scaffolds. Currently, potential cell sources are hepatocytes and MSCs. A porcine hepatic dECM containing liver-derived growth factors was inoculated with hepatocytes derived from induced pluripotent stem cells (iPSC) and then transplanted into rats with the graft's express hepatocyte markers (Park et al. 2016). At present, a variety of liver decellularized hydrogel materials have shown obvious effects whether for the treatment of liver fibrosis or the transplantation of advanced liver failure. However, the core factor of decellularized liver scaffold is the vascularization in the clinical application. Moreover, there are a series of problems such as anticoagulation and endothelialization (Zhou et al. 2015). Stem cell-related dECM liver tissue engineering is still in the early stage of development, in which further studies are needed on large animal models. More data and

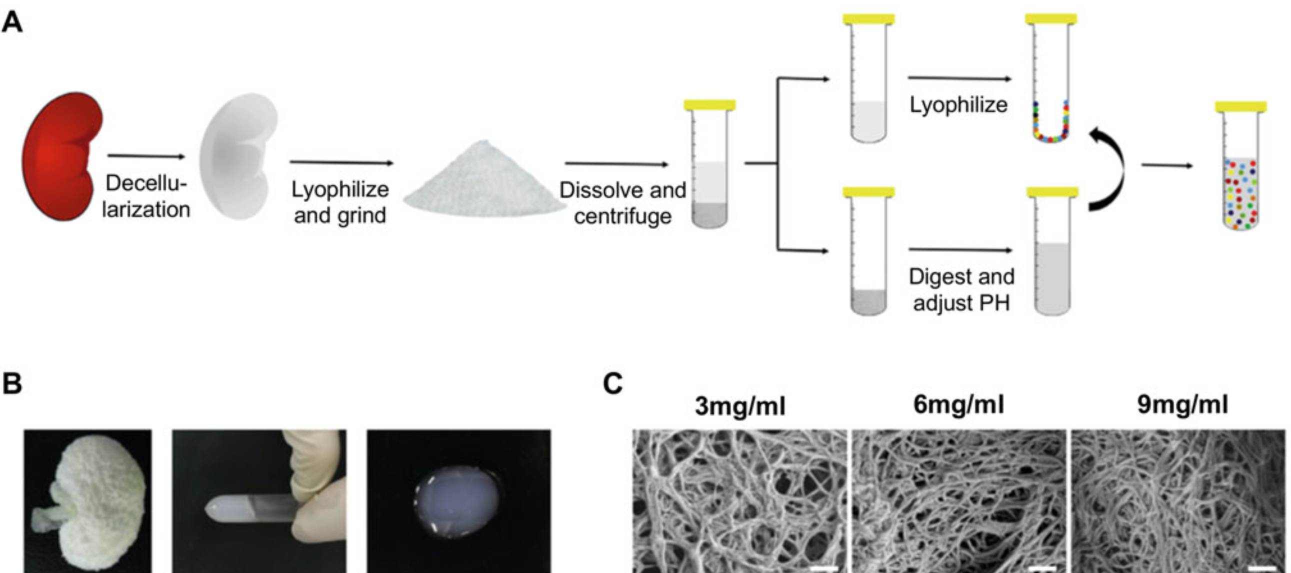

Fig. 23.6 The decellularization, preparation, and characterization of the kidney ECM hydrogel. (**a**) Schematic illustration of the fabrication of kidney ECM hydrogel. (**b**) ECM was self-assembled into a gel structure at 37°C for 30 min. (**c**) The microscopic structure of the kidney ECM hydrogel at different concentrations (3, 6, 9 mg/mL) by scanning electron microscope. (Reproduced with copyrights permission from Zhou et al. 2020)

results are needed to determine the potential therapeutic effects and ultimately apply them in the future for clinical transplantation.

In addition to the abovementioned dECM related to stem cells such as the lung, kidney, liver, etc., it has also been considerable research results in tissues such as blood vessels, muscles, small intestine, cornea, pancreas, spinal cord, and nerves, leading to the satisfactory repair and healing potential. Decellularized vascular tissue has potential application value in engineered small-diameter blood vessel transplantation. In the decellularized porcine coronary artery, rat ADSCs were grown well and maintain good cell viability. With the ADSC-seeded scaffold, the luminal side had a exhibited patency without visible narrowing (Lin et al. 2019). When studying the regeneration of muscle tissue, a composite biological construct composed of decellularized muscle tissue, muscle stem cells, and muscle resident cells has a greater degree of fibrosis in the damaged muscle compared to using decellularized muscle tissue alone. This suggested the superiority of the composite stem cell scaffold to repair muscle damage (Quarta et al. 2018). After decellularization of the human cornea, human adipose-derived adult stem cells (h-ADASC) are implanted on it. h-ADASC has a better ability to regenerate cells on the corneal dECM and has the function of differentiation the tendency of the epithelial cells (del Barrio et al. 2015). The severely damaged small intestine has a higher morbidity and mortality rate, which is generally caused by a variety of factors, such as congenital diseases, surgical complications, inflammation and infections, etc. The tissue-engineered small intestine has been extensively studied for tissue repair and reconstruction, so there is no doubt that the ECM associated with stem cells can repair the small intestine.

23.5 Future Challenges and Perspectives

In recent years, donor organs such as the heart, liver, lung, and kidney have been reported to provide dECM-based scaffolds through the decellularization processes as well as the recellularization potential of stem cells have been proven. Although the potential of decellularized scaffolds in tissue engineering has been confirmed, the molecular mechanism of the interaction between stem cells and decellularized scaffolds is still unclear, which means the signal network of their interaction has not been well explained (Kawecki et al. 2018). There are still some problems needing further discussion in the processes of decellularization, such as the standardization of decellularization methods, sterilization effects, the maintaining of bioactive ingredients, the colonization of stem cells, and the effect on adjacent tissues.

Moreover, some natural polymers are not stable and easy to decompose. It is difficult to maintain a specific shape and size, which will affect the role of stem cells in dECM, ultimately affecting the therapeutic effect of tissue damage (Wen et al. 2019). Many problems are caused by decellularized technology. Clinical trials usually focus on the safety and effectiveness of treatment using specific types of MSCs, as well as complications such as the potential risk of infectious disease, and

the transmission of materials from natural biological sources. For example, the effect of decellularization insufficiency will cause severe immune-inflammatory responses doing great harm to patients. Most human grafts have been tested for recellularization, but there are still many issues to consider regarding method optimization. Because MSCs and induced pluripotent stem cells can differentiate along different lineages based on ECM stimulation, they are the most promising cell types for the recellularization of dECM (Xu et al. 2019). Although it has achieved good results on the decellularization and in vitro recellularization of various human tissues/organs, there is still a lot of work to be done on the methods of surgical implantation. Because of the functional complexity, we develop human transplants of very complex organs such as the liver, kidney, or lung, which is difficult to achieve in in vitro regeneration since it is coming from tissue structure. In the future, it will be critical to evaluate the adaptability of these cells to decellularized organs. Besides, the direct effect of some types of stem cells has not been extensively studied. For example, human amniotic membrane tissue has been widely studied due to its easy availability and thin tissue, while the placental tissue needs more research to explore the great medical value of it.

23.6 Conclusion

Because of its unique biomechanical and chemical properties, dECM material can provide a suitable microenvironment for the adhesion, proliferation, and differentiation of cells in vitro, becoming a platform for cell culture, in line with the principles of high-quality, safe, and efficient treatment. The preparation methods of dECM are complicated; choosing an appropriate decellularization method according to different tissue types can completely remove cells while retaining the structure and composition of the ECM to the maximum. The enzyme modification method of decellularized tissue can improve tissue flexibility, elasticity, friction resistance, etc., which can further remove the immunogenicity of the tissue so that it provides an ideal solution for the widespread application of decellularized tissue products in the future. With the continuous innovation and advancement of decellularization technology, treatment strategies using human iPSCs or non-autologous tissues and organs will gradually improve, thereby helping to eliminate the growing demand for organ or tissue transplantation in the medical community.

Therefore far, stem cell-related dECM tissue engineering has achieved practical research results in the skin, cartilage, periodontal, cornea, tendon, blood vessels, heart, liver, kidney, and so on. Although there are some limitations for the applications, the current research on MSC-ECM involves different fields and different directions, mainly focusing on promoting directional differentiation and performance improvement. It is expected to become one of the most common materials in tissue engineering in the future. Improving the in vitro engineering of ECM and advancing the exploration of advanced equipment, personalized treatment will be greatly promoted. In the next few years, the greater research hotspots will be focused on the development of bioactive materials that can actively induce and stimulate the

regeneration and repair of human tissues and organs, blood-compatible artificial organ materials, and multifunctional nanoscale biomaterials. With the rapid development of materials science and technology, the research on natural biomaterials used in tissue engineering and regenerative medicine will become increasingly in-depth. The application of stem cell-related dECM in tissue engineering will develop expeditiously, which will also bring new technologies and treatments to the future of regenerative medicine and tissue engineering. It is believed that it will promote the development of the biomedical industry and better serve the protection of human health.

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Biomaterials and Scaffold Fabrication Techniques for Tissue Engineering Applications

24

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Abstract

Tissue engineering is a new field of study that seeks to create artificial tissue that can heal and replace damaged or diseased tissue. Materials, cells, growth factors, and other small molecules play a role in the artificial tissue's construction. Scaffolding systems can help the target tissues by acting as an extracellular matrix. Various scaffolding fabrication techniques were used for the construction of artificial tissue. For the fabrication of biomaterials for tissue engineering applications, the freeze-drying process, solvent casting and particulate leaching, gas foaming, 3D bioprinting, electrospinning, and thermal-induced phase separation are constantly used. Considerable pore size, mechanical strength, surface topography, cell proliferation, and cell adhesion are all provided by these methods. The established scaffolding can be used as a personalized medication to treat the patient due to its high precision. As a result, in this chapter, we've presented the fabrication scaffold techniques for tissue engineering applications.

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Keywords

Biomaterials · 3D bioprinting · Freeze-dry method · Electrospinning · Thermal-induced phase

24.1 Introduction

Tissue engineering (TE) is a multidisciplinary field that assembles engineering and life science tools to develop an artificial substitute. The substitute can replace and repair the damaged tissues or organs (Zhao et al. 2018). Even after decades, TE remains a flourishing area of research. The developing technologies are making it much simpler and more accessible by overcoming the limitations of conventional treatments (Yan et al. 2018). TE includes three main traits such as materials, cells, and signaling molecules. The basic approach of TE comprises (1) the isolation of cells from patients' body, (2) seeding of isolated cells on scaffold matrices, (3) providing growth factors for tissue development, and (4) and, finally, placing the developed tissue in the damaged area (Howard et al. 2008). The scaffolds act as an extracellular matrix where the cells can adhere and promote regeneration. Scaffolds are made of biodegradable materials, which get degraded after a certain period of implantation without any side effects. Thus, the scaffold must own some of the major properties such as biocompatibility, mechanical strength, and scaffold architecture as per the desired tissue (Ambekar and Kandasubramanian 2019). The materials used for the fabrication are from both biological and nonbiological sources, which are grouped as ceramics, natural polymers, and synthetic polymers (Keane and Badylak 2014). Diverse biomaterials have been utilized for the development of 3D scaffolds that includes collagen (Chen et al. 2017), laminin (Chen et al. 2018), fibroin (Rosadi et al. 2019), elastin (Vázquez and Martínez 2019), etc. which are proteins found in the connective tissue of the bone, skin, muscle, and cartilage. Hyaluronic acid structured nonsulfated glycosaminoglycan present in ECM, also have the role in cell signalling and wound repair. Natural polymers from different sources such as chitosan (Ahsan et al. 2018), alginate (Rastogi and Kandasubramanian 2019), etc. which are nontoxic, antimicrobial, and antioxidant, respectively. In addition, the ceramics such as hydroxyapatite, tricalcium phosphate, and bioglass are also used (Ribas et al. 2019). These are the well-studied and highly recommended biomaterials in TE applications. The development of these biomaterials as scaffolds needs some specialized techniques. The established techniques are associated with less efficient scaffolds. Thus, still, there is a demand for the development of a much more precise scaffold that can actually mimic preferred tissue (Viera Rey and St-Pierre 2019). The current chapter summarizes the main techniques which are involved in scaffold fabrication for TE applications. In brief, we discuss the concepts of both conventional and additive manufacturing technologies followed by its major benefits and limitations.

24.2 Conventional Fabrication Techniques

24.2.1 Freeze-Drying Method

Freeze-drying, also known as lyophilization, is the most extensively used method in the food industry, pharma industry, biomaterial engineering, nanotechnology, etc. (do Vale Morais et al. 2016). It is widely accepted because it is relatively simple and less likely to affect the sensitivity of the liable bioproduct (Haaparanta 2015; Fereshteh 2018). In tissue engineering, freeze-drying is subclassed under the conventional method for the fabrication of 3D porous scaffolds. It functions based on the sublimation phenomenon (Eltom et al. 2019). Generally, the freeze-drying process is divided into three steps: freezing, primary drying, and secondary drying periods. Primarily, dissolve the biomaterial in the respective solvent, and later it will be frozen down (step 1), leading to the formation of ice crystal, which will be further evaporated via sublimation (step 2), followed by removal of strongly bound water representing secondary drying and (step 3), finally, leaving back the dry scaffolds with interconnected porous architecture (Haaparanta 2015; Roseti et al. 2017).

The developed artificial scaffold must mimic the original tissue by accomplishing properties such as biocompatibility, cell adhesion, cell proliferation, and differentiation (Hutmacher et al. 2014). The freeze-drying (FD) technology stands promising as it fabricates the scaffolds with porosity beyond 90% and a pore diameter range of 20–400 μm (Fereshteh 2018). Freeze-drying not only supports specific geometries but also tailoring of its structural and biological properties. Lara Lopes Reys et al. studied the influence of freeze-drying temperature on the performance of freeze-dried chitosan scaffolds toward cartilage tissue engineering. Their lyophilized scaffold at −196 °C has a compact structure with smaller pores, while at −80 °C it showed a lamellar structure with larger pores (Reys et al. 2017) which suggests that the morphology of the pores varies as per the freezing temperature prior to the lyophilization. The addition of biomolecules such as collagen and elastin prior to the freeze-drying showed enhanced mechanical and elastic properties (Brougham et al. 2017). In addition, the stiffness can also be altered by crosslinking scaffold techniques. The in vitro and in vivo studies by Ma et al. reported glutaraldehyde reinforced chitosan-collagen porous scaffold by the freeze-drying method with improved bio-stability and biocompatibility (Ma et al. 2003). Jayachandran et al. used this technique for the development of porous fucoidan-loaded chitosan-alginate scaffold and observed improved porosity and water uptake ability (Venkatesan et al. 2014). Thus, freeze-drying with the benefit of non-requirement of high temperature nor in separate leaching step (Raeisdasteh Hokmabad et al. 2017) stands as a promising method for the fabrication of artificial tissue such as the bone (Sharifi et al. 2016), skin (Rahmani Del Bakhshayesh et al. 2018), muscle (Basurto et al. 2020), tendon (Beldjilali-Labro et al. 2018), and nerves (Chiono and Tonda-Turo 2015).

24.2.2 Solvent Casting and Particulate Leaching

Solvent casting and particulate leaching (SCLP) is the most preferred technique as it is relatively easy with less operative time and low cost. In principle, the polymer is dissolved in a solvent with uniformly distributed salt particles of a certain size, which is then poured into a predefined 3D model (Bano et al. 2010), where the first step involves the solvent evaporation leaving behind the salt embedded matrix. The uniform porosity is created in the next step, when the salt particles are leached out by dipping the matrix into water (Zhu and Chen 2013). The key feature of the technique is the porosity which can be molded by choosing the porogen of the desired size. The developed scaffold can show porosity from 50% to 95% (Prakasam et al. 2017).

A study by Ong Chia et al. did a modification in the conventional SCLP method by introducing increased centrifugal speed through which salt particles was well distributed and in where they have developed a scaffold using bioactive glass and polyurethane. Here they used SCLP method by altering centrifugal speed and found that porosity increased with the centrifugation speed. Thus, the reported polyurethane-bioactive glass scaffolds was highly porous with good interconnectivity (Ong et al. 2019). In similar the standard SCLP method was used by sola et al. for the development of a 3D polymer scaffold composed of polymethyl methacrylate and polyurethane with rich interconnected porosity (Sola et al. 2019), which was controlled by polymer-to-salt ratio. On the other hand, the method faces difficulty in the distribution of the particles within polymer solution and supports only the fabrication of a thin membrane of 3 mm thickness, as it is hard for the soluble particles to get separated from the matrix (Eltom et al. 2019; Ong et al. 2019). Thus, it is considered the major drawback of the method.

24.2.3 Gas Foaming

Gas foaming is a process widely used in industries, while in bioengineering, it has been employed to overcome problems related to solvent approaches involved in the fabricated scaffold (Barbetta and Costantini 2017). The basic principle of this technique is the development of a porous polymeric matrix through a nucleation-growth mechanism of gas bubbles (Santos-Rosales et al. 2020), where the first step is the dispersion of porogen in a polymeric matrix; here, the porogen can either be any chemical blowing agent, such as sodium bicarbonate, which can decompose substance into an inert gas by chemical reaction or by any physical blowing agent such as inert gas (nitrogen or carbon dioxide) or volatile liquid held back in the polymer. In the second step, the outflow of the porogen in the form of gas bubbles results in pore formation. The final step is the hardening of the polymer matrix (Costantini and Barbetta 2018).

Gas foaming method derived scaffolding systems have reported up to 85% of porosity. A study by Mishra et al. where they have developed a gelatin-PVP polymer composite scaffold using gas foaming technique reported 61.5% of porosity with

2.08 ± 0.02 MPa of tensile strength for bone tissue engineering (Mishra et al. 2019). A porous biodegradable scaffolds poly (propylene carbonate) was developed using this technology by Manavitehrani et al. the obtained pore sizes ranged from 100 to 500 μm. They also reported that the technique is suitable for semicrystalline and amorphous polymers but not crystalline polymers as it is unable to decrease the glass transition temperature (Manavitehrani et al. 2019). Each method has its advantage and disadvantage. Here, the method is simple and easy with one main evident drawback, i.e., unrestrained porous structure and interconnected size distributions (Barbetta and Costantini 2017).

24.2.4 Electrospinning

Electrospun is an extensively used technique for the fabrication of nanofibrous scaffolds. This technique has gained greater attention as the generated nanoscale fine fibrous provides structural similarity with collagen fibers of the bone (Wahid et al. 2018), and the developed higher surface to volume ratio of nanofibers highly favors cell adhesion and provides enhanced mechanical property (Ranganathan et al. 2019). The technique utilizes the electrostatic force of interactions to produce a uniform diameter of nanoscale fibers. In the electrospinning process, the polymer solution is loaded into the syringe with a needle. The syringe is placed at a certain distance away from the oppositely charged collector. Once the voltage is applied to the needle tip, the electrostatic force breaks down the surface tension of the polymer droplet resulting in eruption. Further, the electrically charged liquid jet erupts and undergoes bending instability leading it to whip rapidly forming loops toward the direction of a collector (Kishan and Cosgriff-Hernandez 2017). In the final step on the collector, the polymer-solvent evaporation and drying of nonwoven fibers occur resulting in dried nanofibers (Chahal et al. 2019). The nanofibers can be fabricated by adjusting certain parameters that include working distance, voltage, flow rate, collector geometry, and temperature. Besides, polymer properties such as viscosity, surface tension, and molecular weight are even necessary (Prakasam et al. 2017; Keirouz et al. 2020). Several studies have used this technique for the fabrication of scaffolds in tissue engineering. For example, Kim et al. fabricated PCL/collagen composite transparent hemispherical 3D nanofibrous scaffolds. They designed a peg-top collector where the nonconductive hemispherical device with a metal pin was attached to copper wire. The developed scaffold was obtained by inducing the change of an electric field between a rotating collector and a needle (Kim et al. 2018).

Movahedi et al. generated a uniform core-shell structured polyurethane/starch/hyaluronic acid nanofiber with coaxial electrospinning technique. They reported 83% of porosity with 7.18 ± 0.28 MPa of tensile strength. The polymers used were biocompatible and hydrophilic and exhibited improved mechanical strength. Thus, polyurethane/starch/hyaluronic acid was described as a novel scaffold for wound healing and skin tissue engineering (Movahedi et al. 2020). Similar studies have been fabricated nanofiber scaffolds for both hard and soft tissue engineering

such as blood vessel tissue engineering (Xie et al. 2020; Vatankhah et al. 2014), nerve tissue regeneration (Wang et al. 2011; Li et al. 2015; Wu et al. 2017), bone (Dalgic et al. 2018; Yu et al. 2017), cartilage (Chen et al. 2020; Mirzaei et al. 2020), tendon/ligament tissue engineering (Maghdouri-White et al. 2018; Li et al. 2020a). Beyond its advantages, the technique has some major limitations: where electrospinning provides relatively poor cellular infiltration into the scaffolds due to small pore size, inadequate mechanical strength for load-bearing applications, use of diverse polymers in electrospinning is limited (Shi et al. 2015), issues related to the toxicity of chemical residues in fibers (Khorshidi et al. 2016; Kishan and Cosgriff-Hernandez 2017), slow production rate, etc.

24.2.5 Thermal-Induced Phase Separation (TIPS)

Thermal-induced phase separation is a conventional scaffold fabrication technique that has gained attention for its one feature, i.e., the generation of the interconnected porous matrices (Guo et al. 2017). TIPS works using thermal energy for dividing the homogenous polymer solution into two different phases: the polymer-rich phase and solvent-rich phase, which is obtained by either mixing the polymer with immiscible solvent or quenching it into its bimodal region (Martinez Perez et al. 2011). The bimodal region is where the liquid-liquid and solid-liquid phase mechanism occurs. Certain parameters need to be focused on to fabricate scaffolds with desired porous morphology that includes polymer concentration, solvent composition, quenching temperature and time, coarsening process, and ceramic content (Yousefi et al. 2014). Various scaffolds fabricated different tissue regeneration that includes both hard and soft tissues (Salehi et al. 2021; McKenna et al. 2019; Biswas et al. 2017). The only drawback is the technique is limited to certain polymers (Akbarzadeh and Yousefi 2014).

24.3 Rapid Prototyping Technique

Rapid prototyping (RP) is a group of advanced technologies adopted to overcome the challenges associated with porosity, scaffold geometry, and difficulty in the insertion of bioactive molecules during conventional scaffolding (Wang et al. 2020a). The evolving rapid prototyping techniques emerge as an alternative for conventional techniques. RP generates three-dimensional objects using predefined models. Other terminologies such as additive manufacturing, solid freeform manufacturing, rapid tooling, and layered manufacturing describe rapid prototyping. The process involves the creation and slicing of the computer-aided design (CAD) model followed by layer-by-layer fabrication (Chua et al. 2020). Various biomaterials are used, such as polymers, ceramics, metals, and composites. Based on their initial form, the rapid prototyping is divided into three categories, liquid-based, solid-based, and powder-based, where stereolithography is a liquid-based system and fused deposition model is a solid-based system, whereas bioprinting and

selective laser sintering is a powder-based system (Peltola et al. 2008; Touri et al. 2019).

24.3.1 Stereolithography

As mentioned, CAD explains the size and geometry of the constructs. These are either obtained by using software, mathematical equation, or clinical imaging technologies such as magnetic resonance imaging (MRI) and others (Abdelaal and Darwish 2013). The potential of constructing a structure similar to patient scans made this technology beneficial for spectra of biomedical applications (Peltola et al. 2008). The technique uses a photopolymerization process activated by movable photons to fabricate the 3D structure. Briefly, the photopolymers on which the ultraviolet light is irradiated undergo curing and layer-by-layer construction in a precise pattern till the three-dimensional structure completes. Further, remove the non-polymerized resin followed by post-curing to reduce the irregularities (Melchels et al. 2010; Bagheri and Jin 2019). The irradiation is performed by two different methods, laser-based and digital light projection (Kumar and Kim 2020). The laser-based represents the first-generation stereolithography where the scanned UV beam is used to cure the polymer resins. Besides, the process has two approaches to build a construct that involves bottom-up and top-down methods. Bottom-up is where the laser beam builds up in support of a movable platform located next to the resin. At the same time, the top-down approach comprises a transparent, nonadhering platform located below the bottom surface of the resin (Huang et al. 2020; Qu 2020). In the digital light projection, a digital mirror device adds to the setup making it second-generation stereolithography. It exhibits higher efficiency when compared to the laser-based device concerning the layer thickness and needed exposure time. Based on projection stereolithography, continuous stereolithography was developed for better yield by creating a dead zone, which reduces the printing time (de Beer et al. 2019). Currently, a new approach called a volumetric technique (Kelly et al. 2019) has the best potential for high throughput of >105 mm^3/h and supports the processing of high viscous photopolymers. Researchers are exploring for better yield by combining methods such as scanning projection stereolithography (Zheng et al. 2016). The progress proceeds related to higher resolution, higher printing speed, and multi-material usage.

Based on the properties and application of a variety of polymers, the SLA technique has been widely used in bone, cartilage, and skin tissue regenerations (Skoog et al. 2013). High-resolution printing capabilities of stereolithography were used by researchers to develop a suitable 3D structure, a study from Aisenbrey et al., where they used this technique to develop a 3D structure using poly(ethylene glycol) hydrogel. The results demonstrate the feasibility of the novel hydride scaffolds and their potential to prevent long-term cartilage degeneration (Aisenbrey et al. 2018), a similar study on cartilage tissue engineering where elastic poly(trimethylene carbonate) structures were used to fabricate the scaffold. Additionally, after 6 weeks of cell seeding, the constructs increase 50% of compression moduli, approximately

100 kPa. Thus, it appeared as a promising scaffold for cartilage regeneration (Schüller-Ravoo et al. 2013). The stereolithography in bone regeneration has been heavily studied. The polymers such as hydroxyapatite scaffold with 500 μm of pore diameter and elastic modules ranging from 2.4 to 5.9 GPa were observed (Wang et al. 2020b). A similar nanohydroxyapatite with polytrimethyl was used to develop calcium phosphate-loaded polymeric porous scaffolds with controlled architecture (Guillaume et al. 2017). The technique was even well established in soft tissue engineering using different polymers that include polyethers, polyesters, alginate, hyaluronic acid, collagen, gelatin, and so on (Mondschein et al. 2017). However, the process has well accepted the limitations in photopolymerization process, which include the cytotoxic effect of the photoinitiators and less availability of photosensitive polymers (Kengla et al. 2020).

24.3.2 Selective Laser Sintering

Selective laser sintering works in the same way as any other layer-by-layer for the predesigned computer-aided design. Besides, it overcomes the major limitations of the rest of the techniques, i.e., the usage of toxic solvents. The principle of the technique says the laser fuses the powder materials together by heating them below the sintering temperature (Santos-Rosales et al. 2020). Initially, the process begins by slicing 3D CAD data into cross-sectional layers. Then the machine starts to create the first layer after leveling the powder bed with a thin layer of powder materials. Usually, the CO_2 laser is used as a power source, which trances and scans the cross-section, together selective heating fuses the material (Wang et al. 2020a; Sing et al. 2017). Once the single layer is complete, the powder bed goes down to give space for the next layer. Every layer is sintered deeply to bond with each other. The process is repeated until the predesign is achieved.

SLS is widely used in biomedical engineering for the fabrication of implants. A study from Gayer et al. developed a polylactide/calcium carbonate composite with decreased viscosity and low micro-porosity of 2% that leads to the increased strength of 75 MPa (Gayer et al. 2019). Ramu et al. optimized interconnected porous scaffold made of polyamide/hydroxyapatite using SLS technique. The obtained results showed suitable porosity and mechanical strength of 40–70% and 28.1 MPa, respectively, and also ensured using finite element analysis of a human femur bone under various physical activities (Ramu et al. 2018). The main advantage of the technique is the development of microstructures.

24.3.3 Fused Deposition Modeling

Fused deposition model works based on extrusion strategies to develop highly interconnected porous structure (Zein et al. 2002). Initially the step of the process is the same as the other additive manufacturing techniques, i.e., the "slicing" CAD data into layers. Here, the thermoplastic materials are used in the form of the thin

filament of which undergoes phase change in a temperature-controlled extruder. In exact, the filament is melted or liquified in the extruder head, which is then deposited onto the platform through a nozzle located in the bottom of the extruder (Mohan et al. 2017). The two rollers will be used for the movement of the filament. Further, the deposition of the semi-molten material occurs in the *X* and *Y* direction, forming layers. After each layer, the platform is lowered for the deposition of the new layer. The obtained geometry is influenced by the processing parameters, which vary as per the materials. The parameters include raster angle, air gap, layer thickness, road width, build orientation, and raster width, where raster angels refer to the direction of deposition of raster filling and roads mean the cross-sections which create layer (Zein et al. 2002; Benwood et al. 2018). On the other hand, the temperature is set above the melting point of the material, and the width of roads depends on the set temperature, extrusion velocity, and used nozzles or extruder tip diameter (Garg et al. 2016; Sun et al. 2008). The main limitation of the technique was the weak mechanical strength of the FDM part (Rahim et al. 2019). In recent times several studies have opted this technique to achieve better mechanical strength by altering the parameters (Domingo-Espin et al. 2015; Dorigato et al. 2017; Benwood et al. 2018).

24.3.4 3D Bioprinting

Various advantages of bioprinting over conventional scaffolding methods account for the rapid progress of the technique in the field of tissue engineering. Bioprinting has the potential to develop a 3D structure of patient-specific spatial geometry, controlled microstructure along with homogenous distribution of cells, and active biomolecules (Datta et al. 2018). Thus, these possibilities made it to achieve the 3D structures that can mimic bodily tissue. The bioprinting process usually occurs in three stages, pre-processing, processing, and post-processing (Vijayavenkataraman et al. 2018). Initial pre-processing is a common step of additive manufacturing which involves imaging, converting image to 3D models, and slicing. In the next processing step, the cells are harvested and cultured from the patient's body, which is further expanded to ex vivo for the bioprinting process. The cultured cells are suspended into an additional material that can mimic purposed tissue to form a cell-laden bioink. The bioprinter processes this bioink according to the designed 3D model. The final post-processing step is where the bioreactor provides the tissue-specific physiological in vitro environment for maturation of the developed tissue before being transplanted into patients (Vijayavenkataraman et al. 2018; Kačarević et al. 2018).

Right now, inkjet-based, laser-assisted, and micro extrusion-based bioprinting methods are widely used methods to patterning bioinks (Eltom et al. 2019). Inkjet is a technology that has the ability to generate droplets in picoliter volume. The technique is widely used in the printing industry, as the technology developed researchers find the picoliter-level printing technique is suitable for depositing biological components (Li et al. 2020b). The main categories of this technique are

continuous inkjet and drop on demand inkjet technology (Bishop et al. 2017). Due to the mechanism of continuous production of droplets, drop on demand method was accepted over continuous inkjet, as it is most suitable for the delivery of biomaterials. Depending on the type of droplet triggers, it is further divided into thermal, piezoelectric, and electrostatic systems (Li et al. 2018). In thermal, droplets are generated by the heat bubbles produced by a heat actuator in the chamber, whereas in piezoelectric and electrostatic, the piezoelectric ceramics and circuit connected chamber wall, respectively, triggers the droplet ejection. Even though the method has several advantages, there are some major limitations as well. The common drawback is that the bioink viscosity and high cell concentration causes clogging; thus achieving properties like liquidity viscosity is a challenge. Besides, droplet form of printing is again a loophole for the development of large size structure (Saunders and Derby 2014; Li et al. 2020b).

Extrusion-based bioprinting is a technique capable of depositing biomaterials and cells in the form of filaments, fibers, or droplets (Ning and Chen 2017). Thus, the methods benefit the fabrication of large-scale scaffolds. The extrusion bioprinting system assembles a fluid dispensing head controlled by an automated robotic system. The dispensing head moves along the X, Y, and Z axis to deposit the bioink in the layer-by-layer pattern. In relation to the depositing mechanism, the method can be divided into three categories, pneumatic-, piston-, and screw-based bioprinting (Hospodiuk et al. 2018). The pneumatic method utilizes compressed air to drive biomaterials and cell solutions from syringe to nozzle. The precise deposition is highly determined by the viscosity of the solution. Regarding the piston and screw method, the moving piston and rotating screw, respectively, characterize the technique. Here, the large mechanically controlled driving force of solution may lead to rupture of the cell membrane. Furthermore, the disadvantages of the method include limited printing resolution, reduced cell number, and clogging tip (Ning and Chen 2017). All the existing bioprinting methods are struggling to achieve a high cell density and difficulty in dispensing of cell aggregates. This problem was overcome from laser-based bioprinting, which is capable of generating scaffold of desired cell density (Koch et al. 2013). The system setup consists of two coplanar glass slides. The upper glass slide is made of three layers, support layer, laser absorbing layer, and bioink layer. The glass slide parallel to the donor slide is the collector slide. Laser focus triggers evaporation, and that generates high gas pressure, which sends bioink toward the collector slide (Guillotin et al. 2013). The technique made it possible for accurate deposition by achieving it without any clogging, with the usage of diverse viscosity materials and of high resolution (Ning and Chen 2017).

24.4 Conclusion

The techniques used for the development of 2D and 3D scaffolds for tissue engineering have been explained in the chapter. All the fabrication methods have advantages and disadvantages. The concepts explain the advantages of the technologies to achieve an accurate scaffold using these fabrication techniques.

Currently, the additive manufacturing techniques with computer-aided design have increased feasibility, as it generates patient-specific 3D structure. However, advanced technologies remain a flourishing area for researchers to overcome the challenges of developing new materials with higher manufacturing efficiency.

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