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Heung Jae Chun · Chan Hum Park
Il Keun Kwon · Gilson Khang *Editors*

Cutting-Edge Enabling Technologies for Regenerative Medicine

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Cutting-Edge Enabling Technologies for Regenerative Medicine

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Preface

So far, numerous papers and books on the research and clinical trials related to regenerative medicine have been published, and this field is continuously developing on the basis of the accumulated experience and knowledge. This volume is a series of book I published under the title of "Novel Biomaterials for Regenerative Medicine" and comprehensively reviews "Enabling Cutting Edge Technology for Regenerative Medicine."

This book consists of 4 main sections and 25 chapters containing recent topics reported by a number of prominent researches in these fields. **The first section of this book consisting of five chapters reviews technologies on 3D printing and 3D eletrospun for regenerative medicine.** Chapter 1 introduces a 3D bioprinting technique of adipose-derived stem cells for organ manufacturing. In Chaps. 2 and 3, 3D bioprinting technologies and electrospun 3D scaffolds for tissue engineering applications are reviewed, respectively. Chapter 4 introduces electrospun scaffolds prepared by natural polymers/composites for bone tissue regeneration. As a final chapter of this section, Chap. 5 summarizes electrospun and electrosprayed scaffolds for tissue engineering applications.

The second section consists of five chapters and provides information on intelligent nanocomposite biomaterials for regenerative medicine. In Chap. 6, as promising options for hard tissue regeneration, grapheme-based nanocomposites are introduced. For applications in orthopedic surgery, modified poly(methyl methacrylate) cements are reviewed. Chap. 8 researches trends of intrinsically conductive polymer nanocomposites for cellular applications. As another approach, the materials and applications for smart diagnostic contact lens systems are reviewed in Chap. 9. The last Chap. 10 of this section includes a review on advances in protein-based materials from origin to novel biomaterials.

The third section consisting of chapters 11 to 16 reviews drug delivery systems for regenerative medicine. In Chap. 11, crosslinking biopolymers are introduced for advanced drug delivery and tissue engineering applications. Chapter 12 introduces bone tissue engineering strategies in co-delivery of bone morphogenetic protein-2 and biochemical signaling factors. Delivery systems of growth factors for tissue engineering and regenerative medicine are described in Chap. 13. For biomedical applications, new combined polymer-based nanoparticles are introduced in Chap. 14. Content depicted in Chap. 15 includes reactive oxygen species responsive naturally occurring

phenolic-based polymeric prodrug. In Chap. 16, the feasibility of biodegradable polymer nanocarrier-based immunotherapy for hepatitis vaccination is introduced.

The fourth section consisting of chapters 17 to 25 reviews future enabling technologies for regenerative medicine. Chapter 17 introduces biomaterials for brain tissue engineering. Synthesis, biofunctionalization, potential applications, and challenges of polypyrrole as an electrically conductive biomaterial are introduced in Chap. 18. In Chap. 19, design of temperature-responsive cell culture surfaces for cell sheet-based regenerative therapy and 3D tissue fabrication is reviewed. Chapter 20 reviews harnessing nanotopography of electrospun nanofibrous nerve guide conduits (NGCs) for neural tissue engineering. Review of Chap. 21 includes biomechanics in annulus fibrosus degeneration and regeneration. Chapter 22 introduces nanopatterned scaffolds for neural tissue engineering and regenerative medicine. In addition, in Chap. 23, process system engineering methodologies for tissue development and regenerative medicine are reviewed. Chapter 24 reviews biomimetic extracellular matrices and scaffolds prepared from cultured cells. Finally, Chap. 25 introduces tissue scaffolds as a local drug delivery system for bone regeneration.

This volume was designed while envisioning the applications of biomaterials to implement cutting edge technology for regenerative medicine in twenty-first century. We hope that this book will make a positive contribution to the future development of clinical applications using biomaterials for regenerative medicine. We are grateful to all the contributors who have participated in the preparation of this book. Finally, we deeply thank Dr. Sue Lee, who is the publishing editor of biomedical sciences of Springer Nature, for her helpful suggestions and discussion on the organization of this book. Also we would like to appreciate Mrs. Ok Kyun Choi and Yong Woon Jeong at Gilson's Lab for e-mailing to all authors, editing, pressing, and so on as boring and tedious works. Without their support, this huge work would not have been possible.

Acknowledgement We offer a special thanks to all participants who have generously devoted their time, energy, experience, and intelligence for successful completion of this book. Their efforts will contribute to next generation who studies regenerative medicine based on biomaterials. Finally, we really appreciate the effort of Dr. Sue Lee, the publishing editor of biomedical sciences of Springer Nature, who made a great effort to publish this book. Also we would like to appreciate Mrs. Ok Kyun Choi and Yong Woon Jeong at Gilson's Lab for e-mailing all authors, editing, pressing, and so on as boring and tedious works. Without their support, this huge work would not have been possible. Prof Khang appreciate the financial supports from NRF-2017R1A2B3010270 and H15C2996.

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Part I

3D Printing and 3D Electro-Spun for Regenerative Medicine

1 3D Bioprinting of Adipose-Derived Stem Cells for Organ Manufacturing

Xiaohong Wang and Chang Liu

Abstract

Organ manufacturing is an attractive high-tech research field which can solve the serious donor shortage problems for allograft organ transplantation, high throughput drug screening, and energy metabolism model establishment. How to integrate heterogeneous cell types along with other biomaterials to form bioartificial organs is one of the kernel issues for organ manufacturing. At present, three-dimensional (3D) bioprinting of adipose-derives stem cell (ADSC) containing hydrogels has shown the most bright futures with respect to overcoming all the difficult problems encountered by tissue engineers over the last several decades. In this chapter, we briefly introduce the 3D ADSC bioprinting technologies for organ manufacturing, especially for the branched vascular network construction.

Keywords

Organ manufacturing · Three-dimensional (3D) bioprintng · Rapid prototyping · Tissue engineering · Biomaterials · Stem cells

1.1 Organ Manufacturing

Organ manufacturing is a long historical dream of human beings. The concept of organ manufacturing was first put forth by Professor Xiaohong Wang in 2003, at the Center of Organ Manufacturing, Department of Mechanical Engineering, Tsinghua University [1]. From then, a series of unique organ manufacturing protocols, technologies, and theories have been proposed [2–5]. With the rapid development of rapid prototyping (RP) technologies, it is now possible for us to manufacture bioartificial organs mimicking the natural counterparts with multi-cellular constituents, hierarchical structures (especially the branched vascular networks), and sophiscated functions [6–10].

Theoretically, the concept of organ manufacturing can be defined either in a broad or a narrow sense. In a broad sense, organ manufacturing is to produce organ substitutes with any molecular materials. In a narrow sense, organ manufacturing is to produce bioartificial organs using heterogeneous adult cell types or stem cells/heterogeneous growth factors along with other biomaterials and enabling processing technologies,

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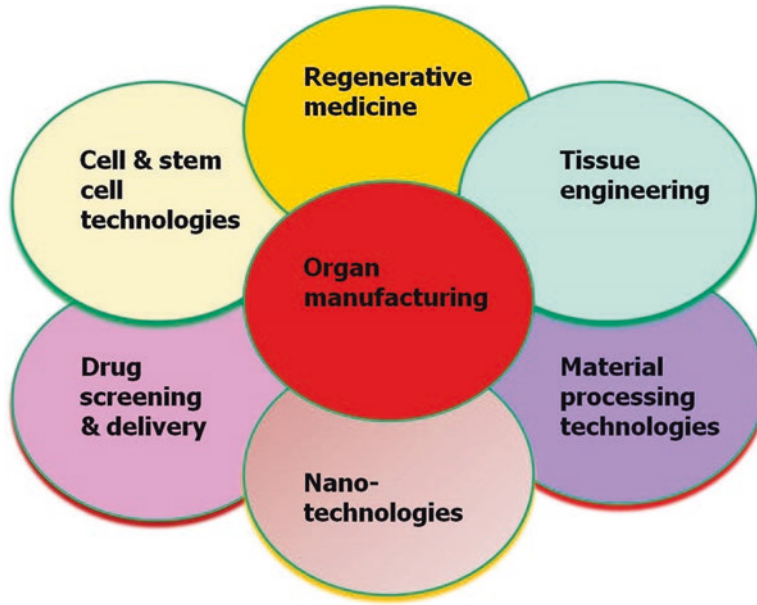

Fig. 1.1 Relationships of organ manufacturing with other sciences and technologies

such as the advanced RP and combined mold technologies [11–13]. Different from those *in vivo* organ generation at molecular or gene level, organ manufacturing is a crossing discipline which comprises many branches of sciences and technologies, and can be completed *in vitro* at cellular level [14–16].

Thus, organ manufacturing is a multidisciplinary field that has evolved in parallel with recent advances in biomaterials, biology, chemistry, computer, medicine and mechanics (or mechanology) (Fig. 1.1). Its main target is to build bioartificial organs using advanced processing tools, polymeric biomaterials, hetereogous cell types and/or stem cells/growth factors [17–19]. The produced bioartificial organs can be used for failure organ restoration, drug screening as well as metabolism analysis [20–22].

1.2 Three-Dimensional (3D) Bioprinting

Three dimensional (3D) bioprinting is the utilization of RP technologies to print cells, growth factors and other biomaterials in a layer-by-layer fashion to produce biomedical parts that maximally imitate natural tissue/organ characteristics [23]. It is also named as additive manufacturing (AM). Based on the working principles, there are three major types of 3D bioprinting technologies: extrusion-based, inkjet-based and laser-based (Fig. 1.2) [2–4].

Among these technologies, inkjet-based bioprinting, usually adapted from the commercial two dimensional (2D) printers, can print cell-laden liquid drops for fast and small-scale products. Extrusion-based 3D bioprinting can create large scale-up 3D constructs in layers using cell-laden hydrogels [24–27]. Meanwhile, laser-based 3D bioprinting can provide high-resolution structures utilizes lasers as the power [28–32]. There are many disadvantages for inkjet-base or laser-based 3D bioprinting technologies for organ manufcturing, such as low loading cell density, drying of droplets during printing, or time-consuming, limited heights of the 3D constructs [33–36].

In 3D bioprinting, cells are normally encapsulated in a polymeric hydrogel as 'bioinks' before being printed. The polymeric hydrogel which acts as the cell-loading matrix should has some unique characteristics, such as, 3D printable when a printer is employed, cellular compatible when inorganic solvents are used, structural tunable (i.e. sol-gel or liquid to solid phase transition) when temperature is changed, crosslinkable when chemical crosslinkers are imposed [2–9].

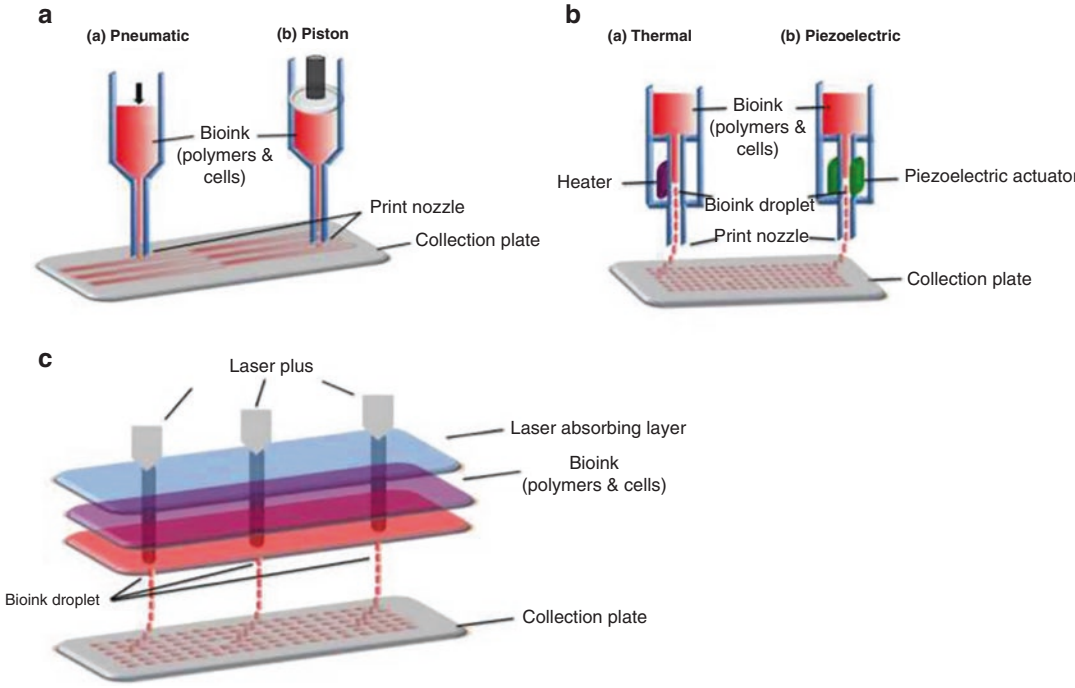

Fig. 1.2 A schematic diagram of the three major types of 3D bioprinting technologies for organ manufacturing: (**a**) extrusion-based bioprinting (*a*: Pneumatic; *b*: Piston); (**b**) inkjet-based bioprinting (*a*: Heater; *b*: Piezoelectric actuator); (**c**) laser-assisted bioprinting

Hydrogel is a kind of gel that uses water as the dispersion medium. There are many types of hydrogels which have been employed in 3D bioprinting technologies as cell-laden 'bioinks'. These hydrogels have different biological, chemical and physical properties depending on their origins, polymer chain structures and gelation mechanisms. The most commonly used 'bioinks' in 3D bioprinting are polymeric hydrogels, such as gelatin, fibrinogen, alginate, collagen, extracellular matrices (ECMs), algae (agar), chitosan, cellulose, casein, gelatin/alginate, gelatin/alginate/chitosan, gelatin/alginate/fibrinogen, gelatin/alginate/hyluroan, chitosan-hydroxyapatite, titanium, peptide (DNA), polycaprolactone (PCL), polyethylene glycol (PEG), polyurethane (PU), pluronic acid and poly(lactide-co-glycolide) (PLGA), poly(methyl methacrylate) (PMMA), gelatin/methacrylate (GelMA), polyester resins, polyvinylpyrrolidone, that contain either water or other specific biological fluids [37–48]. These hydrogels can be 3D printed with good shape fidelity, creating complex 3D structures that mimic *in vivo* cell growth macro- or microenvironment.

The hydrogels are either physical thermoresponsive or chemical/enzyme/photo (incldluing ultra violet, UV) crosslinkable. The encapsulation of living stem cells within a semi-permeable hydrogel is an attractive procedure for most of the 3D bioprinting technologies. Especially, naturally derived polymers, such as gelatin, alginate, hyluroan, collagen and fibrinogen, can form hydrogels and entrap cells at mild conditions using cell-friendly inorganic solvents and are capable of being 3D printed within controlled volumes. These hydrogels are advantageous for soft organ manufacturing as they are highly permeable to cell culture media, nutrients, growth factors, and waste products during tissue/organ generation stages. The cell-laden hydrogels can be printed into customized shapes with various material constituents and biological functions [2–9]. Meanwhile, synthetic polymers, such as PLGA and PU have strong mechanical properties

which can be used for hard organ manufacturing as well as vascular/neural network support.

Modern RP technologies, especially the extrusion-based multi-nozzle rapid prototyping (MNRP), allow the bioartificial organs to be automatically manufactured using computer-aided design (CAD) models [2–9]. Heterogeneous adult cell types and/or stem cells/growth factors can be printed simultaneously in a free-scalable 3D pattern incorporated with a hierarchical vascular and/or neural network [49–51]. The heterogenous cell types or growth factors can be embedded in different natural polymeric hydrogels for different physiological functionality realization.

Till now, 3D bioprinting that uses cell compatible polymeric hydrogels is an easy-to-use protocol allowing assessing the cell viability, cytotoxicity, tissue morphology, and organ maturation in the predefined constructs. While the number of approaches utilizing cell-, especially stem cell -laden hydrogels is constantly growing, it is essential to provide a framework of their typical hydrogel preparation, physiological function realization and target biomark evaluation for various bioartificial organs.

1.3 Cells for 3D Bioprinting

Various cell types have been used for 3D bioprinting technologies. These cell types include adult cells, such as hepatocytes, human umbilical vein endothelial cells (HUVECs), dermal skin fibroblasts, cardiomyocytes, myoblasts, and stem cells, such as embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), bone marrow-derived mesenchymal stem cells (BMSCs), neural stem cells (NSCs), adipose-derived stem cells (ADSCs), human induced pluripotent stem cells (iPSCs), glioma stem cells, and amniotic fluid-derived stem cells [52–60]. For example, in 2008 Phillippi et al. reported the printing results of proliferative cell types including adult stem cells toward muscle- and bone-like subpopulations [61–63]. The 3D bioprinted cells had high cell viability, retained their phenotypes and could self-assembled into vessel-like conduits when co-cultured with endothelial cells. In 2013, Hsieh et al. printed neurons using inkjet and microextrusion 3D bioprinting technologies [64, 65]. The cells maintained their basic cellular phenotypes and functionality in the 3D constructs for more than 2 weeks and developed voltage-gated potassium and sodium channels. In 2010, Choi et al. demonstrated that ESCs printed using a laser direct-write technique formed embryoid body cellular aggregates with neural differentiation capacity [66, 67].

Since most of the autologous or primary adult cells, such as nephrocytes, cardiomyocytes and hepatocytes, are difficult to isolate, propagate and culture *in vitro*, more and more stem cells have been used in 3D bioprinting technologies for organ manufacturing [68].

1.4 Adipose-Derived Stem Cells (ADSCs) for Organ Manufacturing

Stem cells have the properties of self-renewal potency, representing an unlimited cell source for 3D bioprinting and organ manufacturing. ADSC is one of the adult stem cells which can be collected from patients' adipose tissues. It is known that ADSCs are easy to culture *in vitro* and have the potential to improve current understanding of disease mechanisms, while minimizing rejection when transplanted back into the host for the purposes of organ manufacturing [69–72].

We and others have proven that ADSC is an effective building material for various bioartificial organ manufacturing. In our previous studies, ADSCs have been printed in some free-scalable 3D constructs by means of a series of self-developed RP (i.e. 3D bioprinting) technologies. It has been demonstrated that neither the proliferation ability nor the differentiation behaviour of the ADSCs are affected by the 3D bioprinting procedures. ADSCs in the 3D constructs have been differentiated into endothelial, smooth muscle, hepatic and adipogenic lineage pathways with different growth factor engagement protocols. Quantitative assessments of endothelial, smooth muscle, hepatic, and adipogenic markers have been verified. These results have indicated that

bioartificial organ manufacturing using ADSC-laden polymeric hydrogels, growth factor engagements and MNRP technologies is practicable [73–75].

One central object of bioartificial organ 3D bioprinting is to print stem cells, especially ADSCs, along with different growth factors to generate multiple heterogenous tissues in a predefined construct [76–78]. The construction of vasculature (including large blood vessels, small blood vessels and capillary vessels) in the 3D constrcts is a complex process that depends on the interaction and coordination of many growth factors (or cytokines) with the polymeric hydrogels. Deficiencies in any of the essential growth factors for ADSC differentiation to the target tissues may disrupt the organ formation process. The delivery ways of the growth factor combinations we have created are currently promising approaches for bioartificial organ manufacturing [76–78].

We have also verified by quantitative assessments of various adipogenic biomarkers secreted by the ADSCs in the 3D printed constructs resembling cell lineages present in natural organs. Additionally, we have provided the proof that ADSCs in the 3D constructs can be utilized for the generation of vasculatures with a full coverage of endothelial cells, which cannot be achieved by other existing technologies. These results indicate that 3D bioprinting of living bioartificial organs resembling their native counterparts either has already be realized or is within reach [76–78].

1.5 3D Bioprinting of ADSCs for Organ Manufacturing

1.5.1 Organ 3D Bioprinting Technologies

Globally, 3D printing technologies have been applied to nearly every organ in human beings. For example, in 2005 the first 3D printed hepatic tissues was reported by our own group [7–9]. The bioprinted hepatocytes were positive for secreting albumin and other materials. In 2008, the first large scale-up vascularized adipose tissue was created in our laboratory [69–72]. Confocal microscopy was used to demonstrate that the extrusion-based 3D bioprinting technique allowed for micro to macro scale arrangement of ADSCs and other cells for generating multicellular organs with a complexity similar to native vascularized adipose tissues. In 2012 a functional jawbone replacement was produced using a laser sintering technique for an 83-year-old woman suffering from a lower jaw infection [79]. This jaw implant could foster muscle attachment, nerve/vein ingrowth and surgical recovery. At the meantime, a cardiac patch was printed using a laser direct write bioprinting technology by printing human cardiac-derived cardiomyocyte progenitor cells in an alginate hydrogel scaffold [80]. This cardiac patch had the potential to function *in vivo*. Another study found that the time-dependent stiffening of thiolated hyaluronic acid hydrogels could promote the differentiation of primary chicken embryonic cardiomyocytes [81]. In 2017, Kang et al. investigated the photocrosslinking hydrogels on effects of the extrusion bioprinted aortic valve interstitial cells, aortic valve sinus smooth muscle cells and ADSCs [81]. In 2013, a biodegradable PCL tracheal splint was printed using a extrusion-based 3D printing technique for a 2-month-old child who required endotracheal intubation to sustain ventilation [82]. After surgery, an immediate improvement was observed, and the patency was retained for 1 year without complications. Later in 2015 3D inkjet bioprinting was used to print full-thickness skin equivalents with a dermal fibroblast cell suspension [83]. The cells showed high viability after printing. Nevertheless, the use of bioprinted techniques for organ manufacturing is still an emerging concept, and further work is needed to advance these strategies and successfully translate them into clinical applications.

1.5.2 Extrusion-Based Organ 3D Bioprinting Technologies

Back to 2003, the first extrusion-based organ 3D bioprinter was developed in Professor Xiaohong Wang's laboratory, the Center of

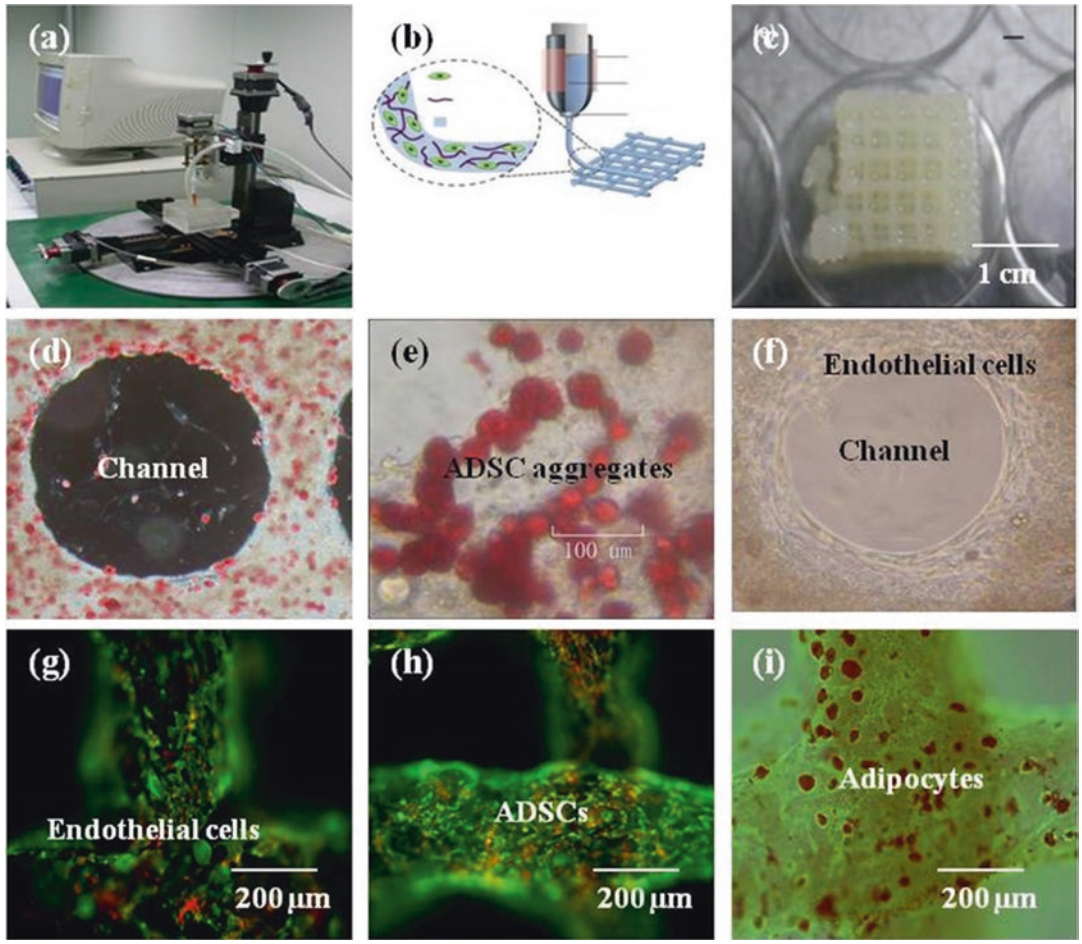

Fig. 1.3 3D bioprinting of ADSCs for organ manufacturing: (**a**) a pioneered 3D bioprinters made in Tsinghua Unversity, Prof. X Wang' group; (**b**) schematic description of the ADSCs encapsulated in the gelatin-based hydrogels being printed into large scale-up 3D constructs using the single-nozzle 3D bioprinter; (**c**) a grid 3D construct made from the ADSC-laden gelatin-based hydrogel; (**d**) ADSC aggregates encapsulated in the gelatin-based hydrogels; (**e**) a magnified photo of (**d**); (**f**) endothelialized ADSCs on the channel surface; (**g**) immunostaining of the endothelizlized ADSCs with mAbs for CD31+ cells in green and PI for cell nuclei (nucleus) in red; (**h**) immunostaining of the endothelizlized ADSCs for CD31+ cells in green and adipose cells in red; (**i**) all the ADSCs in the 3D construct differentiated into target adipose cells after 3 days treatment with insulin, dexamethasone and IBMX, but no EGF

Organ Manufacturing at Tsinghua University (Fig. 1.3a) [7]. In 2004, a cell-laden hydrogel was printed into 3D constructs using this technology. Tailor-made nozzles or thin syringe needles were adapted to print cell- and/or growth factor-laden gelatin-based hydrogels layer-by-layer under a piston-driven force (pressure) [9, 10]. It is a major breakthrough technology that integrates living cells, polymeric hydrogels and a robotic material dispensing device to create large scale-up organs. Since then, a series of gelatin-based hydrogels, such as gelatin, gelatin/alginate, gelatin/chitosan, gelatin/hyaluronan, gelatin/matrigel, gelatin/alginate/fibrinogen and gelatin/alginate/fibrinogen/hyaluronan, have been employed as 'bioinks' for numerous studies, such as high throughput drug screening, energy metabolism model establishment, and bioartificial organ manufacturing.

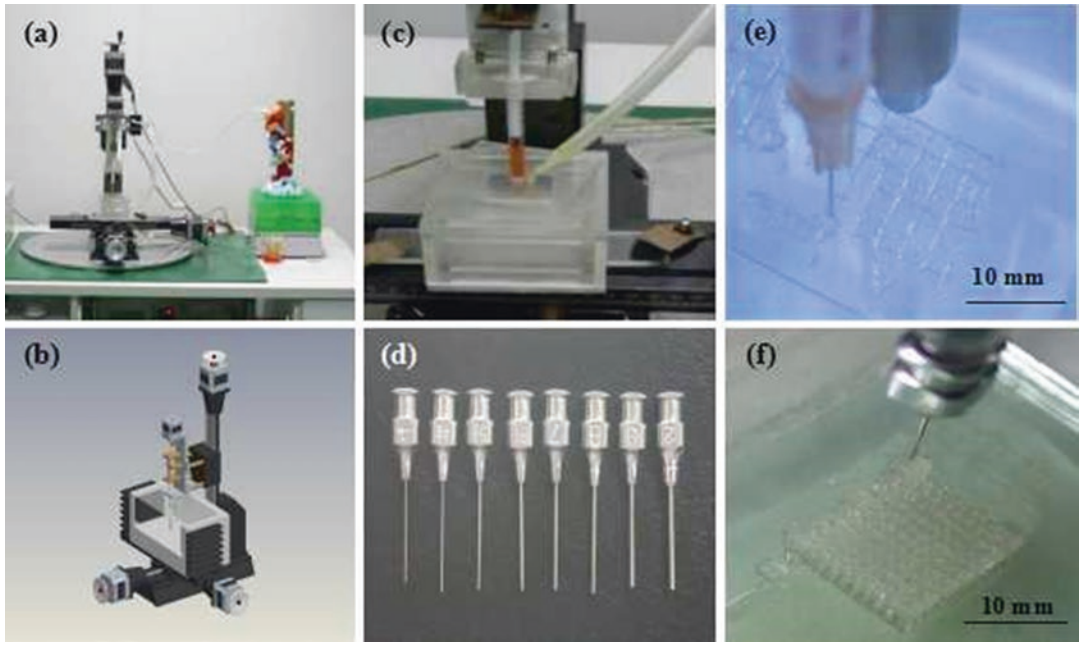

Fig. 1.4 Decomposition chart of Fig. 1.3a: (**a**) the major driver elements of the single-syringe 3D bioprinter; (**b**) a CAD model of the major driver elements; (**c**) the working platform; (**d**) tips of the syringe from sharp to flat; (**e**) a flat tip collateral with a nozzle; (**f**) a working flat tip with several layers of cell-laden hydrogel

Typically, the single-nozzle extrusion-based 3D bioprinter consists of a spiral squeezing unit equipped with a flat tip syringe (as shown in Fig. 1.4) [7–10]. When the cell- and/or growth factor-laden gelatin-based hydrogels are extruded through the flat tip to a working platform at certain rate, a driving force is created to deliver the cell- and/or growth factor-laden hydrogels from the flat tip to the working platform (e.g. polyvinyl chloride board). The deposited cell- and/or growth factor-laden hydrogels take shapes on the working platform as thin fibers and overlap layer-by-layer (in layers).

In 2007, a double-nozzle organ 3D bioprinter was innovated at the center of organ manufacturing in Tsinghua University, professor Wang's laboratory [84, 85]. Using this technology, two cell types with large population of cells have been simultaneously printed into large scale-up living organs. With the updated hard- and software, both the hierarchical branched vascular template and interconnected grid channels have been properly integrated into the living organs under the instructions of the CAD models.

The 3D bioprinted ADSCs, had high cell viability, retained their proliferation capacity for more than 2 months in the gelatin-based hydrogels. Since then, the study of organ manufacturing has been aided by a series of two and multi-nozzle extrusion-based 3D bioprinting (or MNRP) technologies. The extrusion-based 3D bioprinting technologies have provided a high-tech platform that can be used to duplicate the cell living environments *in vivo* and evaluate normal and pathologic conditions *in vitro* beside to produce complex bioartificial organs for implantation.

Most of the studies were performed using different cell-laden composite gelatin-based hydrogels, such as the gelatin/alginate, gelatin/chitosan, gelatin/alginate/fibrinogen. The composite hydrogels is suitable for any cell types, especially for ADSCs with rapid proliferation velocity [86–88]. Compared with single polymeric hydrogels, such as the gelatin, alginate, finrinogen, chitosan, the composite hydrogel formulation demonstrates improved cell-material interactions and adipogenesis by providing an optimized microen-

vironment for the bioprinted cells. These studies have certified that the extrusion-based 3D bioprinting technologies are successful in manufacturing large scale-up bioartificial organs.

1.5.3 ADSC Engagement

Since the ADSCs were first printed into large scale-up 3D constructs and subsequently induced into vascularized organs (e.g. vascularized adipose tissues) with a cocktail growth factor engagement (using the pioneered single-nozzle extrusion-based 3D bioprinter) [89–92]. The 3D bioprinted ADSCs have a very high cell viability (≈100%), retained their commitment to the original phenotypes and have biological functions under various culture conditions. Especially, the 3D bioprinted ADSCs proliferate exponentially and form large aggregates in the gelatin-based hydrogel in 2 months (Fig. 1.3d-i).

There are two key factors which determine the ADSCs whether proliferate into large aggregates or differentiate into heterogeneous tissues. The double crosslinked gelatin-based hydrogels have provided the encapsulated stem cells not only stable accommodations (structural integrity), sufficient nutrients, active spaces (i.e. activity rooms), but also cocktail growth factors, gradient ECM compositions, and spatial engagement effects [69–72].

Before 3D bioprinting, rat and rabbit ADSCs (including fat–derived stromal vascular fraction cells) were mixed with a gelatin-based solution and loaded into the 'bioink' barrel or syringe. Gelatin has a special thermoresponsive property at about 28°C which makes the gelatin-based hydrogels can be piled up in layers during the 3D bioprinting processes. Other natural polymers, such as alginate, chitosan, and fibrinogen, can be incorporated into the gelatin solution for various other purposes, such as the 3D structure stabilization, stem cell nich identification. The 3D bioprinting results are mainly determined by the unique thermoresponsive property of the gelatin molecules [2–9].

The printing procedure has not affected the proliferation and differentiation capabilities of the ADSCs. The ADSCs maintained their triggered lineage behaviors, as measured by expression of adipogenic markers [69–72]. Confocal microscopy was used to demonstrate that the extrusion-based 3D bioprinting technologies allow for macro- and microscale arrangement of the ADSCs in a predefined 3D construct. After different growth factor engagements, multicellular tissues generate in the predefined 3D constructs with a complexity similar to native organs, such as the breast with vascularized adipose tissues, and the pancreas with endothelialized islets.

Furthermore, 3D bioprinting of bioartificial organs that mimic the natural architectures of their counterparts allow for autologous *in vivo* organ replacement, as well as for *in vitro* biomedical investigation, such as pathological diagnoses and high throughput drug screening. In particular, the studies of organ manufacturing using 3D printing of ADSCs allow for *in vitro* modeling of the branched vascular/neural networks with more biomimetic microenvironmental architectures [2–9].

1.5.4 Limitations and Challenges

3D Bioprinting technologies have important implications for complex organ manufacturing, as these technologies can provide an accurate, fast manner to arrange multiple cell types and/or growth factors. Bioartificial organ 3D bioprinting has enormous implications in biomedical fields, especially given the acute need for failure organs and the limited numbers of viable donors [93, 94]. The functional bioartificial organs can also be used for *in vitro* drug screening and as well as disease studies. Nevertheless, there is still no such a powerful 3D bioprinter which can manufacture all the organs in human body.

Organ manufacturing often face multiple tissue design and construction issues. One of the challenge is to recapitulate the complex material, structural and morphological components of the native organs. The primary single or double nozzle 3D bioprinters till now cannot provide all the necessary materials and structures in a single native organ for different physiological function

realization. It is challenging to develop combined MNRP to print more types of multiple adult autologous cells together with other biomaterials, in combination with stem cells, such as BMSCs, ADSCs, peripheral blood stem cells, or MSCs, to create much more complicated organs with additional constructs, such as the bile ducts in the liver and the glomeruli in the kidney beside the branched vascular and nerve networks.

Emphases should be given to the ADSCs, a special MSCs, which have demonstrated their advantages in bioartificial organ 3D bioprinting technologies with high proliferation and differentiation capabilities [69–72, 84–88]. ADSCs in the 3D printed constructs has differentiated into endothelial cell, smooth muscle cell, adipose cells, fibroblasts and hepatocytes. Further work is needed to understand the molecular pathways involved in these processes. Additional sources of human derived or patient specific stem cells, especially ADSCs, are needed for clinical purposes or applications.

1.6 Conclusions and Perspectives

3D bioprinting of heterogeneous cell types is a promising technology to construct bioartificial organs for implantation and other purposes. Combined MNRP 3D bioprinting of ADSCs along with other biomaterials holds much more promise when considering personalized organ regeneration. Heterogeneous multi-cellular bioartificial organs with hierarchical architectual structures could be built using ADSC-laden gelatin-based hydrogels and multi-nozzle 3D biopinters according to the predesigned CAD models. Spatial pattern (or alignment) of ADSC-laden hydrogels with proper growth factor engagement could solve all the bottleneck problems for organ manufacturing encountered by tissue engineers over the last several decades. Distinguishly, fully endothelialization of the inner surfaces of the branched vascular network could be achieved through ADSC inducement using special combinations of vascular cell growth factors. It is therefore a great revolution in biomedical field that can significantly improve the life quality and prolong the average life-span of human beings.

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2 3D Bioprinting Technologies for Tissue Engineering Applications

Bon Kang Gu, Dong Jin Choi, Sang Jun Park, Young-Jin Kim, and Chun-Ho Kim

Abstract

Three-dimensional (3D) printing (rapid prototyping or additive manufacturing) technologies have received significant attention in various fields over the past several decades. Tissue engineering applications of 3D bioprinting, in particular, have attracted the attention of many researchers. 3D scaffolds produced by the 3D bioprinting of biomaterials (bio-inks) enable the regeneration and restoration of various tissues and organs. These 3D bioprinting techniques are useful for fabricating scaffolds for biomedical and regenerative medicine and tissue engineering applications, permitting rapid manufacture with high-precision and control over size, porosity, and shape. In this review, we introduce a variety of tissue engineering applications to create bones, vascular, skin, cartilage, and neural structures using a variety of 3D bioprinting techniques.

Keywords

Bioprinting · Scaffold · Bio-ink · Tissue engineering

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2.1 Introduction

In recent decades, regenerative medicine and tissue engineering research has been directed toward the regeneration, replacement, or restoration of injured functional living tissues and organs, such as bone, vascular, skin, neural, and cartilage [2, 22, 37, 40, 87]. These tissue engineering applications require the insights of researchers from a number of fields, as well as specialized knowledge of biomaterials, cell biology, biocompatibility, imaging, and the characterization of scaffold surfaces [27, 64]. One of the most important aspects of tissue engineering is the fabrication of porous three-dimensional (3D) scaffolds that provide the appropriate environment for regenerating tissues and organs. 3D scaffolds for use in tissue engineering field are fabricated using a various manufacturing methods and biomaterials [41, 79]. Several important characteristics must be considered in these applications [11]: First and most importantly, a tissue engineering scaffold must be biocompatible. Second, fabricated 3D scaffolds should be biodegradable or bioabsorbable so that tissue ultimately replaces the scaffold. Third, the ideal scaffold should have mechanical properties con-

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sistent with the tissue to be implanted. Fourth and finally, the 3D scaffold should be readily manufacturable in a variety of shapes and sizes. Several methods have been developed for fabricating 3D scaffolds using synthetic and natural polymers, including gas foaming, phase separation, electrospinning, and melt molding [20, 40, 55, 65]. These scaffold fabrication methods cannot precisely control the pore size, shape of the scaffold, or the internal channel configuration within the scaffold. Moreover, there are limits on the ability to fabricate scaffolds using cells.

In recent years, 3D bioprinting methods that can readily control the size and shape of a 3D scaffold and the capacity to produce scaffolds together with cells have attracted attention [19, 21]. In this review, we describe 3D bioprinting and its use in adjusting the shape and size of a 3D scaffold. 3D bioprinting was first introduced by Charles W. Hull in 1986 [45]. 3D bioprinting is the process of making 3D solid or gelation objects using 3D modeling software based on computer aided design (CAD) or computer tomography (CT) scan images [7]. 3D bioprinting refers to the technique associated with creating a 3D structure using metals, ceramics, synthetics, or natural polymers [73]. Typically, 3D bioprinting equipment consists of an X-, Y-, Z-axis drive machine, computers, 3D modeling software, and bioprinting materials. After creating a design using CAD or CT images, the 3D bioprinting equipment connected to a computer creates a 3D scaffold structure [91]. 3D bioprinting methods typically consist of stereolithography (SLA) [70], digital light processing (DLP) [25], multi jet modeling (MJM) [89], fused deposition modeling (FDM) [86], selective laser sintering (SLS) [48], and laminated object manufacturing (LOM) [1] components. The SLA and DLP methods use liquid photopolymer resins and ultraviolet (UV) lasers to create 3D structures [25, 70]. The MJM method involves feeding materials through a small diameter nozzle in a material injection process that operates in a manner similar to typical inkjet printers [89]. MJM is an inkjet bioprinting process that uses print head technologies to deposit photo-curable plastic resins or casting wax materials in a layer-by-layer method. The FDM 3D bioprinting method is a common and simple method of using thermoplastic filaments as a bioprinting material [86]. Filaments are melted in the head of a 3D printer by heating and then used to create 3D structures. The SLS bioprinting technology fuses small particles, such as polymers and ceramics, by heating using a high-power laser to form a 3D structure [48]. LOM is a method of creating 3D models by stacking layers of defined sheet materials, such as polymers, plastics, and metals [1]. These methods are used in a variety of fields, including architectural modeling, art, lightweight machinery, as well as in 3D biomaterials used in tissue engineering.

Among the 3D bioprinting methods available SLA and DLP laser-based bioprinting methods, inkjet bioprinting, and the FDM bioprinting of 3D structure used in tissue engineering field have attracted the most attention from researchers. Figure 2.1 illustrates the various 3D bioprinting methods used for tissue engineering applications. 3D bioprinting of biomaterials is a new technology aimed at developing new organs and tissues. 3D bioprinting technologies can adjust the size and shape of a 3D scaffold to control cell proliferation, differentiation, and attachment within the 3D construct. This review introduces a various of tissue engineering field to produce bone, vascular, skin, neuron, and cartilage tissue using 3D bioprinting methods. Table 2.1 summarizes the 3D bioprinting technologies that have been used to produce bio-inks (biomaterials) and cell types.

2.2 Bone Tissue Engineering Using 3D Bioprinting

Bone has a highly specialized organic–inorganic structure that can be classified as a micro- and nano-composite structure that is maintained adjacent to complex cellular components [9]. Researchers have investigated efficient approaches to replacing lost or defective bones and developing good bone substitutes for a very long time [61]. The 3D artificial bone scaffold is used in the clinic for bone regeneration. Bone displays excellent self-healing capabilities if defects are small;

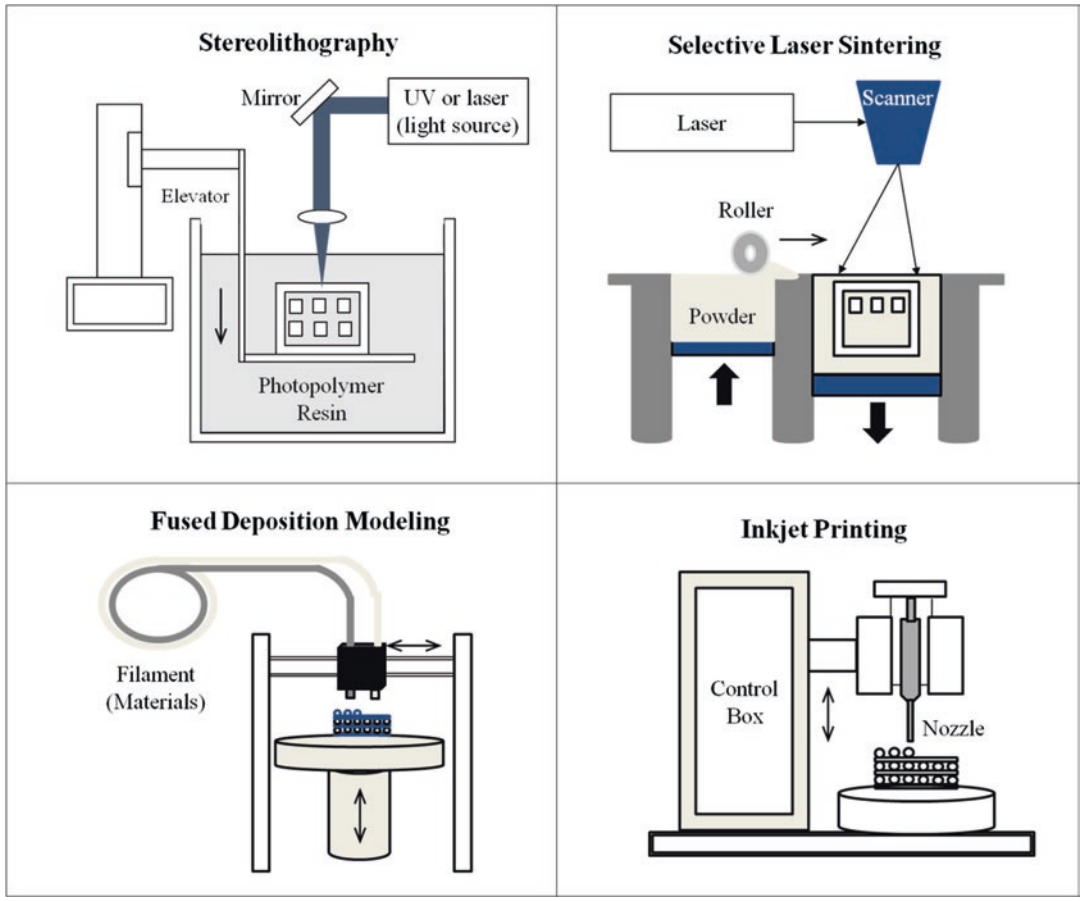

Fig. 2.1 Various 3D printing methods for tissue engineering applications

however, large-scale bone losses or defects cannot be completely healed by the body's innate regeneration systems [82]. In such cases, surgery is needed to replace or repair lost or defective bone. Researchers have attempted to manufacture bone substitutes and to develop restoration methods ([24, 78, 92, 93]). Bones may be classified into two types of structures: cancellous bone (inner part of the bone), which has a spongy structure with a porosity of 50–90%; and cortical bone, which forms a dense outer layer with a porosity of less than 10%. Differences between the internal and external structures of bone require careful consideration for scaffold design for use in bone regeneration. Scaffolds can be used to deliver biomolecules, such as TGF-beta, BMP, IGF, FGF, or VEGF, to promote bone regeneration [49]. Recently, researchers have been interested in 3D bioprinting technologies that can create structures in one step using a various of biomaterials [10, 60].

This chapter examines bone tissue engineering field based on various 3D bioprinting techniques. Rath et al. performed preliminary *in vitro* tests to evaluate the effects of dynamic versus static 3D culture conditions during the seeding of osteogenic cells (bone marrow-derived stromal cells and osteoblasts) onto biphasic calcium phosphate (CaP) 3D scaffolds under osteoinductive and basal culture conditions [71]. Dong et al. integrated poly(ε-caprolactone) (PCL) and chitosan thermogels to form a hybrid 3D scaffold for bone regeneration [17]. The 3D PCL scaffolds were fabricated by FDM bioprinting method. The *in vitro* study shows that the hybrid 3D scaffold could enhance cell proliferation and improve the

Table 2.1 Summarizes of the 3D printing technologies for various tissue engineering applications

Organ	Printing method	Bioink composition	Crosslinking	Cell type	Refs.
Bone	Inkjet-based	Hydroxyapatite/β-TCP Dextrin (Inkjet)	UV	Osteoblasts/bone marrow derived stromal cells	[14, 17, 71, 90]
		Alginate/Collagen (Inkjet)	$CaCl_2$	Human osteogenic sarcoma cells	
	Extrusion based	Chitosan/Polycaprolactone (FDM)	–	Rabbit bone marrow MSC	
		Poly(lactic acid)/Hydroxyapatite (FDM)	~180 °C		
Neural	Inkjet-based	Fibrinogen (Inkjet)	Thrombin	Primary hippocampal Cortical cell	[21, 28, 32, 52, 66, 95]
		Biologically active macromolecules (Inkjet)	–	NSC	
		Collagen Fibrinogen (Inkjet)	Thrombin	NSC	
		Collagen (Inkjet)	37°C	Schwann cell/Bone marrow stem cell	
		Polyurethane (Inkjet)	37°C	NSC	
		Alginate Chitosan/Agarose (Inkjet)	$CaCl_2$	NSC	
Vascular	Inkjet-based	Fibrinogen (Inkjet)	Thrombin	HMVEC	[16, 35, 44, 63, 69, 94]
		Cell spheroid (Inkjet)	–	HUVSMC/Fibroblast	
		Pluranic F127 diacrylate (Inkjet)	UV	–	
		Gelatin methacrylate (Inkjet)	UV	HUVEC/Fibroblast	
		Gelatin methacryloyl/Alginate PEG-tetra-acrylate (Inkjet)	$CaCl_2$UV	HUVEC/hMSC	
	Extrusion-based	Poly(vinyl alcohol)/Gelatin (FDM)	Transgultaminase	Hepatocellular carcinoma	
Skin	laser-based	Blood plasma/Alginate (SLA)	$CaCl_2$	Fibroblast/Keratinocyte hMSC	[15, 26, 42, 43, 50, 59]
		Collagen/Blood plasma Alginate (SLA)	$CaCl_2$	Fibroblast/Keratinocyte	
		Collagen (SLA)	37 °C	Fibroblast/Keratinocyte	
	Inkjet-based	Collagen (Inkjet)	37 °C	Fibroblast/Keratinocyte	
		Collagen (Inkjet)	$NaHCO_3$	Fibroblast	
		Blood plasma/Fibrinogen (Inkjet)	$CaCl_2$	Fibroblast/Keratinocyte	
Cartilage	Extrusion-based/ Inkjet-based	Polycaprolactone/Alginate (Inkjet, FDM)	$CaCl_2$	Chondrocyte	[39, 47, 72]
		Gellan/Alginate/BioCartilage (Inkjet)	$SrCl_2$/4 °C	Chondrocyte	
		Collagen (Inkjet)	37 °C	Chondrocyte	
Cancer	Inkjet-based	Gelatin/Alginate/Fibrin (Inkjet)	$CaCl_2$/Thrombin	Cervical tumor cell	[29, 81, 98]
		Matrigel (Inkjet)	37 °C	Human hepatic carcinoma cell/Human mammary epithelial	
	Laser-based	Poly (ethylene glycol) diacrylate (SLA)	UV	Cervical tumor cell Murin fibroblast	

osteogenesis of rabbit bone marrow mesenchymal stem cells (MSCs). They hypothesized that the PCL/chitosan 3D scaffolds could improve osteoinductivity, cell seeding efficacy, and provide excellent mechanical properties compared to the PCL or chitosan-thermogel 3D scaffold alone. Corcione et al. developed a solvent-free process for producing a hydroxyapatite and poly(lactic acid) composite material suitable for 3D bioprinting processes (using the FDM method) to realize customized scaffolds for bone tissue engineering [14]. In their study, a clinical image of maxillary sinus obtained by cone beam computer tomography were converted into a suitable format and successfully used to fabricate a 3D maxillary sinus model using 3D bioprinting of the composite material. Wang et al. explained that a cell-laden collagen/alginate scaffold could be supplemented with bioglass particles, a well-fabricated, porous, hard material used to fabricate bone replacement scaffolds, to increase the material stiffness and stimulate cell growth and mineralization [90].

2.3 Neural Tissue Engineering Using 3D Bioprinting

More than a billion people around the world are thought to suffer from nervous system disorders [5]. Chronic degenerative diseases or traumatic injury of the nervous system affect central nervous system (CNS) function. Neurodegenerative diseases due to aging are also becoming increasingly important. Despite many studies, treatments that can fully restore neuronal function are not yet available, and our molecular understanding of pathogenic mechanisms is limited. These limitations arise from the lack of models suitable for simulating complex environments *in vivo*. 2D cultures are primarily used for their cost-effectiveness, ease of handling, and applicability to various cell types; however, 2D cultures are not able to support the cell–cell and cell–extracellular matrix (ECM) interactions present *in vivo* [33, 84, 96, 97]. By contrast, 3D tissue engineering is thought to provide a more human-like environment for cells. A variety of 3D tissue engineering systems have been studied for their ability to integrate multiple cell types and create complex neural tissue organization structures [23, 36, 38, 83]. 3D bioprinting can accurately mimic the complexity of our bodies using precisely placed cells and biomaterials based on a desired design [62]. This chapter reviews research into 3D neural tissue models prepared using 3D bioprinting technologies.

Xu et al. used fibrin as a bio-ink to create 3D cellular structures. Whole-cell patch-clamp recordings and immunostaining analysis showed that embryonic hippocampal and cortical neurons maintained their functions and basic cellular properties, including normal, healthy neuronal phenotypes and electrophysiological characteristics, after being printed through thermal inkjet nozzles [95]. Ilkhanizadeh et al. used an inkjet bioprinting system to print biologically active macromolecules onto poly(acrylamide)-based hydrogels that were subsequently seeded with primary fetal neural stem cells (NSCs). They found that the printed macromolecules remained biologically active when printed onto poly(acrylamide)-based hydrogels and influenced the differentiation of multipotent primary fetal NSCs in an efficient and spatially well-controlled manner [32]. Lee et al. reported a tissue engineering scaffold for bioprinting murine NSCs, VEGF-releasing fibrin gels and collagen hydrogels in the construction of an artificial neural tissue. They confirmed the morphological changes displayed by the printed murine NSCs embedded in the collagen and its migration toward the VEGF-releasing fibrin gel. The murine NSCs showed high viability (92.9%) after bioprinting, a viability equivalent to that of manually plated cells. Murine NSCs printed within 1 mm from the border of a VEGF-releasing fibrin gel displayed growth factor-induced morphological changes. The cells printed within this range migrated toward the VEGF-releasing fibrin gel, with a total migration distance of 102 ± 76 μm after 3 days of culture. The results show that 3D bioprinting of VEG-releasing fibrin gel supported sustained release of the growth factor in the collagen scaffold [52]. Owens et al. printed fully biological grafts composed exclusively of cells and cell secreted material. They printed grafts in a rat sciatic nerve injury model of both motor

and sensory function. In particular, they compared the regenerative capacity of the 3D bioprinted grafts with that grafts composed of hollow collagen tubes or autologous grafts by measuring the compound action potential (for motor function) and the change in the mean arterial blood pressure as a consequence of electrically eliciting the somatic pressor reflex [66]. Hsieh et al. used a thermo-responsive hydrogel as a bio-ink and 3D bioprinted NSCs. The stiffness of the hydrogel could be easily fine-tuned based on the solid content of the dispersion. The NSCs in the PCL/poly-DL-lactide (PDLLA) hydrogels displayed differentiation and excellent proliferation but not in the PCL/poly-L-lactide (PLLA) hydrogels. Moreover, the NSCs-laden PCL/PDLLA hydrogels injected into a zebrafish embryo neural injury model rescued the function of the impaired nervous system; however, the NSCs-laden PCL/PLLA hydrogels only displayed minor repair effects in the neural injury model. The function of an adult zebrafish with traumatic brain injury was rescued after implanting the 3D bioprinted NSCs-laden PCL/PDLLA constructs [28]. Gu et al. produced neural tissue by 3D bioprinting human NSCs that were supporting neuroglia and differentiated in situ into functional neurons. The bio-ink incorporated novel clinically relevant polysaccharide-based biomaterials comprising agarose, alginate and carboxymethyl chitosan. Differentiated neurons formed synaptic contacts, established networks, were spontaneously active, showed a bicuculline-induced increased calcium response, and predominantly expressed gamma-aminobutyric acid [19, 21].

2.4 Vascular Tissue Engineering Using 3D Bioprinting

3D tissue engineering to mimic human body functions has been pursued for a long time. As the thickness of a 3D tissue increases, blood vessels become essential components. In a 2D cell culture having a cell population thickness of approximately 20~30 μm, nutrients and oxygen readily diffuse. However, when the 3D tissue thickness exceeds 100 μm, it is difficult for oxygen and nutrients to diffuse to every corner of the tissue [13]. Therefore, vascular tissue guided into the 3D tissue serves to supply nutrients, oxygen and remove waste products. New blood vessel formation in highly vascularized tissues (e.g., liver, kidney, lung, spleen, heart, pancreas, or thyroid) is essential [74, 75]. Thus, there is general consensus that the ability to reconstruct complex vascular networks is crucial to 3D tissue engineering [53]. However, this issue remains a major stumbling block to efforts to create 3D engineering structures with the volume and complexity of human organs [50, 51, 67]. 3D bioprinting technologies that create objects of a desired shape using a variety of bio-inks and cell types have emerged as attractive approaches to designing small-diameter vessels. The advantages of 3D bioprinting method are that researchers can produce heterocellular tissue constructs that readily control the cell density. This provides researchers with finer tools for addressing angiogenic problems in 3D tissue engineering [57]. These techniques can create biomimetic microenvironments in 3D tissues and produce vessels with ideal functions and structures. This chapter introduces research using 3D bioprinting technologies to form blood vessels in 3D tissues.

Cui et al. fabricated fibrin micro-channels using an inkjet-based bioprinting method. When bioprinting human microvascular endothelial cells (HMVEC) laden fibrin hydrogel, they confirmed that the cells aligned themselves within the fibrin channels and proliferated to form confluent linings. A 3D tubular structure was obtained from the printed patterns. They concluded that simultaneously bioprinting both the cells and scaffold promoted HMVEC proliferation and microvasculature formation [16]. Norotte et al. printed small-diameter multilayered tubular vascular grafts that were readily perfused for further maturation. Agarose was used as a bio-ink to print smooth muscle cells and fibroblasts, aggregated into discrete units, either multicellular spheroids or cylinders of a controlled diameter (300–500 μm). The post-bioprinting fusion of the discrete units resulted in single- and double-layered small diameter vascular tubes [63]. Wu et al. used an omnidirectional bioprinting method to fabricate 3D micro-

vascular networks embedded within a pluronic F127 hydrogel scaffold. Using this method, they fabricated 3D microvascular networks using a hierarchical, 3-generation branching topology to form microchannels of diameter 200–600 μm, in which two large parent channels were subdivided into many smaller microchannels [94]. Kolesky et al. reported a new 3D bioprinting method for fabricating 3D tissue constructs replete with vasculature, ECM and multiple types of cells. They confirmed the printability of these structures using two materials, a silicone elastomer and pluronic F127, and confirmed cell viability using a cell-laden gelatin methacryloyl (GelMA) hydrogel. They found that human neonatal dermal fibroblasts and human umbilical vein endothelial cells (HUVEC) proliferated over time [44]. Jia et al. [35] developed perfusable vascular structures with highly ordered arrangements in a single-step process. 4-arm poly(ethylene glycol)-tetra acrylate (PEGTA), GelMA, and alginate were used in combination with a multilayered coaxial extrusion printing system to achieve direct 3D bioprinting. The rheological properties of the bio-ink and the mechanical strengths of the resulting constructs were tuned by introducing PEGTA, which facilitated the precise deposition of complex multilayered 3D perfusable hollow tubes. This blend bio-ink also displayed favorable biological characteristics that supported the proliferation and spread of encapsulated endothelial and stem cells within the 3D bioprinted constructs, leading to the highly organized, formation of biologically relevant, perfusable vessels [35]. In other approaches, perfusable systems and 3D bioprinting have been integrated to achieve 3D tissue vascularization. Pimentel et al. [69] fabricated thick (1 cm) and densely populated tissue constructs using a 3D 4-arm branch network with stiffness comparable to that of soft tissues. This construct could be directly perfused on a fluidic platform over long periods of time (>14 days). They used poly(lactic acid) (PLA) as the support structure and poly(vinyl alcohol) (PVA) as the water-soluble main material. The PLA was selectively removed, and the PVA structure was used to create a artificial 3D vascular network within the ECM that mimicked the stiffness of the liver and encapsulated hepatocellular carcinoma (HepG2) cells. These hybrid constructs were directly perfused with medium to induce the proliferation and formation of HepG2 spheroids. In this study, the highest spheroid density was obtained with perfusion, but overall, the tissue construct displayed two distinct zones: one with a high cell death rate and almost no cell division and one of rapid proliferation. The model, therefore, simulated tissue gradients within necrotic tumor regions [69].

2.5 Skin Tissue Engineering Using 3D Bioprinting

In human body, the skin is the largest organ and protects other tissues from external stimuli. Skin damage leading to infections, or other genetic or physical ailments, can produce chronic ulcers. Skin injuries can expose other tissues to the external environment, including bacteria and viruses. Skin loss can disrupt body temperature regulation. Pathological components of normal skin flora can proliferate in the presence of a broken skin barrier. Skin loss in humans can lead to death in severe cases [12]. Thus, skin damage is a major problem with far-reaching effects on other tissues. Autologous grafts obtained directly from the patient are often used to avoid immune rejection and restore skin function and wound healing after skin damage. Unfortunately, skin damage wounds over large areas or with a significant depth are not adequately healed using autologous grafts [3, 54, 76]. For this reason, there is a need to produce artificial skin substitutes using novel approaches to skin regeneration [34, 58]. These studies have developed sophisticated skin substitutes that interact with human tissues after *in vitro* maturation and transplantation [56, 77, 85]. It has been difficult to mimic the skin of a person while accommodating nerve endings, capillaries, multi-layered 3D structures, and the numerous derivative structures, such as sebaceous glands, sweat glands, and hair follicles. Complex skin structures require accurate signaling systems. Without such systems, the skin structure is lost [80]. In this respect, 3D bioprinting is a very

attractive method that enables the construction of human skin structure mimics using the spatio-temporal patterning of various bio-inks and cells. This chapter introduces research into human skin models using 3D bioprinting technologies.

Koch et al. fabricate a skin model containing dermis and epidermis layers using 3D laser-based bioprinting system. They used an alginate hydrogel as a bio-ink and printed fibroblast, keratinocyte, and hMSC. They then evaluated the influence of the laser-based bioprinting system on the cellular proliferation, survival rate, and apoptotic activity. Modifications of the cell surface markers and DNA damage were assessed and statistically evaluated over several days. The cells survived the transfer procedure with a viability exceeding 98%. All cell types tested maintained their proliferation ability after 3D bioprinting [43]. A collagen bio-ink, keratinocytes, and fibroblasts were printed to form a simple skin structure. The 3D bioprinted cell constructs were assessed after different culture times using immunohistological methods. The presence of cell–cell channels, which indicated tissue formation, was investigated in the vital 3D structures [42]. Michael et al. fabricated a skin substitute using 3D laser-based bioprinting. The skin substitutes were created using fibroblasts and keratinocytes. These 3D structures were subsequently tested *in vivo*. The bioprinted keratinocytes formed a multi-layered epidermis with initial differentiation and stratum corneum after 11 days of culture. Their proliferation was mainly detected in the suprabasal layers. E-cadherin, an indicator of adherens junctions and, therefore, tissue formation, was found in the epidermis *in vivo* as well as *in vitro*. In mice, some blood vessels were found to grow from the wound edges and the wound bed in the bioprinted direction of the cells [59]. Lee et al. demonstrated the potential utility of 3D bioprinting in tissue engineering using skin model as a prototypical human example. They printed collagen as a bio-ink, and fibroblasts and keratinocytes were used as constituent cells to form the dermis and epidermis. Immunohistological characterization revealed that the 3D bioprinted skin tissue was biologically and morphologically representative of human skin tissue *in vivo*. Compared to traditional methods of tissue engineering for skin, 3D bioprinting offers several advantages in terms of form retention and shape, reproducibility, flexibility, and a high culture throughput [50, 51]. Cubo et al. printed bilayer skin using fibrin bio-inks containing human primary keratinocytes and fibroblasts and human plasma. The structure and function of the 3D bioprinted skin was analyzed using immunohistochemical methods, both in 3D cultures *in vitro* and after long-term transplantation into immunodeficient mice. In both cases, the regenerated skin was very similar to human skin and, furthermore, was indistinguishable from the bilayered dermo-epidermal equivalents that were handmade in laboratories [26]. Nanoparticles have recently emerged as a transdermal delivery system. Their surface properties and size determine their efficacy and efficiency in penetrating the skin tissue. Hou et al. utilized 3D bioprinting technologies to generate a simplified artificial skin model useful for rapidly screening nanoparticles for their transdermal penetration capacity. A collagen hydrogel was used as a bio-ink, and fibroblasts were printed into the structure. The effectiveness of this platform was evaluated by using a 3D scaffold using one layer of fibroblasts sandwiched between two layers of a collagen hydrogel to screen silica nanoparticles with different surface charges for their penetration ability. Positively charged nanoparticles demonstrated deeper penetration, consistent with observations from previous studies involving living skin tissue [15].

2.6 Cartilage Tissue Engineering Using 3D Bioprinting

Cartilage, which is only a few millimeters thick, prevents friction between joints and endures extreme load stresses during limb movements. The cartilage defects due to aging, degenerative diseases, trauma or other several factors inevitably lead to arthralgia and chronic disorders [4, 31]. Despite numerous attempts, artificial cartilage that can fully mimic the composition of

the tissue, ECM, and mechanical properties has not yet been developed [30]. 3D bioprinting, which can fabricate products of a desired shape using various materials and cells, presents a great opportunity in cartilage tissue engineering. This chapter discusses research into cartilage tissue engineering using 3D bioprinting.

Kundu et al. fabricated cell-printed 3D scaffolds using the PCL and chondrocyte- encapsulated alginate hydrogel. Cell-based biochemical *in vitro* assays were performed to measure the DNA, total collagen content, and glycosaminoglycans (GAGs) in the different alginate/PCL gel constructs. Alginate/PCL gels containing transforming growth factor-β (TGF-β) displayed greater ECM formation. The cell-printed 3D alginate/PCL gel scaffolds were implanted in the dorsal subcutaneous spaces of female nude mice. Immunohistochemical analyses revealed enhanced cartilage tissue and collagen type II fibril formation in the alginate/PCL gel (TGF- β) hybrid scaffold after 4 weeks [47]. Kesti et al. developed a cartilage-specific bio-ink for use in 3D bioprinting applications based on a blend of alginate and gellan mixed with commercially available BioCartilage particles. They imaged bioprinted scaffolds using magnetic resonance imaging (MRI) to compare the 3D shapes with the original model, and evaluated the utility of MRI in detecting changes in the water relaxation times as they related to ECM production in tissue-engineered grafts. To evaluate cartilage formation, cell-laden Bioink and Bioink/BioCartilage disks were cultured for 8 weeks *in vitro* with and without TGF-β3 supplementation. All of the characteristics of the 3D bioprinted scaffolds were superior to those of native articular cartilage [39]. Ren et al. used collagen hydrogel as a bioink and fabricated 3D cartilage constructs. The chondrocyte density gradient indicated a zonal distribution throughout the ECM. They evaluated the effect of the chondrocyte density gradient on the formation of the regional distribution of ECM in the bioprinted 3D structure. The ECM production was positively correlated with the cell density during the early stages of culture, and the biosynthetic abilities of chondrocytes were affected by both the cell density and the cell distribution in the bioprinted 3D structure [72].

2.7 Cancer Model Using 3D Bioprinting

Cancer is a leading cause of disease and death throughout the world. Despite advances in cancer treatment, many challenges remain, and the characteristics of the tumor microenvironment must be considered [6, 68]. Cancer research commonly relies on 2D cultures and animal models; however, it is difficult to imitate 3D human tissues in 2D cultures, and animal model results cannot necessarily be extrapolated to the human response [8, 18, 46, 88, 96, 97]. These problems may be addressed by furthering our understanding of complex cancers tissue using 3D tumor models that can mimic the microenvironment of native cancer. This chapter introduces research into bioprinted 3D cancer models.

Snyder et al. fabricated a 3D liver cancer model and studied the radiation protection functionalities and drug conversion of living liver tissue analogs. They used matrigel as a bio-ink onto which were printed human hepatic carcinoma cells. Cell-laden bioprinted matrigel and microfluidic chips were used to evaluate the radiation shielding properties of the liver cells using the amifostine pro-drug. The benefits of radiation protection and the conversion of pro-drug by multiple cell types were best realized using a dual-tissue model [81]. Huang et al. created a *in vitro* 3D micro-chip in a hydrogel using 3D projection bioprinting. The micro-chip featured a honeycomb branched structure that mimicked a 3D vascular morphology useful for monitoring, and analyzing differences in the behaviors of cancer cell lines (Hela) versus normal cells (fibroblasts). Fibroblasts exhibited greater morphological changes due to channel width than HeLa cell lines; however, the channel width had a limited influence on fibroblasts migration, whereas HeLa cells migration increased as the channel width decreased [29]. Zhao et al. reported a HeLa cells laden 3D bioprinting alginate/fibrinogen/gelatin hydrogels to construct *in vitro* cervical tumor models. Cell viability was

exceeding 90% using the defined bioprinting technology. Comparisons between the 2D and 3D culture models revealed that the HeLa cells showed a higher proliferation rate in the bioprinted 3D culture model and tended to form HeLa cells spheroids, whereas they formed monolayer cell sheets in the 2D culture system. HeLa cells in the bioprinted 3D models also displayed higher chemoresistance and higher matrix metalloproteinase (MMP) expression than those in the 2D cultures [98].

2.8 Challenges and Future Directions

Thus far, a variety of 3D bioprinting technologies have been used to study tissue engineering applications intended to mimic a variety of tissues and organs. 3D bioprinting has paved the way combining biomaterials, imaging, modeling, and computational technologies in the biomedical and tissue engineering field. The 3D bioprinting technologies permits adjustments to the shape, porosity, and size of the 3D scaffolds, attracting much attention in the tissue engineering field. Several challenges remain, for example the development of biomaterials (bio-inks) for use in bioprinting tissues or organs. Conventional 3D bioprinting focuses on 3D structure creation without cells, whereas recent 3D bioprinting technologies have quickly and accurately produced 3D structures using cells in one step. 3D bioprinting with cells requires materials with excellent biocompatibility and cell affinity. For this reason, the development of bio-inks is very important for bioprinting with cells. In the future, it will be necessary to develop new biomaterials and increase the precision of bioprinting equipment to quickly create accurate 3D structures.

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Electrospun 3D Scaffolds for Tissue Regeneration

3

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Abstract

Tissue engineering aims to fabricate and functionalise constructs that mimic the native extracellular matrix (ECM) in the closest way possible to induce cell growth and differentiation in both *in vitro* and *in vivo* conditions. Development of scaffolds that can function as tissue substitutes or augment healing of tissues is an essential aspect of tissue regeneration. Although there are many techniques for achieving this biomimicry in 2D structures and 2D cell cultures, translation of successful tissue regeneration in true 3D microenvironments is still in its infancy. Electrospinning, a well known electrohydrodynamic process, is best suited for producing and functionalising, nanofibrous structures to mimic the ECM. A systematic control of the processing parameters coupled with novel process innovations, has recently resulted in novel 3D electrospun structures. This chapter gives a brief account of the various 3D electrospun structures that are being tried as tissue engineering scaffolds. Combining electrospinning with other 3D structure forming technologies, which have shown promising results, has also been discussed. Electrospinning has the potential to bridge the gap between what is known and what is yet to be known in fabricating 3D scaffolds for tissue regeneration.

Keywords

Tissue engineering · Electrospinning · Dynamic liquid bath collectors · 3D scaffolds

3.1 Introduction

Tissues in our bodies are broadly classified into four types: epithelial, connective, muscle and nervous. A precise and intricate arrangement of these tissues make up the various organs of our body and the seamless connections between them thereby maintain optimum physical and mental health necessary for leading a normal life. Most of these tissues are composed of more than one cell type and the extracellular matrix (ECM) components that are secreted by these cells. The nanoscale dimensions of the natural ECM result in an intricate nanofibrous web, having a high surface area to volume ratio, favourable for the growth and proliferation of cells [61]. A fixed microscale arrangement of these cells and ECM is necessary in order to perform specific functions of the tissue type [80]. During tissue damage through disease, trauma or wear and tear, there is disruption in the tissue architecture and function. The first line of defence in such situations is the body's inherent healing mechanism. Body's healing

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mechanism forms a clot over the lesion, and the necessary stem cells, growth factors, etc. are transported through blood, to the specific injury site and start forming the new tissue. If this natural process is delayed or is insufficient due to numerous reasons, external intervention becomes necessary. Although autografts (tissue taken from a part of person's own body and transplanted in another part) and allografts (tissue taken from a donor) were used for tissue substitutes, they are often associated with drawbacks such as limited availability, donor site morbidity and longer rehabilitation time.

Porous, mechanically stable and biocompatible scaffolds having scope for incorporating bioactive molecules into their structure so as to mimic the natural ECM in the closest possible way are the need of the hour. Techniques such as freeze drying, rapid prototyping (3D printing, melt deposition, additive manufacturing etc.) and electrospinning are being extensively studied for forming such scaffolds. The shortcomings of each process are being overcome with innovative enhancements to the existing systems and also through a synergistic approach by combining the advantages in one system with that of the other. A review on the recent developments in 3D electrospun scaffolds for tissue regeneration is presented in this chapter.

3.1.1 Tissue Regeneration and the Need for Tissue Engineering

Tissue regeneration from a biological perspective, is not just an aftermath of trauma, but a cyclical process part of everyday adult biology [50]. Common examples are renewal of intestinal lining, generation of new neurons in the brain, preservation of skin, hair and bone. However, the significant regeneration of body parts or limbs as found in rest of the animal kingdom, such as full body regeneration from tiny body pieces in invertebrates such as earthworms; tail, limb and other body part regeneration found in vertebrates like lizards, frogs (tadpoles), newts and fishes, is not so prevalent in mammals. But it is also not completely absent; tissue re-growth has been observed in finger tips of children [64], regenerative capacity of human liver is well documented [65], the innate regenerative ability of bones is widely studied [18].

The functionality of a tissue is related with its complex architecture and hence, tissue engineers have been trying to mimic this complexity *in vitro,* in order to successfully fabricate artificial scaffolds for tissue regeneration. Recapitulating the structural hierarchies, well defined vascular networks, interfaces between the biological and mechanical cues and other complex functional features present in the human tissue architecture is one of the most challenging aspects of tissue regeneration [43]. Tissue engineering is an integral part of tissue regenerative studies focussing more on the scaffold fabrication areas. Tissue engineering necessitates the conjoined efforts of biotechnologists and material scientists, to produce tissue constructs outside the body, functionalise it by seeding cells, growth factors and/or other biomolecules and finally placing it inside the body so as to augment the healing of injured sites. This is often represented by the tissue engineering triad as shown in Fig. 3.1.

The notion of cells functioning as an individual entity is obsolete. It is now understood that in addition to its genome, a cell must be evaluated in the context of its ECM, growth factors and other biomolecules that are responsible for the regulation of its functions resulting in the ultimate formation of an organ and in turn the organism as a whole [72]. In fact it is reported that a cell's genetic traits (genotype) and its expression (phenotype) may vary depending upon its interaction with the dynamic ECM, through a series of signalling cascades [5].

Unfortunately, the tissues that are studied extensively are those with relatively simple architectures and cellular organization such as skin epidermis, cartilage and corneal epithelium [80]. Engineering scaffolds for mimicking metabolically active organs such as bone, liver or heart which are characterized by hierarchical and multiscale 3D organization, well developed vasculature and functionally complex 3D architectures is still a challenge [79]. 3D structures

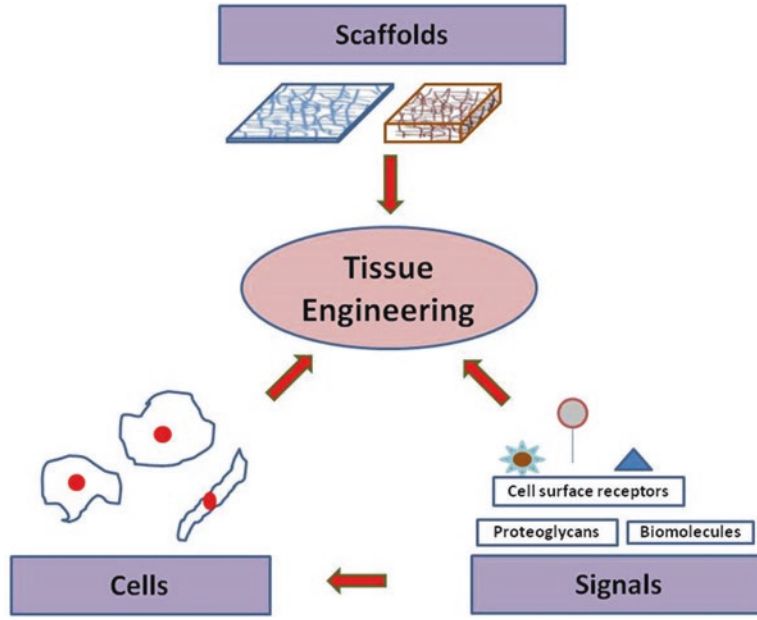

Fig. 3.1 The tissue engineering triad

mimic the native ECM by providing the necessary microenvironment and the appropriate mechanical cues for successful growth and proliferation of cells used in tissue engineering [38]. However, there is a need for simple but effective systems for fabricating 3D architectures that can function as tissue substitutes and control cellular alignment without the need for external stimulation or guidance systems [3].

Cell culture studies play an important role in evaluating the efficacy of the developed scaffolds. The drawbacks inherent in the traditional 2D cell culture techniques have been realised and a shift towards 3D culture is also noted in literature [32]. A good part of the literature currently focuses on developing bioresorbable 3D scaffolds that are able, to reproduce the ECM structure and topography, to provide a biological support guiding cell attachment and proliferation and capable of inducing differentiation of stem cells into the necessary cell lineages [11]. Successful translation of tissue engineering from laboratory research to clinically viable products highly depends upon the fabrication of 3D scaffolds and evaluating them using appropriate test conditions and methodologies which replicate the actual conditions present *in vivo*.

3.1.2 3D Culture

Julius Petri invented the "Petri dish", over a century ago [78] which introduced a paradigm shift in the culture, manipulation and analysis of biological cells. It paved way for the traditional 2D culture systems which were later joined by other substrates like the micro-well plates and tissue culture flasks. However their applicability in tissue engineering is plagued by the following drawbacks:

(a) 2D substrates are greatly limited in emulating the complex 3D microenvironments because of the lack of structural architecture [33].
(b) The architectural mismatch between the rigid 2D substrates and the unique ECM microenvironment of the particular cells, which may force the cells into adapting to the flat, rigid surface thereby misrepresenting the data.
(c) The 2D architecture, which prevents cells from forming spheroids (three dimensionally organised cellular aggregates) which develop into the intended tissue with appropriate functionality.

These drawbacks can alter cell metabolism and are also likely to misrepresent findings to some degree [33]. The inadequacies in representing a cell's microenvironment, while using 2D substrates for cell cultures [78], and the need to re-examine biological signalling and cellular communication in the light of 3D matrix – cell interaction [16] have steered research in the direction of developing appropriate 3D matrices for 3D cell culture.

In the body, virtually every tissue cell resides in an ECM consisting of a complex 3D fibrous network with varied/hierarchical distribution of fibers and gaps that provide complex biochemical and physical signals [33]. The variation in the composition of proteins such as collagen, elastin and laminin in the ECM is responsible for the different mechanical properties of each tissue. They also influence the intracellular communication happening within the ECM. Cell surfaces have receptors known as integrins which determine the cells' interpretation of the biochemical cues in and around the ECM. Thus there is a complex interplay between the mechanical and biochemical properties. This defines the biological properties of the tissue as a whole. Using 2D cell cultures, as conveniently used for maintaining cells, to evaluate cell behaviour *in vitro*, is bound to impose abnormal mechanical and geometrical constraints on the cells, forcing them to adapt to the surroundings thereby compromising the findings of the study [5]. The differences in cell behaviour observed between 2D and 3D cultures has been attributed to how the cell experiences its microenvironment differently in 2D as compared to the natural 3D surroundings [72]. 3D cell cultures can help in overcoming these drawbacks. To establish a 3D culture, cells need to be grown within a 3D fibrous structure mimicking the natural ECM along with the necessary biomolecules that aid in its growth and proliferation, as close as possible to the *in vivo* conditions.

3.2 3D Scaffolds

Creating environments that successfully support cell growth without loss in functionality of the injured tissue is the primary goal of tissue engineering. Success of tissue engineering largely depends on the role played by the scaffolds which act as the synthetic ECM. Ideally these scaffolds should be able to provide a micro-environment that is conducive for the cell to attach, grow and ultimately develop into the tissue lineage it is required to grow into [60]. Compared to the work done on understanding and replicating the chemical environment in which cells reside, less is known about how architectural cues affect cells [47]. The various scaffold fabrication techniques are shown in Fig. 3.2. The methods on the left hand side (those represented in brown background), are the conventional methods. Solvent

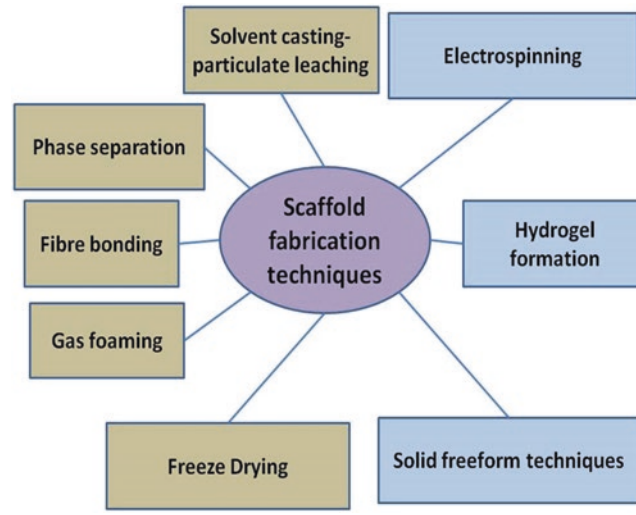

Fig. 3.2 Various techniques used for fabricating tissue engineering scaffolds

casting/particulate leaching involves the casting of polymer/composites solutions with porogens into predefined shapes followed by the selective removal of the porogens, thus resulting in a porous structure. Phase separation techniques involve the rapid cooling and subsequent sublimation of polymer solutions made with volatile organic solvents with low melting points, forcing the polymer solutes into interstitial spaces and subsequently forming porous structures. Fiber bonding techniques utilise physical bonding of prefabricated fibers through heating, needle punching or knitting technologies to form porous fibrous meshes. Gas foaming utilises high pressurized gases to saturate polymers and rapidly drop the pressure to induce a thermodynamic stability which results in the formation of pores and in the final structure. Freeze drying induces freezing of polymer solutions followed by lyophilisation leading to formation of structures with highly interconnected porosity.

A growing body of evidence suggests that 3D scaffold structures may be critical to induce cellular organization and replicate anisotropic tissues, such as cardiac muscle or bone [32, 49]. Micro-scale scaffold architecture has a decisive role in guiding 3D cellular organization and the subsequent recovery of tissue functions [33]. Hence in addition to studying interactions between cells and scaffolds, it is necessary to examine and understand the formation of scaffold architecture in order to better engineer future tissue engineering scaffolds [47].

3.2.1 3D Architectures and their Fabrication Methods

Fabricating 3D tissue scaffolds (as shown in Fig. 3.2.) to meet a variety of structural and biological requirements has always been a challenge in tissue engineering. For complex tissue architectures, precise control over material deposition, porosity and pore interconnectivity is required, in order to provide the required structural strength, transport properties, and the right microenvironment for cellular growth [60]. Evaluating and assessing a particular 3D scaffold should be done keeping in mind its end use. For example, the high tensile properties needed for in bone tissue applications may not be necessary in soft tissue applications. The internal architecture of scaffolds will need to be designed to allow specific 3D configurations of cell–cell and cell-matrix interactions [33].

The native ECM is a complex and bioactive hydrogel scaffold which, apart from providing structural stability, guides cell adhesion, proliferation, differentiation and gene expression [72]. A complex framework of fibrous proteins such as collagen, fibronectin and laminins, are responsible for the mechanical properties of the ECM. Tissue engineering aims at recapitulating these features to the closest extent possible through the help of various manmade 3D structures. There have been a plethora of structures fabricated from various techniques, evaluated as scaffolding materials. These can be classified into three categories, namely hydrogels, porous solids and nanofibrous structures.

3.2.1.1 Hydrogel Scaffolds

Hydrogels are insoluble cross-linked hydrophilic polymers networks, either naturally derived or synthetic, that swell by absorbing large amounts of water. They can be formed from a wide array of natural and synthetic polymers. The large amounts of water they absorb, improves their biocompatibility over bulk polymers and provides a porous environment through which cells can migrate and proliferate. This also simulates the hydrated structural feature of naturally occurring ECM [53]. Hydrogels can also handle the stresses needed for proper mechanotransduction [72] and the ease of incorporation of cells and bioactive agents [39]. However, a major drawback of some hydrogels is that the hydrogel matrix lacks the cellular-scale architecture which promotes strong cellular adhesion and subsequent cellular infiltration for the rapid repair of tissues [19]. Also the mechanical fragility of conventional hydrogels limits their application as substitutes in load bearing tissues. Interpenetrating network (IPN) hydrogels and double network (DN) IPN hydrogels have superior mechanical properties, however the processes used to form

these materials do not allow the successful encapsulation of cells [9].

3.2.1.2 Porous Solid Scaffolds

All the structures apart from hydrogels and nanofibrous scaffolds, which are explored for tissue engineering applications, fall under this classification. Hence scaffolds obtained using the conventional scaffold forming techniques, such as solvent casting-particulate leaching, gas foaming, phase separation, fiber bonding, and those obtained using the most recent solid freeform fabrication (SFF)/rapid prototyping technologies fall under this category.

Limitations of the conventional scaffold fabrication techniques are [52]:

1. Inability to precisely control pore size, pore interconnectivity, spatial distribution of pores.
2. Also only thin scaffold cross sections are possible due to difficulty in removing salt particles from larger cross sections.
3. Usage of organic solvents and difficulty in their complete removal which may be toxic to the cells during culturing.
4. Majority of structures are foam structures, with insufficient nutrient passage to the interiors, hindering the migration of cells into the structure

Solid freeform fabrication (SFF) collectively refers to a technologies that build 3D structures based on layer by layer manufacturing strategies. Though there are variations in the process for each technique, the basic principle involves producing a 3D physical object from a computer generated model based on computer aided design (CAD), computed tomography (CT) or magnetic resonance imaging (MRI) data [36] in a layer by layer fashion where each layer corresponds to a cross sectional division [52]. The attractive features of SFF techniques are the precise control over scaffold parameters such as pore size, porosity and pore distribution, as well as the ability to incorporate an artificial vascular system, that can increase the mass transport of oxygen and nutrients into the interior of the scaffold so as to support cellular growth in that region [52]. However rapid prototyping techniques are also burdened by certain shortcomings, such as complex equipment, costly setup and the limited number of materials they can process.

3.2.1.3 Nanofibrous Scaffolds

Among the different material types, fibrous materials like nanofibrous non woven mats, are more desirable for tissue regeneration applications, due to their high porosity, variable pore size distribution, high surface area to volume ratio, and most importantly, architectural similarity to the natural ECM [35, 66]. 3D nanofibrous environments promote *in vivo* like cellular phenotypes and overall tissue morphogenesis [73]. A synthetic ECM should not only meet the morphological similarities to the ECM but also the biochemical properties that are required to guide tissue development and overall homeostasis [42]. The continuous and ultrafine nature of the fibers makes them an ideal substrate for cell adhesion and proliferation. Functionalization of the surface so as to make it favourable for a particular cell type also becomes possible. Loading of biomolecules or drugs to enhance its application in tissue engineering applications also becomes a viable option. The commonly used techniques for fabricating nanofibrous scaffolds are phase separation, self assembly and electrospinning (ES). ES has emerged as a versatile fabrication technique in the past 3 decades.

3.3 Electrospinning

The method of producing polymeric filaments using electrostatic forces was known since, 1902 [15, 44]. However, a revived interest in ES related technologies has started from the mid-1990s with advances in nanotechnology [51]. ES now has become a popular and versatile electrohydrodynamic technique for the fabrication of continuous thin (micro/nano) fibers, by subjecting solutions or melts to high voltages. This simple method of producing polymeric nanofiber nonwoven mats has led to the extensive study on the development of scaffolds for tissue engineering applications. In its conventional form, ES setup

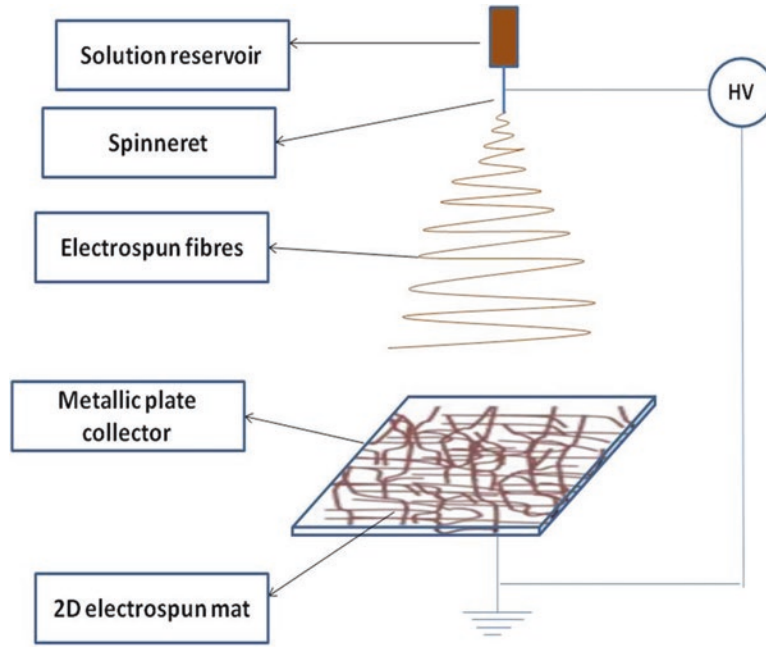

Fig. 3.3 Schematic diagram of the conventional ES setup

comprises of a solution reservoir, a spinneret, a high voltage setup and a collector for the electrospun fibers as shown schematically in Fig. 3.3.

The fundamental process parameters that are often manipulated during ES are (a) solution concentration (b) distance between the collector and needle tip (c) flow rate of the solution (d) applied voltage. The reason for its widespread usage in fabricating tissue engineering scaffolds is summarised in the following points:

(a) The process of setting up an ES unit is relatively simple and cost effective.
(b) The nanofibrous architecture of an electrospun structure resembles the fibrous networks of the natural ECM. More specifically the nano dimensions of electrospun fibers grant them an extremely high surface area to volume ratio thereby making them conducive for cellular proliferation.
(c) Applicability of the technique for a broad spectrum of materials, such as synthetic and biological polymers and polymerless sol-gel systems [40]
(d) The possibility of blending natural and synthetic polymers for overcoming their inherent drawbacks such as inferior mechanical properties and biocompatibility issues, respectively.
(e) Electrospun fibers have the potential to be collected into various configurations by the use of different collectors such as metallic plates, patterned surfaces, collectors with air gaps, rotating mandrels, liquid bath collectors etc.
(f) The prospect of expanding its versatility in fabricating novel structures through innovative control of process parameters and system modifications.
(g) ES technique allows for the functionalization of the fibers through the addition of growth factors, drugs, ceramic nano fillers, etc.
(h) Nanofibers can be incorporated into large scale cell culture protocols such as cell reactors/fermentors or roller cultures and can function of scaffolds for *ex vivo* culturing of cells for use in tissue engineering and cell based therapies.

The past 3 decades have seen the tremendous potential of the ES process being utilised for producing many nanofibrous structures made up of a variety of polymers and composite materials. The electrospun non woven mats have been found to

be suitable for a variety of applications such as tissue engineering (bone [57], cartilage [71], cardiovascular [30], neural [22]), drug delivery [21] and wound healing [31] and certain non-medical applications such as energy storage, wearable electronics and water filtration [13, 51]. Polycaprolactone (PCL) has emerged as one of the most versatile electrospinnable polymer [14] especially in the field of tissue engineering scaffolds [4]. However, most of the studies utilise the conventional ES setup with little modifications and are mostly capable of forming only 2D non woven mats with or without fiber alignment, porous fibers and the inability to form 3D structures which better mimic the ECM, remains a concern.

3.4 3D Electrospun Scaffolds

In spite of the nanofibrous structure, the conventional electrospun mats lack the architectural similarity with the natural ECM in terms of thickness, hierarchical fiber organisation, tissue specific profile and contour, large pores which allow for cellular infiltration [1]. The two prime limitations of ES are the chaotic fiber deposition arising due to the whipping instabilities and the difficulty in forming thicker, self supporting structures. Literature indicates an increasing number of studies focussed on innovations in ES setups in order to achieve novel structures that can be used as tissue engineering scaffolds [62, 76]. Primarily the focus has been on producing structures other than the nanofibrous 2D non woven electrospun mats. For producing non 2D electrospun structures, either enhancement in the process parameters and/or the collector systems or post processing of 2D non woven mats are essential. This section will give an account of such structures categorized in the following manner.

3.4.1 3D Scaffolds from Post Processing of 2D Electrospun Structures

Post processing of electrospun mats have been attempted to produce porous and thicker structures. These are shown schematically in Fig. 3.4.

Simple methods include folding or rolling the electrospun mats into tubular structures [56]. Mechanical expansion, with the help of metallic combs, of as spun mats into high porosity mats has also been reported [55]. Though the overall

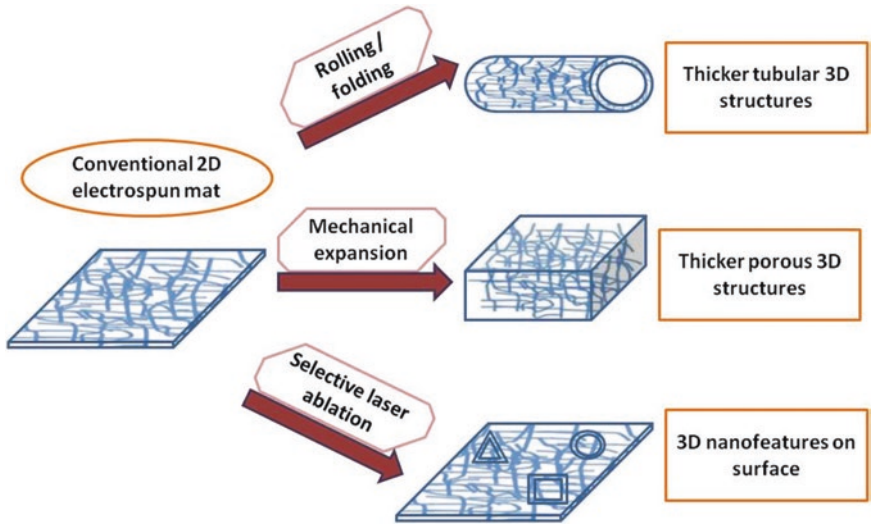

Fig. 3.4 Schematic diagram of post processing techniques of 2D electrospun mats for producing 3D structures

structure becomes more porous and fluffier, the process of disrupting the architecture, is bound to make the resulting structure weak and unstable. Patterning of fiber mat surface by phase modulated femtosecond laser pulse [23], has been claimed to result in a 3D topography in an inherently 2D mat structure. Desired features can be formed on the surface that may be beneficial for guided growth of cells during culturing. While folding, compressing or rolling of electrospun mats does increase the overall thickness of the obtained structure, on the micro/nano scale, the distances between the adjacent layers remains large and are identified as separate 2D surfaces by the cells. Thus, the cells only spread on each surface and 3D cell growth is prevented [45].

3.4.2 3D Scaffolds through Parameter/Process Modification

3D structures have also been fabricated by modifications to the process or by playing around with the electrostatic properties of the electrospun fibers. The various techniques reported in literature is shown schematically in Fig. 3.5. Straightforward methods of increasing thickness in an electrospun mat include increasing the spinning time or multilayering the mat. Multilayering has been achieved through sequential ES [48], co-ES [59] and also by using auxiliary electrical fields to confine the electrospun fibers into the collecting area [48]. During multilayering, sequential ES of nano/microfibers has been carried out with the help of a metallic ring as an auxiliary electrode [48]. This helped in focussing the electrospun fibers into a smaller area thereby increasing the thickness of the final structure. The final bilayered structure was found to have hierarchical porous structure as a result of the variation in the fiber diameter. The nanofibers between the microfibers favoured mesenchymal stem cells (MSC) spreading [48]. Similarly, nanofibers in combination with microfibers showed significantly higher adhesion and viability rates of MSC, than nanofibers alone [59]. Adding of salt during ES has resulted in a thicker, 3D structure, which on salt leaching formed a porous 3D structure [29]. Chondrocyte adhesion and proliferation was significant and its characteristic roundness was maintained. Alternatively, a metallic disc has also been used as an auxiliary electrode, to accumulate highly conductive polyacrylonitrile/multiwall carbon nanotubes composite fibers, vertically so as to form a fluffy web with low packing density [76]. Reducing surface resistivity of polymer by the addition of sodium dodecyl sulphate (SDS) has also shown to increase repulsion between fiber edge and collector surface and result in a loosely packed fibrous structure [10]. Likewise, utilising the electrostatic interaction between electrospun fibers and ES jet, polystyrene composite fibers have been self assembled into conical spongiform structures [63]. A more intricate and dynamic self assembly of the electrospun PCL fibers into honeycomb structures has also been achieved [1]. This was shown to be possible with the use of an insulating mask with a small hole, over the metallic collector, which helped in focussing the deposition of fibers.

3.4.3 3D Scaffolds Using Modified Collectors

Another approach of producing electrospun 3D structures involves the use of modified innovative collectors. Both solid and liquid assemblies have been successfully utilized. They may either be static or dynamic collectors. A list of techniques developed with the solid mechanical collectors and the corresponding 3D structure obtained are shown schematically in Fig. 3.6. A rotating mandrel has been explored (Fig. 3.6a), as a collector, to produce tubular structures. When the speed of rotation is optimised it is possible to produce aligned fibers which are found to be beneficial for neural tissue engineering [34]. At higher speeds of rotation, the electrospun fibers reaching the collector surface are subsequently aligned along the direction of rotation. A low speed rotating drum filled with dry ice, used as a collector for electrospun poly(lactide-co-glycolide) (PLGA)/tricalcium phosphate (TCP) composite fibers,

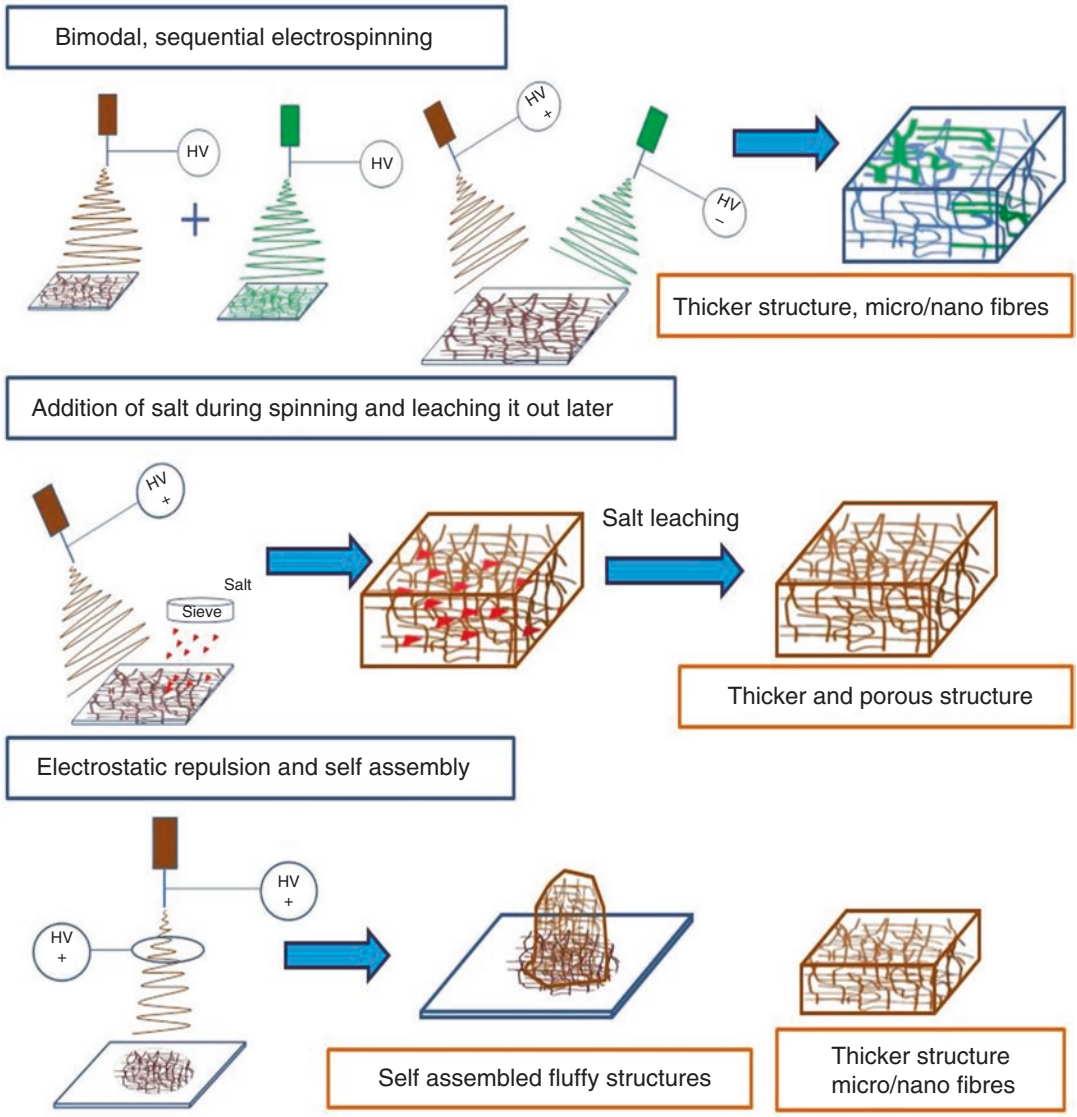

Fig. 3.5 Schematic diagrams of the various process/parameter variations during ES for producing 3D structures

resulted in a cottonwool like scaffold with high compressibility and mouldablity suitable for bone filling applications [54]. High bioactivity combined with good surgical handling for *in vivo* studies in non load bearing areas were reported. A perforated mandrel (Fig. 3.6b) with a provision for pressurized air exit through the holes has been used to form a highly permeable graft [41]. The graft had adequate mechanical properties and displayed higher cellular penetration compared to scaffolds obtained using a solid mandrel.

When two conducting structures/points separated by an airgap (Fig. 3.6c), such as rings [17], poles [24] or blades [68], are used as collectors, fibers tend to accumulate between the them. This results in bundles of aligned fibers [17, 68] or twisted mutifilament yarns [24] when one end of the fiber bundle is rotated. This technique is often termed as airgap ES. The alignment obtained is not dependent on the fiber diameter as is the case when using rotating collectors for alignment [24]. The intra-fiber distance is also suitable for

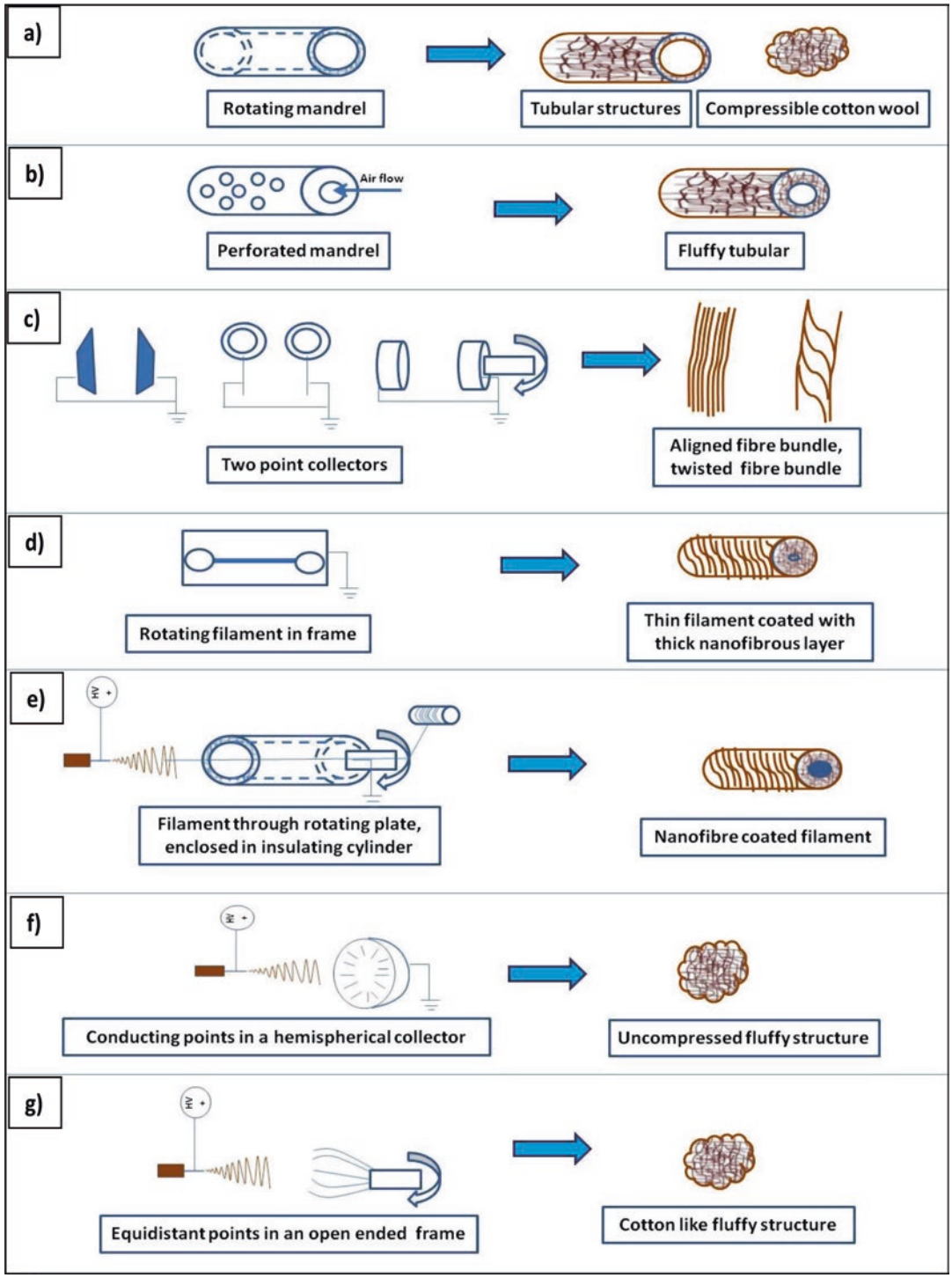

Fig. 3.6 Schematic diagrams of the modified solid collectors used for ES 3D structures

nerve conduits Custom made collectors (Fig. 3.6d) with provision for coating a rotating polyglycolic acid (PGA) filament with electrospun fibers, have been used to form thick yarn like scaffolds mimicking the osteon structure in bones [2]. This structure allowed for the growth, proliferation and production of calcium phosphates by mouse pre-osteoblastic cells (MC3T3-E1). A rotating plate collector with a provision for a filament passage through its centre (Fig. 3.6e) and subsequent winding, was used to coat electrospun PCL fibers on polylactic acid (PLA) microfibers [71]. The collector assembly was placed inside a teflon tube to focus the electrospun fibers.

Using multiple conducting points placed in an organised manner has shown to produce 3D fluffy structures. These include a hemispherical collector with multiple metallic points placed within it (Fig. 3.6f) [6] and an array of equidistant point electrodes at the periphery of an open-ended frame made from flexible metallic rods (Fig. 3.6g) [25]. In both cases collection of fluffy cotton ball like fibrous masses happened between the conducting points when ES was carried facing the metallic points. Additionally in the latter, the collector was rotated and using spinnerets of opposite polarities positioned at 45°, with respect to collector axis, ES was carried either simultaneously or consecutively, to produce composite or core sheath yarns respectively.

Liquid surfaces can also act as substrates for collecting electrospun fibers and can even manipulate the fibers better due to their fluid nature [76]. Moreover the electrospun structure can be collected from the liquid surface with minimal disruption of the nanofibers, compared to a conventional solid substrate wherein the fiber substrate interaction may lead to tearing of nanofibers [76]. Hence liquid baths have also been explored as collectors for electrospun fibers. A schematic diagram of the representative ES techniques reported using liquid bath collectors is shown in Fig. 3.7. Non solvent baths, of the polymer being electrospun, are used as reservoirs to collect the fibers and wind them up into aligned fiber bundle yarns [58] or directly lyophilize them into 3D nanofiber foams [28] or spongiform fabrics [75]. A slightly varied setup, wherein the level of liquid in the bath was manipulated, has been used to produce size controlled, shape stable porous 3D structures [20]. Dynamic water baths have also been developed as collectors for electrospun fibers. They utilize vortex forming mechanisms to produce diverse 3D structures. The first dynamic liquid collector system consisted of one liquid bath, with a drain at the centre, placed over another reservoir. The draining of the liquid produced a vortex in the top liquid bath, over which the ES was carried out. The liquid from the bottom reservoir was pumped back to the top so as to maintain the liquid level [69]. By varying the fiber deposition and mode of collection of fibers from the liquid surface, different 3D structures have been produced. These include aligned [37] or twisted yarns [77], 3D cotton like structures [27], 3D nanofibrous meshes with hierarchical structures [67], ring and spindle shaped [76] structures. Another method for inducing a vortex formation in a liquid bath is the use of magnetic stirrer. This has also been tried as a collector but succeeding in only producing a fibrous mass, but not a shape stable 3D structure.

The structures produced using liquid baths are either spongy fibrous structures or fibers aligned/bundled to form yarn like or other shapes. While these structures are definitely more porous and suitable for cellular infiltration, self supporting structures, with controlled fiber alignment, in the size range required for implant/tissue substitute applications, e.g. hard tissue applications have not been explored. In this regard, the authors have developed a unique dynamic liquid collection system for producing 3D structures [12].

A schematic of the modified collector and the ES process followed is shown in Fig. 3.8. The liquid bath collector is a bowl shaped container with a metallic base. Vortex formation is achieved using a magnetic stirrer. As ES of PCL and PCL/hydroxyapatite (HA) composite fibers proceeds, collection of fibers occurs inside the vortex and a novel structure is obtained as shown in Fig. 3.9.

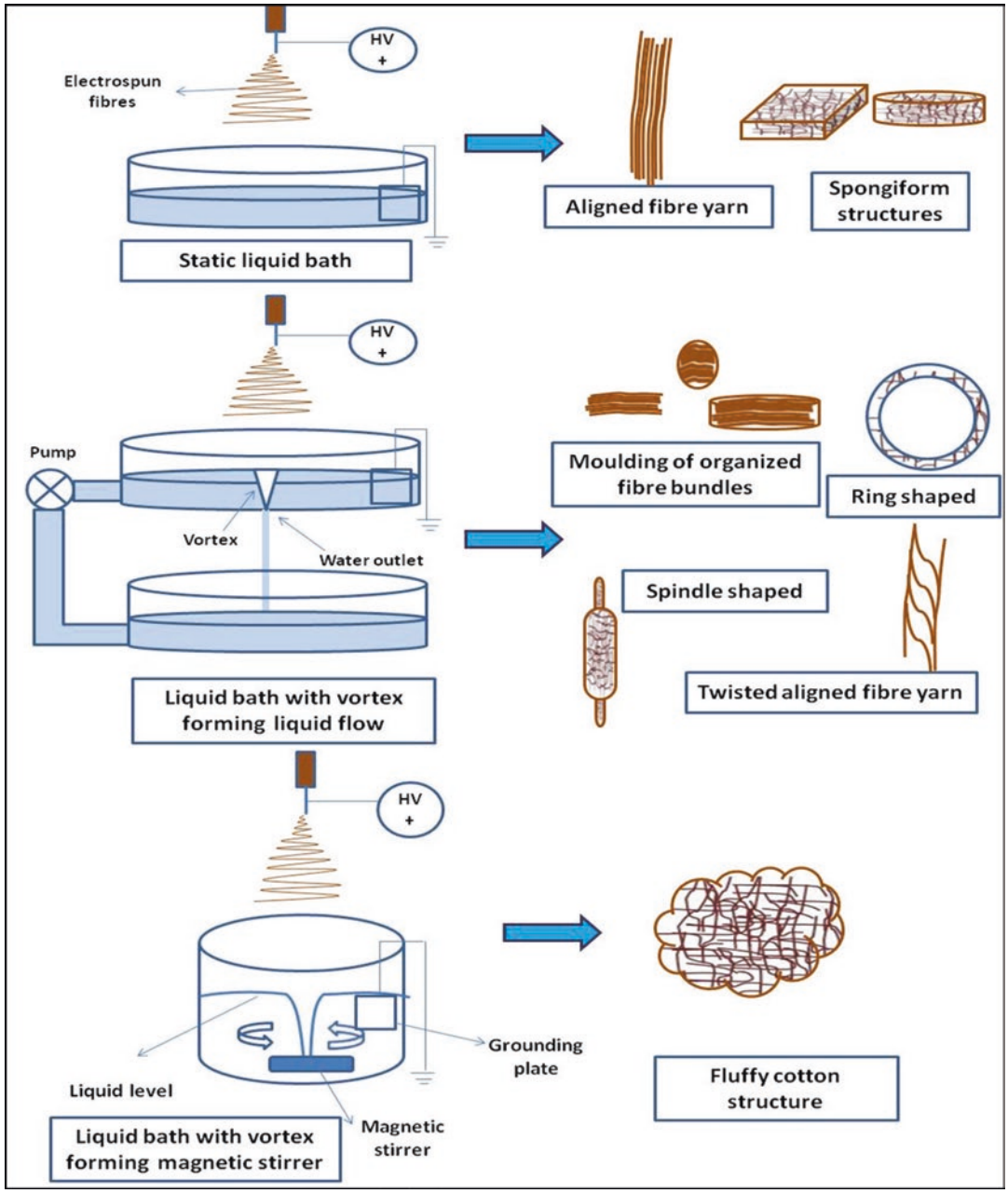

Fig. 3.7 Schematic diagrams of the modified liquid collectors used for ES 3D structures

Three distinct sections (head, stem and base) were identified in the 3D structure. The SEM images of the surface of the head and stem portion is shown in Fig. 3.10. While the base portion had random fibers, the head and stem portion had fibers aligned but perpendicular to each other. The head portion was in the size range necessary for filling critical size defect of the craniofacial region. Human osteosarcoma (HOS) cells proliferated well with higher metabolic activity on the 3D composite (PCL/HA) structure.

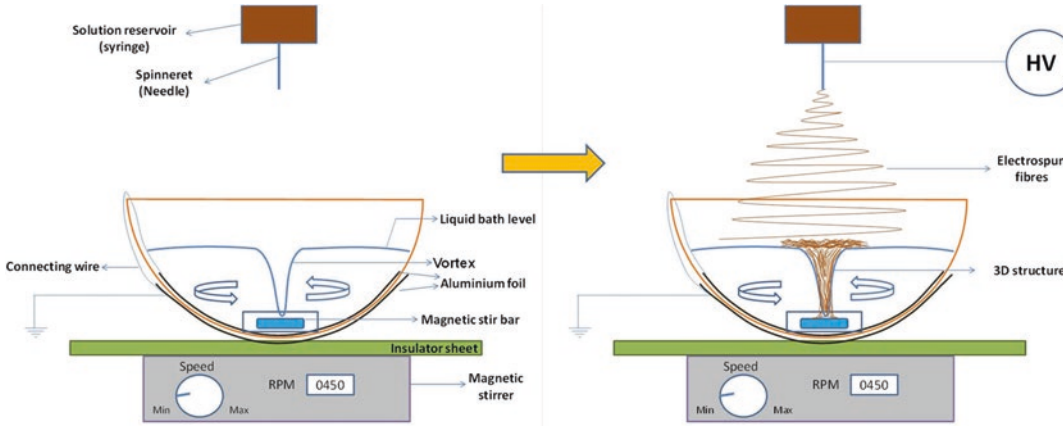

Fig. 3.8 Schematic of the modified liquid collector based ES system used for obtaining a unique 3D structure

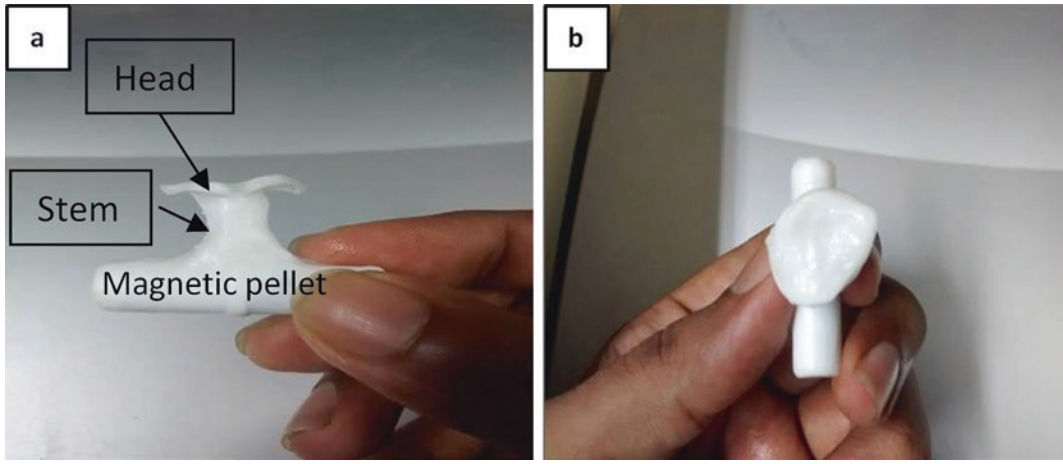

Fig. 3.9 Photographs of the 3D structure obtained on the magnetic pellet (**a**) side view and (**b**) top view

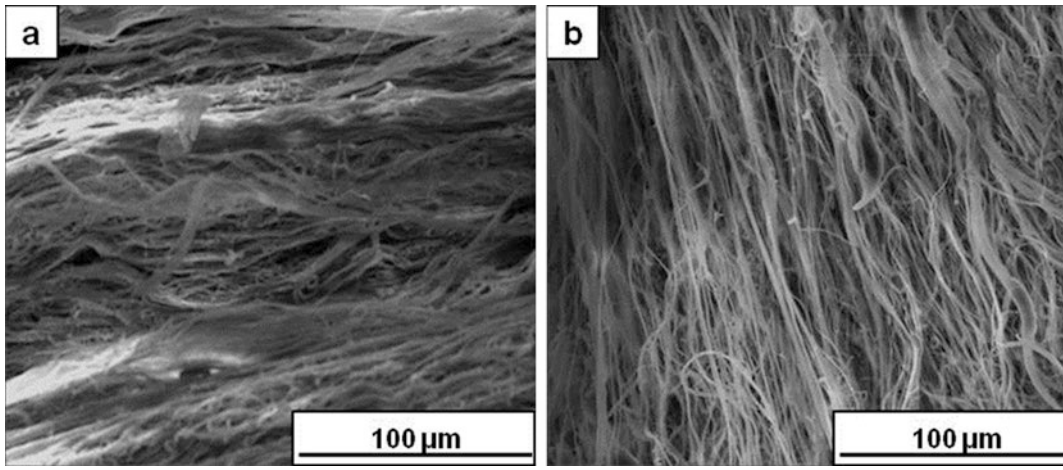

Fig. 3.10 SEM images of the surface of (**a**) head and (**b**) stem section of the 3D structure

3.5 3D Scaffolds by Combining ES with Other Fabrication Techniques

The versatility and scope for innovation in ES has been established beyond any doubt. The two major limiting factors, namely the uncontrolled chaotic deposition of electrospun fibers and the difficulty in forming 3D structures are also being overcome by combining ES principles with other scaffold forming technologies, such as hydrogel formation, textile and additive manufacturing technologies. These amalgamations are discussed in this section.

3.5.1 ES with Hydrogels

Natural ECM is a complex fiber meshwork of collagen and elastic fibers embedded in a highly hydrated gel-like material mostly composed of glycosaminoglycans, proteoglycans, and glycoproteins [33]. Conventional electrospun mats and hydrogels, though accepted as scaffolds for tissue engineering, are associated with drawbacks such as limited cellular penetration and inferior mechanical properties which can be overcome by the formation of composite structures of electrospun fibers and hydrogels [7]. The fibers in such composites are mostly obtained through ES [9]. True biomimicry is not possible by the fabrication of hydrogel or electrospun fiber scaffolds separately, as the extracellular matrix is itself a composite structure combining fibrous and gel-like components [26]. Concurrent ES and electrospraying of hydrogels has also resulted in the formation of 3D scaffolds [7]. Nanofiber reinforced hydrogels are reported to be biologically and mechanically similar to the gel component (nucleus pulposus) of intervertebral disc, and useful in improving degenerated discs [70].

3.5.2 ES Combined with Additive Manufacturing Technologies

Focussed and controlled deposition of electrospun fibers is needed in order to make nano patterned structures suitable for cell culture applications. Melt ES is an area where in ES principles are combined with those of direct writing [8]. ES has also been coupled with microfluidic systems to develop microenvironments *in vitro* that are better suited for understanding the interplay between cells and substrate topography [74]. A hybrid process of direct polymer melt deposition and ES has resulted in 3D structures with higher porosity. This is due to the fiber diameter and interfiber distance arrangement that can be produced through this unique combination [46].

3.6 Summary

The importance of 3D culture systems and 3D scaffolds for enhancing the tissue regeneration has been highlighted. The various structures that can function as manmade ECM and their fabrication method have also been explained. No single conventional scaffold fabrication method seems to result in the fabrication of scaffolds that are truly 3D in nature, with a completely interconnected pore network and a microstructure which varies hierarchically across the scaffold matrix [36]. Also it is evident that a nanofibrous structure with the necessary biochemical and mechanical cues is the closest way of mimicking the natural ECM. ES has the potential to be the most versatile option for producing such structures. Innovations made in the electrospinning setups and collector systems are producing fibrous micro/macro structures that are becoming more and more functional with scope for further improvement in performance. However, producing tissue specific scaffolds for tissue regeneration purposes is a huge challenge ahead of us. Some ways of tackling this issue are listed below:

1. Reassessing the wide database available on 2D cell culture results in the light of the evolving 3D culture systems, so as to identify areas where improvisations and extrapolations should and should not be performed respectively.
2. Understanding the mechanisms guiding the cell matrix interactions and the inherent biochemical, mechanical and structural cues

present in the different types of tissues, so that properties of the scaffolds can be controlled more precisely to recapitulate these features in the tissue engineered scaffolds.

3. Formulating experimental plans after identifying a particular challenge in tissue engineering scaffolds and subsequently conducting experiments would greatly benefit the research community as a whole.
4. Improving the efficiency of ES systems through innovations focussed on reducing the chaotic nature of fiber production and enhancing the collection of fibers with the use of novel collector systems, such as dynamic liquid collectors.
5. Combining ES with technologies such as additive manufacturing, melt spinning and hydrogel formation, so as to eliminate the drawbacks in each process and develop highly effective scaffold fabricating techniques.
6. Improving the functionality of the electrospun scaffolds by incorporating the most essential biomolecules, to the maximum extent possible, into them.

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4 Scaffolds Fabricated from Natural Polymers/Composites by Electrospinning for Bone Tissue Regeneration

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Abstract

Naturally bone is a hierarchical and highly integrative dynamic tissue that is continuously remodeled by osteoblasts and osteoclasts. Deformities in bone due to trauma and/or disease are highly prevalent and mostly need surgical intervention. However, the methods of surgical treatments are associated with donor site morbidity, infection and/or complete rejection. Bone tissue-engineering provides a platform for growth of new bone tissue by fabricating scaffolds along with cells, growth factors and other dynamic forces. The polymeric materials especially natural polymers in their nanofibrous forms have been developed and introduced for bone tissue regeneration. At the nanoscale, natural polymers possess tunable properties and can be surface functionalized or blended with other polymers to provide juncture for cell-seeding, proliferation, differentiation and further resulting in regenerated tissue formation. These scaffolds fabricated from natural polymers and additives by electrospinning are bio-inspired to mimic the natural extracellular matrix resembling the native collagen of bone. This chapter focuses on the fabrication techniques as state of art nanofibrous scaffolds from natural polymers/additives during the recent years by the process of electrospinning for use in bone tissue regeneration. Further on, this chapter highlights the development in the scaffold fabrication from natural polymers like silk fibroin, chitosan, collagen, gelatin, cellulose, starch and, zein. The importance of add-on materials like stem cells, hydroxyapatite, apatite-wollanstonite, growth factors, osteogenic cells, bone morphogenic proteins and osteogenic drugs have been discussed and illustrated by various examples for enhancing the formation of new bone tissue. Furthermore, this chapter explains how these natural polymers influence the several signaling pathways to regulate bone regeneration.

Keywords

Bone · Scaffolds · Electrospinning · Regeneration · Nanofibers · Hydroxyapatite

4.1 Introduction

4.1.1 Hierarchical Structure of Bone

The structure of bone is hierarchical in nature and is comprised of both organic and inorganic components. At the lowest level, the inorganic component (i.e., hydroxyapatite (HAp)) is the

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main solid component formed between the aligned organic collagen fibers. Anatomically bone comprises of a dense compact shell cortical bone and a porous core composing of spongiosa/trabecular bone. Basically, bone tissue comprises mainly of the collagen fibrils, HAp particles and proteoglycans [143]. The HAp provides good mechanical support, which eventually transfer stress back to the healing tissue. However, this process is difficult to achieve with HAp alone as far as the ideal scaffolds are concerned. The collagen fibrils are helical in shape with a length of 10 nm and a diameter ranging from 50 to 500 nm. These fibrils have the ability to self-assemble into fibrous networks and are determinants of mechanical and biological properties of the bone [74]. The presence of different nano-level structures in the hierarchy allows the bone to perform different functions and provides it distinct physical and mechanical properties. At the microstructure level of hierarchy, the cancellous bone has an irregular and porous structure, while as cortical bone has ordered microstructure and is composed of very tightly packed osteons called Haversian systems [23]. Moreover, these osteons are cylindrical structures and are composed of lamellar collagen fibers aligned in concentric layers. The collagen fibers are themselves composed of aligned fibrils and are formed from self-assembled triple helical collagen molecules [106]. The collagen molecules are arranged in such a fashion at the nano-level, that they create an anionic gap where HAp is nucleated as crystals [37, 106]. These crystals align and elongate along with collagen fibrils and enhance the mechanical properties that give bone its toughness and strength [136]. It's the cross-linking framework between collagen and HAp components that provides the viscoelasticity to the bone and hence maintains its load bearing functions and/or shock absorbing characteristics [8, 111]. Such an arrangement at the bio-nano interface plays a significant role in bone mineralization, cell response and behavior. The osteoblasts and osteoclasts are present on the mineralized bone whereas the bone matrix hosts the osteocytes. The signaling cascade between these cells and other specific signaling inputs are responsible for extra cellular matrix (ECM) secretion, bone absorption, bone resorption and mineralization. The osteoblasts secrete the collagen matrix and helps in deposition of HAp. The collagen matrix also provides framework for the osteocytes to attach, align and proliferate [132] [ref]. This level of structural integrity makes bone a complex living tissue and provides structure, mechanical support and flexibility to the body. Besides these functions bone tissue is also involved in mineralion homeostasis [134], blood pH regulation [4], storing of healing cells [57] and many other functions [66].

4.1.2 Types of Bone and Bone Formation

Bones can be classified as trabecular and cortical bone depending upon the mechanical properties. The former is involved in movement of limbs and joints while as the later provides the mechanical support and acts as protective shield [35, 105]. Long bones comprising mostly of the cortical bone possess modulus of elasticity (i.e., 17–20GPa) and a compression strength of (131–224 MPa) [45]. In contrast, the elastic modulus of cancellous bone is much lower due to its high porosity [41, 97]. The mechanical strength can also vary from bone to bone depending upon the magnitude of mineralization and testing conditions [24]. The formation of bone involves intramembranous and endochondral ossification wherein mesenchymal stem cells (MSCs) are differentiated into cartilage and later on replaced by bone cells. As aforementioned, although bone is a tissue of strength and represents superior mechanical properties however, bone defects are commonly persistent in all age groups.

4.1.3 Bone Injuries and Approaches of Treatment

Bone injuries and defects due to trauma, disease and congenital deformity pose significant medical challenge and often require clinical assistance [48]. The severe traumatic bone usually cannot

heal itself and mostly require surgical interventions [22]. Moreover, surgical resection is also needed in cases of osteosarcoma where large portions of the bone need to be removed and replaced appropriately. Current clinical practices to treat bone injury and/or deformity suggest the use of bone grafting and transplantation. The autologous transplants are considered as golden standard for the treatment due to non-immunogenic nature of the transplant and histocompatibility with the native tissue [11]. However, it involves extraction of the bone from patient's own body which results in donor-site morbidity. On the other hand, allografting involves extraction of the bone tissue from the cadaver. However, limitations are graft rejection due to the immunogenic response [15].

4.1.4 Bone Tissue-Engineering

The predicament in clinical treatments had motivated researchers for modern approaches like bone tissue-engineering, that can actually regenerate the deformed and injured bones. The intriguing approach is to implant a temporary biodegradable and biocompatible scaffold that will be eventually replaced by the native tissue and more comforting is that it will require only one surgical procedure. The biomaterial scaffolds come up with the add-on properties that cells, growth factors or osteogenic drugs can be incorporated systematically. Further, these can be surgically placed at the site of bone tissue injury [137]. These biomaterials being osteoconductive, osteoinductive and osteointegrative provide a prototype for engineering scaffolds necessary for cellular regeneration [1].It is imperative to mention that stem cells and growth factors can directly be incorporated into these scaffolds for osteoconductivity and thus by inducing the MCSs to differentiate into osteoblasts [42]. At the biophysical level, these scaffolds provide extracellular micro-environment, thereby operating as a framework to support and stimulate cell-driven regeneration. Such scaffolds have a prime requisite to possess required porosity for cellular infiltration, nutrient transport and removal of toxic metabolites. Furthermore, these scaffolds should have certain degree of surface roughness and wettability to promote cell adhesion, proliferation and differentiation [124]. The seeded cells after division proliferate into a new tissue and in the meantime the degradation of bio-polymeric scaffold takes place, resulting in formation of complete tissue without the used scaffolds. The cells seeded onto these scaffolds depend on the type of tissue to be regenerated and the required application. For example, stem cells seeded onto the scaffold matrix can be directed to differentiate towards a particular lineage and osteoblasts seeded onto these scaffolds will develop into new tissue for regeneration of the damaged bone. It is well know that different stem cells are capable of osteogenic differentiation. These include adipose derived stem cells (ADSCs) dental pulp stem cells (DPSCs) and MSCs of various origins (e.g., bone marrow, periosteum, trabecular bone, pericyte, dermis, synovial membrane etc.) [13, 20, 25, 60, 85]. The induction of stem cells towards an osteogenic lineage can be done by supplementing the scaffolds with certain differentiating factors and other biomolecules [95]. The growth factors incorporated into these scaffolds are released in controlled manner only to recruit the endogenous stem cells for instigating them to differentiate into a desired lineage for regeneration of the injured tissue [14]. Other prerequisites for the bone tissue regeneration scaffolds include; ability of osteoid deposition, malleability, resilience to irregular wound-site, sterilizability, devoid of side-effects to the surrounding tissue and easy availability [50].

4.1.5 Materials for Bone Tissue Engineering

Different materials are being investigated to be used as scaffolds for bone tissue engineering. These include scaffolds fabricated from ceramic and biocompatible polymers [121]. The ceramics being structurally similar to the composition of natural bone and possessing desired mechanical properties are considered an explicit choice. However, the increased brittleness due to loose

powder nature and needle-like appearance and lack of biodegradability makes them incompatible. On the other hand, polymers are versatile in their properties, which are biodegradable and are biocompatible. They also possess the requisite mechanical properties and can also be blended with the ceramics to form scaffolds for bone grafts. For example, the incorporation of the nano-sized HAp into polymer scaffolds has shown advancement in mechanical properties such as improved strength and crack-resistance [61, 96, 150]. The dual advantages of these composites have led to the development of polymer-ceramic scaffolds for application in bone tissue engineering. A number of bioactive inorganic phases besides the natural HAp are available to form the non-crystalline matrix (e.g., calcium phosphate, apatite-wollanstonite glass ceramics and bioactive glass). Among these, HAp being a native constituent of the bone is the most widely used matter [12]. However, intriguing results have demonstrated that bioactive glass containing Si ions and/or doped with ceramics have better bone apatite inducing potential than the pristine HAp [47]. The phenomenon is ascribed due to the role of Ca and Si in the bone mineralization process [87]. It has also been investigated that the size of the bioactive glass in the polymeric dispersions affects the morphology and mechanical strength of the nanofibers [56]. A wide variety of polymers are used for the fabrication of these scaffolds including natural, synthetic, and/or their composites. On one hand, natural polymers possess the ability to bio-mimic the natural cellular niche due to its resemblance with the ECM. On the other hand, the synthetic bio-polymers have the advantage of mechanical toughness and durability for fabrication of scaffolds in bone tissue engineering. However, a combination of the two allows tailoring of specific properties into a more versatile version. Moreover, the combination of the polymers is also advantageous for mineralization of the scaffold with HAp using techniques such as co-precipitation, electrospraying and blending [40, 131, 98]. However, recently natural polymers are used alone and also in combination with other natural polymers because these can be surface tailored or blended with elastic and mechanically durable polymers like collagen [51].

Herein, in this chapter, we have presented a brief outlook with the robust examples of bone tissue engineering from recent years. Briefly, we will be focusing on the scaffolds fabricated from natural polymers, their blends with natural and/or synthetic polymers. Furthermore, their subsequent functionalization with other polymers and bioactive compounds are investigated. The natural polymers such as chitosan, silk fibroin, collagen, elastin, fibrin, hyaluronic acid, cellulose, starch, etc. possess built-in properties like biocompatibility and biodegradability. They are also devoid of immune rejection and do not evokeany histocompatibility reactions [46, 114, 115].

4.1.6 Techniques for Scaffold Fabrication in Bone Tissue Engineering

Many techniques are known till date for the fabrication of scaffolds for bone tissue engineering. These techniques include phase separation [76], solvent casting/particulate leaching [16, 100], freeze-drying [116], gas foaming [141], porogen leaching [84], melt molding [21], membrane lamination [79], fiber bonding [19], electrospinning [122], and rapid prototyping [70] methods. Some of these produce the scaffolds with decreased mechanical strength, porosity, yield, less-thickness and poor degradation rates of the polymer. Among these techniques, electrospinning is one of the most versatile and promising approach to produce nanofiber scaffolds that can mimic the ECM of many variant tissues, including that of the multifaceted structure of the bone [125]. Electrospinning is a simple fabrication technique based on the fact that when an ample voltage is supplied to a polymer droplet extruding from a fine needle, it becomes charged. The repulsion between these charges destabilizes the droplet and leads to fiber formation which is deposited at the collector end connected to other terminal of the voltage supply [104]. The electrospinning apparatus is simple and comprises

of a high voltage power supply, a spinneret (usually a hypodermic needle), a syringe and a collector [123]. In the process of electrospinning, the ultra-thin jet emancipating from the needle finally settles and dries at the collector, leaving the solvent aerosolized [29]. The electrospinning process has the unique ability to reproduce fibers of nanometer range that can actually mimic the ECM of many tissues, including that of the complex hierarchical structure present in the bone. This method has been utilized to create highly aligned nanofibers from natural, synthetic polymers or a combination of the two [73]. The bone tissue engineering using electrospinning process presents a versatile way of repairing and regenerating bone tissue [46, 74].

The process of bone tissue-engineering is a dynamic which regulates cell adhesion, migration, proliferation and differentiation. This accelerates the formation of bone matrix at the site of injury or transplant and as a result shortens the healing time as compared to other traditionally available procedures (i.e., auto or allografting) [39, 75]. Thus, these biomimetic scaffolds provide an osteogenic environment and promote the process of ossification, thereby improving the outcomes of clinical therapy [83]. The prime focus in this field is to design scaffolds that are frontrunners in bio-mimicking the natural bone matrix present in human body. This is usually achieved by mineralizing the electrospun nanofibers fabricated from natural polymers and/or their blends. Loading the mineralized growth factors and other signaling biomolecules and then creating 3D bone-like structures is an assuring strategy for bone tissue-regeneration. In the following sections, we will review the current research based on the fabrication of nanofiber scaffold from natural polymers or their blends for bone tissue engineering. We will also analyze how electrospinning and bio-mineralization techniques are simultaneously used for creation of scaffolds for regeneration of bone tissue including *in-vivo* applications. Furthermore, we will briefly discuss how some of these natural polymers are involved in signaling cascade to stimulate bone-regeneration.

4.2 Natural Polymers and their Composites in Bone Tissue Engineering

The examples of natural polymers that we will describe in this chapter for fabrication of scaffolds in bone tissue engineering include proteins such as silk fibroin, collagen, gelatin and polysaccharides like cellulose, starch and zein.

4.2.1 Silk Fibroin Scaffolds in BTE

Silk fibroin, a core structural protein, is an easy to process natural polymer with the state of art in bone tissue engineering. It's mostly extracted from silk cocoons from *Bombyx mori* and is characterized by its distinct amino acid composition [91]. The fibrous protein has a strong resemblance with collagen I, the main constituent of bone ECM. The secondary structure of silk fibroin comprises of β-sheets with amorphous linkages, resembling the non-collagenous proteins that augment in deposition of HAp [82]. The silk-based biomaterial can be molded into diverse forms resulting in wide-applications for delivering bone growth factors and drugs to diseased and fractured sites for the process of regeneration. Silk fibroin as a biomaterial for bone regeneration is not only because it mimics the ECM, possesses cellular compatibility, but also because of its ability to stencil the growth of HAp crystals leading to osteointegration [129]. Silk fibroin as an osteogenic biomaterial has the potential to induce differentiation of stem cells by inhibiting the Notch pathway, a signaling transduction pathway which controls cell fate determination and apoptosis [99]. Notch is exhibited in human bone marrow derived MSCs (hBMSCs) and an activation signal leads to early induction of osteogenic differentiation. The termination of that signal leads to the formation of mature osteoblasts by inhibiting the Runx2 activity [26, 44]. A number of sources of silk fibroin like silk worms (*Bombyx mori*), spider silk (*Nephilaclavipes*) and *Antheraeamylitta* have been used to produce nanofiber scaffolds using the process of electrospinning [55, 93, 151]. In

order to potentiate and improvise the cell differentiation and proliferation property, it's often electrospun with other polymers like collagen, gelatin and chitosan etc. [62, 102]. In this section, we will discuss about the novelties in these scaffolds since last 5 years. We will also explore how different types of silk fibroin scaffolds are developed and how incorporation of different materials like Ca, Si, BMP etc., leads to development of scaffolds with better performance in bone tissue engineering.

The biphasic scaffolds of silk fibroin were developed to mimic the topography of collagen-alignment present at the surface of tendons and bones. The two different arrangements of the pores were obtained and the pores were anisiotropic at the tendon/ligament side, while as they were isotropic at the bone side. The hBMSC scultivated on these scaffolds were influenced by the pore geometry. Moreover, it was observed that biphasic scaffolds based on pore alignment supported the cell attachment and commanded cytoskeleton organization [36]. The 3D scaffolds of silk fibroin/chitosan/HAp were fabricated for osteogenic differentiation and bone regeneration. The scaffolds seeded with BMSCs presented good attachment and proliferation. These scaffolds when implanted at the lesion site in a femur defect of rabbit revealed regeneration of chondral and subchondral layers as observed in a micro-CT shown in Fig. 4.1 [110].

The preparation of core-shell nanofiber scaffolds for bone tissue engineering allows incorporation of different bone inducing factors along with polymer and also provides sustained release of these factors. The co-axial electrospinning allowed the fabrication of 3D nanofiber scaffolds loaded with BMP-2, IGF-1 and BSA. In this case, the silk fibroin and poly (L-lactide-co-caprolactone) (PLLACL) were electrospun along with these bone inducing factors. The polymer occupied the shell and BMP-2/IGF-1/BSA was encapsulated into the core. The release of these factors was analyzed by ELISA microplate reader and it was found that the scaffolds showed a gradual increasing release with varying degrees of burst and sustained release profiles. The scaffolds were then seeded with BMSCs and showed good viability and high ALP activity. The findings suggest that these scaffolds can promote early osteoinduction and can be used in guided bone-regeneration [149]. 3D nano-composite fiber scaffolds of silk fibroin and active carbon containing dual growth factors (i.e., BMP-2 and TGF-β1) showed sustained release and early attachment, growth, proliferation and differentiation of the cultured osteoblasts and mesenchymal cells. The minimal release of pro-inflammatory cytokines as evidenced from *in-vitro* and *in-vivo* studies from these scaffold ssuggested that they were immunocompatible. Radiological fluorochrome labelling and histological investigations of these scaffolds during *in-vivo* studies showed their potential in mimicking the physiological bone niche and supported the osseointegration process [89]. The hBMSCs cultured on silk fibroin scaffolds containing BMP-2 were prepared by electrospinning and have been used for *in-vitro* bone formation by supporting mineralized tissue formation [72]. These scaffolds supported mesenchymal cell growth and differentiated into osteogenic outcomes. Moreover, silk fibroin scaffolds incorporated with BMP-2 supported higher calcium deposition and enhanced transcription levels of bone specific markers.

Composite nanofiber scaffolds were prepared by electrospinning of silk fibroin/chitosan and studied for osteogenic differentiation of hBMSCs. The cells were viable on these composite scaffolds as determined by MTS assay. The cells attached and differentiated into osteogenic lineage as reported by high ALP activity and expression of osteogenic marker genes. It was shown in this study that blending these two natural polymers to form composite nanofiber scaffolds retains the osteogenic nature of chitosan without compromising the cell proliferative effect of silk fibroin. The dual advantage of these two natural polymers makes these nanofiber scaffolds suitable for bone tissue engineering [69].

Composite nanofiber scaffolds were prepared from silk fibroin and carboxymethyl cellulose using free liquid surface electrospinning method [126]. The scaffolds have the ability to assist in nucleation and biomineralization of nano-sized calcium phosphate for use in bone tissue engi-

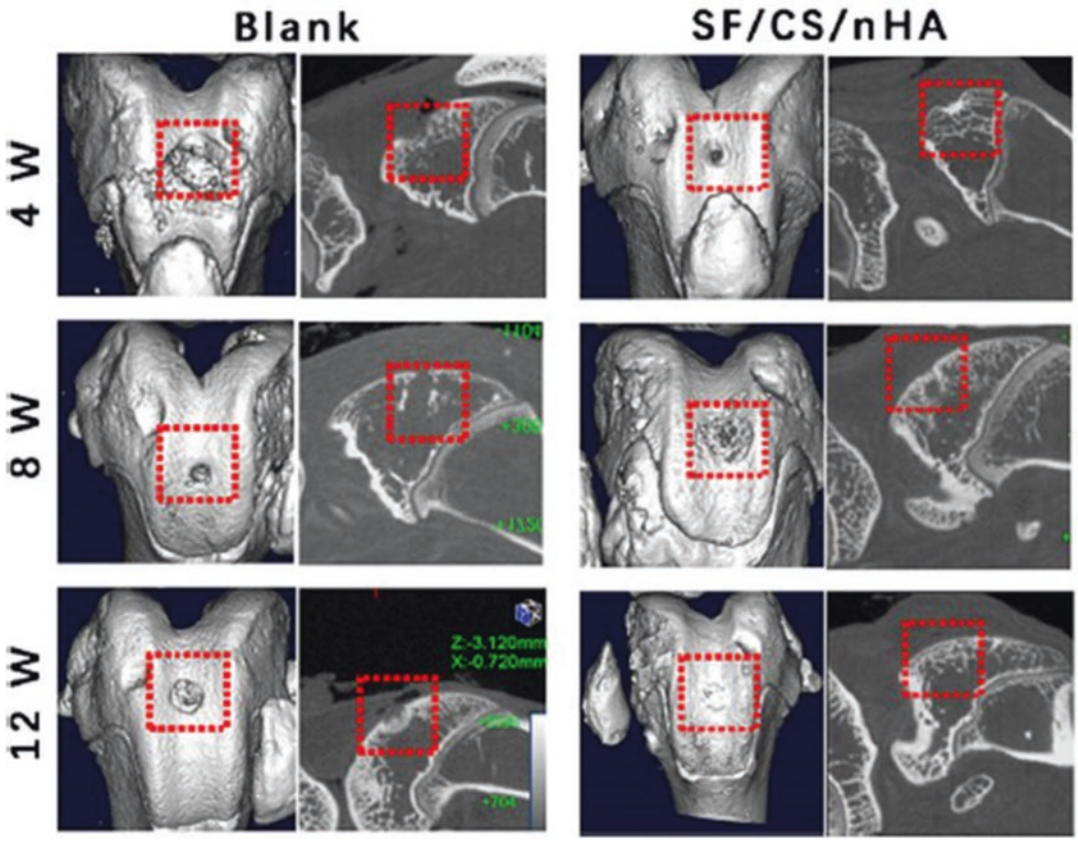

Fig. 4.1 Micro-CT images showing regeneration of chondral and subchondral layers in a rabbit femur defect on implantation with BMSC-biphase-scaffold after 4, 8 and 12 weeks post treatment. The blank group received no implants while as the treated group received Silk fibroin/Chitosan/Hap 3D scaffolds seeded with BMSCs. (Reprinted with permission from Springer nature [110])

neering applications. The apatite-like crystals are dispersed uniformly throughout these scaffolds as determined by XRD and EDX analysis. Compared to pure silk fibroin nanofiber scaffolds, these composites show early differentiation of umbilical cord blood derived human mesenchymal stem cells to osteogenic lineage. This shows that scaffolds fabricated using carboxymethyl cellulose assists in the early differentiation of stem cells. The high ALP activity, expression of runt-related transcription factor, osteocalcin and alizarin staining further confirms the bone-regeneration ability of these composite scaffolds [126].

The development of functionalized scaffolds is the novel trend in scaffold fabrication for bone tissue engineering. In this connection, electrospun silk fibroin scaffold has been functionalized by HAp using a two-stage process. During the initial stage, the HAp nanoparticles are electrospun with silk fibroin dispersion and then in the second stage HAp nanoparticles are surface immobilized on scaffolds using mussel adhesive-inspired polydopamine chemistry. As such this scaffold contains HAp both inside the fiber as well as onto the surface of the fiber (Fig. 4.2). The method has improved the mechanical properties (Fig. 4.2) of the scaffold and provides an environment mimicking to the bone tissue matrix. Moreover, genetically modified hADMSCs seeded on the scaffolds boosted the process of bone repair by enhancing osteogenic differentiation. The hADMSCs were genetically modified with the transcriptional co-activator with PDZ

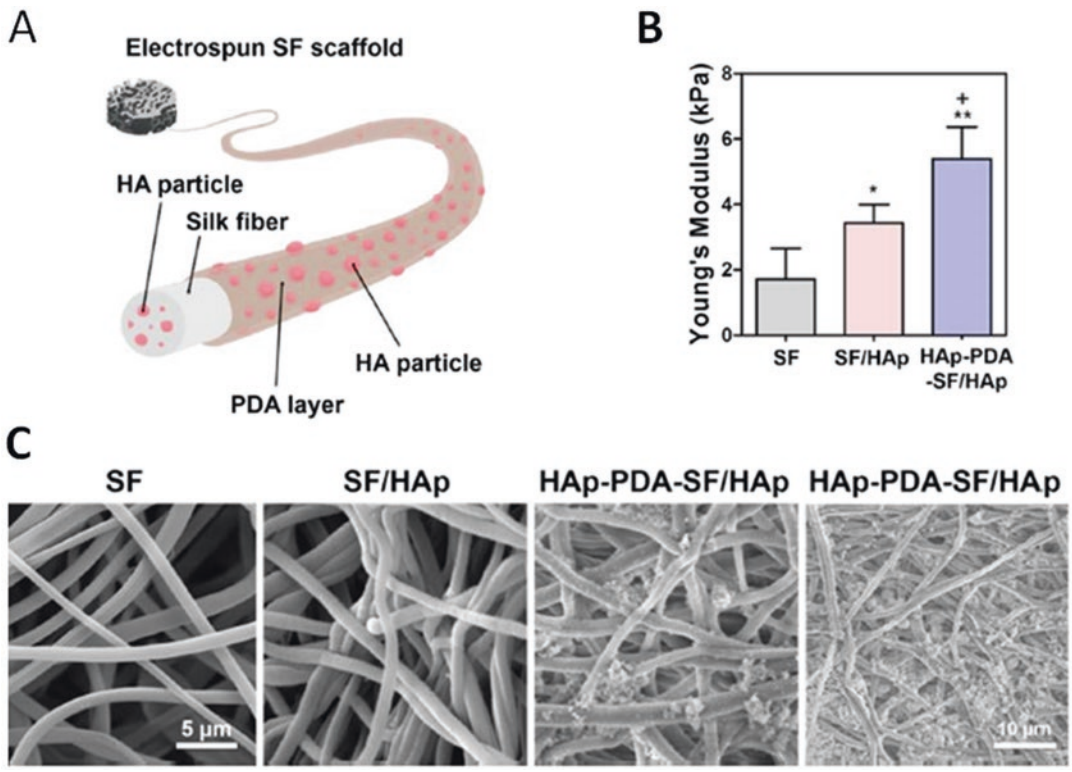

Fig. 4.2 Two-stage engineered HAp functionalized electrospun silk fibroin (SF) nanofibrous scaffolds. (**a**) Schematic representation of the silk fibroin scaffold illustrating two-stage HAp functionalization in the core and periphery. (**b**) Young's modulus values of different SF scaffolds measured by universal testing machine describing the improvised mechanical strength of the functionalized electrospun fibers (**c**) Scanning electron microscopy images of SF, SF/HAp, and HAp-PDA-SF/HAp scaffolds. (Reprinted with permission from American Chemical society [65])

binding motif (TAZ). The TAZ is a known transcriptional modulator that sparks the osteogenic differentiation of MSCs. The potential of these scaffolds in bone tissue engineeering was explored in a critical-sized calvarial bone defect model. The enhanced mineralization of the bone tissue was observed using micro-CT after 8 weeks of the treatment as compared with the group receiving no treatment as seen in Fig. 4.3. Bone regeneration volume and ratio of regenerated bone volume to total bone volume calculated from the micro-CT studies also confirms that these scaffolds have huge potential in bone-regeneration and remodeling [65].

The concept of guided bone regeneration (GBR) utilizes membranes for restoration of alveolar bone tissue and mandible defects thereby preventing the formation of non-functional scar-tissue layer by acting as a barrier tissue. The two layered membranes prepared by electrospinning of polymers have played an important role in GBR. A sandwich structure comprising of electrospun silk fibroin-polycaprolactone and polyethylene glycol-polycaprolactone incorporated with nano-calcium phosphate in one layer and polycaprolactone in other layer, was developed for GBR. The nano-sized-calcium phosphate, synthesized by flame spray pyrolysis improved the mechanical strength and osteoconductivity of these scaffolds. Human dental pulp stem cells seeded on these scaffolds showed surpassing cell adhesion, ALP activity and proliferation upon increasing the nano-calcium phosphate content in the scaffolds [135]. Silk fibroin from non-mulberry sources like *Antheraeamylitta* can be grafted onto aminolysed and polycaprolactone and

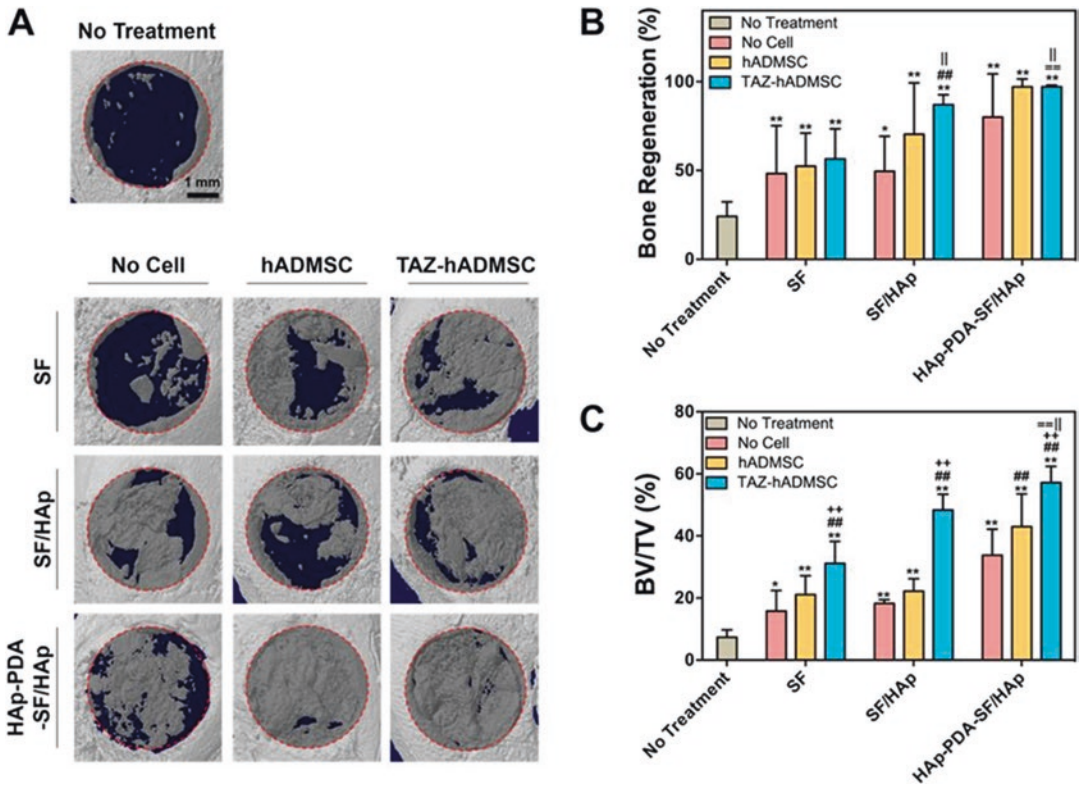

Fig. 4.3 Enhanced mineralized of the bone in a critical-sized calvarial bone defects post 8 weeks after transplantation with two-stage hydroxyapatite (HAp)-functionalized silk fibroin (SF) scaffolds containing hADMSCs. (**a**) Micro-CT images of the calvarial bones treated with three different scaffolds: SF, hydroxyapatite (HAp)-containing SF scaffold (SF/HAp), and HAp-containing SF scaffold with polydopamine (PDA)-mediated HAp surface coating (HAp-PDA-SF/HAp) and three different cell conditions: No Cell, hADMSC, and TAZ-transfected hADMSC (TAZ-hADMSC). Significantly improvised mineralization compared to control groups and other scaffolds can be seen in HAp-PDA-SF/HAp scaffolds (**b**) The percentage of bone regeneration area to total defect quantified from micro-CT images in different groups describing significant bone regeneration in defects receiving HAp-PDA-SF/HAp compared to the other groups. (**c**) The percentage of regenerated bone volume (BV) to total volume (TV) quantified from micro-CT images in different treatment groups and the control group also explaining the potential of the two-stage functionalized scaffolds in bone regeneration. (Reprinted with permission from American Chemical Society [65])

nanofibers, as this contains tri-peptide (Arg-Gly-Asp) integrin binding RGD sequence [135]. The sequences inherent to the fibroin are found to augment cell adhesion and proliferation [94]. The grafted fibroin scaffolds have been found better than fibroin-polycaprolactone blended scaffolds in terms of supporting cell adhesion, growth and proliferation of human osteoblast-like cells. These grafted scaffolds have even performed better as they possess improved hydrophilicity, surface roughness and mechanical strength [10]. The presence of flavanoids like papryflavonol-A, broussochalcone and high calcium hydroxide content in the extract of *Broussonetiakazinoki*, a Chinese traditional herb makes it a suitable material for osteogenic differentiation of stem cells and osteoconductivity [130]. The incorporation of this herbal extract with silk fibroin has resulted into fabrication of silk fibroin/*Broussonetiakazinoki* scaffolds for application to defective bone areas. This scaffold is an efficient cell carrier and promotes cell adhesion, migration, division and proliferation of BMSCs. Up-regulation of genes related to osteogenic dif-

ferentiation and bone regeneration has been observed both in *in-vivo* and *in-vitro* studies. The implanted scaffolds after 8 weeks post-surgery in a defective bone model (i.e., rat calvarial defect) has demonstrated bone tissue bridging, restoration of mechanical properties and new bone formation as evidenced from micro-CT and histological studies [63].

Tussah silk fibroin is also rich in Arg-Gly-Asp amino acid domains and these motifs are known to promote cell adhesion and proliferation [86]. Fabrication of scaffolds from Tussah silk fibroin and composites has been recently introduced in bone tissue engineering. A multilayered scaffold composed of Tussah silk fibroin and poly(lactic acid) were fabricated mimicking the lamellar architecture of the natural bone. Blends of Tussah silk fibroin and poly(lactic acid) were electrospun into nano-scale woven yarns and it was found that a 9:1 mixture of the two had excellent mechanical properties with tensile strength of 180.36 MPa. These scaffolds were found to support the adhesion and proliferation of mouse MSCs as well as enhanced mineral deposition. Application of these scaffolds in damaged femoral condyle in rabbits significantly improved the formation of new femur bone after the 12 weeks of implantation, compared to the untreated group as shown in Fig. 4.4 [118]. Similar studies have been conducted in scaffolds fabricated from Tussah silk fibroin and polycaprolactone [9]. Composite scaffolds of Tussah silk fibroin and HAp present as core and the core being encased in a shell of Tussah silk fibroin has been proposed to mimic the complex hierarchical structure of the bone. These scaffolds produced by electrospinning can

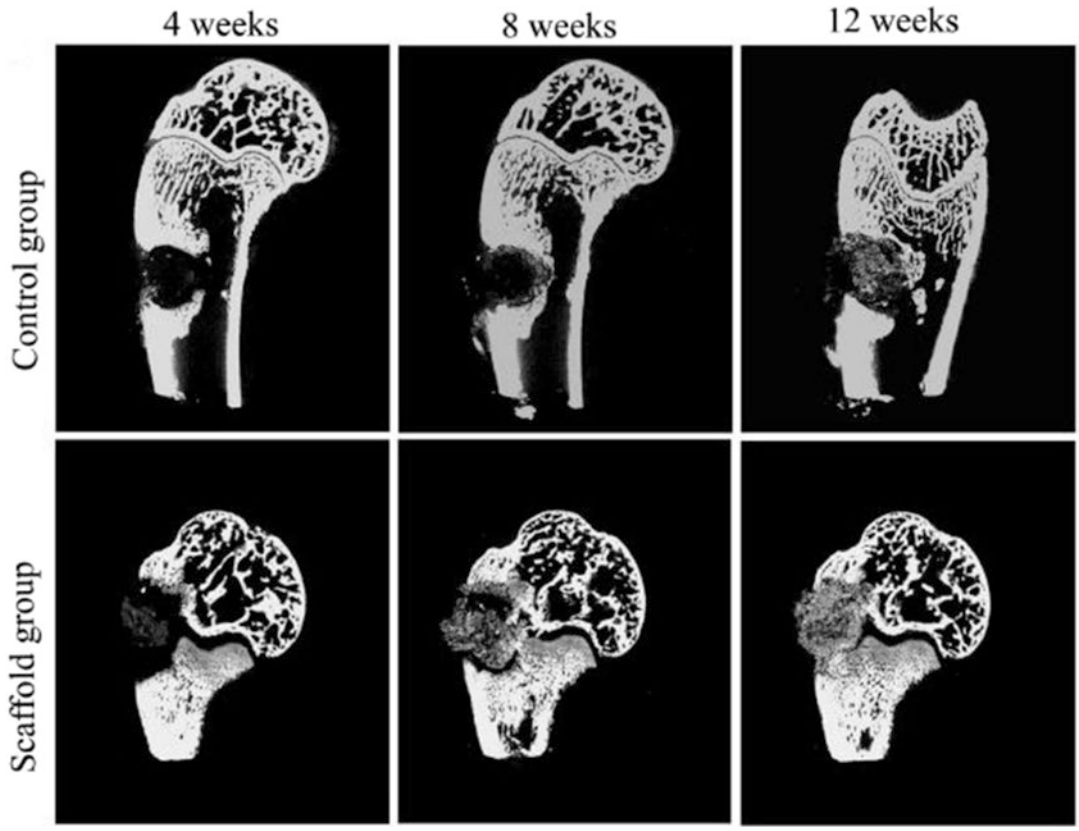

Fig. 4.4 Representative micro-CT images of damaged femoral condyles in rabbits, taken post 4, 8, and 12 weeks of the treatment with or without a PLA/TSF scaffold. Significant improvisation of the defect can be seen in the micro-CT images post 12 weeks of the treatment as compared to the control groups. (Reprinted with permission from Elsevier [118])

support the adhesion and proliferation of osteoblast-like MG-63 cells [119].

Icarin, a main constituent from Chinese traditional herb, (*Epimediumbrevicornum* Maxim) is an active flavanoid with a potential of having bone defect regeneration relating to dental implants. In this regard, the icarinwasincorporated into the nanofiber membranes of silk fibroin and poly(lactide-co-epsilon-caprolactone) by co-axial electrospinning. This acted as an osteoinducting factor and was released in two stages; with initial burst and other with sustained release at constant stage for over a period of 30 days. These scaffolds were biocompatible with BMSCs and promoted osteogenic activity. The bone defects in rat cranium covered by these scaffolds healed most of the defected region (volume and density) after 12 weeks of implantation. The experimental procedure in rat and application of these scaffolds on bone defects is shown in Fig. 4.5. The implanted scaffold will degrade with time as the natural tissue replaces it. This scaffold has been found to degrade neither too rapidly to allow cells proliferate and secrete new matrix, and nor too slow for homogeneity and biological function of the regenerated bone [148].

Developing scaffolds with aligned nanofibers are known to induce early osteogenesis in bone tissue engineering. When these aligned nanofibers are loosely packed, it provides more chances for migration, infiltration and diffusion for bone forming cells. Wet-collection electrospinning is known to produce such type of scaffolds by collecting the nanofibers on a rotating mandrel placed in an ethanol bath. Such a scaffold allows 3D culture of the MC3T3-E1 pre-osteoblasts. The ethanol bath in this method allows formation of uniform and aligned fibers with increased thickness and porosity. The osteoblasts grown on these scaffolds exhibited more ordered arrangement with elongated morphology and deeper

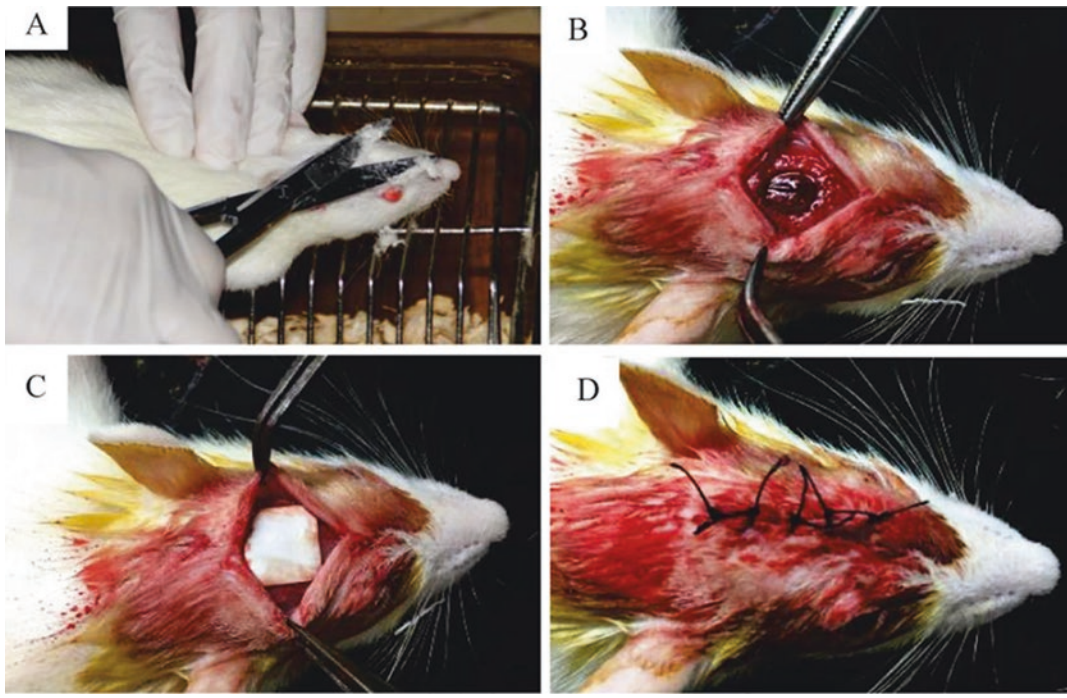

Fig. 4.5 The surgical experimental procedure for implantation of scaffold in experimental animals (Rat). (**a**) The preparation of rats for nanofiber membrane implantation. (**b**) Creating a complete defect on the cranium. (**c**) Implanting the nanofiber membrane and coverage of the defects by the membrane. (**d**) Closing the soft tissues and by suturing layer by layer after implantation [148]

infiltration. This resulted in establishment of 3D cultures with enhanced proliferation and differentiation of pre-osteoblastic cells. These scaffolds could have significant potential in bone-regeneration [28]. Incorporating bioactive glass in the polymeric scaffolds has led to development of engineered constructs with hierarchical structure resembling from nano- to micro-scale to that of the bone tissue ECM. Bilayer deposition of bioactive glass with the nanofibrous scaffolds fabricated from silk fibroin and poly(vinyl alcohol) was done by free liquid surface electrospinning and stabilization through ethanol washing. Stabilization with etanol washing leads to the densification and Si-O-Si network formation of bioactive glass. The composites contained the bioactive glass particles across the surface as well as within the fibers. This leads to controlled bio-mineralization of the scaffold and hence regulates apatite formation. Such a scaffold possesses superior hydrophilicity, stiffness, tensile strength and greater osteogenic potential. The biocompatibility and osteogenic potential of these scaffolds were evaluated by culturing them with cord blood derived-MSCs. The *in-vitro* studies have explored potential of these scaffolds for regeneration of bone tissue [127]. Delivering the inducing factors along with the bone regenerating scaffolds allows early osteogenic induction. Recently, silk fibroin scaffolds cross-linked with glutaraldehyde was used for delivering rhBMP-2 in different ratios. The introduction of rhBMP-2 growth factor not only improves the mechanical robustness of the scaffold, but also the water retention capacity as evident from the swelling studies. The hMSCs were viable on these scaffolds and were also found to have high ALP activity under both *in-vitro* and *in-vivo* conditions [30]. While the osteogenesis is on its way, it's important that angiogenesis proceeds parallel to it for provision of nutrients, oxygen and growth factors to the osteogenic cells. This will ensure the sustenance, survival and subsequent differentiation of the osteogenic cells. Keeping in view this fact, the researchers are adding VEGF along with the osteogenic factors like BMP for neovascularization [58]. The VEGF loaded silk fibroin/poly(lactic-co-glycolic acid)/Calcium phosphate composite nanofibers fabricated by electrospinning has the ability to deliver this factor for a period of over 28 days. The system has been found to significantly heal rabbit calvarial defects [33]. Magnetic nanoparticles can play an important role in mediating ion-channels in cell membranes, thereby, affecting cell behavior. Incorporation of these magnetic nanoparticles in the scaffolds of silk fibroin and chitosan along with synergistic application of external magnetic field has improved the growth and proliferation of MG-63 osteosarcoma cell lines. In the MTT assay, the scaffolds containing magnetic nanoparticles were more viable than the non-magnetic scaffolds [2].

4.2.2 Collagen Scaffolds in Bone Tissue Engineering

Collagen I is a natural constituent of bone ECM [112]. It is easily degraded in the human body and is known to possess good mechanical strength as well as cell adhering properties [15]. This section about collagen as a biomaterial will describe the recent prospectus of this biomaterial in bone tissue-regeneration. Collagen is often blended with other natural and/or synthetic polymers to improvise the mechanical strength and biomimicking properties. Reinforcing collagen and chitosan nanofibers with synthetic polymers like polyurethane and poly (vinyl alcohol) by the process of electrospinning. This improvised the tensile strength of collagen/chitosan nanofibers. The improved mechanical strength has been found to be of great importance in bone tissue engineering. Porcine iliac endothelial cells cultured on these scaffolds have been found to exhibit biocompatibility with composite scaffold [155]. Collagen extracted from jelly fish has been fabricated along with keratin, obtained from human hair and nano-sized egg shell derived HAp into porous scaffolds for use in bone tissue engineering. The isolated hAMSCs were characterized for different cell markers like CD29, CD44, CD73 and CD90 and multilineage capacity by flow cytometry. The cell markers expressed by these cells on the scaffolds revealed that during *in-vitro* studies these cells were directed towards osteogenic, chondrogenic and adipogenic lin-

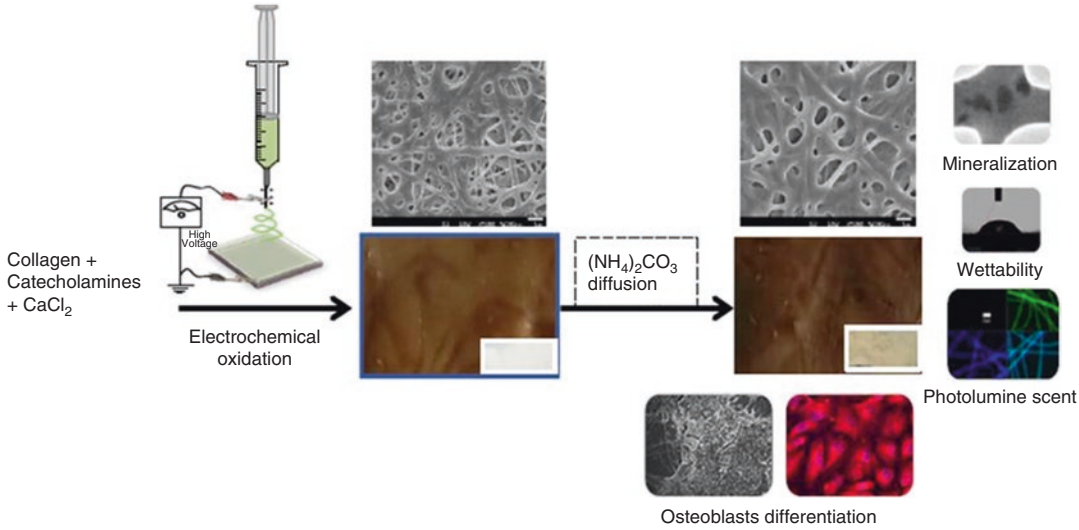

Fig. 4.6 Schematic representation of electrospinning of a dope solution containing collagen (8% w/v), dopamine (10% w/w of collagen) and 20 mM $CaCl_2$ in 90% HFIP. The polydopamine formation is depicted by the appearance of brown coloration in the mats. The subsequent exposure of the polydopamine mats to $(NH_4)_2CO_3$ vapors resulted in intensification of brown coloration and precipitation of $CaCO_3$. For comparison purpose the photograph of mat electrospun without $CaCl_2$ is also shown in the lower bottom. (Reprinted with permission from Elsevier [27])

eages, respectively [5]. Commercially available periodontal collagen membranes are modified by electrospinning fibers of synthetic polymers over the surface along with antibacterial agents and other growth factors for enhanced efficacy in bone-regeneration. The resulting membrane known as functionally graded membranes possess different layers with different applicability. Likewise in one of the experiments where poly (L-lactide-co-d, l-lactide) encapsulating metronidazole was electrospun to form a scaffolds sandwiched between the commercially available collagen. The metronidazole release from the scaffold studied showed that the drug was released over a time period of 28 days. These functionally graded scaffolds were studied in preclinical efficiency for alveolar ridge regeneration and it was found that these scaffolds improved the bone-regeneration as compared with the control collagen only membranes [5].

Collagen being a natural constituent of the bone can be modified to exactly mimic the formation of vertebrate bone especially during the early stage of bone development. In this connection, the bio-inspired formulations of collagen were prepared by electrospinning of collagen with catecholamines (e.g., dopamine and norepinepherine along with Ca^{2+} supplements) as presented in the schematic Fig. 4.6. It has been found that the presence of Ca^{2+} induces polymerization of catecholamines as well as cross-links the collagen fibers. The mineralization of the scaffolds was done by ammonium carbonate diffusion method which causes complete oxidative polymerization of catecholamines and precipitation of amorphous $CaCO_3$. Mechanically robust and photoluminiscent scaffolds have been obtained by this method with Young's modulus value approaching towards the cancellous bone. While comparing the development of human fetal osteoblasts on the pristine collagen nanofibers or tissue culture plates with that of the composite, the scaffolds displayed an amplified cell adhesion, migration, differentiation and expression of osteogenic matrix proteins (e.g., osteocalcin and osteopontin) making them suitable candidates for bone-regeneration. Furthermore,comparing the scaffolds within, it was found that collagen scaffolds cross-linked with norepinephrine demonstrated superior mechanical strength and cell proliferation than the dopamine-linked collagen scaffolds [27].

Bio-mimicking scaffolds have also been developed for bone tissue engineering by taking insights from muscle inspired cell adhesion through protein secretion. A lamination model in which collagen-HAp composite nanofibers have been successfully glazed over polydopamine coated microbifrous poly(lactic-co-glycolic acid) fabrics. The lamination is carried by electrospinning a sol. of collagen containing L-glutamic acid grafted with HAp nanorods over the fabric of polydopamine coated poly(lactic-co-glycolic acid). These scaffolds in correlation with that of pristine poly(lactic-co-glycolic acid) fibers or dopamine-coated poly(lactic-co-glycolic acid) fibers or collagen-HAp composites produced more wettable surfaces and displayed excellent cell adhesion of MC3T3-E1 osteoblasts. The enhanced bioactivity of these scaffolds for MC3T3-E1 cells is attributed to ligand-receptor interactions [68]. The use of harsh organic solvents is often a problem in fabricating scaffolds for tissue-engineering [125]. These solvents are toxic and the residuals left over in the scaffolds may cause death of the culturing cells. It's often recommended to use greener and eco-friendly solvents to prevent cytotoxicity. Zhou et al., developed a greener method for fabrication of collagen-HAp nanofiber scaffolds which does not require any harsh organic solvents to process. Instead the collagen I is dissolved in eco-friendly phosphate buffer saline/ethanol solution followed by desalination and co-electrospinning the collagen with HAp sol. The nanofibers produced by this method had a mechanical strength 40 times higher than those synthesized by other routes requiring organic solvents. More interestingly, it was found that nano-HAp needles were found to be aligned along the longitudinal direction of collagen fibers, which is quite akin to the natural bone architecture. The human myeloma cells (U2-OS) were viable on these scaffolds and differentiated into osteoblasts as demonstrated by CCK-8 assay and high ALP activity [154].

Dual extrusion electrospinning provides an arrangement for fabrication of multilayered stacked 3D nanofiber scaffolds. The schematic representation of fabrication of dual stacked 3D nanofiber scaffolds from poly(lactic-co-glycolic acid) and collagen solutions is shown in Fig. 4.7. To fabricate scaffolds, 35 wt% of poly(lactic-co-

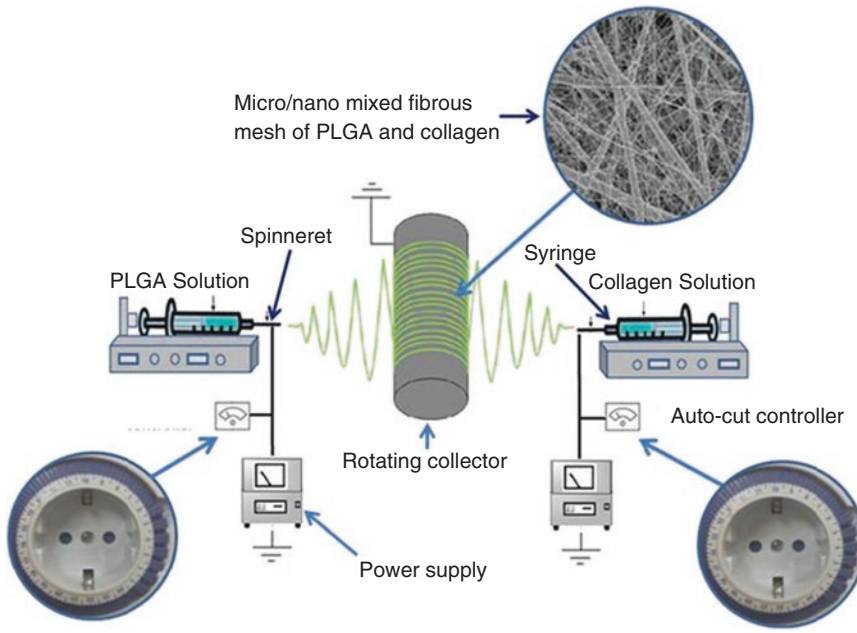

Fig. 4.7 Schematic representation of dual electrsospinning technique to develop 3D multilayerd stacked scaffolds for bone tissue engineering [67]

glycolic acid) in a binary mixture of THF-DMF and collagen with or without nano-HAp rods in HFIP were prepared. These solutions were electrospun such that the poly(lactic-co-glycolic acid) meshes were layered in alternate fashion to nanofibrous meshes of collagen. Glutamate functionalized HAp nano-rods were prepared to enhance the dispersity and mixing in collagen sol. The resulting homogenous dispersions and the density of the collagen fibers enhanced the adhesion, proliferation and osteogenic differentiation of MC3T3-E1 cells cultured on these scaffolds [67].

Collagen and gelatin composite nanofibers when fabricated along with sub-micron bioglass fibers has been found to mimic the natural composition of the bone. The collagen/gelatin mixture acts as the organic phase while as the bioglass acts as the inorganic phase of the bone. The scaffold is prepared by a combination of freeze-drying and electrospinning methods. In this study, genipin was used as a cross-linker instead of glutaraldehyde. Genipin is a *Gardenia jasminoides* fruit extract that is capable of cross-linking the polypeptide structures through nucleophilic attack of amine residues on lysine and arginine moieties [120]. The use of genipin as cross-linker comparable to glutaraldehyde not only increases the gel strength of the polymers but is almost 10,000 times less cytotoxic than the glutaraldehyde [120]. Moreover, the Cu ions at a concentration of less than 1 mM were also incorporated in these scaffolds as these are known to promote angiogenesis in the new bone as well as provide antibacterial action. The Cu containing scaffolds were found to promote the growth and viability of human osteoblast-like cell line (SaOS-2) more as compared to scaffolds without Cu ions. In a different study, similar results have been obtained by culturing human primary osteoblasts on these scaffolds [138]. Comparative studies have been performed to study the influence of fish collagen and mammalian collagen in bone regeneration. The influence of chitosan and HAp addition to the collagen scaffolds prepared from these two sources by electrospinning has also been investigated. The results have demonstrated that addition of chitosan and HAp improves the viscosity, stiffness and biodegradation rate of the scaffolds whereas, the water binding capacity, shrinkage and porosity are diminished. Furthermore, the stiffness and water binding capacity was elevated in mammalian collagen as compared to the fish collagen. However, both the mammalian and fish collagen efficiently promoted the growth and proliferation of osteoblasts (hFOB12) as well as promoted the differentiation of human acute T-Lymphocyte leukemia cell lines (6 T-CEM) into osteoblasts as evident from the high ALP values. Overall, it was observed that both the composite scaffolds have huge potential for bone-regeneration [32]. The delivery of recombinant bone inducing proteins like BMP-2 is a challenge in bone tissue engineering. Recent studies have been conducted to express clinically used rhBMP-2 inside the cells. The process of electrospinning has been utilized to fabricate plasmid DNA based scaffolds and deliver these plasmids for release of DNA. However, the transfection efficiency of these plasmids was lower than expected. Recently, studies have been conducted to adsorb plasmid DNA transfection complexes onto a collagen-I/poly-L-lactic acid electrospun scaffold for delivery of rhBMP-2. The adsorption process was carried by mixing *β-gal* plasmid DNA with True Fect-Lipo transfection reagent and incubating these with collagen-I/poly-L-lactic scaffolds. During the *in-vitro* studies, pre-osteoblast cell line (MC3T3) was transfected with rhBMP-2 plasmid DNA adsorbed onto the surface of collagen-I/poly-L-lactic scaffolds. The studies demonstrated that transfection efficiency was increased 14-fold due to the robust rhBMP-2 transgene mRNA expression. This plasmid incorporated scaffold was then tested for *in-vivo* in a mouse pouch muscle model to evaluate the gene delivery and ability of this scaffold to induce ectopic bone formation. The results demonstrated by histological and immunohistochemistry studies suggest that the recombinant gene for expression of BMP-2 was expressed efficiently both at mRNA and protein level. This study demonstrated that these complex scaffolds stimulated the ectopic bone formation in the muscle pouch model and further studies desired to be conducted to evaluate the clinical response [152].

4.2.3 Gelatin Scaffolds in Bone Tissue Engineering

Gelatin is a denatured product of collagen and has poor mechanical properties, therefore, cannot be reinforced as a bone tissue engineering material. To overcome this barrier, many ceramic types of nano-fillers are introduced along with the gelatin to increase its mechanical toughness. Recently, boron nitride has been used to reinforce gelatin as a biocompatible scaffold by the process of electrospinning. The incorporation of boron nitride in the gelatin nanofibers has been confirmed by XRD and FTIR spectroscopy. The presence of boron nitride influenced in improvement of Young's modulus. Even after cross-linking with glutaraldehyde, the morphology of the nanofibres remained well preserved and mechanical toughness showed further advancement. These scaffolds were highly active in forming bone-like HAp and supported the attachment, proliferation and growth of human osteosarcoma cell line. The use of these scaffolds as bone tissue engineering materials is further confirmed by osteoblast gene expression and high ALP activity [88]. Fabricating multi-layered cellular stacks of cross-linked gelatin-HAp and pure gelatin by electrospinning has been a recent trend to develop dynamic cell culture seeding method for the human fetal osteoblasts. Different types of stacks were developed (e.g., four-layer stack with cells seeded in the 2nd and 4th layer, and a three layer stack with cells seeded at the bottom of each layer). In these studies, it was observed that the three layered scaffold of regularly seeded gelatin maintained cell-free porous zones in all the layers compared to the four layered stacks. This was due the higher number of pores in the three layered stacks through which the culture medium could easily perfuse. This maintained dynamic culture conditions throughout the stack and could be used to develop highly efficient bone tissue engineering materials [113].

Reinforcing gelatin with polycaprolactone improves the mechanical properties of gelatin and improves the biocompatibility issues. These polymers were co-electrospinned and cross-linked with genipin to develop scaffolds for bone tissue engineering. Smooth structures of these fibers were developed both in 2D and 3D architecture. Culturing these scaffolds with MC3T3-El osteoblasts promoted bone formation in both normal and osteogenic medium [101]. Seeding the ADSC in a layer-by-layer paper stacking scaffolds of gelatin and polycaprolactone fabricated by electrospinning process has improved the bone-regeneration process. The schematic fabrication procedure is shown in Fig. 4.8. Such a stacking method improves oxygen distribution, nutrient mobilization and waste transportation more efficiently. Significant expression of osteogenic related proteins (e.g., ostepontin, osteocalcin and osteoprotegerin) have been observed after seeding ADSCs on these multi-stacked scaffolds. The *in-vivo* application of these scaffolds in rat calvarial defects for a period of 12 weeks showed significant bone regeneration volume and covered almost 90% of the surface area of the defect [139].

Fabricating scaffolds of natural polymers with materials which can enhance the electrical signaling can actually regulate cell adhesion, proliferation and differentiation [107]. Gelatin when fabricated with different blends of aniline pentamer, a conductive material along with poly(L-lactide) have exhibited excellent bone regenerative properties. The mouse pre-osteoblasts M3 T3-E1 cells cultivated on these scaffolds significantly improved the cellular elongation and promoted cell differentiation by electric pulse signaling [80]. The morphology of the nanofibers has a modest effect on the application of the scaffolds. Nest-like fiber arrangement scaffolds were developed from gelatin and poly-L-lactic acid using a weight ratio of 1:1. These scaffolds when seeded with BMSCs displayed superiority over non-woven nanofibers in enhancing osteogenic differentiation and proliferation. The application of these scaffolds in rat cranial defects showed significant formation of new calcified bone after 12 weeks post-operation [103]. More recently, calcium phosphate has been used as ceramic material along with natural polymers to develop scaffolds for bone tissue engineering. Incorporating calcium phosphate in the form of

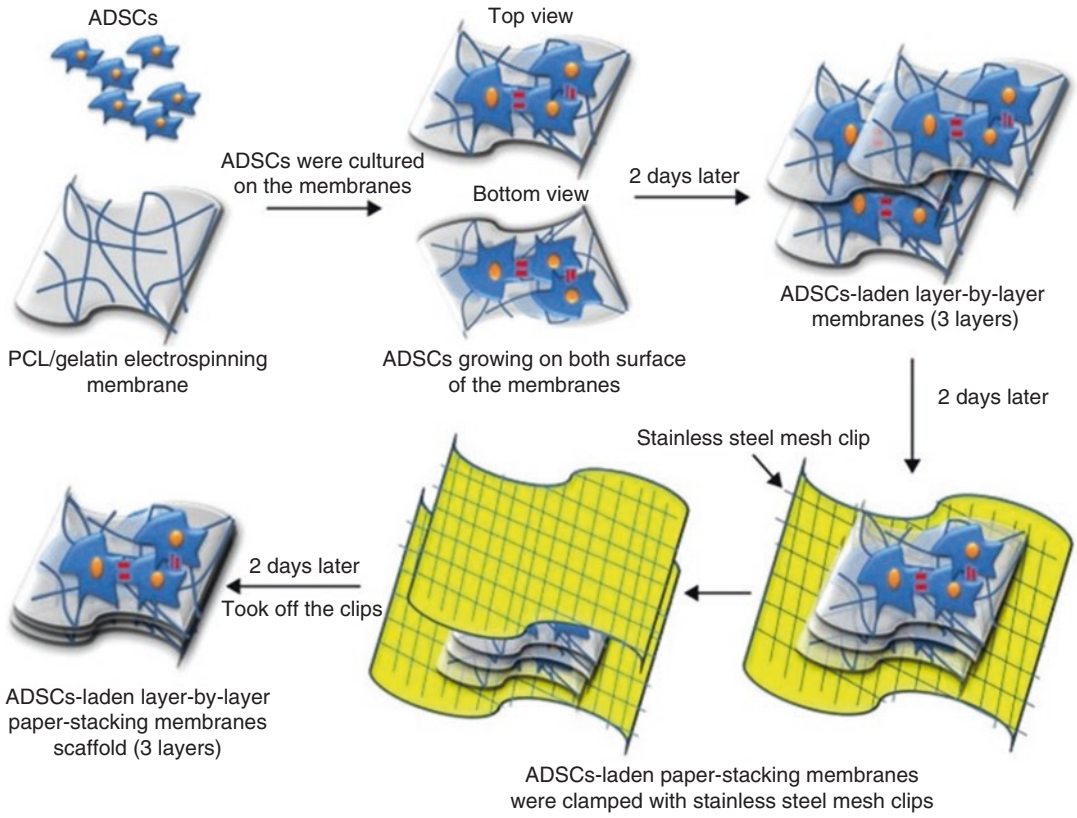

Fig. 4.8 Schematic fabrication procedure for lading ADSCs on a layer-by-layer paper stacking membrane of PCL/Gelatin composite scaffolds [139]

nanoparticles during electrospinning of gelatin has resulted into porous and rough nanofiber surfaces. In contrast with pure gelatin nanofiber scaffolds, these composites display an increased cell attachment, proliferation, osteogenic gene expression and neovascularization. The *in-vitro* studies with BMSCs has promoted osteogenic differentiation and the*in-vivo* studies indicated repairing of rat calvarial defects with advanced bone-regeneration by activating Ca^{2+} sensor receptor signaling pathway [31].

4.2.4 Chitosan Scaffolds in Bone Tissue Engineering

Chitosan, the de-acetylated form of chitin is a biodegradable, biocompatible and non-toxic polysaccharide polymer. This polymer is abundantly found in the shells of crustaceans and has been found to act like collagen in higher vertebrates [142]. The similarity of this polymer with gylcosaminogylcans makes it suitable candidate for applications in tissue-engineering including bone tissue regeneration. The polymer is also described to possess antimicrobial properties [128] and targeted drug delivery applications [117]. Chitosan possess the amine group that can be modified by various chemical reactions to develop functionalized materials. The protonation of amine group makes chitosan a cationic polymer and is the only cationic polymer to possess biocompatibility and biodegradability. However, chitosan is a known immunogen (TLR agonist) [43] and has serious solubility issues. This often requires to blend chitosan with other polymers, functionalize the sur-

face amine group and to counter solubility issues; the use of harsh solvents is required [52]. Studies conducted recently have demonstrated the huge potential of chitosan nanofibers prepared by electrospinning in bone tissue regeneration. Chitosan nanofibers have been found to support the adhesion, migration, proliferation and differentiation of bone forming cells and stem cells [53, 64, 71]. The molecular mechanisms and signal transduction pathways by which this polymers promote the bone regeneration has also been communicated. Chitosan has the potential to activate Wnt canonical pathway, which plays a significant role in embryonic development. The signaling cascade in this pathway includes 19 secreted glycoproteins that transduce the intracellular signaling to control gene expression [7] [ref]. Wnt3a pathway is known to promote proliferation and suppresses osteogenic differentiation of hMSCs [140]. Moreover, the Wnt pathway is generally activated via formation of Wnt3a, Frizzled and Lrp5 proteins, which play an important role in the skeletal homeostasis. Chitosan is also known to stabilize fluorine, a common mineral present in water and food items for stimulatory effect on osteoblasts, thus promoting bone formation [54, 146]. This is done by up-regulating the levels of Lrp5, β-catenin and GSK-3 β proteins while down-regulating the level the level of RANKL, resulting in elevation of osteogenic differentiation [49]. In this section, we will discuss about the recent advancement of scaffolds fabricated from chitosan or chitosan blends in bone tissue engineering.

The use of chitosan scaffolds in bone tissue engineering is well documented. However, the use of this polymer in guided bone tissue engineering is limited due to uncontrolled swelling and instability in the aqueous environment. Chitosan is modified by different methods to overcome these problems. Wu et al., modified the surface properties of chitosan by surface butyrylation. This modification resulted in a 75% decrease in swelling index of the polymer. The NIH 3T3 fibroblasts grown on these scaffolds showed cell-occlusiveness, proliferation, biocompatibility and non-toxicity. The application of these nanofiber scaffolds in critical size defects in rat calvarium showed significant improvement in healing than commercially available collagen membranes. The experimental data overall suggested that these nanofiber scaffolds have potential applications in bone tissue engineering [144]. One of the causes of slow bone healing particularly in large bone defects is the lack of osteointegration which is due to the absence of periosteum. The periosteum is an upper layer of the bone containing osteo-progenitor cells for critical growth and remodeling of the bone tissue. Chitosan nanofiber scaffolds were fabricated by one step electrospinning process along with nano-HAp particles and genipin. The genipin acted as a cross-linker and is thought to emulate the microenvironment of the non-weight bearing bone. Together all these constituents resulted in a scaffold that mimics physical, mineralized structure and mechanical properties of a non-weight bearing bone ECM similar to perisoteum, promoting osteoblast growth and maturation. The cross-linking with genipin increased the mechanical strength and that is comparable to the strength of periosteum as evident from Young's modulus values of 142 MPa [38]. Furthermore, the *in-vitro* studies revealed that these scaffolds supported the adhesion, proliferation and differentiation of mouse 7F2 osteoblast cell lines. The higher rate of osteoinductive activity of these scaffolds was determined by osteonectin mRNA expression. The authors believe that this type of a scaffold is well suited for non-weight bearing bone defects such as that of maxillofacial defects and injuries [38]. Various inorganic materials are incorporated into the polymeric meshes for mineralization of the bone tissue. In a study conducted by Zhou et al., it was shown that chitosan nanofibers incorporated with whitlockite ($Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$) demonstrated significantly better proliferation and osteogenic ability of hMSCs than that of chitosan/HAp scaffolds. Moreover, these porous scaffolds of chitosan/whitlocklite significantly improved rat calvarial defects as compared to porous chitosan/HAp scaffolds. The study suggested better efficacy of whitlocklite as compared to HAp [153].

Chitosan combined with oxidized starch through reductive alkylation process and then electrospun with calcium phosphate coated with

polycaprolactone produced composite nanofiber scaffolds. These scaffolds were highly porous as demonstrated in SEM studies and also supported the adhesion, migration and proliferation of MG63 osteoblast cells. These scaffolds were well adhered monolayer of cells and requisite elasticity are potential candidates for bone tissue engineering [90]. Bi-layered scaffolds of chitosan and collagen were electrospun and evaluated for biocompatibility and differentiation of MSCs in GBR. These scaffolds were found to support higher metabolic activity and proliferation of MSCs compared to pristine chitosan-collagen nanofibers. The qRT-PCR analysis demonstrated that these fibers induced osteogenic genes as evidenced from high calcium content and ALP activity of MSCs. The *in-vivo* studies of these scaffolds were carried on calvarial defects of adult rabbits. The experimental procedure for incorporation of different variants of the scaffolds is described in Fig. 4.9. Results from histomorphometric analysis after 1 and 2 months of the surgery revealed that bone formation was observed mostly in nano chitosan-collagen bilayered scaffolds than pristine chitosan-collagen scaffolds [81].

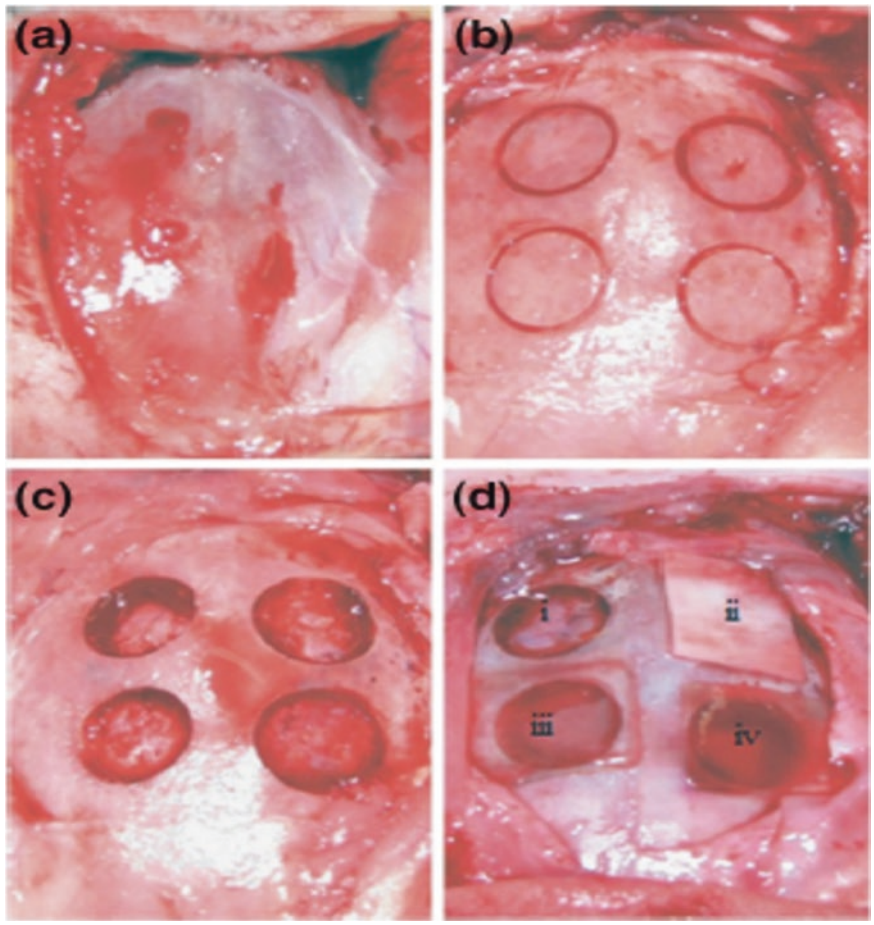

Fig. 4.9 Step wise experimental procedure for application of membranous scaffolds. (**a**) Exposed parietal bone by removal of full thickness skin flap. (**b**) Drilling procedure using trephine for cutting uniform circular (6 mm diameter) defects on the outer cortex of skull. (**c**) Removal of cortical bone without causing brain injury. (**d**) Application of different scaffolds on the defects (*i*) No membrane (*ii*) BioGide membrane (*iii*) Chitosan nanofibrous collagen membrane (*iv*) chitosan collagen membrane. (Reprinted with permission from Springer Nature [81])

Chitosan is sometimes also used along with synthetic polymers to increase biocompatibility of these polymers. A bio-competent scaffold with a core shell configuration was prepared from Poly(lactic acid) and chitosan by electrospinning, having Poly(lactic acid) at its core and chitosan on its shell. Mineralization of this core scaffold was done by HAp and pre-osteoblasts (MC3T3-E1) were cultured along with these scaffolds. It was found that chitosan on the surface of Poly(lactic acid) enhanced the ALP activity of pre-osteoblast cells by enhancing protrusions on the fiber surface. This study shows that natural polymers like chitosan play significant role for bone tissue engineering [147]. Chitosan and poly(vinyl alcohol) when grafted with graphene oxide, the composite nanofibers produced by electrospinning revealed enhanced tensile strength. Uniform dispersion of graphene oxide was confirmed by FE-SEM. The biocompatibility studies of mouse chondrogenic cell line (ATDC5) was studied in this system and it was found that 6% of this blend provided appropriate growth of these cells as compared to 4% concentration. The results suggested that these scaffolds could be used as artificial cartilage [17]. In bone tissue engineering scaffolds of polymers provide architecture for bone forming cells to adhere, penetrate and subsequently proliferate to form new bone tissue. Electrospinning is a versatile technique to develop such scaffolds that can temporarily act as architects for bone formation during bone injury cases. In one of such a study, researchers fabricated electrospun nanofibers of chitosan and silk fibroin and studied the cell growth and proliferation of hMSCs towards osteogenic differentiation. The *in-vitro* studies like cell culture analysis, cytoskeleton analysis, MTT assay suggested proliferation of these cells on chitosan-silk fibroin nanofibers. It was also found in these studies that chitosan guided the differentiation of hMSCs towards osteogenic lineage and induced bone formation [69]. The presence of another bio-polymer with a composite nano-biopolymer fiber results in good mechanical and biological performances including bone-mineralization. In one of the studies, chitin-whiskers enhanced the mechanical properties and osteoblast cell growth using chitosan/poly(vinyl alcohol) nanofibers produced by electrospinning. The nanofibers obtained by electrospinning process allowed mineralization of HAp in concentrated simulated body fluid. This resulted in improvement of tensile strength as evidenced from Young's modulus values. On the other hand, chitosan-whiskers promoted osteoblast cell growth and proliferation. Together this, the presented nano-biocomposite offers great advantage in bone tissue-engineering applications [92]. Bone allografts suffer from the problem of impaired and substandard healing due to loss of periosteum. Artificial membranes fabricated from polysaccharides (Chitosan and heparin) and grafted over the bone allograftscan resemble the natural periosteum. These engineered nanofiber membranes were studied in mouse femur defect model anddeliveredFGF-2, TGF-β1 and ASCs to these fractured bones. The results were quite promising, as implanted ACSs responded very well to the engineered periosteum, resulting in proliferation of these stem cells in the defective area of femur region. Increased bone callus formation was observed in regions implanted with ASCs as compared to their cell-free controls while as periosteal cartilage and bone formation was observed in allografts delivering FGF-2 and TGF-β1 [109].

4.2.5 Cellulose Scaffolds in Bone Tissue Engineering

Scaffolds of cellulose are rare in use for bone tissue engineering because of reduced mechanical properties. Cellulose derived from native cotton was electrospun along with different concentrations of HAp to form scaffolds for bone tissue engineering. The SEM analysis revealed that these fibers due to incorporation of HAp increased the fiber diameter and mechanical strength. This was clearly evident from high tensile strength and Young's elasticity modulus values. The *in-vitro* cell culture of human dental follicle cells on these scaffolds revealed that these were cytocompatible, suggesting their utility in bone tissue engineering [3]. Modified celluloses like hydroxyethyl derivative functionalized with bone-like calcium phosphate have been fabri-

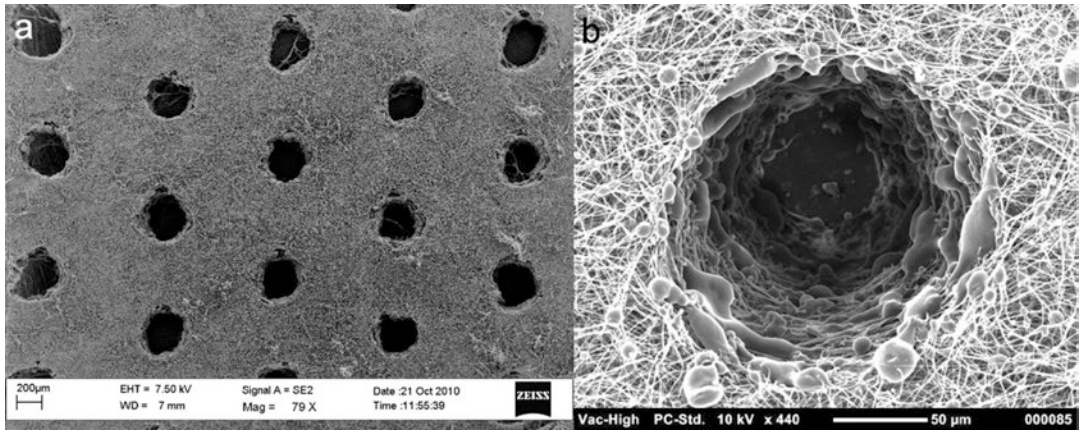

Fig. 4.10 Micro-ablated electrospun scaffolds of cellulose. (**a**) The pores diameter in pattern are 300 µm. (**b**) Magnification of the pore on an edge of 150 µm pore revealing intact fibers of the different layers. (Reprinted with permission from Elsevier [108])

cated into scaffolds using electrospinning. The mineralization was achieved by incubating the polymer in simulated body fluid. The coated layer of the minerals on subjected to XRD reveal that the mineral phase is a mixture of calcium phosphate hydrate and apatite. The mechanical strength of these scaffolds is comparable to that of trabecular and proximal femoral bones. The human osteosarcoma cells evaluated on these scaffolds supported cellular attachment and proliferation. These scaffolds have all the characteristics to aid in bone tissue-regeneration [18].

Basically, the non-woven fibers have a different architecture and make it difficult for the cells to adhere, migrate and proliferate [133]. This is because cells live in complex architecture of pores and ridges and these structures influence the inward diffusion of growth factors, ECM proteins as well as outward diffusion of waste metabolites [59]. As such introducing the porosity in the scaffolds will improve cell movement, migration and nutrient mobilization for tissue engineering applications. In one of such a study, cellulose scaffolds were produced from electrospinning of cellulose acetate. Subsequent saponification of cellulose acetate fibers yielded cellulose nanofiber scaffolds. The micro-architecture and surface chemistry of these fibers was modified by treatment with CO_2 laser and phosphate buffer solution. Due to laser ablation of the irradiated area specific site pores were created in the scaffold, leaving the bulk material unmodified. The macro-porosity induced in the scaffold can be seen in Fig. 4.10. This type of engineering to enhance porosity of the scaffold increased cell adhesion and spreading of seeded osteoprogenitor cells for bone tissue engineering. The role of this technology can be further extrapolated and combined with 3D weaving to enhance porosity and vascularization of the scaffolds [108]. In a similar study, bacterial cellulose which has biodegradability issues was modified by laser ablation to create pores resembling well-defined honey-comb like pore arrays. The bacterial cellulose was also subjected to periodate oxidation yielding biodegradable bacterial cellulose. Mineralization of this oxidized cellulose with HAp resulted into scaffolds that could mimic the native bone structure. Studies demonstrated that hMSCs were adherent on these scaffolds, conforming their potential in bone tissue engineering [34].

4.2.6 Other Polymeric Scaffolds in Bone Tissue Engineering

Polymers like starch and zein have been recently used in bone tissue engineering and have shown promising results for future use. In this section, these polymers have been reviewed briefly.

Starch is electrospun along with graphene oxide to develop starch-based scaffolds for application in bone tissue engineering. Graphene oxide is used as multifunctional additive in polymer composites and is known to promote HAp mineralization [77, 78] and osteoconductivity. The process utilized formic acid both as a solvent and an esterification agent to stabilize and strength the starch nanofibers, which otherwise are difficult to process by electrospinning alone. The electrospinning scheme is shown at Fig. 4.11. The SEM studies revealed that incorporating graphene oxide in the scaffolds decreased the fiber diameter and narrowed fiber distribution besides improving thermal stability and hydrophilicity of the fibers. It was found that 2.5% weight of graphene oxide in the starch nanofiber scaffolds displayed positive biocompatibility to osteoblastic MG-63 cells. Experiments conducted in simulated body fluid revealed that these scaffolds assisted calcium phosphate mineralization [145].

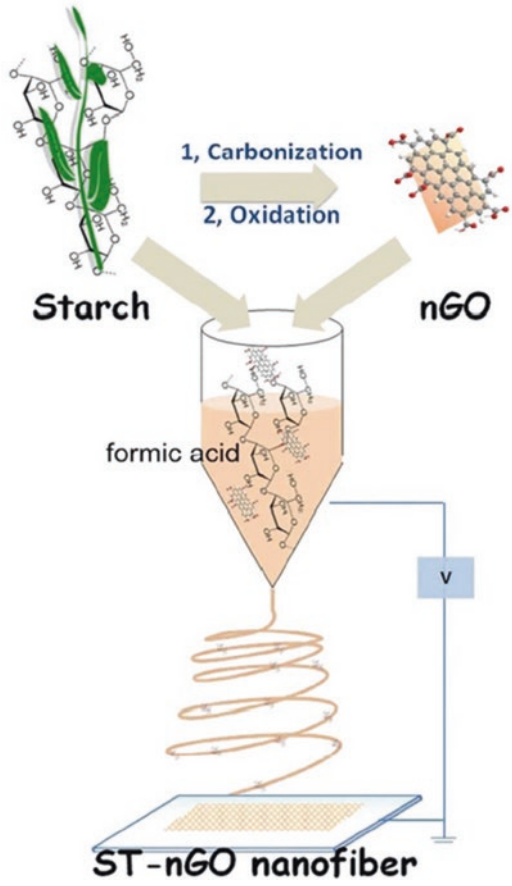

Fig. 4.11 Schematic representation of the process for electrospinning starch-nano graphene oxide nanofibers. (Reprinted with permission from American Chemical Society [145])

The delivery of BMP-2 has also been achieved by fabricating it along with zein/polydopamine nanofiber scaffold for investigating the osteogenic differentiation potential. The BMP-2 was conjugated with TiO_2 nanoparticles to allow its sustained release. The human fetal osteoblasts cultured on these scaffolds were examined for different biochemical cues like osteopontin and topographical stimulation. The sustained delivery of the BMP-2 during the *in-vitro* studies improved cell adhesion, differentiation and mineralization of the scaffold matrix. The expression of osteogenic markers further demonstrated that these scaffolds possessed better cell-biomaterial interactions and can act as a substrate material for bone-regeneration [6].

4.3 What Is Next?

Bone tissue engineering using electrospinning and biomineralization techniques for fabrication of complex bio-inspired scaffolds can prove to be a potential gear for replacement of complex surgeries required for bone deformities. The fabrication of scaffolds from polymers especially of the natural origin allows for the development of scaffolds that can mimic the natural ECM of bone efficiently. Further, tailoring these natural polymers by surface modifications and/or blending with polymers of mechanical strength concedes more natural proximity. Mineralization of the scaffolds makes them ideal substrates to support the attachment, infiltration, alignment, proliferation and differentiation of osteoblasts/osteogenic stem cells. Development of these scaffolds to closely mimic the natural bone niche by incorporating various schemes to surface modify and controlling the delivery of bioactive compounds blended with these polymers would allow calibration of cellular responses for bone tissue regeneration.

The future perspectives in this field are the fabrication of scaffolds with mechanical strength comparable to the strength of the natural bone, fabrication of composite mesoporous scaffolds without the usage of toxic solvents and development of fibrilliary structures resembling native tissue (i.e., osteons). There is a need to understand the coupling between angiogenesis and osteogenesis as the healing bone will need new blood vessels to supply nutrients and waste matter. The current strategies in this field should be focused to create functional blood vessels during the process of new bone formation to vascularize the new tissue. Furthermore, to effectively mimic the natural environment, the scaffolds should have the potential to facilitate both local and systemic biological functions. This can be achieved by proper selection of the biomaterial, the geometry of the pores, and the ability to release biomolecules and drugs at the desired rate. The application of these scaffolds has resulted in positive outcomes during *in-vitro* and *in-vivo* studies. However, the fabrication of 3D structures out of these polymers closely mimicking the natural bone environment further warrants extensive research, especially in the clinical arena. The need of the hour is to convert these lab-born ideas into clinical realization by initiating more and more clinical trials.

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Electrospun and Electrosprayed Scaffolds for Tissue Engineering

5

Natasha Maurmann, Laura-Elena Sperling, and Patricia Pranke

Abstract

Electrospinning and electrospraying technologies provide an accessible and universal synthesis method for the continuous preparation of nanostructured materials. This chapter introduces recent uses of electrospun and electrosprayed scaffolds for tissue regeneration applications. More recent *in vitro* and *in vivo* of electrospun fibers are also discussed in relation to soft and hard tissue engineering applications. The focus is made on the bone, vascular, skin, neural and soft tissue regeneration. An introduction is presented regarding the production of biomaterials made by synthetic and natural polymers and inorganic and metallic materials for use in the production of scaffolds for regenerative medicine. For this proposal, the following techniques are discussed: electrospraying, co-axial and emulsion electrospinning and bio-electrospraying. Tissue engineering is an exciting and rapidly developing field for the understanding of how to regenerate the human body.

Keywords

Electrospray · Electrospinning · Biomaterials · Regenerative medicine · Coaxial · Bio-electrospray

5.1 Introduction

Electrohydrodynamic techniques, namely electrospraying and electrospinning, are very powerful tools for developing and producing materials with the structural features necessary for tissue engineering (TE) applications. By definition, TE is a multidisciplinary field, integrating engineering principles, materials science, with chemistry, biology, and medicine, with the aim of either restoring or enhancing tissue or organ functions [56]. A tissue engineered construct is commonly made of materials and cells. The materials are often presented as porous biodegradable scaffolds which provide structural support for the cells. The materials used to produce the scaffold are a major area of study in TE. Natural materials offer the advantage of presenting structures and sequences that stimulate cell proliferation and adhesion but are highly variable from batch to batch or difficult to obtain on a large scale. Synthetic polymers allow for the control of various parameters, such as molecular weight, hydrophobicity and degradation time, but on the other hand, do not allow for good cell adhesion, proliferation or maintenance of the differentiated state

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[108]. The materials can be processed by various techniques in order to obtain the scaffolds, including electrospinning and electrospraying.

Electrospraying and electrospinning are technologies which use high electric fields for the production of particles and fibers, respectively. In the technique, a polymer solution jet is accelerated and drawn through an electric field. Depending on the apparatus, electric field strength, preparation conditions and physical properties of the solution, the stretched jet can break, causing droplets that produce micro/nanoparticles or remain as a filament which, after drying, produce nanometric/micro diameter fibers [90, 99, 134].

The first study that described the application of high electrical potentials to generate aerosols from drops of fluids was published in 1745 [14]. An application of the electrospraying technique was patented in 1902. Regarding electrospinning, which follows the same physical principles of electrospraying, the first patent which described the technique was reported in 1934 [100].

The conventional setup of an electrospinning and electrospraying process is illustrated in Fig. 5.1. The technology consists of three major components: a source of the electric field (high voltage power supply), a spinneret (nozzle coupled with a syringe pulsed with a pump) and a counter electrode (normally a metal collector plate). The solution is put through a pump and a difference in an electrical voltage is applied between the nozzle and the counter electrode. The flow rate of the polymer solution and the applied voltage need to be optimized, depending on the type of the solution used. Because of the high voltage that is applied (in the range of 1 to 30 kV), the drop of the polymer solution becomes highly electrified and it causes a cone-shaped deformation because of the surface tension, known as Taylor cone [14, 100]. Under the electrostatic forces the electric field becomes greater, the cone-shaped deformation breaks into highly charged droplets and by selecting the suitable conditions, the droplets reach to micro or nanosize level [101, 106]. Along the path that traverses the electrified jet ejector nozzle to the collector, the stretching process takes place and, depending on the physical characteristics of the polymer solution, the jet can break up into drops or remain as a filament [90, 100]. On this route to the counter electrode, the evaporation of the solvent and solidification of the polymer also occurs, leading to the formation of particles or solid continuous filaments with a reduced diameter [14, 90, 100].

The fiber formation in an electrospinning process depends on several parameters, which includes solution parameters (molecular weight, viscosity, surface tension, electric conductivity and dielectric effect of the solvent), ambient condition (humidity, temperature, pressure and type of atmosphere), processing conditions (voltage, flow rate, feed rate, diameter of spinneret and distance between the spinneret and collector) [90].

A video of electrospinning fundamentals regarding optimizing solutions and apparatus parameters in TE can be seen in Leach and collaborators [59] as well as the demonstration of electrospinning/electrospraying polymer solutions for biomedical applications [51].

Several polymers have been used industrially, such as nylon, polyester, polyacrylonitrile, polyvinyl alcohol, polyurethane, polylactic acid etc. Conventionally, the electrospinning technique mainly uses a polymer solution in organic solvents, such as chloroform, formic acid, tetrahydrofuran (THF), dimethylformamide (DMF), acetone and alcoholic solvents.

Currently, various modified electrospinning techniques have been developed, such as coaxial electrospinning, emulsion electrospinning, core-shell electrospinning, melt-spinning, blow assisted electrospinning and post-modification electrospinning [134]. The schematic diagrams show the co-axial electrospinning (Fig. 5.1c) and emulsion electrospinning techniques (Fig. 5.1d).

Electrospraying is a versatile way to make nanoparticles. Electrospraying, also called electrohydrodynamic atomization, represents a modified form of electrospinning and is a technique for the preparation of micro- and nanoparticles instead of fibers. Electrospraying has been widely applied to develop different types of commercial technologies, such as mass spectrometry, focused ion beam instruments and electrostatic precipitation of nanoparticles [11].

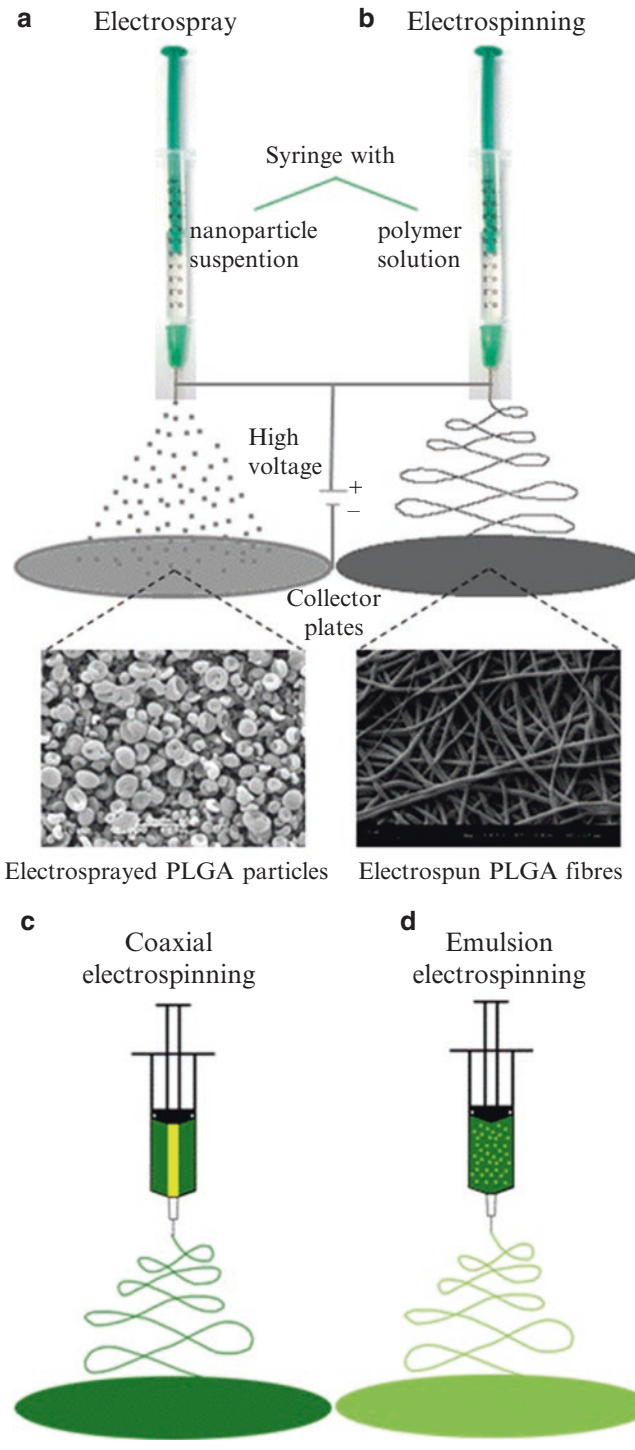

Fig. 5.1 Schematic diagram of the electrospraying (**a**) and electrospinning (**b**) processes for the production of particles and fibers, respectively. The polymer solution is pumped into the syringe and passes through a spinneret. This nozzle is connected to one terminal of the power supply and a metal collector to the opposite terminal. The jets of polymer solution ejected from the capillary tube may form polymeric particles or filaments, depending on the physical properties of the polymer solution. (**c**) co-axial and (**d**) emulsion electrospinning. Photographs kindly provided by the Stem Cell Laboratory archives, Universidade Federal do Rio Grande do Sul

The particles and filaments produced by the electrospraying and electrospinning techniques can be used in various research and industry areas, as in TE in the production of biomaterials which are useful for treatment and also for diagnosis in the areas of pharmaceuticals, food, cosmetics, etc. [53, 134].

5.2 Electrospun and Electrosprayed Scaffolds for Tissue Engineering

The total number of papers published in the *PubMed* database (http://www.pubmed.gov) with the keywords "tissue engineering" and "electrospinning" or "electrospun" in the field search "Title/Abstract" is 2215 since their first use in 2001 until 2017 (see Fig. 5.2). The number of papers containing these keywords has increased each year, especially in 2009, from 108 to 127, 163, 171, 214, 270, 266 and 296 in each subsequent year, up to 373 in 2017. A search in the *PubMed* database with the keywords "tissue engineering" and "electrosprayed" or "electrospray" in the field "Title/Abstract" resulted in 41 original papers from 2001 to 2017.

5.2.1 Types of Electrospun and Electrosprayed Materials for Tissue Engineering

Natural and synthetic polymers, together with their respective blends and composites, can be processed by both procedures of electrospinning and electrospraying, resulting in either fibers or particles with specific engineered properties. There are three individual groups of biomaterials used in the production of scaffolds for TE: natural polymers, synthetic polymers, and ceramics. Each biomaterial groups has advantages and disadvantages, so the use of composite scaffolds comprised of different types of biomaterials is becoming increasingly habitual [108].

This subsection illustrates the types of materials used for electrospinning and electrospraying, using some examples; however, it does not intended to present a list of all the materials belonging to this category.

5.2.1.1 Natural Polymers

In order to serve as a temporary extracellular matrix (ECM) for cells involved in the regenerative processes, the scaffold has to present some of the advantageous features of the natural

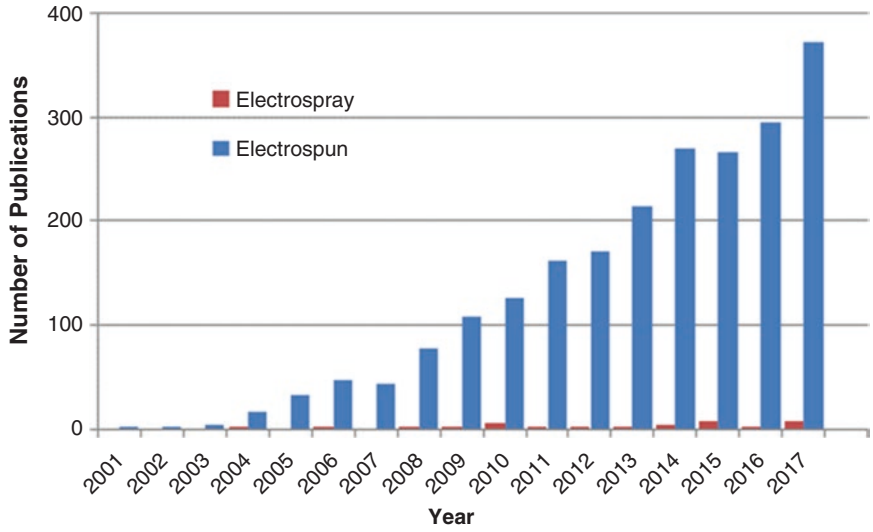

Fig. 5.2 The number of papers published in the *PubMed* database. The keywords "tissue engineering" and "electrosprayed" or "electrospray" (red columns) and "tissue engineering" and "electrospinning" or "electrospun" (blue columns) were used in the field Title/Abstract (Search realized in 31st December 2017)

ECM. There are two types of natural polymers derived from natural sources typically used as scaffolds in TE: (1) proteins such as collagen, gelatin, fibrinogen and silk fibroin and (2) polysaccharides as for example hyaluronic acid, chitosan, and alginate. These naturally occurring proteins and polysaccharides have been extensively used in the production of electrospun fibrous scaffolds [76]. Proteins such as collagen, fibrinogen and silk fibroin, which are able to form fibers in nature, are highly recommended for electrospinning and during the process, they easily assemble in fibers. Moreover, these proteins are also biocompatible and biodegradable, and some of them can also present antibacterial and anti-inflammatory properties [73].

The principal component of the ECM of various tissue types is generally collagen and the ratios of different collagen types and their organization define the mechanical properties and structure of the developing and growing tissue. An ideal scaffold should imitate the structure of the natural collagen found in the target organ [57, 73]. Typically, collagen can be electrospun from solutions based on either fluoroalcohols or water-ethanol mixtures [15]. The first study of electrospinning scaffolds using collagen was carried out by Huang and collaborators in 2001 with the electrospinning of collagen–polyethylene oxide (PEO) nanofibers [42]. Fluoroalcohols are still the solvents of choice, although some concerns have arisen concerning the possible effects of these solvents on collagen denaturation. However, cross-linking procedures are needed due to the fact that collagen nanofibers have poor mechanical resistance and a high degradation rate. In order to enhance the resistance different methods have been tested, as, for example, stabilization with either glutaraldehyde, epoxy compounds, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and exposure to ultraviolet light [31]. These methods can be employed during and after the process of electrospinning. On the other hand, the conversion of collagen into nanoparticles is challenging, but it is possible using one-step electrospray deposition. Gelatin is a mixture of products resulting from the degradation of collagen frequently used for biomedical applications. Fibers of gelatin were produced by electrospinning from solutions using HFIP, TFE, acetic and formic acid, and subsequently crosslinked for better mechanical resistance [44, 76, 86].

In addition to collagen and gelatin, fibrinogen is a candidate for the production of scaffolds by electrospinning for TE. Similar to collagen, fibrinogen is a protein that is present in the blood and plays an important role in wound healing, where during hemostasis, it is converted into fibrin fibers that are insoluble. Fibrinogen was first electrospun by Wnek and collaborators using a mixture of hexaflouroisopropanol and minimal essential medium as electrospinning solvent; the fibrinogen nanofibers presented a strong variation of the fiber diameter in the range of 80–700 nm (Gary E. [31, 32]). A study that used fibrinogen showed that fibers that were electrospun were characterized by elasticity and extensibility higher than collagen fibers [6]. In order to better control the mechanical resistance or the rate of degradation of fibrinogen scaffolds the fibers were either cross-linked with chemical compounds, such as glutaraldehyde or the cell culture medium was supplemented with aprotin.

Silk from the silkworm has been used as a medical suture for many centuries. The silk fibers present particularly remarkable mechanical properties and represent an option for many clinical applications [2]. The protein produced by the silkworm *Bombyx mori* is fibrous. In nature, silk is protected by a coat of sericin which is a glue-like protein. The raw silk fibers need to undergo degumming procedures in order to make the protein available and avoid possible biocompatibility problems due to contamination from residual sericin [2]. It is well known that fibroin possesses excellent biocompatibility and shows minimal immunogenicity and anti-inflammatory activity. This protein also plays important roles in re-epithelialization and elimination of scarring by enhancing the biosynthesis of collagen. For this reason, studies have been directed towards producing fibroin fibers through electrospinning in combination with other active principles, such as vitamin C or green grape seed extracts [28, 65].

Polysaccharides, such as alginate, cellulose, and chitosan can also be electrospun, but the pro-

cess of electrospinning and electrospraying present some challenges and in most cases require additives.

Chitosan is a polysaccharide obtained through the deacetylation of chitin, which is the second most abundant polysaccharide in nature (after cellulose). Besides this, chitin is the major structural component of the exoskeleton of crustacean, as shrimps and crabs, and of the cell walls of fungi [76, 93]. As mentioned above, polymeric additives and also different acidic solutions, such as acetic acid, trifluoroacetic acid, formic acid and hydrochloric acid, are necessary for electrospinning chitosan [76]. Due to the presence of many amino groups in its backbone that gives to the molecule a polycationic character chitosan is difficult to electrospin. This polycationic nature increases the surface tension of the solution and a high electrical charge becomes necessary to produce electrospun chitosan nanofibers. During the electrospinning, process particles are often formed, probably due to the repulsive forces between ionic groups in the chitosan backbone in an acidic solution [60]. The resulting scaffolds are characterized by haemostatic and antibacterial properties, low immunogenicity and biocompatibility. Methacrylate glycol chitosan, carboxymethyl chitosan, and carboxyethyl chitosan are examples of water-soluble derivatives of chitosan that have been synthesized and electrospun, for TE applications [94]. For example, cinnamon oil, which exhibits antibacterial activity has been incorporated into chitosan/poly(ethylene oxide) (PEO) fibers. The electrospun fibers of cinnamon oil/chitosan were able to release the essential oil *in vitro* [92]. Electrosprayed chitosan microspheres were also produced and represent a potential carrier for the controlled release of drugs [111]. As chitosan is a mucoadhesive polymer the chitosan microspheres can attach to the mucosal surfaces and therefore may prolong the residence time and improve specific localization of absorption of the target drug.

Hyaluronic acid (HA) is a polyanionic polymer component of the ECM of connective tissue, such as umbilical cord, synovial fluid, vitreous, etc. The polyanionic surfaces of HA are highly hydrophilic. When the material is used as cell support, HA does not favour cellular interaction and attachment due to its thermodynamical features. Consequently, this material does not promote tissue formation. Therefore, in order to improve cell attachment onto HA-based biomaterials, ECM proteins such as type I collagen and fibronectin are coated onto HA surfaces, and HA microporous scaffolds are produced, which serve to direct the growth of cells within the scaffold [49]. As mentioned already in the case of protein-based biomaterials, HA is also often mixed with other polymers or needs to be dissolved in the suitable solvent mixture be to be able to form fibers when submitted to electrospinning procedures [49].

Alginate is an anionic polysaccharide derived from brown seaweed and produced by bacteria [60]. The sodium salt of alginate is soluble in water and forms highly viscous solutions even at very low polymer concentrations (2–3 wt%). Alginate is a very feasible biomaterial, because it is ableto form beads, sponges, and microfibers, which have been used for many TE approaches, such as cartilage, skin, liver, bone and cardiac tissue regeneration [60]. Alginate has many advantages in its use as a biomaterial for scaffolds, such as excellent biocompatibility, and the fact that it does not stimulate the immune response. It is also low cost, and has low toxicity, and can be transformed into a gel with the help of divalent cations, especially Ca^{2+} and Mg^{2+}. Being of a polyanionic nature, the same challenge, namely the repulsive force among the polyanionic alginate chains, has to be overcome by performing electrospinning only in the presence of synthetic polymers, such as PEO and PVA [9, 68]. Generally, electrospinnability of alginate increases with the increase of synthetic polymers concentration, and nanofibers have been electrospun with compositions that were rich in synthetic polymers, as for example an alginate/synthetic polymer ratio of 50/50. However, the resulting electrospun fibrous alginate scaffolds present a high water solubility which limits their stability in aqueous environments. This instability in water-containing environments is overcome by cross-linking the fibers with glutaraldehyde, divalent ions, and TFA [60, 76].

Polyhydroxyalkanoates (PHA) are biodegradable polymers synthesized by microorganisms such as the bacterium *Burkholderia xenovorans* [1, 63]. The typical production techniques of PHA scaffolds include electrospinning, salt-leaching, solution casting and 3D printing [63]. One study compared the fabrication of a type of PHA, the poly-hydroxybutyrate (PHB), using electrospinning and salt-leaching techniques [74]. It was found that the nanofibrous scaffolds had better mechanical properties and Vero cells proliferated more on the electrospun PHB scaffold when compared to the PHB salt-leached scaffold. It was concluded that nanofibrous scaffolds were a better choice overall [74]. Another study showed that PHB electrospun membrane obtained by bacterial synthesis from a mutant strain of *Azotobacter vinelandii* promoted an increase in the cell density when compared to the cast film, suggesting that the fibrous morphology allows for better nutrient transference [95]. The study comparing electrospun fibers aligned to poly-(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) meshes increased elasticity, tensile stress and MSC proliferation compared with randomly-oriented studies [116].

Decellularized ECM is gaining attention as a biomaterial for TE applications. The goal of decellularization is the removal of all the cells from a tissue or organ once cellular antigens are recognized as foreign by the host and, thereafter, the induction an inflammatory response [87]. Recent studies demonstrated that it is possible to produce hybrid scaffolds based on decellularized ECM and fibrous polymer meshes by electrospinning [113]. For example, Kim and collaborators developed nanofibrous electrospun from heart decellularized ECM-based hybrid scaffolds with poly(l-lactide-co-caprolactone) (PLCL) as a wound dressing for reducing scarring in wound healing [54]. Aslan and collaborators evaluated the use of a combined construct for corneal regeneration consisting of a collagen foam, a poly(l-lactic acid) (PLA) nanofiber mesh and decellularized matrices [5]. Goyal and collaborators developed hybrid scaffolds with decellularization derived from cultivated cells deposited within synthetic polymeric fibers [36].

However, a natural ECM component or its derivatives may not represent the ideal scaffold for biomedical applications because the scaffold should be able to accelerate the regeneration process that is normally encountered during the natural processes.

5.2.1.2 Synthetic Polymers

A great variety of synthetic polymers have been used to produce fibrous scaffolds by electrospinning, such as: poly(caprolactone) (PCL), poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(D,L-lactide-co-glycolide) (PLGA), PLCL, poly(L-lactic acid) (PLLA), poly(carbonate), poly(urethane) (PU), poly(ethylene oxide) and poly(ethylene glycol) (PEG), among others [19]. The most important advantages of synthetic polymers over natural polymers are the possibility of customizing their mechanical properties, the controlled degradation process and the fact that can be synthesized into the desired form. Moreover, they are generally low-cost and have highly reliable characteristics [46].

PGA is the simplest polyester, it is biodegradable, and presents high crystallinity. PGA has slow degradation rate and is water insoluble. Its solubility depends on the molecular weight and typically, high molecular weight PGA is insoluble in most organic solvents except fluorinated solvents (e.g., hexafluoroisopropanol). Another synthetic polymer, PLLA, represents one of the most promising materials in TE because of its excellent mechanical properties, processability, and very good biocompatibility and biodegradability. PLLA is commonly used in various suture and implantation applications and due to its good mechanical properties, it can last for the sufficient time period in mechanical stresses *in vivo* [38]. In addition, PLLA electrospun fibrous scaffolds have been used in different biomedical applications. PLA itself presents high crystallinity (ca. 40%) and rigidity and a slow degradation, which limits its use as a suture material. Therefore, lactic acid is very often copolymerized with other biodegradable monomers as, for example, glycolic acid in order to achieve the required properties [38].

PLGA is a synthetic copolymer that is biodegradable and biocompatible and is very often used in electrospinning and electrospraying. Various characteristics can be obtained using the polylactides/glycolide by manipulating four key variables: the co-monomer ratio, the monomer stereochemistry, the linearity of the polymer chain, and the molecular weight [17]. The mechanism of degradation of the copolymer is the simple hydrolysis of the ester links, with crystallinity and water uptake being two key factors in determining the rates of *in vivo* degradation [17]. The crystallinity of the PLGA is related to its swelling behavior, mechanical strength, biodegradation rate, hydrolysis capability, which further depends on the molecular weight, and the molar ratio of the lactic and glycolic in the polymer chain. The lactide/glycolide polymer chains are cleaved by hydrolysis to the monomeric acids and are eliminated from the body through the Krebs cycle, primarily as carbon dioxide and in urine. The rate of hydrolysis of the PLGA is dependent only on changes in temperature and pH or the presence of a catalyst, therefore very little difference is observed in the degradation rate at different body sites [17]. PLGA is approved by both the European Medicine Agency and the US Food and Drugs Administration as a material that can be used in implantable biomedical applications and also for designing drug delivery systems. Moreover, PLGA is frequently used worldwide for the preparation of intravenous drug delivery systems and biomimetic materials and it has extensive application possibilities in TE.

PCL is a biodegradable and biocompatible polymer, which is chemically stable, mechanically strong, semi-crystalline and with a glass transition temperature of −60 °C. Due to these properties, PCL is approved by the Food and Drug Administration (FDA) for its application in drug delivery systems and TE [46].

The synthetic polymers mentioned here are being widely used in drug delivery systems, biomedical devices, and TE applications in both electrospun and electrosprayed preparations.

5.2.1.3 Polymeric Composites

Most of the polymers used to produce electrospun TE scaffolds are in their pure, single component form. However, very often one polymer does not meet all the requirements for various TE applications [57, 73].

As mentioned earlier, natural polymers, such as collagen, gelatin, silk fibroin, fibrinogen, chitosan, HA and their combinations, have been electrospun into nanofibers. However, these electrospun scaffolds show poor mechanical properties and lose their 3D structure in an aqueous environment. This problem can be overcome by blending the synthetic and natural polymers, which results in composite materials that present a combination of both good biocompatibility and mechanical strength, combining the advantages of two types of materials [22]. The combination of naturally occurring polymers on the surface of the composite nanofibers provides continued cell recognition signals, which is important for cell functioning during regeneration [22]. Natural bone matrix is an example of composite material, consisting of collagen (organic) and mineral components (inorganic), which provides an excellent balance between strength and toughness, superior to either of its individual components. The composite scaffolds with polymer and inorganic part are very advantageous scaffolds for bone TE.

In one study, Cui and collaborators produced PDLLA fibers by electrospinning and investigated their physical properties. They found that the fibers were hydrophobic, and could not support the initial adhesion and further growth of the cells, probably due to the surface erosion and dimensional shrinkage [21]. They, therefore, produced PDLLA and PEG composite electrospun fibers by blending different amounts of PEG into PDLLA [21]. PCL/HA composite nanofibrous scaffolds produced by electrospraying HA on PCL or PCL/collagen nanofibers enhanced the differentiation of mesenchymal stem cells into osteogenic lineage, indicating the use for bone TE [115]. Zheng and collaborators electrospun membranes with different gelatin/PCL ratios. The results show that three kinds of membranes with various gelatin/PCL ratios exhibited bio-

compatibility with chondrocytes and that electrospun gelatin/PCL is a good candidate for cartilage and other tissue regeneration [130].

Composite polymer/carbon nanotubes are another example of a combination of the excellent mechanical and electronic properties of the carbon nanotubes with the biocompatibility and degradability of synthetic polymers. For instance, the single carbon nanotube has a modulus as high as several thousands of gigapascal (GPa) and a tensile strength of several tens of GPa. However, carbon nanotubes are very difficult to align when they are used as mechanical reinforcement in composite fabrication and, therefore, the resulting composite does not exhibit the mechanical properties as one would expect. The alignment is a crucial step and electrospinning is presented as one method to align the carbon nanotubes in fibers [43, 79]. A number of research groups have tried to yield such nanofibers in recent years, by making PCL/gold or ZnO, polyacrylnitrile (PAN)/TiO_2, PVA/Silica, and Nylon6/montmorillonite (Mt) ultrafine fibers, respectively.

5.2.1.4 Inorganic and Metallic Materials

Electrospinning applications are mostly limited to the fabrication of nanofibers from natural and synthetic polymers because of the accessibility in preparing a polymer solution with appropriate physical properties required for electrospinning. Inorganic materials, as for example ceramics, are usually considered not to be suitable for electrospinning. However, it is possible, to electrospin ceramic nanofibers from their melts by using extremely high temperatures [61]. In order to prepare ceramic fibers by electrospinning, some extra steps are required, steps that are normally not necessary when electrospinning natural or synthetic polymers; they are the following: preparation of an inorganic solution containing a matrix polymer together with an alkoxide, polymer precursor or a salt, followed by electrospinning of the solution to produce composite fibers and, finally, calcination, sintering, or chemical conversion of the precursor into the desired ceramic at high temperature, with removal of all organic components from the precursor fibers [61].

Several groups have shown that inorganic sols prepared by hydrolysis and condensation could be directly used for electrospinning [58, 61]. Fibers constructed of Al_2O_3, $PbZr_xTi_{1-x}O_3$, SiO_2, and TiO_2/SiO_2 have been successfully produced in this manner.

Not only fibers but also inorganic nanoparticles have been developed for various biomedical applications due to their nanosize and biological properties. Several studies have reported encapsulation of inorganic nanoparticles such as titanium, silica, alumina, calcium carbonate and magnetic iron oxides onto a polymeric matrix, but their biomedical applications are sparse [40].

5.3 Tissue Engineering Applications

The facility of fabricating micro/nanofibers or particles and the wide variety of biocompatible polymers that can be used in electrospinning and electrospraying have revealed their potential applications in TE. One advantage of scaffolds produced by electrospinning is that their surface can be adjusted by controlling the parameters, thereby allowing for the topography that best fits the application. Another advantage is that nanofiber sheets can be shaped into almost any form-in accordance with the desired application. Electrospun scaffolds have assisted in the regeneration of a variety of tissue, such as skin, vasculature, neural, bone, ligament, and tendon ([105]a; [62]).

Regarding papers using electrospinning for TE published until 2017 in the *PubMed* database, it was possible to verify that approximately 30% have been used for the regeneration or reconstruction of bone; 16% for soft tissue; 13% for vascular; 13% for wound healing/skin/wound dressing; 11% for neural; 6% for cardiac; 6% for cartilage and a lower percentage for suture, bladder, corneal, liver, urinary incontinence and conjunctival regeneration (Fig. 5.3).

For TE, the research carried out on electrospun nanofibers quantified in terms of journal

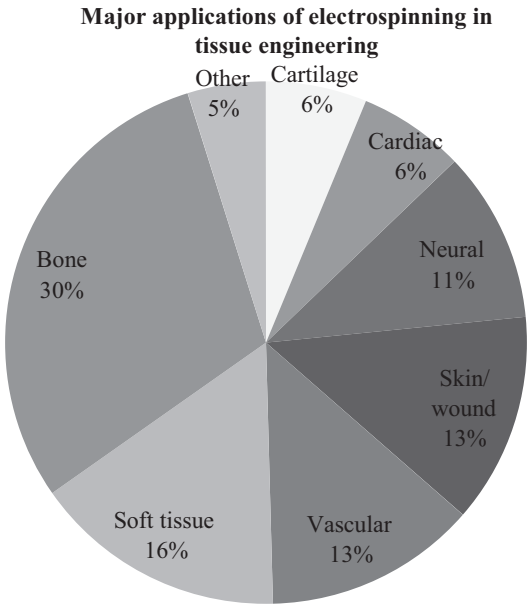

Fig. 5.3 Tissue engineering areas of major application of electrospinning, according to research realized in the *PubMed* database until December 2017. The keywords used in the field Title/Abstract were: "tissue engineering" and "electrospinning" or "electrospun" and the organs and tissue applications ("cartilage" or "trachea", "heart" or "cardiac", "nervous" or "nerve" or "neural", "skin" or "wound healing" or "wound dressing", "vascular" or "vessels", "soft tissue" or "tendon" or "valve" or "muscle", "bone", "suture", "bladder", "corneal", "liver" or "hepatic", "urinary incontinence", "conjunctival")

publications is much more in comparison with that of electrosprayed nanoparticles.

5.3.1 Bone Tissue Engineering

For bone repair, autograft is considerate the gold standard, however, there are supply limitations in its use [26]. In this context, the use of electrospun scaffolds for bone TE has become a rapidly expanding research field. Some of the most current materials and approaches used in electrospinning scaffolds for bone TE are summarized in Table 5.1.

5.3.2 Soft Tissue Engineering

In the field of soft TE, there is an immediate need to develop biomaterials with a high capacity for mechanical and biological performance [3]. A variety of scaffolds are investigated to promote soft TE by electrospinning; some current materials and approaches used are summarized in Table 5.2.

5.3.3 Vascular Tissue Engineering

Patients with cardiovascular disease have greatly benefited from the development of devices such as tissue-engineered vascular graft (TEVG) [30]. Vascular TE usually involves the fabrication of electrospun tubular scaffolds. Some current materials and approaches used in electrospinning scaffolds for vascular TE are summarized in Table 5.3.

5.3.4 Skin Tissue Engineering

Skin grafts are usually autografts, allografts, allogeneic, or xenogeneic. With the use of TE, it is possible to develop an improved approach to wound healing [81]. Some current materials used in electrospinning scaffolds for skin TE wound healing and wound dressing are summarized in Table 5.4.

5.3.5 Neural Tissue Engineering

Peripheral nerve tissue can self-regenerate if the external injuries are small. However, a number of challenges lie in restoring nerve tissue. Various kinds of scaffolds have been applied for neural TE, such as electrospun nanofibers [134] (Table 5.5).

Table 5.1 Current papers with electrospinning scaffolds for bone tissue engineering

Scaffold material	Outcomes	References
Collagen/nano-hydroxyapatite (n-HA)/bone morphogenetic protein-2) (BMP-2)/PCL-PEG-PCL hydrogels wrapped in PDLLA electrospun nanofiber	Scaffold working as a barrier between the hydrogels and the tissue, maintaining the n-HA/BMP-2 in the required places of osteogenesis and exhibiting an excellent effect on the spinal fusion	[89]
Amalgamating chitosan, aniline-pentamer, and hydroxyapatite	Utilization of electrical stimulation or hydroxyapatite-enhanced osteogenesis could lead to more efficient osteogenesis protocol	[85]
PVA based bionanocomposite with nanohydroxyapatite and cellulose nanofibers	Cell viability, cell attachment, and functional activity of osteoblast MG-63 cells showing higher cellular activity for scaffolds with nanofillers	[27]
Blend of PLA/PCL/PEO	The modified process to arrive at a sponge-like 3D scaffold with highly interconnected pores and mechanically viable, exhibited sufficiently large pores and allowed for cell penetration	[16]
Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)/graphene oxide	Easy process of production, appropriate structures of porous, favorable biomechanical properties, and fast osteogenic capability	[133]
Blend of polydioxanone and polysaccharides kappa-carrageenan and fucoidan	Enhanced differentiation of human preosteoblasts	[35]
Poly(vinylidene difluoride – Trifluoroethylene) (PVDF-TrFE)	Piezoelectric fibrous scaffolds selectively promote mesenchymal stem cell differentiation	[24]
Polyglutamate acid conjugated with BMP-2/silk fibroin/PCL	Characterization of mineralized scaffold and cytocompatibility. The polyglutamate motif improved binding properties, and the BMP enhanced expression of osteogenic genes	[70]
PCL/PLA	Osteogenic differentiation of stem cells can begin on PCL scaffolds without specific culture conditions	[8]
PCL/PLGA	A hybrid scaffold constructed by 3D printed and PLGA fibers can be used for the regeneration of hard tissue, such as bone	[75]

5.4 Electrospray as Drug Delivery System

Electrospraying is recognized as an important and one of the most efficient techniques for the preparation of nanoparticles with respect to pharmaceutical applications. The entrapment of drugs into a biocompatible and biodegradable polymer matrix has been the focus of interest for the production of sustained drug release applications. The polymer takes the drug to the target, reduces the metabolic drug degradation, provides a continuous release, increases the active pharmaceutical ingredient activity and reduces the side effects of the drug [106]. Moreover, the nanoparticles have a wider range of applications because of their "zero" dimensional nature, whereas nanofibers are more restricted due to their two-dimensional nature [106].

Many different types of biodegradable polymers have been developed with differences in biodegradability. Drug delivery systems based on polymeric nanoparticles have the advantages of scalability, biodegradability, biocompatibility, cheaper cost, targeted delivery, sustainability in the release of encapsulated drugs that will result in improved efficacy. Among these polymers, polyesters such as PLGA, PCL, PLA and their derivatives, polyorthoesters and polyanhydrides, are being extensively used in a wide range of clinical applications as they are approved by the Food and Drug Administration for their biocompatibility and low toxicity [25, 82]. Bohr and collaborators successfully electrosprayed PLGA fabricated microspheres containing celecoxib, a non-steroidal anti-inflammatory drug; the compound proved to be amorphous and physically stable for more than 8 months [12]. Yu and col-

Table 5.2 Current papers with electrospinning scaffolds for soft tissue engineering

Scaffold material	Outcomes	References
Blend of polycarbonate urethane with ciprofloxacin bound to triethylene glycol	*In situ* drug release for the regeneration of the periodontium	[118]
Trichostatin A -laden PLA	Functional tendon TE	[128]
PCL/PLA	PCL/PLA fibers with increased diameter promoted stem cells towards the tendon lineage without tenogenic factors	[8]
Collagen	Aligned anisotropy nanofiber induces myotube differentiation for musculoskeletal TE	[55]
PLA	Aligned fibrous scaffolds produced by a novel centrifugal melt electrospinning technique could repair and regenerate tendon defects and injuries	[119]
Poly(caprolactone fumarate)/PCL	A novel elastomeric was fabricated with *in situ* photo-crosslinking	[103]
Keratin	Secretome with scaffold improves tendon-bone healing	[102]

laborators prepared nanoparticles by a modified electrospraying process using polyvinylpyrrolidone (PVP) as a hydrophilic polymer matrix and ketoprofen as a model drug [127]. Ketoprofen is widely used for the treatment of inflammation, pain, or rheumatism and its short biological half-life leads to an increase of the frequency of medicine intake. In another example, a coating of nanoparticles composed of carbonated calcium deficient hydroxyapatite and PLA were deposited on a PLA substrate surface via electrospraying [132]. The deposited coating was also applied as a carrier to assist alendronate sodium, an approved bisphosphonate drug used for the treatment of osteoporosis, through local release. Paclitaxel, an antineoplastic chemotherapy drug which is widely used for the treatment of ovarian, breast, lung and pancreatic cancer was also successfully encapsulated into different biodegradable polymers, such as PLA, PCL or PLGA with high encapsulation efficiency through electrospraying [82].

Moreover, natural polymers of either protein or carbohydrate were found to produce stable micro/nanoparticles without any loss of their bioactivity of either the drug or encapsulating biomolecules [25, 37].

Electrospraying has been shown to meet the requirements for production of aerosols because monodisperse particles with controllable size and shape can be produced [82]. Ijsebaert and collaborators developed an aerosol generator of beclomethasone dipropionate by using electrospraying [45]. Electrospray nebulizers were used for producing microparticles of a size range of 2–5 μm and the particles serve the purpose of inhaling drugs through the lungs [106].

Another application of electrospraying as a drug delivery system is its potential in delivering more than one drug. Multiple drugs in a fixed dose combination could be delivered and released at the target sites [82]. The main benefit of the fixed-dose combination is that co-delivery of various therapeutic agents in the same delivery vehicles can bring the advantage of synergy, suppress drug resistance and be more convenient for patients (simplified dose regimen for daily treatment). An example is the work of Sakuma and collaborators who used electrospraying to enhance oral absorption of lopinavir through co-encapsulation with ritonavir [98]. Lopinavir, a human immunodeficiency virus (HIV) protease inhibitor, is used for the treatment of HIV infection. Low bioavailability and fast elimination are observed when lopinavir alone is orally administered; however, co-administration with ritonavir dramatically improves the poor pharmacokinetic properties of lopinavir [98].

Table 5.3 Current papers with electrospinning scaffolds for vascular tissue engineering

Scaffold material	Outcomes	References
Gelatin/oxidized carboxymethylcellulose blend	*In-situ* crosslink. Scaffold surface and mechanical properties validate properties of the native vessel.	[50]
Poly(glycerol sebacate)	Anisotropic membrane for vascular TE	[41]
PLCL/collagen fibers and PLGA/silk fibroin yarns	Tri-layered vascular scaffold consisted of aligned fibers in the inner layer, yarns in middle layer, and random fibers in outer layer with tissue regenerative capability	[120]
PCL and gelatin composite	Cuffs for tissue engineered blood vessels.	[109]
PGA and PLCL	Bone marrow mononuclear cell seeding for tissue-engineered vascular graft	[29]
PCL	Fibrous vascular graft with two layers, one thin and internal made of longitudinally aligned fibers and the other a relatively thick highly porous external layer	[112]
Tecophilic/gelatin	Dynamic cultivation promoted increased accumulation of collagen, indicating a balance between scaffold biodegradation and matrix turnover	[114]
PCL functionalized with heparin and vascular endothelial growth factor	The scaffolds exhibited antithrombogenic properties, mechanical properties compatible with the arteries and favored the development of cells on their surface	[13]

Table 5.4 Current papers with electrospinning scaffolds for skin tissue engineering

Scaffold material	Outcomes	References
Gelatin/cellulose acetate/elastin	The use of the grid-like pattern as a collector increased the swelling ratio, degradation rate, increased the elongation at break and is a biocompatible scaffold	[52]
Silk fibroin/gelatin loaded with thyme essential oil and doxycycline	A characterized mat with antibacterial properties for drug delivery applications and biocompatible	[23]
Cellulose/PLA/polydioxanone	Sugar-cane bagasse derived cellulose	[91]
Nanochitosan enriched PCL with curcumin	A characterized membrane hemocompatible for drug releasing properties with pH stimulus.	[20]
Laminin-functionalized PDLLA	Biomaterials could provide support for the cells and stimulate the healing of the burnt skin in an animal model	[107]
Gelatin and sulfated or non-sulfated hyaluronan and chondroitin sulfate	Characterization and biocompatibility of biomimetic scaffolds	[10]
PLGA/collagen	Characterization and biocompatibility of scaffolds, but mechanical strain needs to be improved	[97]
PCL/chitosan/ gelatin extracted from cold water fish skin	Characterization and biocompatibility of scaffolds. Scaffolds with gelatin (GEL) have better physical properties whereas, without GEL, scaffolds support higher cell adhesion	[34]
PCL/gelatin containing silicate-based bioceramic particles (Nagelschmidtite, NAGEL, Ca7P2Si2O16)	Biocompatibility, characterization and released Si from the conducive biomaterial for diabetic wound healing	[71]

5.5 Co-Axial Electrospinning and Electrospraying

The co-axial electrospinning method (Fig. 5.1c) is a modification of the traditional single spinneret electrospinning set up. This innovative method was first reported by Loscertales and collaborators in 2002 [67]. Loscertales and collaborators produced the capsules with diameters ranging from 150 nm to 10 μm by use of electrospraying. Sun and collaborators first used this set up to prepare nanofibers with core-sheath structures and called this technique 'co-electrospinning' [110]. The main purpose of

Table 5.5 Current papers with electrospinning scaffolds for neural tissue engineering

Scaffold material	Outcomes	References
PLA coated with alginate and gelatin, followed by a multi-wall carbon nanotube coating combined with valproic acid	Characterization, biocompatibility, and differentiation of mesenchymal stem cells into neuron-like cells in the 3D composite nanofiber scaffold for damaged neural tissue	[33]
PLLA	Cytocompatibility and neurite guidance effect on randomly and aligned fibers with induced pluripotent stem cells differentiated into neural stem cells for use in neural TE	[64]
PLLA-poly(glycerol sebacate)	Characterization and biocompatibility of core-shell membranes. The developed materials presented the potential for nerve regeneration and biomedical engineering	[122]
Cellulose acetate/PLA containing citalopram-loaded gelatin nanocarriers	Scaffold with the core-shell structured fibrils using drug loaded coaxial wet-electrospinning (produced by the nanoprecipitation method) for sciatic nerve injury	[80]
PLGA	Aligned fiber scaffold which increased the differentiation of embryonic stem cells into neural precursors	[104]
Poly(γ-benzyl-l-glutamate)-r-poly(glutamic acid)	The polypeptides were synthesized and the scaffold was biocompatible and biodegradable with controlled hydrolysis of glutamic acid for neural regeneration	[117]

co-axial electrospinning is to obtain fibers with a core-shell structure. The single spinneret is replaced by two co-axial capillaries in which two channels are connected to two reservoirs [69]. This technique is mainly used to obtain fibers with a core-shell structure having the specific desired drug encapsulated in the core of the fibers, which, due to the controlled degradation of the shell polymer, leads to a continued and controlled drug release over a long period. For drug delivery purposes various molecules have been successfully loaded into the co-axial fibers, as for example, antibiotics, proteins, enzymes and growth factors, The main advantage of this technique is that the core-shell fiber structure offers protection for the molecule loaded into the core and the bioactivity of the growth factor or drug, remains preserved [19, 105]. The fact of the drug or biological molecule being in the inner jet while the electrospinning process is in progress gives protection and enhances its the enhancing functionality or maintains the bioactivity of unstable compounds. Another advantage of the core-shell system is that it improves the sustained release of drugs [19]. The co-axial fibers present several advantages regarding the materials for the preparation of scaffolds, namely due to the possibility of combining a core with the desired mechanical properties with a shell, prepared from biocompatible materials, which will establish appropriate interactions with the host.

Various parameters can influence the encapsulation of drugs and biomolecules in the core of the co-axial fibers, such as concentrations of the core polymer and shell polymer, the relative flow rate of the core and shell solutions and the molecular weight and drug concentration [101]. Depending on the degradation rate of the shell polymer, when necessary an accelerated transport of core molecules into the environment is achieved by incorporating low molecular weight PEG as a porogen into the shell.

Co-axial electrospraying allows for the production of bilayered nano and microparticles by using a high electric field between the coaxial capillary needle and the ground. In this technique, the resultant electrical shear stress elongates the core and the shell liquid menisci at the needle outlet to form the Taylor cone; after this phenomenon, the jet of the liquid elongates enough until it is broken into multilayer droplets owing to the electrohydrodynamic forces [129].

Table 5.6 Current papers with co-axial electrospinning scaffolds for tissue engineering

Scaffold material	Outcomes	References
PCL/GelMA	Characterization of their chemical/physical properties and their hemo and biocompatibility in vitro for use as vascular grafts	[18]
P3HB4HB/(gelatin + PVA)	P3HB4HB as the core solution and gelatin + PVA mixture into the shell solution. Characterization and testing of the osteogenic and chondrogenic potential	[72]
Kartogenin/ (PLCL/ collagen nanofibers)	PLCL and collagen solution as shell and kartogenin solution as core. The kartogenin scaffold promoted the chondrogenic differentiation of mesenchymal stem cells, being an effective delivery system for kartogenin and a promising TE scaffold for tracheal cartilage regeneration	[126]
Poly(glycerol sebacate)-PCL/ gelatin-dexamethasone	PGS-PCL as core and Gt as shell, scaffolds as being appropriate for soft TE	[78]
Cellulose acetate/PLA	PLA as core solution, cellulose acetate as shell solution. The electrospun scaffold was rolled into a conduit and implanted into a rat model of nerve injury	[80]
Collagen-chitosan-PLCL/heparin	Collagen/chitosan/PLCL as the shell solution and heparin as the core solution. The scaffolds showed excellent biocompatibility and can be used for vascular TE purposes	[125]

Table 5.6 shows some recent examples of possible TE applications of co-axial fibers.

5.6 Emulsion Electrospinning

Emulsion electrospinning (Fig. 5.1d) is a simple variation of electrospinning to produce core-shell nanofibers by using a stable polymer emulsion, which has raised increasing interest, as the process is considered more stable. The advantage of emulsion electrospinning over the other blending techniques is that the drug of interest and the polymer are each solubilized in appropriate solvents, thus eliminating the need for a solvent that is suitable for the drug and polymer at the same time [101].

The emulsion electrospinning technique is a good alternative as it allows for the encapsulation of lipophilic molecules using hydrophilic polymers and avoids the use of organic solvents [83]. Emulsion electrospinning relies on chemical means of separation through the creation of an emulsion within a single solution and the subsequent organization of the emulsified droplets into two distinct phases as the solvent evaporates from the electrospun fibers. However, the method lacks well-defined control over the placement of the therapeutic agent within either the core or shell of the structure.

Yang and collaborators assessed the potential use of emulsion electrospinning to prepare core-shell fibers as carriers for therapeutic proteins [123]. Bovine serum albumin (BSA) was selected as a model protein and PDLLA as a polymer. The ultrafine fibers prepared by emulsion showed higher structural integrity of the core-shell fibers [123]. Table 5.7 shows some of the most recent studies investigating TE applications of scaffolds produced by emulsion electrospinning.

5.7 Bio-Electrospraying

Bio-electrospraying is a development of electrospraying that allows for producing matrices of cells inserted in scaffolds that could form engineered tissues and organs. Bio-electrospraying was first developed in 2005, and since then has been employed in some studies which further refined its use and made it evolve as a novel, direct *in vivo* TE and regenerative medicine strategy [39, 47, 48, 77, 84]. As the name suggests, the bio-electrospraying involves the spraying of living cells under the application of an electrical potential difference. Jayasinghe and collaborators have electrosprayed two types of cells,

Table 5.7 Current papers with emulsion electrospinning scaffolds for tissue engineering

Scaffold material	Outcomes	References
GDNF/PLGA and NGF/PDLLA	Proper physical properties, high encapsulation efficiency, and well-preserved bioactivity exhibiting different release behaviors. Possible candidates for neural TE	[66]
PCL/chitosan	The scaffold is a promising, readily available, cost-effective, off-the-shelf matrix as a skin substitute	[88]
PLA/alginate	The scaffolds show good mechanical properties and are beneficial for cell proliferation and differentiation. Suitable for various TE applications due to the antibacterial properties of the alginate	[121]
Poly(ethylene glycol)-b-poly(l-lactide-co-ε-caprolactone) (PELCL)/microRNA 126	Encapsulation of miR-126 in the electrospun membranes, a sustained release profile, strategy for cardiovascular disease treatment and for blood vessel regeneration	[131]
PELCL/peptide QK	Successful encapsulation of the QK peptide and maintenance of its secondary structure after released. The release of QK peptide could accelerate the proliferation of vascular endothelial cells showing potential applications in vascular TE	[124]
PLGA/VEGF	Nanofibers with sustained release of the encapsulated VEGF. General TE applications with a focus on vascular TE	[96]

namely human blood and Jurkat cells, assessed for their viability after the procedure using trypan blue staining, they demonstrated that the cells could be maintained viable [47, 77]. Bartolovic and collaborators established a protocol for bio-electrospraying hematopoietic stem cells, showing that the cells retained both their viability and stem cell characteristics [7].

Bio-electrospraying has been continuously refined since its development to improve jet stability and continuity, but also to allow for the formation of encapsulations that include cells of various morphologies or multicellular model organisms such as zebrafish. The technique is unique for many reasons, as for example the ability to handle highly concentrated suspensions (>10^6 cells/mL). Moreover, the technique offers the possibility of forming nano/microstructures, including cells while using large needles, which are necessary in order to limit cell damage arising from shear forces on the cells [4]. One advantage of bio-electrospraying over other techniques such as ink-jet printing and aerosol delivery is that it can process denser cell suspensions and also generate finer droplets [48].

The methodology of bio-electrospraying provides a wide range of applications spanning from bio-analytics to diagnostics, but most importantly, it shows the potential for forming synthetic or artificial tissue, repairing and replacing damaged/aging tissue. One of the possible applications is found in the ability to bio-electrospray whole human blood without affecting the genetic make-up, demonstrating that this technique is a possible diagnostic protocol [77].

Bio-electrospraying allows for the design of constructs and this synthetic construct would require significantly reduced bioreactor time. Moreover, the combination of traditional electrospinning with bio-electrospraying offers the possibility of creating nanofibrous scaffolds with a uniform 3D distribution of cells for use in TE and regenerative medicine.

5.8 Conclusions and Perspectives

Electrospinning and electrospraying are electrohydrodynamic techniques used for the production of scaffolds or nano/microparticles in several fields of research. Variations of these techniques include electrospraying for drug delivery systems, co-axial electrospinning, and electrospraying, emulsion electrospinning as well as bio-electrospraying. The materials most commonly used for the production of scaffolds for TE or nano/microparticles for drug delivery are natural or synthetic polymers, polymers composites and inorganic and metallic materials.

The ability to produce fibers and particles with well-defined dimensions is an important characteristic of the electrospinning/spraying methods. The morphology of materials can be manipulated by solution parameters, such as molecular weight of the polymer, viscosity, the concentration of the solution, and by manipulating the processing parameters, such as tip to collector distance, conductivity, applied voltage, etc. The variously modified electrospinning and electrospraying techniques allow for the production of a great variety of nanostructured materials with desired properties for the regeneration of tissue and organs.

TE applied for regenerative medicine is an interdisciplinary subject of medicine, biology, engineering and life science. Numerous studies have been devoted to this rapidly developing field, with the aim of developing scaffolds to provide support for cells. It is with the continuous study of TE that we can successfully achieve the necessary groundbreaking techniques for the rebuilding of human tissue and organs.

There are still many challenges to be met regarding the improvement of the materials in terms of guaranteeing their three-dimensional structure along with vascularization and sufficient porosity to allow for the cells to grow and proliferate in the scaffolds. For drug delivery, the systems need further refinement for optimizing the encapsulation of biomolecules, ensuring they attain the appropriate quantity and their delivery in the desired location. Numerous studies are working to provide realistic solutions for these desired improvements to guarantee the successful development of scaffolds or drug delivery systems. The long-term goal for these studies will be a very high standard system for delivering drugs in small but highly efficient doses in the target with a significant decrease of undesired side effects and the elevation of efficiency in tissue regeneration in terms of the repair of lesions in tissue and organs.

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Part II

Intelligent Nanocomposite Biomaterials for Regenerative Medicine

6 Graphene-Based Nanocomposites as Promising Options for Hard Tissue Regeneration

Yong Cheol Shin, Su-Jin Song, Seung Jo Jeong, Bongju Kim, Il Keun Kwon, Suck Won Hong, Jin-Woo Oh, and Dong-Wook Han

Abstract

Tissues are often damaged by physical trauma, infection or tumors. A slight injury heals naturally through the normal healing process, while severe injury causes serious health implications. Therefore, many efforts have been devoted to treat and repair various tissue defects. Recently, tissue engineering approaches have attracted a rapidly growing interest in biomedical fields to promote and enhance healing and regeneration of large-scale tissue defects. On the other hand, with the recent advances in nanoscience and nanotechnology, various nanomaterials have been suggested as novel biomaterials. Graphene, a two-dimensional atomic layer of graphite, and its derivatives have recently been found to possess promoting effects on various types of cells. In addition, their unique properties, such as outstanding mechanical and biological properties, allow them to be a promising option for hard tissue regeneration. Herein, we summarized recent research advances in graphene-based nanocomposites for hard tissue regeneration, and highlighted their promising potentials in biomedical and tissue engineering.

Keywords

Tissue engineering · Graphene · Nanocomposite · Hard tissue regeneration

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6.1 Introduction

Tissue regeneration is an issue of the first importance for us, because severe tissue injuries caused by physical trauma, infection or tumors are very

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difficult to naturally heal as well as leading to serious health implications. In principle, animal tissues are classified based on their origin and function into connective, epithelial, muscular, and nervous tissues. These tissues can be also categorized into two main groups according to their structures: soft and hard tissues. Soft tissues are tissues that are generally not hardened or calcified, including skin, nerve, fat, muscle, ligament, and tendon. The soft tissues are mainly composed of cells and surrounding extracellular matrices, and their properties are highly deformable and nonlinear significantly depending on their function, composition and structure [6]. On the other hand, hard tissues are mineralized tissues having firm intercellular matrices, such as bone, dental enamel, cementum, dentin, and cartilage. The hard tissues are relatively strong and rigid, but their fracture is the most common traumatic disease due to their intrinsic brittleness [28, 56, 90]. Recently, a lot of efforts have been made to promote the regeneration of hard tissues, among which tissue engineering approaches have attracted a great attention as a novel strategy [23, 52].

With the recent advances in nanoscience and nanotechnology, various nanomaterials have been suggested as novel biomaterials. Among a great number of available candidates, graphene and its derivatives have emerged as promising candidates for biomedical applications. Graphene is a two-dimensional (2D) carbon nanomaterial in which carbon atoms are arranged in a honeycombed lattice structure [10, 31, 32, 82, 129]. It has been reported that the graphene and its derivatives, including graphene oxide (GO) and reduced GO (rGO), have exceptional electrical, thermomechanical, physicochemical, and optical properties [2, 29, 82, 99, 106, 109, 110]. In addition, graphene nanomaterials have recently been found to possess promoting effects on various types of cells [47, 58, 59, 65, 79, 86, 102]. In addition to those unique properties of graphene nanomaterials, they can be used in a combination with other biomaterials, including polymers or biomolecules, which in turn graphene-based nanocomposites have become more and more fascinating [60, 75, 96, 101, 102, 113]. In particular, there have been tremendous attempts to employ graphene-based nanocomposites for hard tissue regeneration because of their exceptional mechanical properties and outstanding biological properties, which are typical advantages of graphene nanomaterials, and very interesting results have been documented in recent years [22, 34, 94]. Herein, we are attempting to summarize recent research advances in graphene-based nanocomposites for hard tissue regeneration, and highlight their promising potentials in biomedical and tissue engineering.

6.2 Bone Tissue Regeneration

Bone is the hardest tissue supporting and protecting various organs of the body. Bone defects are commonly caused by various reasons, such as physical trauma, infectious disorder, aging, genetic disease, or congenital malformation [7, 24]. Currently, immobilization with fixation and bone grafting are generally available therapeutic methods for repairing the bone defects [18, 44, 88, 89]. However, those therapeutic methods have limitations, such as limited supply, a loss of function and a risk of infection. Therefore, many studies have suggested various strategies for promoting osteogenesis as well as bone regeneration through tissue engineering approach as an alternative therapeutic strategy [25, 27, 42, 46, 53–56, 76, 100].

Bone tissue is composed of bone cells and complex matrices mainly consisting of collagens, non-collagenous proteins and deposits of calcium phosphates [9, 114]. Among them, a calcium phosphate plays a central role in the reconstruction and regeneration of bone tissues because it affords mechanical stiffness, strength and structural integrity to bone through extracellular mineralization [19, 35, 68]. However, the intrinsic poor mechanical properties of calcium phosphates, including low fracture toughness, low wear resistance and brittleness are often quoted as their main disadvantages [55, 69, 100]. To address these poor mechanical properties of calcium phosphates, graphene nanomaterials can be incorporated with calcium phosphates.

Graphene nanosheets have been reported to exhibit exceptional mechanical performance, and can be used as reinforcing nanomaterials in polymer or calcium phosphate composites [27, 33, 93, 97, 99, 126]. The graphene-based nanocomposites can be applied to bone regeneration in a variety of forms, such as composite powders, sheets, surface coatings, and composite scaffolds [64]. Graphene-based composite powders or sheets can be readily used as additives in cell cultures, and surface coatings or composite scaffolds can be useful as structural and functional supports for bone cells.

Fan et al. reported that graphene/hydroxyapatite (HAp) nanorod composite sheets can be easily synthesized by a convenient one-pot hydrothermal method, and the morphology and adhesion strength of HAp can be effectively controlled [27] (Fig. 6.1). They presented that the graphene/HAp composite sheets showed higher hardness and Young's modulus than HAp particles alone. In addition, the graphene/HAp composite sheets exhibited better biocompatibility with MC3T3-E1 osteoblasts and increased *in vitro* biomimetic mineralization in stimulated body fluid (SBF).

The reinforcing effects of graphene nanomaterials on mechanical properties are also confirmed in polymer-based composite scaffolds. Mohammadi et al. prepared poly(ε-caprolactone) (PCL) nanofibers with different GO nanosheet contents (ranging from 0.5 to 2 wt%), and showed that the mechanical properties, including tensile strength, elongation and Young's modulus, of composite scaffolds containing 2 wt% of GO nanosheets were significantly increased as compared with pure PCL nanofiber scaffolds [76]. In addition, the inclusion of GO nanosheets into PCL scaffolds could improve both the biomineralization and protein adsorption of composite scaffolds, which leads to improved cell adhesion and proliferation of MG-63 cells (human osteoblastic sarcoma cell line). These results clearly show the feasibility of using graphene nanomaterials in applications to bone tissue engineering.

According to the previous reports, the surface potential of rGO generally varies in −25 to −35 mV due to the negatively charged oxygen-containing surface groups, while calcium phosphate is positively charged due to its calcium moieties [55, 62]. Therefore, graphene nanoma-

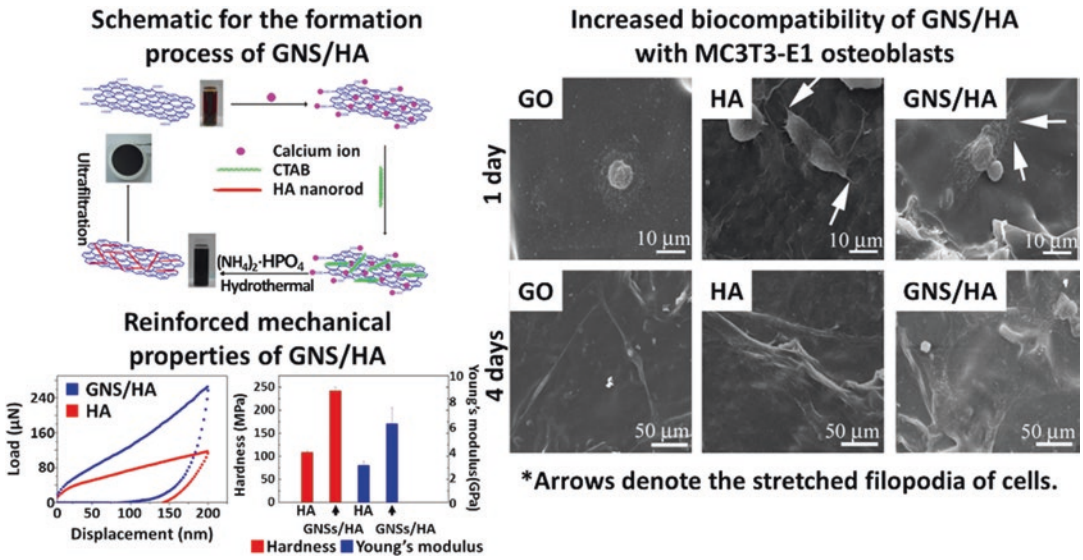

Fig. 6.1 Reinforcing effects of graphene nanosheets on mechanical properties of calcium phosphates (hydroxyapatite, HAp) and increased *in vitro* biomimetic mineralization and biocompatibility of graphene nanosheet/HAp composites. (Reproduced from Ref. [27], Copyright (2013) Elsevier Ltd.). Abbreviations: *CTAB* cetyltrimethylammonium bromide, *GO* graphene oxide, *GNS* graphene nanosheet, *HA*, hydroxyapatite

terial and calcium phosphate composite powders can be readily formed *via* electrostatic interactions between graphene nanomaterials and calcium phosphate particles. Meanwhile, interesting results have been more recently documented by Lee et al., who showed that the negatively charged dicalcium phosphate particles can also be hybridized with rGO because of the unique structural features of graphene nanomaterials [56]. They partially explained by the fact that the negative charges of graphene nanomaterials (i.e. GO and rGO) are mainly distributed on their edge sites, which in turn, the colloidal particles of dicalcium phosphate can be adsorbed on the basal plane of graphene nanomaterials [66, 104, 122]. Moreover, the interfacial interactions between oxygen-containing functional groups of graphene nanomaterials and hydroxyl groups of calcium phosphate particles can also facilitate the good adhesion of calcium phosphate particles to graphene nanomaterials, suggesting that the graphene-based nanocomposite powders can be effectively designed [70, 123]. The stimulating effects of graphene nanomaterial and calcium phosphate composite powders on osteogenic differentiation were also investigated [42, 53, 54, 56, 100]. Shin et al. have revealed that the rGO and HAp nanocomposite powders can synergistically promote the osteogenic activities of MC3T3-E1 osteoblasts. They investigated the cellular behaviors of MC3T3-E1 osteoblasts treated with rGO and HAp nanocomposite powders, and showed that the synergistic effects of rGO and HAp can significantly enhance osteogenic activities, including alkaline phosphatase (ALP) activity, extracellular calcium deposition, matrix mineralization, and the expression of osteogenic protein, without hindering cell proliferation.

Graphene nanomaterials can be also incorporated with other materials such as calcium silicate, bioactive glass, carrageenan, and strontium [25, 30, 48, 67, 72, 118]. Gao et al. prepared graphene and 58S bioactive glass (58 mol% of SiO_2, 33 mol% of CaO and 9 mol% of P_2O_5) nanocomposite powders, and showed that the addition of graphene can improve the mechanical properties and *in vitro* mineralization activity of bioactive glass scaffolds, while maintaining good biocompatibility for MG-63 cells [30]. It has been also demonstrated by Kumar et al. that nanocomposite scaffolds composed of PCL and rGO-decorated strontium nanoparticles can significantly increase osteoblast proliferation and differentiation [48].

More recently, the *in vivo* effectiveness of graphene-based nanocomposites on bone tissue regeneration was studied [46, 55, 80]. Lee et al. prepared rGO and HAp nanocomposite powders, and investigated their *in vivo* effects on critical-sized calvarial defects in a rabbit model [55] (Fig. 6.2). The results showed that the rGO and HAp nanocomposite powders can not only spontaneously stimulate the osteogenesis of MC3T3-E1 osteoblasts, but can also effectively enhance new bone formation with no inflammatory responses.

Meanwhile, 3D bioprinting has recently emerged as a novel approach for developing 3D bone tissue engineering scaffolds with desirable structure and biochemical properties [11, 40, 41, 64, 125]. It has been reported that the graphene nanomaterial-incorporated bio-inks can be readily prepared by blending with biocompatible polymers or ceramics while maintaining unique properties of graphene nanomaterials, such as exceptional mechanical, electrical and biochemical properties [40]. Jakus et al. successfully prepared osteogenic biomaterial inks using graphene nanoflakes and HAp microspheres, and fabricated 3D printed constructs [41]. They revealed that the 3D printed graphene-HAp hybrid scaffolds can support the cell growth of human bone marrow derived mesenchymal stem cells and upregulate osteogenic gene expression, including osteocalcin, osteopontin and collagen type I. These findings suggest that the graphene-based nanocomposites can be employed as osteogenic bio-inks. Zhang et al. and Boga et al. also supported the fact that the 3D bioprinted scaffolds have a great potential for bone tissue engineering [11, 125]. They prepared graphene nanomaterial-based scaffolds by 3D bioprinting, and demonstrated that the 3D bioprinted scaffolds containing graphene nanomaterials exhibit outstanding osteoinductive and osteoconductive properties beneficial to bone tissue regeneration.

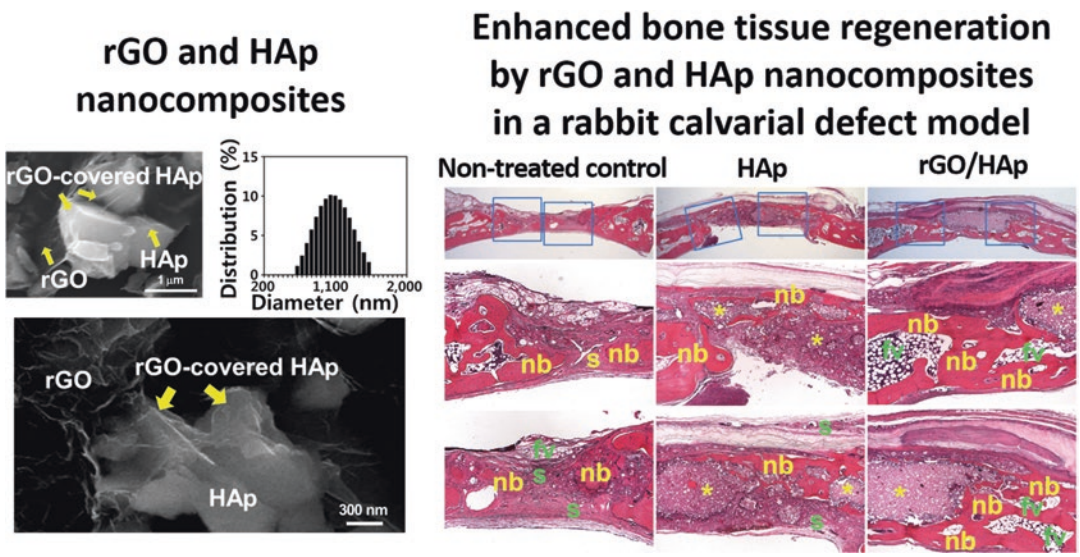

Fig. 6.2 Enhanced bone tissue regeneration by rGO and HAp nanocomposites in a rabbit calvarial defect model. (Reproduced from Ref. [55], Copyright (2015) Springer Nature). Abbreviations: *fv* fibrovascular tissue, *HAp* hydroxyapatite, *nb* new bone, *rGO* reduced graphene oxide, *s* soft tissue, * graft materials

Collectively, it is noteworthy that the 3D bioprinting is a promising and novel strategy for bone tissue regeneration because it can realize native bone-like scaffolds by manufacturing biomimetic scaffolds similar to the native bone structure and composition, while retaining the osteogenic capabilities of graphene nanomaterial-based nanocomposites.

According to the recent findings, the exceptional mechanical properties and outstanding biological properties of graphene-based nanocomposites make them possible to predict a bright future for bone tissue engineering.

6.3 Dental Tissue Regeneration

Graphene-based nanocomposites have been also proposed as promising candidates for dental tissue engineering due to their superb mechanical and biological properties [83, 91, 107, 116, 117]. Xie et al. revealed that the differentiation of periodontal ligament stem cells (PDLSCs) was enhanced on the graphene-coated glass slides, and their differentiation was further promoted when they were cultured in an osteogenic medium [116] (Fig. 6.3). It has been reported that the graphene nanomaterials differently interact with chemical growth factors depending on their types [4, 59]. Graphene-coated substrates can readily interact with osteogenic inducers, such as dexamethasone and β-glycerophosphate, via π-π stacking interactions, whereas GO-coated substrates can more favorably interact with adipogenic inducers, including insulin, through electrostatic hydrogen bonding interactions. This phenomenon could be due to the differences in surface moieties of graphene nanomaterials. GO has abundant oxygen-containing surface functional groups on its surface, leads to favorable electrostatic and hydrogen bonding interactions with chemical growth factors rather than π-π stacking interactions.

In addition, the mechanical properties of substrates can directly affect cellular behaviors [14, 26, 36]. Because of excellent mechanical strength of graphene nanomaterials, graphene-coated substrates exhibit relatively high stiffness, which can be a factor in promoting osteogenic differentiation [59, 92, 95]. It has been extensively acknowledged that higher mechanical properties of scaffolds are favorable to the osteogenic differentiation than lower ones [20, 38, 92, 95]. The underlying mechanism for the beneficial effects of high mechanical properties of substrates can be partially explained by the fact that the increase

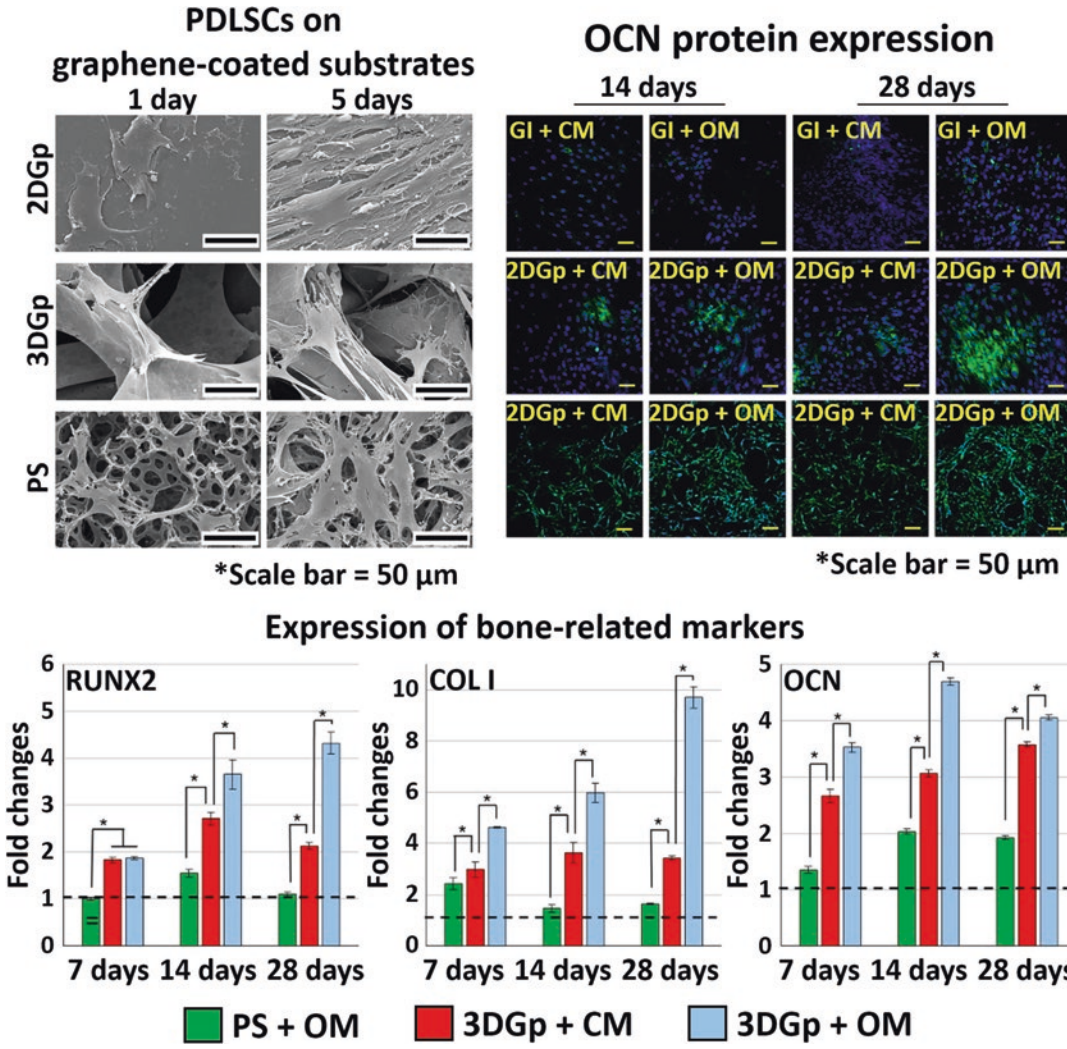

Fig. 6.3 Magnified osteogenic differentiation of periodontal ligament stem cells (PDLSCs) on graphene nanomaterial-based substrates. (Reproduced from Ref. [116], Copyright (2015) Elsevier Ltd.). Abbreviations: *2DGp* glass slide coated with graphene, *3DGp* three-dimensional graphene scaffold, *CM* culture medium, *COL I* collagen type I, *Gl* glass slide, *OCN* osteocalcin, *OM* osteogenic differentiation induction medium, *PS* polystyrene scaffold, *RUNX2* runt-related transcription factor 2

in stiffness of substrates can activate mitogen-activated protein kinase (MAPK) and Ras homolog gene family (Rho) signaling pathways, which results in enhanced osteogenic differentiation [38, 45, 78]. MAPK and Rho signaling pathways play a central role in regulating the osteogenic differentiation of mesenchymal stem cells and preosteoblasts [39, 95, 115, 119]. Khatiwala et al. have reported that the increasing the substrate stiffness can activate MAPK pathway, followed by the stimulation of runt-related transcription factor 2 (RUNX2), a transcription factor essential for osteogenic differentiation [45]. In addition, the increase in the stiffness of underlying substrates can also stimulate RhoA, followed by the up-regulation of bone morphogenetic protein (BMP)-dependent Smad and/or extracellular signal-regulated kinase (ERK) phosphorylations, and subsequently the osteogenic differentiation is promoted through the activation of RUNX2 [38]. Therefore, graphene-coated substrates can effectively facilitate the osteogenic differentiation of

PDLSCs by providing both chemical and physical cues.

GO-based substrates can also promote the differentiation of dental pulp stem cells (DPSCs) [91]. The surface topography of substrates plays a crucial role in regulating cellular behaviors [1, 12, 37]. Graphene nanomaterials have unique surface topographical features, such as wrinkles and ripples [4, 98, 130]. Therefore, graphene nanomaterial coatings can increase surface roughness of substrates. The rough surface is able to provide anchorage sites for cells, which allows cells to easily adhere to the substrates. Moreover, surface functional groups of GO are able to adsorb serum proteins in culture medium [47, 59]. Hence, GO-based substrates can effectively promote the differentiation of DPSCs because of those unique physicochemical properties.

Titanium (Ti) and its alloys have been extensively used in dental and orthopedic applications due to their good biocompatibility, mechanical properties, chemical stability, and corrosion resistance [15, 49, 103]. Therefore, graphene nanomaterial and Ti composites have been also actively investigated in dental tissue engineering [21, 61]. In addition, Ti substrates coated with graphene-based nanocomposites have been proposed to improve both mechanical properties and bioactivities [63, 124]. Li et al. prepared GO/HAp composite-coated Ti sheets using cathodic electrophoretic deposition process, and found that the adhesion strength and corrosion resistance of GO/HAp composite coatings were increased as compared with pure HAp coatings. In particular, the addition of GO into HAp layers diminished the mismatch of thermal expansion coefficients of coating layers and Ti substrates, which could strengthen coating adhesion of GO/HAp on Ti substrates. Moreover, the GO/HAp-coated Ti substrates showed good *in vitro* biocompatibility.

In order to further improve bioactivities of Ti, biomolecule-loaded graphene nanomaterials can be incorporated with Ti and its alloys [43, 50, 51]. It has been suggested by Jung et al. that the commercially available Ti implants with dexamethasone-loaded rGO can significantly promote the growth and osteogenic differentiation of osteoblasts, and can be employed for clinical dental applications [43] (Fig. 6.4). They coated rGO on Ti alloys (Ti13Nb13Zr), followed by

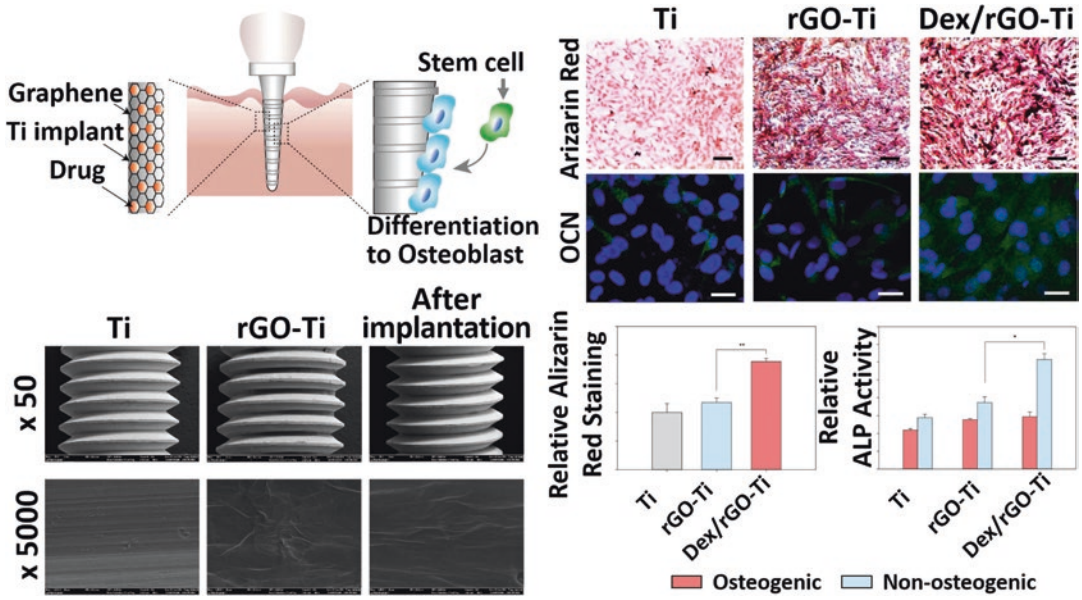

Fig. 6.4 Feasibility of Ti implant materials with drug- or biomolecule-loaded graphene nanomaterials for dental tissue engineering. (Reproduced from Ref. [43], Copyright (2015) American Chemical Society). Abbreviations: *Dex* dexamethasone, *OCN* osteocalcin

loading with dexamethasone. It was shown that the dexamethasone was stably loaded on rGO-coated Ti implants via π-π stacking, and showed long-term release behaviors suitable for osseointegration post-implantation. In addition, the cellular behaviors of MC3T3-E1 osteoblasts, including growth and osteogenic differentiation, were significantly promoted on dexamethasone/rGO-Ti alloys. Furthermore, they demonstrated the feasibility of Ti implant materials with drug- or biomolecule-loaded rGO for clinical dental applications, by showing that the rGO coating layers were stably adhered on the surface of Ti alloys even after implantation.

In addition to several applications described above, graphene nanomaterials can be used for guided bone regeneration (GBR) membranes. In the work of Park et al., the potent effects of GO-coated Ti membranes on bone tissue regeneration were elucidated [85]. An ideal GBR membrane should have both promoting effects on osteogenesis and ability to guide bone tissue regeneration. They fabricated GO-coated Ti membranes by meniscus-dragging deposition technique, and investigated their *in vitro* and *in vivo* potentials as GBR membranes. The GO-coated Ti membranes have been found to possess good biocompatibility with osteoblasts as well as suitable physicochemical properties for GBR membranes. In addition, *in vivo* studies in a rat calvarial defect model have provided evidence that the GO-coated Ti membranes hold great potentials as GBR membranes by accelerating new bone formation in calvarial defects. An underlying mechanism for the stimulating effects on osteogenesis by GO coating was suggested by Wu et al. [112]. They suggested that the osteostimulation effects of GO involve the activation of Wnt-related signaling pathway, which regulates cellular behaviors, including morphogenesis, proliferation and differentiation [71, 111]. The GO could promote the binding of Wnt3a with lipoprotein receptor-related protein 5 (LRP5), followed by the up-regulation of intracellular axis inhibition protein 2 (AXIN2) and catenin beta (CTNNB) gene expression, and subsequently resulted in the enhanced osteogenesis. Taken together, these findings strongly support the fact that the graphene-based nanocomposites are promising candidates for hard tissue regeneration.

6.4 Cartilage Tissue Regeneration

A cartilage tissue is a type of fibrous connective tissue composed of chondrocytes and collagenous extracellular matrix. Because cartilage tissues intrinsically lack self-repair capabilities, there have been significant efforts to promote cartilage tissue regeneration [5, 8, 74, 105, 108]. It has been revealed that graphene nanomaterials have stimulating effects on the differentiation of various cell types towards specific lineages, including chondrogenic lineage, and graphene-based nanocomposites are promising candidates as novel biomaterials for cartilage tissue engineering [16, 58, 81, 84, 87, 121].

Much research concerning cartilage tissue engineering has mainly focused on the development of biocompatible and biofunctional hydrogel scaffolds. Cong et al. have reported the hybrid composite hydrogels consisting of GO and poly(acryloyl-6-aminocaproic acid) (PAACA) that possess both high mechanical and self-healing properties [16]. The incorporated GO can form double networks in PAACA hydrogels, as well as forming 3D hydrogen-bonding network between polar groups of PAACA and oxygen-containing functional groups of GO. These interactions endow GO/PAACA composite hydrogels with improved mechanical properties and stretchability, while maintaining pH-sensitive self-healing properties. These results indicated that the GO-based composite hydrogels can be used as drug delivery carriers and tissue engineering scaffolds.

In addition, the dual roles of graphene nanomaterials as delivery carriers and cell-adhesive substrates have been revealed [58, 121]. In general, the chondrogenic differentiation of adult stem cells is induced through the culture of cells in pallets [3]. However, in the cell pellet culture

system, cells are difficult to efficiently interact with surrounding microenvironments due to the diffusional limits of biomolecules [73]. To address this limitation, graphene nanomaterials loaded with biomolecules, such as growth factors or proteins, can be used for cartilage tissue engineering. Yoon et al. and Lee et al. prepared biomolecule-loaded graphene nanomaterials, and formed graphene-cell biocomposites by incorporating of biomolecule-loaded graphene nanomaterials into pellets of stem cells [58, 121]. The results showed that the biomolecule-loaded graphene nanomaterials can significantly contribute to the effective cellular accessibility to the biomolecules, which results in substantially enhanced chondrogenic differentiation of stem cells. In addition, the graphene nanomaterials can adsorb various proteins on their surface, indicating that the graphene nanomaterials are able to serve as a novel platform for stem cell culture and cartilage tissue engineering [121].

Three-dimensional graphene-based composite scaffolds have been also explored for cartilage tissue engineering [81, 84]. The graphene nanomaterial and polymer composite scaffolds showed not only higher mechanical properties but also better biocompatibility as compared with their polymer equivalents. In particular, the 3D graphene-based composite foams can provide uniform 3D environments for coherent interactions between cells and graphene nanomaterials [13, 17, 57, 77, 120, 127]. In addition, 3D graphene foams can be used in a combination with polymer to achieve suitable physicochemical and reinforced mechanical properties. The graphene foam/polymer hybrid composites can be readily obtained by dip-coating methods. An interconnected network structure is organized within the hybridized composites by polymer bridge formation [81]. Moreover, polymer materials can heal defect sites of graphene foam, such as microcracks, microvoids and branch discontinuities. Hence, the mechanical properties of graphene foam/polymer composites, including compression and tension strength, were increased. Meanwhile, the 3D graphene-based composite foams enable to provide a 3D microenvironment for cells that can allow cells to mimic cellular behaviors found in the *in vivo* circumstances. Furthermore, the human mesenchymal stem cells could successfully grow within the composite foams, and their chondrogenic differentiation was enhanced by unique mechanical and structural properties of 3D graphene-based foams.

More recently, 3D bioprinted GO-based scaffolds have been reported by Zhou et al. [128] (Fig. 6.5). They prepared photocrosslinkable ink composed of GO, gelatin methacrylate (GelMA) and poly (ethylene glycol) diacrylate (PEGDA), and successfully fabricated customizable GO-GelMA-PEGDA composite scaffolds by 3D bioprinting. It was confirmed that the 3D bioprinted GO composite scaffolds have outstanding biocompatibility as well as favorable mechanical properties, and possess good ability to adsorb proteins in media due to the incorporated GO. In addition, the cellular behaviors of human bone marrow mesenchymal stem cells, including cell adhesion, proliferation and chondrogenic differentiation, were significantly promoted, and the chondrogenic gene expressions, such as type II collagen, SOX-9 and aggrecan, were especially improved on GO-GelMA-PEGDA composite scaffolds. Collectively, according to this series of findings, it is indicated that the graphene-based nanocomposite holds a highly promising potential for cartilage tissue regeneration.

6.5 Conclusions and Perspectives

During the past decade, significant advances have been devoted to the development of graphene-based nanocomposites for tissue engineering and biomedical applications. Herein, we summarized some of recent research advances concerning graphene-based nanocomposites for hard tissue regeneration, and highlighted their promising potentials in biomedical and tissue engineering. Considering the fascinating results described here, it is apparent that the graphene-based nanocomposites can be readily used in a variety

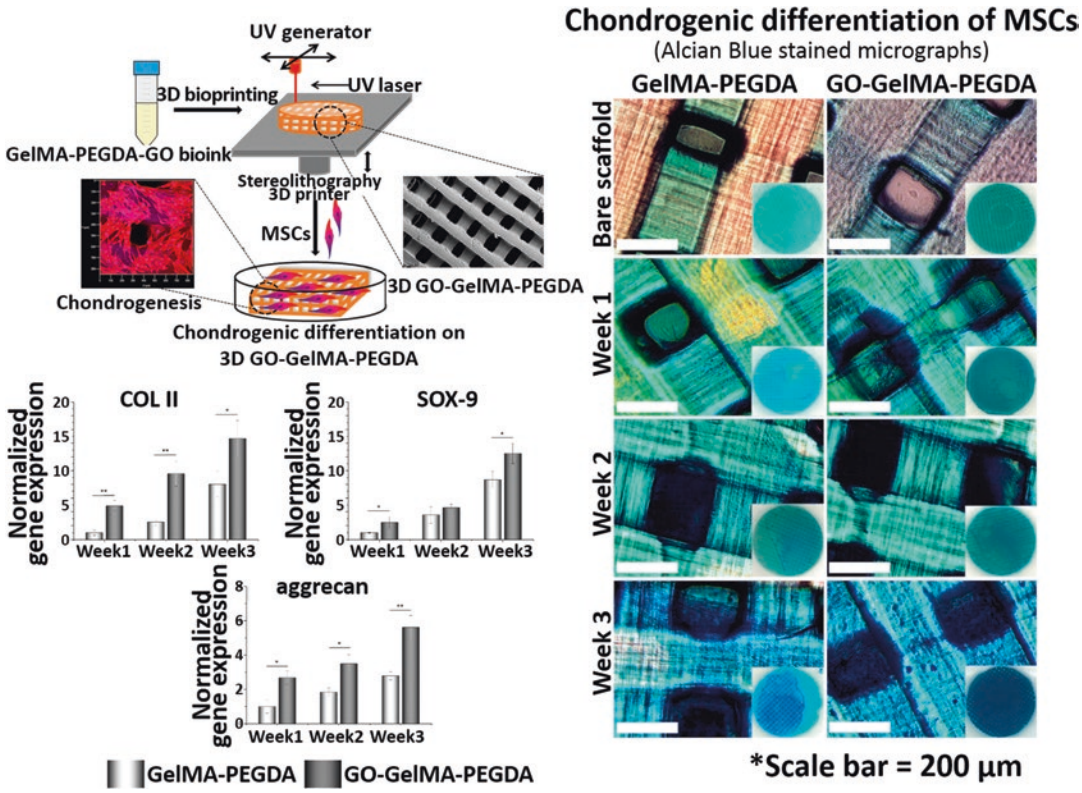

Fig. 6.5 3D bioprinted graphene-based nanocomposite scaffolds for cartilage tissue engineering. (Reproduced from Ref. [128], Copyright (2017) Elsevier Ltd.). Abbreviations: *COL II* type II collagen, *GelMA* gelatin methacrylate, *GO* graphene oxide, *MSC* mesenchymal stem cell, *PEGDA* poly (ethylene glycol) diacrylate

of ways to tissue engineering and biomedical applications. Cells and tissues are directly affected by surrounding environments, and immediately response to them through complex and sensitive interactions by various stimuli. In particular, cellular responses are largely different according to the types of tissues. Given that the hard tissues are relatively strong and rigid, the exceptional mechanical properties of graphene nanomaterials are highly beneficial not only to reinforce other biomaterials, but also to favorably regulate hard tissues and cells belonging to them. Moreover, their outstanding biocompatibility and stimulating effects on cellular behaviors are especially valuable characteristics to employ them for tissue regeneration. Even though elucidating the potential biological effects of graphene nanomaterials remains crucial challenges, we envision that the extraordinary properties of graphene-based nanocomposites can afford unprecedented opportunities for tissue engineering and biomedical applications.

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7 Modifications of Poly(Methyl Methacrylate) Cement for Application in Orthopedic Surgery

Yue Sa, Fang Yang, Yining Wang, Joop G. C. Wolke, and John A. Jansen

Abstract

Even with the emerging of newly-developed bone substitutes, poly(methyl methacrylate) (PMMA) cement is still a widely-used bone replacing biomaterial in orthopedic surgery with a long history. However, aseptic loosening, infection of the prosthesis and thermal necrosis to surrounding tissue are the common complications of PMMA. Therefore, additives have been incorporated in PMMA cement to target those problems. This chapter summarizes different additives to improve the performance of the PMMA cement, i.e.: (1) bioceramic additives; (2) filler additives; (3) antibacterial additives; (4) porogens; (5) biological agents, and (6) mixed additives. To improve the biological and mechanical performance of PMMA cement, mixed additives aiming to fabricate multifunctional PMMA seem the most suitable choice. Although *in vivo* animal studies have been conducted, long-term and clinical studies are still needed to evaluate the modifications of multifunctional PMMA cement for matching a specific clinical application.

Keywords

Poly(methyl methacrylate) cement · Additive · Bioceramic · Porogen · Antibacterial

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7.1 General Introduction and History of Poly(Methyl Methacrylate) (PMMA)

7.1.1 General Introduction of PMMA

Undoubtedly, PMMA or PMMA cement is a successful biomaterial in orthopedic surgery. Even with the emerging of newly-developed bone sub-

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stitutes, like calcium phosphate cement [22, 37, 41, 111], bioglass [68, 74], and calcium sulfate cement [17, 77, 110], PMMA is still a widely used bone substitute in cemented arthroplasty, vertebroplasty, and osteoporosis fractures due to its excellent biocompatibility, low cost, easy handling characteristics, sufficient strength and moldability *in situ* [21, 49, 59, 80–82, 86, 107]. According to a recent survey, PMMA was used in around 65% of primary total hip joint replacements in Sweden and 80% of total knee joint replacements in the United States [56], indicating that PMMA is an irreplaceable material in orthopedic surgery.

However, long-term clinical observations have indicated that PMMA cement is also beset with some drawbacks [54, 61]. Aseptic loosening, infection of the prosthesis and thermal necrosis to surrounding tissue are the most common complications with PMMA in orthopedic applications [61]. In particular, aseptic loosening accounts for almost two-thirds of the revision hip arthroplasties and one-half of the revision knee arthroplasties [93]. The major cause of aseptic loosening is the lack of ideal biological and mechanical characteristics of PMMA cement, which results in a weak PMMA-bone interface [9, 97]. Infection of the prosthesis is a devastating complication for the patient. Especially in individuals receiving total joint replacements, the incidence of PMMA infections even increases to 13% [69]. Furthermore, the polymerization of PMMA cement can induce a high exothermic temperature between 67 and 124 °C [105], which causes necrosis of the surrounding tissues and finally results in prosthesis failure [38, 58, 60, 91]. To overcome the aforementioned shortcomings, roughly six types of additives, were investigated over the last decades to address the problems of plain PMMA, i.e.: (1) bioceramic additives; (2) filler additives; (3) antibiotic agents; (4) porogens, (5) biological agents and (6) mixed additives (Fig. 7.1). Bioceramic additives were used to increase the bioactivity of PMMA cement aiming to enhance the conjunction between cement and the bone. Furthermore, the added bioceramic particles would also change the bulk mechanical property of the PMMA cement, which may benefit the load transfer from the PMMA to bone. Therefore, bioceramics were added to overcome the aseptic loosening of PMMA. Filler additives were mixed with PMMA matrix to improve the mechanical property of PMMA, which also showed beneficial effects on overcoming the aseptic loosening of PMMA. Antibiotic agents were directly added to control the PMMA infection. Porogens were used to create the porosities in PMMA, which would change the mechanical property of PMMA cement and encourage the bone ingrowth to avoid the aseptic loosening. Moreover, porogens were normally hydrogels, which would significantly reduce the exothermic temperature to prevent the thermal necrosis. Biological agents were used to increase the biocompatibility of cement, modify the mechanical property and reduce the polymerization temperature of PMMA cement, which showed beneficial effects on reducing the risk of aseptic loosening and thermal necrosis. Mixed additives, the combinations of two or more aforementioned additives, were added to PMMA to create the multifunctional cement to fulfill the requirement in the clinic.

The current chapter summarizes all the above-mentioned additives and aims to provide information for future PMMA application in orthopedic surgery. First, the history of PMMA cement is described, followed by a review of the various PMMA cement additives. Finally, the chapter provides future perspectives for the optimization of PMMA cement. It has to be emphasized that the review part only reports about additives related to the aseptic loosening, infection of the prosthesis and thermal necrosis to the surrounding tissue. The use of additives to reduce other drawbacks of PMMA, *e.g.* cement shrink and monomer release, were beyond the scope of this chapter.

7.1.2 History of PMMA Cement

The pioneering work of PMMA started as an industrial synthesized acrylic in the early 1900s. In 1933, the German chemist Dr. Otto Röhm patented a PMMA product with the trade name

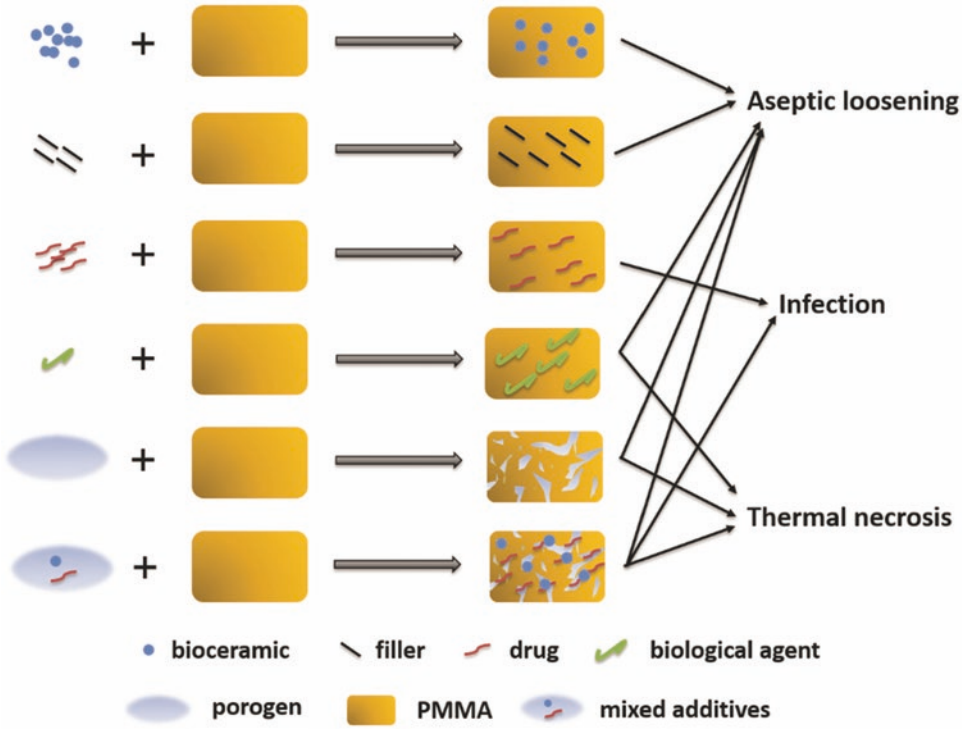

Fig. 7.1 A schematic diagram showing the relationship between the incorporation of additives into PMMA cement and the associated clinical problems

Plexiglass. This material was first applied in submarine periscopes and airplane canopies [73]. The demand and interest for this material increased enormously during the pre-war and war era. Later, PMMA was clinically used for hard contact lenses due to its biocompatible behavior, which was discovered by accident. In World War II pilots got splinters of the PMMA canopy in their eyes, which showed no inflammatory response. PMMA found also its use in dentistry as an essential material to manufacture dentures [6, 84]. In the 1950s, a British surgeon from the University of Manchester, Dr. John Charnley, was the first to adopt "dental acrylic" for the cementation of an orthopedic prosthesis in total hip arthroplasty surgery [61]. However, the initial clinical results were poor because of mechanical and biological reasons [50]. After material modification, Dr. Charnley developed a new product called "bone cement" showing improved characteristics. From then on, modern PMMA bone cement evolved into a new stage, and the use of PMMA bone cement quickly expanded to the global orthopedic community. Especially after the Food and Drug Administration (FDA) approved the use of the bone cement technology in the United States [6].

Commercial PMMA cement is sold as a two-phase compound, consisting of a solid and a liquid part. The solid phase consists of the polymer, the catalyst of the polymerization reaction and the radio-opacifier, whereas the liquid phase is a mixture of the monomer, the reaction accelerator, and the stabilizer [64, 85]. The hardening time for most of the PMMA cements varies from 10 to 20 min [24]. In some cases, PMMA hardening lasts for weeks after implantation [32, 52].

7.2 Modifications of PMMA

7.2.1 Bioceramic Additives

The lack of bioactivity is one of the main reasons for aseptic loosening. Plain PMMA is bioinert [13, 29, 36]. Its surface is not favorable for adhesion, proliferation, and differentiation of osteoblasts [21]. Therefore, the fibrous layer forming at the PMMA-bone interface hampers the formation of a direct bone contact at the bone-PMMA interface, which leads to aseptic loosening (Fig. 7.2) [3, 36, 93]. To establish a direct chemical bonding between bone and PMMA cement, various osteoconductive bioceramics, like hydroxyapatite (HA), α-tricalcium phosphate (α-TCP), bioglass and calcium acetate, have been incorporated in plain PMMA to enhance the bioactivity of cement. HA is a well-known calcium phosphate ceramic that resembles bone mineral. Dalby et al. added HA to PMMA and investigated the biological response *in vitro*. They found that HA increased the proliferation and alkaline phosphatase (ALP) activity of primary human osteoblast-like (HOB) cells [26–28]. Further, energy dispersive X-ray analysis showed the calcium peak was absent for the plain PMMA, while present for PMMA/HA. HOB cell were found to adhere preferentially to HA particles exposed at the cement surface rather than the polymer matrix for PMMA/HA samples. Additionally, HOB cells appeared to show greater expression of vinculin adhesion plaques on PMMA/HA compared to PMMA [26, 27]. An increased HA amount in PMMA led to enhanced osteoblast adhesion and response [28].

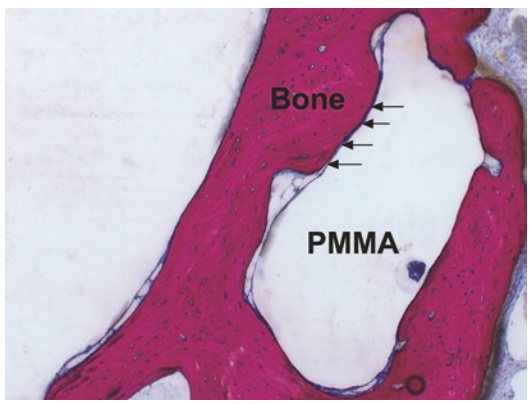

Fig. 7.2 Light micrograph showing PMMA cement implanted in the mandible bone of the rabbit. The PMMA became surrounded by a thin fibrous capsule, as indicated by the arrows

Itokawa et al. investigated the osteoconductivity and biocompatibility of HA-PMMA cement *in vivo*. HA-PMMA cement was implanted in the cranium of eight full-grown beagles with HA cement as a control. After 12 months, no inflammatory reaction was seen in any of the specimens. New bone formed at the interface of the HA-PMMA composite and adhered to the surrounding bone. Mixing HA and PMMA did not interfere with the physicochemical properties of the HA component and the newly formed bone was always found preferentially to HA particles exposed at the HA-PMMA composite surface rather than the PMMA, which indicates that the added HA enhanced osteoconductivity of the cement [42]. Other bioceramics, such as α-TCP, bioglass and calcium acetate, were also investigated and they showed similar improvement of the PMMA bioactivity as HA additives (Table 7.1). Fini et al. synthesized composite polymeric matrix (PMMA+α-TCP) and evaluated the *in vitro* and *in vivo* biological properties of composite with PMMA as the comparative material in the control group. The authors found PMMA+α-TCP significantly and positively affected osteoblast viability, synthetic activity and interleukin-6 level as compared to PMMA. Further, the PMMA+α-TCP implants in rabbit bone successfully osteointegrated in trabecular and cortical tissue after12 weeks. D. Arcos et al. incorporated bioglass into PMMA and found the modified cement kept the bioactive behavior of the glass. After soaking into SBF, nanocrystalline HCA layer was formed on the surface of PMMA-bioglass cement [5]. Sugino, A. et al. added calcium acetate into PMMA and found calcium acetate developed the osteoconductivity of PMMA [92].

Besides the improved bioactivity, the incorporation of bioceramic additives was also found to influence the bulk mechanical of characteristics of the cement (Table 7.1). Vallo et al. observed that the added HA particles acted as rigid fillers,

Table 7.1 Summary of PMMA cement additives for biological modification

Additive	Evaluation model	Results
Hydroxyapatite (HA)	*In vitro* primary human osteoblast-like (HOB) cells [26–28], human bone marrow cells (HBMC) [43], bone marrow mesenchymal stem cells (BMSC) [101]/*in vivo* full-grown beagles [42], *in vivo* ovine cancellous bone [101]/Mechanical testing at room temperature [97], Servohydraulic mechanical testing system [4]	HA increased proliferation, differentiation and alkaline phosphatase (ALP) activity of HOB and HBMC cells. Favorable bone response was observed on HA-PMMA interface of the cement. HA reduces fatigue failure of the surrounding bone. HA influences the flexural modulus and fracture toughness of cement.
α- tricalcium phosphate (α-TCP)	*In vitro* MG63 cells [34, 96]/*in vivo* rabbit [8, 34]	The added α-TCP affected positively osteoblast viability and expression of transforming growth factor beta 1 of MG63 cell. Favorable bone was observed at α-TCP-PMMA interface
Modified ceramics: Bioglass [5, 71, 88]/ Calcium acetate-MPS [92]	*In vitro* SBF soaking [5, 92]/*in vivo* rat [71, 88], in vivo rabbit [92]/Mechanical testing under wet condition test [71, 88]	The modified cement showed spontaneous apatite formation in SBF and better osteoconductivity when implanted *in vivo*. Bioglass-PMMA increased the mechanical characteristics.

which enhanced the fracture resistance and flexural modulus of PMMA [97]. This favorable effect occurred only when maximally 15 wt% HA was added. Mousa et al. reported that the incorporation of bioglass increased significantly the compressive strength, elastic modulus and fracture toughness of PMMA, but decreased the tensile strength [71].

7.2.2 Fillers

The significant difference in mechanical properties between PMMA and the surrounding bone is another major reason for aseptic loosening. After implantation, loading of the prosthesis results in transfer of mechanical forces from the prosthesis to the bone as well as from the bone to the prosthesis [31, 84]. However, plain PMMA is a stiff material showing a higher compressive strength [59, 107], lower fracture toughness [79, 94], lower fatigue strength and higher Young's modulus than bone [51, 54, 56, 59]. Such a mechanical mismatch between PMMA and bone causes the occurrence of interface fractures and aseptic loosening. To solve the problem, various fiber fillers were introduced as solid additives into PMMA. As the PMMA-fiber filler composite increased significantly fracture toughness and fatigue strength, it was supposed that the added fibers would enhance the transfer of external load as well as hinder crack propagation through the cement.

Different types of fibers were used as additives into PMMA. For example, Topoleski et al. incorporated 1, 2 and 5% biocompatible titanium-fibers by volume into PMMA and found that 5% titanium fibers-reinforced cement increased the fracture toughness by up to 56% [94]. The underlying mechanism is the energy absorption contribution of plastic deformation and ductile fracture of titanium fibers, which leads to high energy required at break. In the other study, Khaled et al. introduced 0 to 2 wt% nanostructured titania fibers into the cement matrix, hypothesizing that nanostructured titania would provide a substantially higher interfacial area for load transfer compared to their micro counterparts. The authors found that 1 wt% titania fibers were the optimal concentration, which resulted in a significantly increased fracture toughness (63%), flexural strength (20%) and flexural modulus (22%) with maintenance of the handling properties and *in vitro* biocompatibility of PMMA in comparison with the fiber absence cement [47]. To increase the biocompatibility, they further modified titanium dioxide nanotubes (n-TiO_2) with strontium oxide (SrO) and added 1,

2, 4 and 6 wt% of the n-TiO_2-SrO into PMMA matrix. PMMA-2 wt% of n-TiO_2-SrO was found to be the optimal combination showing a more uniform dispersion and stronger adhesion of the nanotubes in the PMMA matrix. This formulation significantly increased the fracture toughness (20%) and flexural strength (40%) in comparison with the control PMMA matrix (unfilled n-TiO_2-SrO). The use of nanotubes reinforced the brittle PMMA matrix by bridging the crack at the interface. Moreover, the incorporated SrO exhibiting a significant enhancement of osteoblast cell proliferation *in vitro* [46].

Besides titania-based fibers, carbon and rubber fibers have also been used as additives into the PMMA matrix. Bowman et al. found that carbon fibers [10] could prevent the formation of cracks in PMMA. Moseley et al. dispersed rubber particles into the PMMA matrix and reported that the fracture toughness of the experimental material was significantly greater than a non-toughened control. However, the elastic modulus and compressive strength of rubber-incorporated PMMA were reduced [70, 79].

7.2.3 Antibacterial Agents

Deep infection is a serious complication of total joint arthroplasty (TJA). According to literature, periprosthetic joint infection (PJI) is responsible for 16.8% of the failures of all total knee arthroplasty (TKA) revision surgeries and 14.8% of the failures of all hip revision surgery [11, 12, 23, 109]. Therefore, the use of antibiotic-loaded bone cement (ABC) for the prophylaxis and treatment of PJI has been documented since the pioneering work of Buchholz and Engelbrecht, who incorporated antibiotics in PMMA in the 1970s [14, 15]. From then on, numerous antibiotic-impregnated cements were tested and PMMA cement was found to be a good carrier for the release of antibiotics at the site of infection [2, 35, 67] (Table 7.2). There is general consensus that the use of ABC is a prophylactic and lowers the deep infection rate in primary TJA [55, 103, 104, 106]. Due to these convincing clinical results, ABCs have become the standard of care in reducing primary infection and improving the success rate of orthopedic implants in most parts of the world [61].

Based on the dose level of antibiotics, ABC can be used for treatment as well as prophylaxis. As a rule, at least 3.6 g of antibiotic per 40 g of PMMA cement is required for treatment to achieve a sufficient therapeutic level of antibiotics during their elution from the PMMA cement [44, 76]. In contrast, prophylaxis requires low doses of antibiotics, which is defined as ≤1 g of powdered antibiotic per 40 g of PMMA cement [44]. Gentamicin was the first and is still the most common antibiotic incorporated into PMMA cement, which is based on its broad-spectrum activity, low allergy profile, high water solubility and superior thermal stability [6, 61, 108]. Clinical data proved that a significant level of gentamicin is obtained in the vicinity of the cement, even up to 5.5 years after implantation [102]. Other less commonly used antibiotics are cefuroxime, vancomycin, tobramycin, oxacillin, cefazolin, erythromycin and colistin (Table 7.2). They are used alone or together with gentamicin. It is known that the antibiotic release from bone cement is a complex process [53, 76]. Type of antibiotic could affect the elution characteristics of antibiotics. Penner et al. designed three groups of antibiotic loaded PMMA. There were two control groups, one incorporating vancomycin and the other tobramycin, and one experimental group in which the two antibiotics were combined. The elution of tobramycin from the disks in the study group was increased by 68% over that of the tobramycin control group. The release of vancomycin from the study group disks was increased by 103% over the vancomycin control group. Combining two antibiotics in bone-cement improves elution of both antibiotics *in vitro*.

Moreover, type of bone cement also influenced the elution effect of antibiotics [16, 75]. Penner et al. compared three different bone cements, namely. Palacos-R, CMW 1, and CMW 3. Vancomycin and tobramycin were loaded into different cements. During the study period, the cement disks were placed in saline baths for 9 weeks and the baths were periodically refreshed.

Table 7.2 Summary of antibiotic-impregnated PMMA cement

Antibiotics	Evaluation model	Results
Gentamicin	Gentamycin bone cement (GBC) in infected bone [48]/Prophylactic effect of GBC in 1688 THA patients [45]/GBC in 1542 Charnley low-friction arthroplasties [63]/GBC in 15 patients with THA [102]/GBC in 10 patients with THA[95]	Prophylactic effect of gentamicin cement against deep infection after THA was effectively proven. Further, GBC allows local antibiotic therapy in high concentration. Gentamicin can be continually released from cement for a long time.
Cefuroxime	178 patients with cefuroxime-impregnated cement compared with 162 patients with cefuroxime free cement [18]/41 patients with cefuroxime-impregnated cement compared with 37 patients with cefuroxime free cement [19]	Cefuroxime-impregnated cement was effective in the prevention of deep infection in primary TKA patients
Other antibiotics	Vancomycin [20, 39]/colistin [90]/gentamicin combined with vancomycin [7, 67]/Gentamicin combined with colistin/erythromycin [33]/Vancomycin, tobramycin [66]/Vancomycin, tobramycin and their combinations [76]/Oxacillin, cefazolin, and gentamicin [65]	Acrylic cement with antibiotic had a bacteriostatic effect. Antibiotic-loaded cement showed improved prophylaxis effect than antibiotic-free cement. The antibiotic can be released for several months from the cement *in vivo*. The use of combination therapy may lead to a better synergistic antibiotic effect and improve the long-term elusion profile *in vivo*.

For both antibiotics, there was no significant difference in antibiotic release between CMW 1 and CMW 3 cement. But the *in vitro* elution characteristics of Palacos-R are superior to CMW1 and CMW2. Cerretani et al. compare the elution characteristics of vancomycin alone and in combination with imipenem-cilastatin from 3 bone-cements (CMW1, Palacos R, and Simplex P). The cement disks were placed in saline baths for 5 weeks and e baths were periodically refreshed. Results showed CMW1 had better elution characteristics than the other cements when treated with vancomycin alone; the elution of Palacos R and Simplex P were better than that of CMW1 when vancomycin was combined with imipenem-cilastatin.

Furthermore, the mixing condition would influence the elution effect of antibiotics [57, 72]. As known, the antibiotic is manually blended with the cement powder at the initial stage of the surgery procedure in the United States. While in Europe, the antibiotic was blended using an industrial mixer and pre-packaged for the commercial sell [57]. Lewis et al. investigate the influence of the method of blending gentamicin sulphate with the powder of the PMMA cement [57]. The blending methods used were manual mixing (the MANUAL Set), use of small-scale mechanical powder mixer (the MECHANICAL Set) and use of large-scale industrial mixer (the INDUSTRIAL set). It was found the elution rates of antibiotic from the MANUAL and the MECHANICAL Sets were about 36% lower than from the INDUSTRIAL averagely. This finding showed consistency with the study by Neut et al. [72]. They investigated the effect of mixing method of gentamicin powder (manual versus industrial) with three commercially available PMMA cements. The manual mixing cement leaded to a lower release of antibiotics than that in corresponding pre-industrial mixing antibiotic-loaded cements. These results were probably due to the difference between the distribution of the antibiotic in the cured MANUAL or MECHANICAL cements and the INDUSTRIAL cements.

7.2.4 Porogens

The inclusion of porogens to generate open porosity was considered as an effective way to improve the inert bone behavior of PMMA [62]. The advantage of introducing porosity in PMMA is that the pores allow the migration of cells, create sufficient space for nutrient transportation, tissue infiltration, vascularization and ultimate bone ingrowth. This creates interlocking and

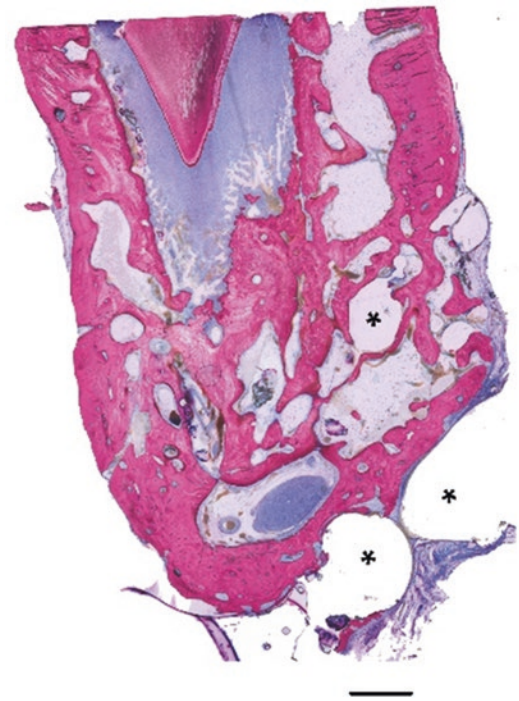

Fig. 7.3 Histological evaluation of bone formation and tissue response to porous poly(methyl methacrylate) (PMMA) cement in a rabbit mandibular model. The image shows bone ingrowth within porous PMMA. Black asterisks indicate PMMA component, appearing as empty voids. The scale bar stands for 1000 mm. (Reproduced from Sa Y et al., [83]. Reprinted with permission from TISSUE ENGINEERING: Parts C, May 2017, by Sa Yue, et al., published by Mary Ann Liebert, Inc., New Rochelle, NY)

anchorage of the PMMA into the bone and prevents the aseptic loosening [13, 25, 103, 104, 106] (Fig. 7.3). Moreover, due to the typical porosity-mechanical property relationship in materials, the mechanical properties of cement can be easily tailored by the amount of porogen [86, 87, 103, 104, 106]. Last but not least, porogens, being often water-soluble materials or hydrogels, can effectively reduce the temperature increase during the PMMA polymerization by acting as a heat sink.

Porous PMMA cement can be produced by mixing the highly viscous, aqueous biodegradable carboxymethylcellulose (CMC) gel with the hydrophobic PMMA cement. This method was developed by de Wijn et al. [29]. The produced porosity was shown to have a beneficial effect on decreasing the stiffness as well as polymerization temperature of the PMMA cement. Van Mullem et al. evaluated the *in vivo* biological response of CMC-based porous PMMA cement [98–100]. They implanted porous PMMA cement into the frontal and parietal bone of pigs with dense PMMA cement as control. Cell colonization, blood vessel ingrowth, and bone deposition were observed within pores. In contrast, the dense PMMA became encapsulated by connective tissue [100]. Bruens et al. evaluated the application of porous PMMA cement for the correction of human craniofacial defects. The CT-scan results confirmed that most of the porous PMMA cement specimens showed calcification at the outer PMMA surface, while one out of four showed deeper calcifications into the cement at a maximum of 4.5 mm. These results confirmed the occurrence of bone ingrowth into the porous PMMA. However, it was also noticed that parts of the porous PMMA showed no calcification at all. This can be because porous PMMA per se lacks osteoconductivity.

Besides the aforementioned application as bone cement orthopedic devices or augmentation of cranial defects, porous PMMA was also tested as a temporary space maintainer for two-stage craniofacial reconstruction. The Mikos' group performed a series of *in vivo* and *in vitro* studies for such an application [49, 89, 103, 104, 106]. In agreement with de Wijn et al. [29], they observed that porous PMMA decreases maximum polymerization temperature and reduces compressive strength as well as bending modulus compared to solid PMMA [103, 104, 106]. *In vivo* evaluation showed a beneficial effect of porous PMMA on soft tissue coverage. In comparison with dense PMMA, porous PMMA constructs for temporary space maintenance can provide a template for soft tissue regeneration, priming the wound bed for a definitive repair of the bone tissue with greater success [89].

7.2.5 Biological Agent

In addition to the above-mentioned components, biological agents were added to PMMA cement to increase its bone behavior. Growth hormones are known to play a central role in the development and growth of skeletal tissues as well as in the control and local regulation of calcium and phosphorus concentration [30] [78]. Downes et al. mixed 2.5 mg human Growth Hormone (hGH) with 10 g of PMMA to prevent cement loosening. The release of hGH from PMMA blocks into elution fluid was monitored *in vitro* and the hGH-loaded PMMA was inserted into the femur of rabbit to evaluate the *in vivo* performance of cement. The *in vitro* release of hGH continued to 40 days, indicating the successful loading of hGH into PMMA. Histological analysis of the rabbit specimens indicated that after 1 month of implantation a greater percentage of osteoid was present at the hGH-loaded cement surface than at the hGH-free cement. Evidently, the hGH-loaded cement enhanced the interaction between bone and cement [30]. Pritchett et al. evaluated the effect of hGH-loaded PMMA in a clinical study. They mixed 20 mg hGH with 40 grams of PMMA. The hGH-loaded PMMA and hGH-free PMMA cements were used in 30 patients for hip replacement treatment (n = 15). The levels of hGH secretion were high at the beginning of cement implantation, but then fell rapidly within the first 72 h. No complications or adverse reactions were noted by the addition of hGH[78]. However, the clinical data could not confirm that hGH-loaded PMMA enhanced the fixation of the hip prostheses.

Blood was also used as an additive into PMMA. Ahn et al. mixed different volumes of human blood with PMMA [1]. They found that blood reduced significantly the Young's modulus of PMMA to that of the osteoporotic vertebral body and also decreased the polymerization temperature. This effect of blood can decline the level of stress to the adjacent vertebrae and prevent thermal injury to the nerve tissue.

7.2.6 Mixed Additives

To fulfill the clinical need, frequently two or more additives were combined to create a multifunctional PMMA composite (Fig. 7.3 and Table 7.3). For example, Arcos et al. mixed bioglass and gentamicin with PMMA. The PMMA/bioglass/gentamicin composite demonstrated a sustained drug release and a favored the osteogenic response of bone cell cultures due to the bioglass [5]. However, no *in vivo* study was performed to confirm these *in vitro* results. He et al. mixed bioglass and calcium sulfate (CS) with dense PMMA and investigated the *in vitro* and *in vivo* reactions of this composite [40]. The bioglass enhanced the attachment, proliferation and osteogenic differentiation of preosteoblast cells. The dissolution of CS created porosity on the surface of PMMA, which allowed the ingrowth of bone in a rabbit femoral condyle model. However, the bone ingrowth was limited to the surface and bone did not penetrate into the internal structure of PMMA cement. Therefore, recently a series of novel cements consisting of PMMA, porogen and functional fillers were fabricated and evaluated. Lopez-Heredia et al. [59] and Sa et al. [80–83] fabricated PMMA/CMC porogen/CaP cement. The CMC hydrogel acted successfully as a porogen, which decreased the T_{max} during cement polymerization and adjusted the mechanical properties of cement close to the cancellous bone. The added CaP increased the biomineralization ability of PMMA cement. Furthermore, an *in vivo* study showed that after 12 weeks of PMMA/CMC porogen/HA implantation, the majority of the porosity was filled with newly formed bone. The presence of HA in the PMMA enhanced bone formation and bone was always in direct contact with the HA particles. Shi et al. mixed colistin with CMC porogen. The modified PMMA/CMC/colistin showed proper mechanical characteristics and a safe T_{max}. The cement released colistin even up to 5 weeks *in vitro*. Inspired by the PMMA/CMC porogen approach, Shi et al. [87], Sa et al. [80, 82] and Wang et al. [107] fabricated gelatin

Table 7.3 Summary of multifunctional modifications in PMMA cements

Purpose	Modifications	Results
To enhance the bioactivity and antibacterial property	Added gentamicin sulfate and SiO_2-CaO-P_2O_5 bioactive glass into the PMMA cement [5].	The novel cement could be used as drug delivery system to release the drug sustainably without weakening the bioactive behavior of the glass.
To promote the bone ingrowth into cement, improve the bioactivity and mechanical property of PMMA cements.	Added bioglass and CS into PMMA to produce a composite with porous surface. *In vitro* cellular behaviors test and *in vivo* bony response were evaluated [40]	The PMMA-CS-bioglass cement showed proper handling characteristics and adequate mechanical properties. Further, PMMA-CS-bioglass cement showed increased degree of attachment, proliferation and osteogenic differentiation of preosteoblast cells *in vitro* and higher interfacial bonding strength *in vivo*.
To establish the multifunctional PMMA cement	Carboxymethylcellulose (CMC) hydrogel with/without functional additives was mixed with PMMA [59, 62, 80–82, 86, 103, 104, 106]	Interconnected porosity was successfully imparted by CMC hydrogel. Functional additives could be loaded into CMC hydrogel. Modified PMMA cement showed improved performance by the beneficial effects of additives on bioactivity, mechanical property and drug release.
To establish the multifunctional PMMA cement	PMMA cement was mixed with gelatin hydrogel [87]/CS–glycerophosphate (CS–GP) hydrogel [80, 82]/CS–poly (vinyl alcohol) (CS–PVA) hydrogel[107]	Gelatin hydrogel and CS-based thermosensitive hydrogel can also produce interconnected porous structure. Desired property can be obtained by the loaded additives.

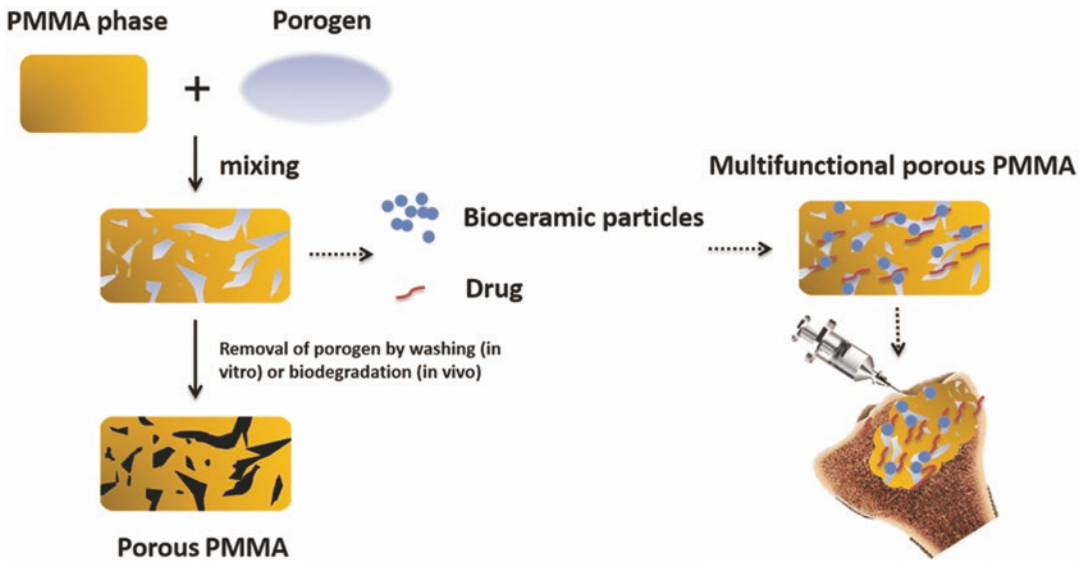

Fig. 7.4 Schematic diagram for the preparation of porous as well as multifunctional porous PMMA

hydrogel or CS based thermo-sensitive hydrogel (CS-GP and CS-PVA hydrogel) as porogens and these porogens meantime load antibiotic or nano-HA for multifunctional properties (Figs. 7.4 and 7.5). It was found that interconnected porous structures were created by the porogens. Porogens and the loading nano-HA particle and antibiotics produced multifunctional PMMA cements with the proper mechanical property, safe T_{max}, prolonged working time, increased mineralization capacity and enhanced anti-bacterial activity. However, data are from *in vitro* studies and more animal studies with these multifunctional PMMA cements have still to be performed.

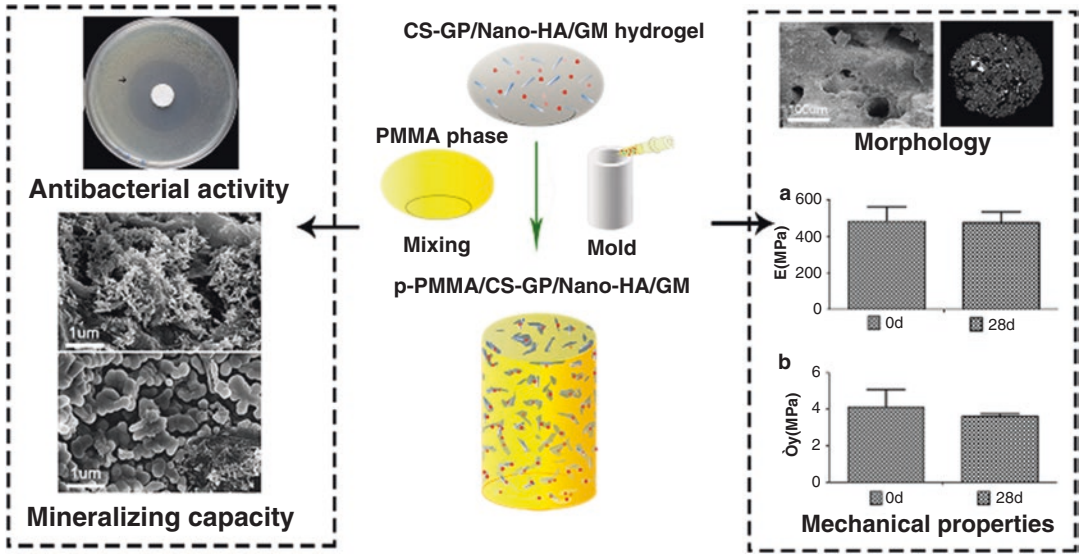

Fig. 7.5 To improve the performance of PMMA, artificial extracellular matrices like chitosan–glycerophosphate (CS–GP) thermosensitive hydrogel and osteoconductive nano-sized hydroxyapatite (nano-HA)/antibiotic gentamicin (GM) were introduced into PMMA. CS–GP effectively created open pores at the surface of the PMMA cement and the obtained cement had a proper modulus of elasticity and a compressive yield strength. Furthermore, the incorporated nano-HA particles sufficiently increased the mineralization capacity of the cement without compromising its mechanical properties and the incorporated GM remarkably enhanced the anti-bacterial activity of the cement. (Reproduced from Graphical Abstract of Sa Y et al., [80, 82])

7.3 Conclusion and Future Perspectives

Several recently developed bone cements are available in orthopedic surgery, but the application of PMMA-based cement in arthroplasty, vertebral augmentation procedures and bone filling treatment after tumor resection has still a major impact on orthopedic surgery. PMMA cement is a highly flexible biomaterial with desirable characteristics, i.e. sufficiently strong to provide mechanical support, moldable to fill complex defects, cost efficient, and FDA-approved. Although the drawbacks associated with MMA cement can cause clinical complications, the use of additives, such as bioceramics, fillers, antibiotics, porogens and biological agents, can produce a material with much more favorable properties. The beneficial effect of porous PMMA cement on bone ingrowth, drug release and as space maintainer have already been proved in the field of reconstructive surgery.

Therefore, more attention should be paid to design PMMA based cement with added functionality. An elaborated and improved selection of porogens and functional additives will further enhance the targeted function of porous PMMA based cement. By all means, long-term, prospective, multicenter and randomized clinical trials have to be performed to confirm the favorable effect of these multifunctional PMMA cements.

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8 Intrinsically Conductive Polymer Nanocomposites for Cellular Applications

Özge Lalegül-Ülker, Ayşe Eser Elçin, and Yaşar Murat Elçin

Abstract

Intrinsically conductive polymer nanocomposites have a remarkable potential for cellular applications such as biosensors, drug delivery systems, cell culture systems and tissue engineering biomaterials. Intrinsically conductive polymers transmit electrical stimuli between cells, and induce regeneration of electroactive tissues such as muscle, nerve, bone and heart. However, biocompatibility and processability are common issues for intrinsically conductive polymers. Conductive polymer composites are gaining importance for tissue engineering applications due to their excellent mechanical, electrical, optical and chemical functionalities. Here, we summarize the different types of intrinsically conductive polymers containing electroactive nanocomposite systems. Cellular applications of conductive polymer nanocomposites are also discussed focusing mainly on poly(aniline), poly(pyrrole), poly(3,4-ethylene dioxythiophene) and poly(thiophene).

Keywords

Intrinsically conductive polymers · Conductive polymer composites · Tissue engineering · Electrical stimulation

8.1 Introduction

Intrinsically conductive polymers (ICPs) have attracted considerable interest in biomedical applications due to their unique functions. Electrical stimulation can be delivered directly to cells by using ICPs and their composites in biomaterial form. Not every electrically conductive material is suitable for use in biomedical applications. Most fundamentally, materials must display biocompatibility and biodegradability for biomedical use. In addition, tunable semiconductor and conductor properties of these polymer systems make them an important class of materials that can be used in many applications, including artificial tissue development [38], controlled drug release [26, 72], neural therapy [66] and the development of biosensors [21, 91].

Conductive polymer systems used for biomedical applications are developed in order to provide the desired functionality. Uniformly dispersed components in conductive polymer

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composites provide the essential mechanical, electrical, optic and chemical functionalities. There are two different strategies used to obtain a conductive polymer system. In the first approach, conductive polymer system can be obtained by incorporating conductive fillers such as graphene, carbon nanofibers and gold nanoparticles into the polymer matrix [1, 83, 95]. These materials ensure the transmission of electrical signals from an external source, to the cells. However, long term effects of these particles in the biological systems are not yet known. Besides, it is difficult to achieve uniform dispersion of fillers in the polymer matrix for at least two phase composite systems, so conductive properties of these polymer composites are affected negatively. In the second approach, conductive polymer systems can be obtained by using intrinsically conductive polymers which are soluble in organic solvents and miscible with other polymers or inorganic components prior to processing [7, 40, 41, 64, 94]. Polymer composites gain a homogeneous structure, since the polymers can be dissolved in one another. Thus, the electrical signals can be transmitted more efficiently and each cell is equally affected by the electrical stimulation. Intrinsically conductive polymers and their composites have considerable potential in biomedical applications due to these outstanding features. There are many prominent reports that involve ICPs as biomaterials.

In 1977, Hideki Shirakawa et al. found that the conductive poly(acetylene) (PAc) films doped with chlorine, bromine and iodine vapor were 10^9 times more conductive than their undoped state [79]. The discovery of conductive polymers was awarded the Nobel Prize in Chemistry in 2000 [57, 78]. Addition to PAc, the intrinsically conductive polymer family contains poly(pyrrole) (PPy), poly(aniline) (PANI), poly(thiophene) (PTh), and poly(3,4-ethylenedioxythiophene) (PEDOT), etc. which have high potential due to their ease of synthesis, chemical stability, biocompatibility and mechanical properties (Fig. 8.1).

Tissue engineering is a branch of regenerative medicine, which is the interdisciplinary field including biology, medicine, chemistry and materials science. Scaffold design for tissue engineering applications is specific for characteristic cell properties, such as attachment, migration, proliferation and differentiation [20]. The use of ICPs as biomaterials is particularly important for electrically sensitive cells, including nerve, muscle, bone, and cardiac cells. This chapter focuses on the structure and unique properties of ICPs, and different fabrication routes of ICPs for tissue engineering applications.

8.2 Intrinsically Conductive Polymers

8.2.1 Conductivity

Metals such as copper and silver have the high electrical conductivity in the range of 10^4–10^6 S cm^{-1} while this value is less than 10^{-12} S cm^{-1} for conventional polymers. Polymers have been known as electrical insulators since their first use [e.g. poly(ethylene)]. Due to their insulating nature, they are still widely used for sheathing of electrical cables [15]. But, this view has changed with the discovery of poly(acetylene) which is an intrinsically conductive polymer (ICP) which conducts electricity almost as efficient as a metal [79]. The structure of ICPs is considerably different from conventional polymers. Unlike other polymers, ICPs show electrically conductive features due to consecutive π-electron bonds along the polymer chain. Alternating double and single bonds lead to higher electrical conductivity by facilitating the movement of the electrons and charge transfer. Valence electrons of sp^3-hybridized carbon atom in carbon-carbon single bond are located in the four hybrid orbitals, since the stimulation of electrons in the C-C bond requires relatively higher energy and these compounds have a fairly wide band gap [29]. However, sp^2 and sp-hybridized compounds containing double and triple bonds have also p orbitals containing electrons that do not participate in hybridization.

In polymers with long conjugation chain, π bonds overlap the p orbitals. Hence, π electrons of the π bond provide metallic conductivity.

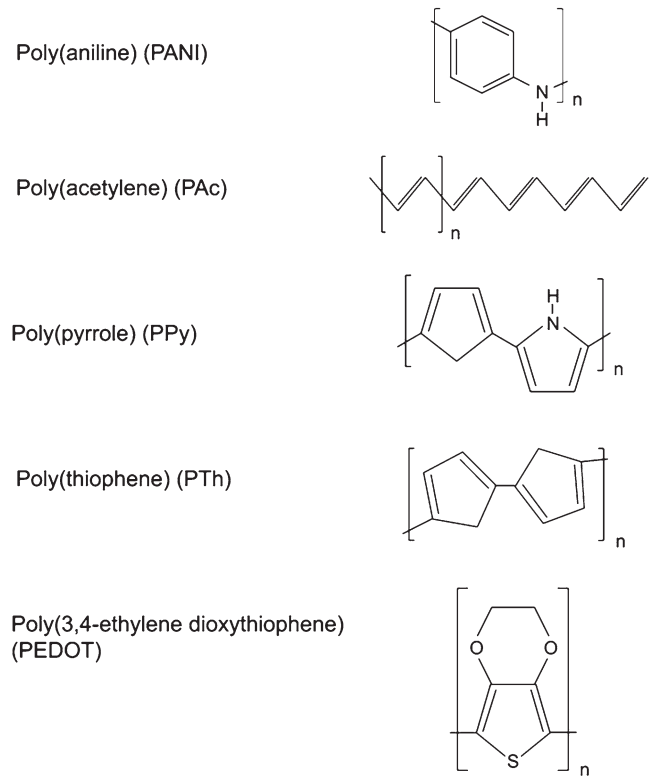

Fig. 8.1 Intrinsically conductive polymers

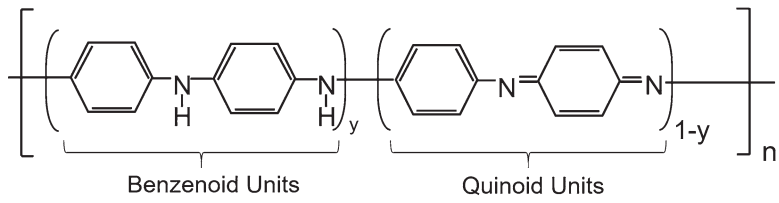

Fig. 8.2 Chemical structure of poly(aniline)

There are many electrically conductive polymers however; few of them can be processed for biomaterial applications (e.g. PANI, PTh, PPy).

Among π-conjugated ICPs, PANI draws considerable interest due to its superior features such as good environmental stability, doping/dedoping chemistry, easy synthesis, and its monomer being inexpensive. The main structure of PANI comprises two basic parts. The fully reduced part 1 contains repeating units that have two benzenoid rings. The fully oxidized part 2 contains repeating units having a benzenoid ring and a quinoid ring (Fig. 8.2).

While benzenoid rings react with oxidative agents, the quinoid rings react with the reducing agents. PANI consists of nitrogen heteroatoms in either amino or imino forms. There are three main forms of PANI associated with its oxidation state; reduced leucoemeraldine, oxidized pernigraniline and semi-oxidized emeraldine forms where nitrogen atoms are in the form of amino, imino and amino/imino, respectively (Fig. 8.3).

PANI is an electrical insulating structure in Leucoemeraldine base (y = 1), which consists of a series of benzenoid rings connected by secondary amines. Leucoemeraldine form can be oxidized giving two electrons, each tetramer unit and transform into the conductive emeraldine salt in acidic medium. The fully oxidized pernigranilin (y = 0) can be synthesized by the oxidation of emeraldine (y = 0.5). The partially oxidized emeraldine-base becomes the conducting

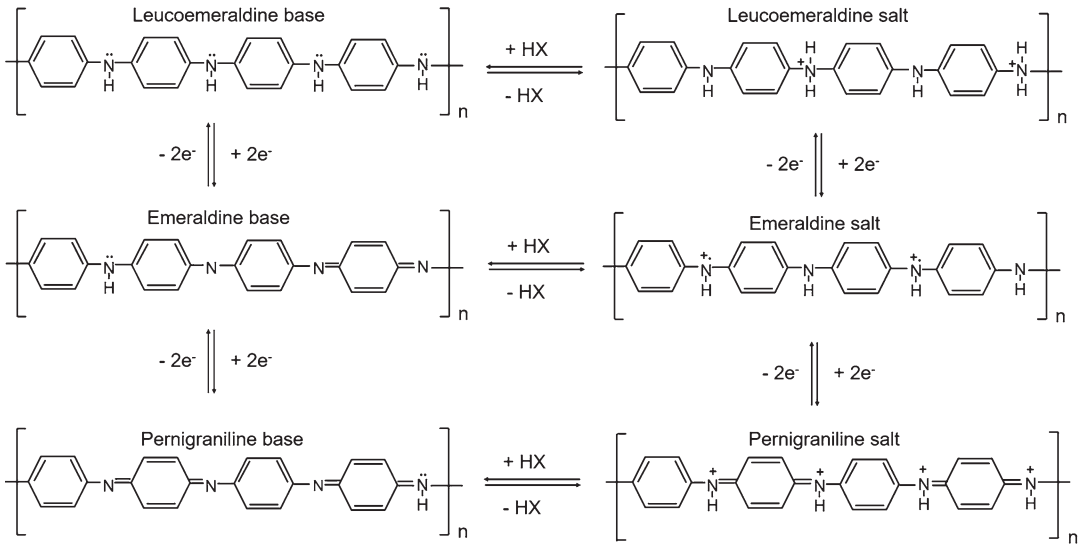

Fig. 8.3 Three major oxidation states of poly(aniline) (PANI)

emeraldine-salt and conductivity increases from 10^{-10} ohm^{-1} cm^{-1} to 100 ohm^{-1} cm^{-1} after protonation. The three major oxidation states of PANI are required for the doping process to gain electrical conductivity [53].

8.2.2 Doping Process

Conjugated polymers have semiconductor properties. Conjugated structure is not sufficient alone to demonstrate high conductivity. "Doping" process is used to improve the conductivity of the ICPs. Chemical or electrochemical methods lead to either formation of positively charged gaps corresponding to oxidation (p-doping) or formation of negatively charged areas corresponding to reduction (n-doping) in the lattice [49]. Conductivity increases with the increase in the capacity movement of electrons and gaps. Chemical doping methods use dopants such as, AsF_5, I_2, SbF_5, $AlCl_3$. Conductive polymer salts can be incorporated into the structure, while electrochemical methods provide formation of cations and anions by applying a potential difference.

The main mechanism of charge transport in conducting polymers is based on polarons and bipolarons (Fig. 8.4). Oxidation breaks double bonds in the polymer chain, causing the formation of positively charged radicals called the polaron. The number of polarons increases in the presence of excessive amounts of dopant, as a consequence, polarons interact to form bipolarons. In brief, with the doping process, polarons and bipolarons are formed on the energy levels in the band gap.

8.3 Intrinsically Conductive Polymer Nanocomposites

8.3.1 Processing

ICPs have a wide potential for biomedical applications (e.g., biosensors, drug delivery systems, cell culture technologies, and possibly tissue engineering). Some materials are insufficient alone to provide the desired purposes, limited by specifications such as biocompatibility, mechanical strength, conductivity, processability. Composites are prepared by combining two or more such components to overcome these challenges. Nanocomposite materials comprise of at least one component in the nano-scale range. ICPs can be combined with nanostructured com-

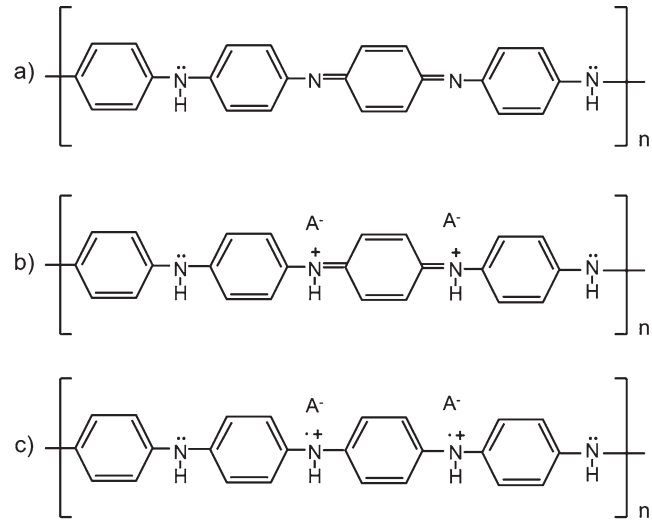

Fig. 8.4 (**a**) Unprotonated emeraldine base; (**b**) bipolaron; (**c**) polaron

ponents and other polymers to add properties and functionalities that surpass individual components. Composites or blends of ICPs can be enhanced and tuned to improve processability, mechanical properties, biocompatibility and conductivity of conventional polymers [5, 11, 51]. ICPs can be doped with nanoparticles (NPs), nanotubes, nanowires or several biomolecules to improve their electrical, optical, mechanical, and biological properties [36, 39, 90]. Intrinsic properties of the conductive polymer and the combined materials in question are factors that define the suitability and functionality of the composite. Low solubility in common solvents, poor processability and sub-par mechanical properties restrict the potential applications of conjugated polymers.

PANI is considered a unique ICP due to its adjustable electrical properties and metal-like electron transport characteristics at both room temperature and lower temperatures. PANI has received considerable attention both in the academia and industry. Nanostructured PANI composites have been used to develop useful devices with enhanced performance, including chemical and biological sensors [34, 42, 106], microelectronic devices [45, 81], electromagnetic shielding devices [22, 59], anticorrosion coatings [50], light emitting diodes [33], electrocatalysts [60, 97], biosensors [30] and biomaterials [54, 65, 67, 68].

There are three different approaches for the synthesis of conductive composite materials depending on the aim of use. The first approach is to dope ICPs with nanostructured materials [48, 102]; the second approach is to dope polymer matrix with nanostructured ICPs [8, 13]; and the third approach is to prepare ICP-polymer blends [10, 75]. In the first and second approaches, in order to obtain a homogeneous structure, nano-sized components must be dispersed in the matrix. However, in the third approach, the polymer-polymer blends mix more homogeneously within one another.

Chen et al. [12] have shown that increasing concentrations of PANI in poly(ε-caprolactone) (PCL) solutions caused an increase in the conductivity of PANI/PCL composite scaffolds from non-detectable for pure PCL fibers, up to 63.6 ± 6.6 mS cm^{-1} for PCL with 3 wt. % PANI mass ratio. On the other hand, increasing concentrations of PANI causes an increase in the Young's modulus of PANI/PCL composite scaffolds from 7.2 ± 5.0 MPa for pure PCL fibers, and up to 55.2 ± 3.6 MPa in 3 wt.% PANI mass ratio, PANI/PCL. This is because PANI has a rigid backbone and low processability; causing reduction of elasticity of the nanofibrous PANI/PCL scaffolds [12]. It is crucial to blend PANI with elastic polymers to overcome mechanical limitations to develop conductive biomaterials.

8.3.2 Biocompatibility and Biodegradation

Since biological systems interface with biomaterials and biosensor surfaces, ICPs containing conductive scaffolds must be non-cytotoxic, should support viability and proliferation of cells. Maráková et al. [52] compared the cytotoxic effects of PPy and PANI, which were used as coating materials for cotton fabric. The cytotoxicity results showed that viability of cells cultured in the presence of PANI reduced compared to cells cultured in presence of PPy. However, in the presence of silver NPs, both of PANI and PPy-coated groups showed cytotoxic effects, proportional to the amount of silver. PANI coatings were significantly cytotoxic because of the low molecular-weight impurities contained in PANI [52]. PANI has a unique molecular structure among other ICPs. It can be modified by reprotonation/deprotonation process to both arrange the conductivity and remove by-products. This process also provides significant increase in the biocompatibility of PANI. Various oxidation states and concentrations of PANI samples were evaluated in relation to biocompatibility were also investigated in the work of Humpolíček et al. [31]. It was found that PANI samples caused allergic response, induced cytotoxicity in terms of metabolic activity in two different cell types, *i.e.* human immortalized non-tumorigenic keratinocyte cells and human hepatocellular carcinoma cells. However, cytotoxic effect was attributed to by-products and residues rather than to the structure of polymer. This study showed that use of PANI is advantageous as it enhances biocompatibility by deprotonation/reprotonation cycles, which removes by-products and residues [31]. Nanomaterials have a crucial role for biomedical applications. They can affect cell internalization, inflammatory response, and cell properties such as adhesion, migration and differentiation. The form and size of nanostructured ICPs have also considerable effects on their cytocompatibility. Oh et al. [62] evaluated the cytotoxic effects of PEDOT's morphology on lung fibroblasts (IMR90) and alveolar macrophage (J774A.1) cell lines. They demonstrated shape-dependent cytotoxicity of PEDOT nanomaterials by measuring the amount of intracellular LDH released as a marker of membrane integrity and showed that cytotoxicity decreased from ellipsoids to rods and tubes [62]. Likewise, morphology of PANI nanomaterials has similar effect with PEDOT nanomaterials on cytotoxic response in lung fibroblast cells (IMR90) [61].

ICPs may cause biological toxicity depending on their morphology and surface characteristics; and a decrease in cytotoxicity may be expected if the ICP surface is not directly associated with cellular components. Researchers have developed new strategies to solve these problems, and have been able to improve the biological properties of ICPs for potential tissue engineering applications. These strategies are based on their combination with biocompatible and biodegradable components (organic or inorganic) to form composite systems. Xu et al. [98] have reported the toxic effects of PANI on PC12 cells. However, the toxicity level decreased with the use of PANI/poly(L-lactic acid-co-ε-caprolactone) (PLCL) copolymer in the composite nanofiber structure [98]. Electrically conductive polymer composites or blends are usually prepared by combining ICPs with various substances to improve biological, chemical and physical properties such as biodegradability, biocompatibility and processability. By physical and chemical modifications, ICPs can strengthen the polymer matrix while also providing new electrical properties to the composite structure.

The developed material must be biocompatible and biodegradable for biomedical applications. Biodegradable synthetic polymers such as, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolic acid) (PLGA) have advantages due to their ease of synthesis and modification. In addition, the mechanical properties of these synthetic polymers are atop of natural polymers. However, biocompatibility depends on polymer-tissue interaction. Natural polymers have a more efficient interaction with the biological environments, as the natural polymers can support and direct cell behavior through their specific molecular domains. In a study by Rajzer and colleagues, electrospun PCL/gelatin scaffold

modified with calcium phosphate NPs and osteogenon were used to prepare a bilayer composite scaffold. Structure was formed by incorporation of the conductive PANI. It was demonstrated that the conductive hybrid scaffold directs some aspects of cell behavior including attachment, proliferation, migration, and differentiation [70]. It is necessary to improve the crucial properties of conductive polymer systems, such as biodegradability, biocompatibility and mechanical strength, for their use in biomedical applications. Due to lower biodegradability of conductive polymers, addition of hydrolyzable side groups to the monomer and development of conducting polymer–biodegradable polymer composites could be important for tissue engineering [101]. Composite systems containing ICPs allow improvement in both biodegradation and biocompability of the material. To date, great numbers of studies have been carried out to improve ICP's biodegradability, for their potential use in tissue engineering applications. Limited biodegradability of ICPs prohibits their application in biological environments such as the human body. However, this limitation can be overcome by blending ICPs with biodegradable polymers and modifying with various side groups [2].

Jaymand et al. [35] fabricated a biodegradable, biocompatible ICP-composite, made up of hyper-branched aliphatic polyester, PTh, and PCL. As PTh is difficult to process and has poor mechanical properties, its sole use as a biomaterial is limited. Conductive PTh was blended with PCL and hyperbranched aliphatic polyester in order to improve the mechanical properties and biodegradability for regenerative medicine applications. Results showed that composite material containing PTh did not show cytotoxic effect on mouse osteoblast MC3T3-E1 cell line, and showed an improvement in cell proliferation as compared to non-treated group [35]. PPy modified gold surfaces could maintain the adhesion and proliferation of bone marrow derived multipotent mouse stromal cells, and no toxic effects were reported for the duration of the study [89]. These results show that PPy may be used to maintain stem cell viability in tissue engineering applications for electroactive tissues.

Since ICPs have a conjugated and rigid backbone, they are high in stiffness and hard to process. For this reason, it is difficult to use them for soft tissue engineering applications. There are some approaches for developing ICP/hydrogel composites to overcome this problem. Hydrogels are highly hydrophilic polymer networks and can be easily modified, however they are mechanically weak and have poor conductivity. Composite materials containing conductive polymers and easily processable hydrogels bring together these advantageous features. Accordingly, Yang et al. [99] fabricated PPy-incorporated conductive hyaluronic acid (HA) hydrogels at different concentrations and showed the correlation between mechanical and electrical properties with increasing PPy content. It was found that fibroblasts could easily adhere and proliferate (6-fold in 5 days) on these conductive hydrogels. Thus, cell adhesion property was attributed to the PPy component since HA was non-cell adhesive [99]. There are also some studies on fabricating complex 3-D geometries based on bio-printing technology using conductive hydrogels containing ICPs. It is a challenge processing ICPs due to their rigid p-conjugated bonds. Wu et al. [96] developed gelatin methacrylate (GelMA)-PANI by interfacial polymerization which occurs at the interface between the hydrophilic GelMA hydrogel and the hydrophobic organic solution. This method has advantages over conductive hydrogels obtained by blending hydrogels with conductive particles. In this study, the conductive hydrogel is printable in a complex user-defined hexagonal geometry by digital stereolithography [96].

The production of ICP composites is particularly promising in terms of the development of key biomaterials for the repair of electroactive tissues. Recently, several studies have been conducted with biodegradable polymers, such PCL, PLA, PLGA, and with polysaccharides, such as dextrin in order to obtain biodegradable composites of ICPs [47, 73, 88].

Many cell types including rat pheochromocytoma (PC12) cells, multipotent stromal cells (MSCs) and Schwann cells can maintain viability on the surface of ICP composites. Cell attachment and in-vitro compatibility of PPy–alginate

(Alg)/chitosan (Cs) scaffold were analyzed using MG-63 cells (a cell line which serves as a model for primary neuronal cells). Conductive PPy is required to blend with natural components to enhance cell interactions, and with pliable polymers to increase processability. Chitosan enhances mechanical strength of the scaffold, while alginate supports cell attachment and viability [73]. Incorporation of ICPs with natural polymers and ECM components has positive effects on cell properties, such as proliferation, migration and differentiation. Li et al. cultured H9c2 rat cardiac myoblasts on nano-fibrous scaffolds containing electro-active PANI. They reported that PANI-gelatin blend nanofibers were biocompatible and promoted cardiac myoblast proliferation and attachment [46].

Accordingly, many studies have shown that PANI, PTh, and PEDOT are also cyto-compatible, especially when they are incorporated with functional molecules or natural polymers. Sirivisoot et al. [80] showed that collagen-incorporated conductive PANI and PEDOT nanofibres were cyto-compatible with PC12 cells and human skeletal muscle cells. High concentration of conductive polymers in the collagen gel has an inhibitory effect on cell proliferation in comparison to collagen-only positive control. Collagen type I can mimic natural extracellular matrix, moreover cell proliferation and viability increases on conductive collagen gels combined with PANI and PEDOT at certain concentrations [80].

8.4 Cellular Applications of Conductive Polymer Composites

Tissue engineering and regenerative medicine is used for the treatment of damaged tissues and organs due to disease and trauma. An ideal tissue engineering treatment aims to develop complementary or alternative tissues (or organs) in the laboratory environment to allow the damaged tissue to renew itself by using biomaterials. Biomaterials are used as "carrier scaffolds" in tissue engineering applications. Scaffolds provide structural support to the cells, allowing them to adhere and proliferate. The basic properties required for a scaffold are its biocompatibility, biodegradability, mechanical strength and being capable of providing tissue delivery. Biocompatible and biodegradable polymeric biomaterials are frequently used for tissue regeneration. In addition to these properties, mechanotransduction, *i.e.* transformation of physiological stimuli into biochemical signals between biomaterials and cells, has great importance in terms of functional tissue engineering. In this respect, development of mechanotransducing biomaterials is the main goal of recent biomaterial studies for tissue engineering application [16, 74]. ICPs offer unique possibilities to provide physiological stimulation facilitating tissue regeneration and wound healing. Since some tissues such as neural, cardiac, muscle, and bone exhibit electrical activities modulating cellular behavior; the use of substantial ICPs, PANI, PPy, PEDOT, PTh draws considerable interest for the treatment of these electroactive tissues. Electrically conductive biomaterials present a great potential as they allow the local delivery of electrical stimulation to cells. A number of recent studies have demonstrated that ICPs with/without other bioactive molecules can direct stem cell behaviour, such as differentiation and proliferation [32, 44, 84]. Electrical charges play a significant role in stimulating stem cell behaviour. External electrical stimulation can modulate cellular activities re-distributing membrane receptors, altering levels of calcium ions, cAMP and decreasing cell membrane potential.

8.4.1 Neural Tissue

Neurons create complex connections similar to electrical circuits. Neuronal electrical activity allows the specific ion channels to be opened and closed in the neuronal plasma membrane. This phenomenon generates action potential at the synapse providing information flow between nerve cells. Neurotoxins such as tetrodotoxin or saxitoxin, and the blockage of the ion channels cause a decrease in the electrical activity leading to the loss of electrical properties and neurode-

generation. The targeted delivery of electrical stimulation leads to activation of target neural populations. So, it is desirable to follow biomimetic approach that has the capacity to supply optimal mechanical and biochemical signals for tissue regeneration.

Neural tissue engineering approaches have significant potential in the peripheral, and possibly in the central nervous system injuries facilitating neurite regeneration. Scaffold materials should be electrically conductive for promoting neuronal regeneration. ICPs have been used in various neural tissue engineering applications, where they were combined with natural polymers such as gelatin, collagen, hyaluronic acid, and with synthetic polymers, such as PCL, PLA, PLGA (Table 8.1). In-vitro studies have demonstrated that ICP composites can promote neurite outgrowth by providing suitable environment and electrical signals for axonal growth [66, 76, 87].

Table 8.1 In-vitro neural tissue engineering studies of ICP composites

Polymer and form	Dose and duration of external electrical stimulus	Conductivity of scaffold	Cell type	Determinants	References
PPy/SF-PLCL (electrospun nanofibers)	0.1 mV cm^{-1} (1 h/day, for 5 days)	1.36×10^{-4} S cm^{-1}	Rat PC12 cells	β-Tubulin III, GAP43, Synapsin I, cell viability (MTT)	[87]
PANI/PCL-gelatin (electrospun nanofibers)	1.5 mV cm^{-1} (15-30-60 min, for 1 day)	2.0×10^{-8} S cm^{-1}	NSCs	Cell viability (MTS)	[23]
PPy(DBS) (polymer film)	−0.25 mA/cm^2 (250 Hz pulsed) (8 h/day, for 3 days)	Surface conductivity (SECM)	Human NSCs	β-Tubulin III, GFAP, Ki67, Nestin, Sox2, Vimentin,	[85]
PEDOT:PSS	1 V (100 Hz pulsed) (24 h/4 days, then >12 h in the following 8 days)	5.8×10^{-4} S cm^{-1}	Human NSCs	β-Tubulin III, GFAP	[66]
PPy/PLA (alligned electrospun nanofibers)	None	1.0×10^{-4} S cm^{-1}	Rat Schwann cells, Human UC-MSCs	P75, S100	[105]
PPy/PCL (electrospun nanofibers)	None	1.9×10^{0} S cm^{-1}	Rat PC12 cells	β-Tubulin III	[76]
PEDOT/Cs/Gel (hydrophilic hydrogel)	None	6.22×10^{-1} S cm^{-1}	Rat PC12 cells	GAP43, SYP	[92, 93])
PANI/PLCL/SF	100 mV cm^{-1} (1 h/day, for 5 days)	3.5×10^{-2} S cm^{-1}	Mouse Schwann cells, rat PC12 cells	F-Actin, Cell viability (MTT)	[103]
PANI/PEGDA	None	1.1×10^{-6} S cm^{-1}	Human MSCs, Rat PC12 cells	GAP43	[24]
PPy/PLA	100 mV for 2 h (steady) 100 mV for 4 h (biphasic) per day, for three days	~ 1×10^{1} S cm^{-1}	Rat hippocampal neural cells	β-Tubulin III, Nestin, c-fos	[86]

Abbreviations: *PPy* poly(pyrole), *PLA* poly(L-lactic-acid), *SF* silk fibroin, *PCL* poly(ε-caprolactone), *PLCL* poly(L-lactic acid-co-ε-caprolactone), *PANI* poly(aniline), *DBS* dodecylbenzenesulfonate, *PEDOT* poly(3,4-ethylenedioxythiophene), *PSS* poly(styrene sulfonate), *Cs/Gel* chitosan/gelatin, *PC12* pheochromocytoma cells, *NSCs* nerve stem cells, *UC* umbilical cord, *MSCs* mesenchymal stem cells, *GFAP* Glial fibrillary acidic protein, *SYP* synaptophysin

For instance, PANI modified PCL/gelatin (Gel) nanofibers were developed by the electrospinning process to enhance nerve regeneration [23]. PANI/PCL/Gel composite scaffold gains essential features such as mechanical strength from PCL, enhanced cell proliferation through gelatin and conductivity from PANI. Ghasemi-Mobarakeh et al. [23] applied a potential difference of 1.5 V (equal to 100 mV = mm electrical field) to neural stem cells (NSCs) seeded onto conductive scaffold for up to 60 minutes. Application of electrical stimulus to NSCs seeded on PANI/PCL/Gel scaffold significantly enhanced the neurite outgrowth (increase in neurite length from 22 ± 0.97 mm without electrical stimulation to 30±1.1 mm). It was found that conductive nanofibrous PANI/PCL/Gel scaffolds allow the electrical stimulation of NSCs and support cell proliferation, proving to be suitable substrates for nerve tissue engineering.

In another study, Stewart et al. evaluated the induced differentiation potential of multipotent hNSCs to neurons and glia cells by electrical stimulation on laminin coated-PPy doped with dodecylbenzenesulfonate (DBS). However, differentiation into both cell types was found to be minimal [85]. On the other hand, polyelectrolyte complexation fibers based on PPy-collagen supported the neural differentiation of human bone marrow-derived MSCs, under external electrical stimulation. PPy provided electrical communication between cells and stimulated neural differentiation of MSCs, while collagen promoted cellular adhesion and proliferation [100].

8.4.2 Muscle Tissue

Skeletal muscle tissue has a remarkable self-repair capacity especially in acute injuries. However, in cases such as major injuries, muscular dystrophy, tumor ablation, the regeneration potential of skeletal muscle is limited. For such cases, conventional medical and surgical treatments are not always effective to cure the damaged skeletal muscle tissue. Muscle tissue engineering scaffolds provide a biological alternative, which supports the damaged skeletal muscle regions while providing faster healing. Since electrical stimulation is required for skeletal muscle tissue functions, the investigation of the effects of electrical stimuli on muscle cells has become an interesting topic for in-vitro skeletal muscle tissue engineering (Table 8.2). There are a number of studies suggesting that electrical stimuli contributes to skeletal muscle regeneration by affecting cellular microenvironment and altering gene expression related to skeletal muscle functions. Park et al. [63] examined the effects of electrical signals on collagen type I deposition stimulating C2C12 cells at different voltages and frequencies. They showed increased accumulation of type I collagen proportionate to changes in electrical frequency, while the differentiation of muscle sarcomeres in excitable cells was observed at a specific voltage value [63]. Besides using electrical stimulation alone, muscle tissue formation can be stimulated by using electroactive scaffolds. Electrically-conductive materials may have significant potential as mediator between the skeletal muscle cells and electrical stimulation, and as a tissue construct for damaged skeletal tissue. Combination of both electrical stimulation and well-oriented conductive materials may have a synergistic effect on muscle formation. Muscle cell alignment plays an important role in musculoskeletal myogenesis. Biomaterials with aligned patterns are useful for guiding myotube formation for skeletal muscle regeneration. Aligned nanofibers incorporated with ICPs could be a promising scaffold for muscle tissue engineering. Random or aligned PCL/PANI nanofibers were fabricated by electrospinning process to evaluate their effect on C2C12 myoblasts. As the result, combined effect of alignment and conductivity promoted myoblast orientation, myotube formation and further myotube maturation [12]. Similarly, Zhang and Guo [104] developed electroactive composite scaffold by incorporating silk fibroin with water-soluble PANI. Myosin heavy chain expression levels enhanced with increasing PANI concentration, indicating that it supports myotube formation. It was observed that the expression levels of myogenic regulatory factors including MyoD1 and myogenin were significantly higher in PANI-

Table 8.2 In-vitro non-neural tissue engineering studies with ICP composites

ICP	Scaffold form	Components	Application	Cell type	Outcomes	References
PANI	Electrospun micron-fibers	PANI/PCL	Vascular tissue engineering	HUVECs	Enhanced cell adhesion and viability	[47]
					Much better spreading and proliferation of cells under electrical stimulation	
	Electrospun fibers	PANI/Gelatin	Preliminary study for cardiac tissue engineering	H9c2 cells	Promotion of cell attachment, migration, and proliferation	[46]
					Biocompatible	
	Electrospun sub-micron fibers	PANI/PCL	Skeletal muscle tissue engineering	C2C12 cells	Promotion of myogenic differentiation of myoblasts but minimal effect on cell proliferation	[37]
					Increased expression level of myogenic genes	
					A model system to determine the effect of electrical signals on muscle cells	
	Electrospun conductive micro-nanofibers	PANI/PDLA	Neuromuscular regeneration	Primer rat muscle cells	Development of a biodegradable, biocompatible, and electrically conductive scaffold	[54]
					A suitable coating material rather than as a primary scaffold component due to its degradability features	
	Multilayered core-shell fiber webs	PMMA/Gold/PANI	Preliminary study for artificial muscle development	hAFSCs	Electrospinning technique combined with DC sputtering	[4]
					Controllable electrochromic properties depending on applied potential	
					High cell adhesion and good biocompatibility	
	Composite films	PGS/PANI	Cardiac tissue engineering	C2C12 cells	Good attachment, growth and proliferation of myoblasts	[69]
					Biocompatible	
					Potential delivery vehicle of functional cells to the myocardial infarct	
	Inkjet printed pattern on electrospun composite	PANI – PCL/Gelatin modified with osteogenon and CaP NPs	Bone tissue engineering	Human osteoblasts	Appropriate stimulator for cellular functions including attachment, proliferation, migration and differentiation	[70]
					Electrically conductive environment for cells	

(continued)

Table 8.2 (continued)

ICP	Scaffold form	Components	Application	Cell type	Outcomes	References
PPy	Lyophilized scaffold	PPy-Alginate/ Chitosan	Bone tissue engineering	MG-63 cells	Cytocompatible	[73]
					Interactive substrate between seeded cells and external electric field	
	Hydrogel	PPy-Hyaluronic Acid	Tissue engineering (general)	NIH-3 T3 cells	Promotion of cell attachment and growth	[99]
	Hydrogel	PPy – Xanthan	Tissue engineering (general)	Human fibroblasts	Increased fibroblast proliferation	[9]
					Non-toxic, biodegradable, biocompatible	
	Hydrogel	PPy – Chitosan	Cardiac tissue engineering	Neonatal rat cardiomyocytes	Enriched Ca^{2+} signal conduction	[56]
					Improved electrical conduction	
PEDOT	Disc scaffold	PEDOT:PSS	Bone tissue engineering	MC3T3-E1 cells	Enhanced ALPL, COL1A1 and RUNX2 expression	[25]
					Osteogenic differentiation of pre-osteogenic precursors	
	Nanocomposite scaffold	PEDOT:PSS/ nHA-CS	Tissue engineering (general)	L929 cells	Promotion of cell attachment and growth	[43]
					Non-toxic to cells	
	Electrospun nanofibers	PEDOT – PNIPAm	Tissue engineering (general)	L929 cells	Increased cell proliferation	[77]
					Biocompatible	

containing scaffolds compared to pure silk fibroin scaffold [104]. In addition to studies underlining conductive polymers and electrical stimulation triggering skeletal muscle regeneration, some other studies have focused on stem cell differentiation into skeletal muscle cells by the effect of conductive polymers and electrical stimulation. For example, it has recently been shown that adipose stem cells differentiate into skeletal muscle cells on PPy-coated scaffolds [6]. In another study, satellite cells seeded on PANI/poly(acrylonitrile) scaffold were induced into mature muscle cells owing to combined effect of conductivity, and mechanical properties of the scaffold. Skeletal muscle markers including MyoD1, MHC and B-actin showed higher expression levels on PANI/poly(acrylonitrile) electrospun scaffolds, compared to that of pure PANI scaffold [27].

8.4.3 Cardiac Tissue

Conduction of electrical impulses allows cardiac tissue to contract and relax [18]. Since electrical impulses are imperative for synchronous mechanical contraction of cardiac cells, which provide effective cell–cell interactions, the use of ICPs for cardiac tissue engineering applications have also attracted attention [19].

Any problem that may occur during the transmission of electrical signals leads to cardiovascular diseases [71, 107]. Conduction is significantly reduced at the infarct site. Conductive polymer nanocomposites allow control of the electrical stimulus. Qazi et al. [69] found that conductive PANI-poly(glycerol-sebacate) (PGS) composites supports adhesion, growth and proliferation of C2C12 cells in addition that they provide transfer of electrical signals to cells. Electrical conductivity increased from 0 S cm^{-1} for pure PGS to 0.018 S cm^{-1} for 30 vol.% PANI–PGS composite and conductivities were preserved for at least 100 h post-fabrication. Results showed that 3-D porous scaffolds fabricated using PGS hold promise to serve as carrier and delivery vehicles of functional cells to the myocardial infarct [69]. Mihardja et al. obtained injectable alginate–PPy polymer blend to repair ischemic myocardium after myocardial infarction [55]. Spearman et al. [82] created conductive films comprising PPy and PCL, which showed the same electrical properties with native cardiac tissue. Results demonstrated that HL-1 atrial myocytes seeded on the PPy/PCL films presented increasing cardiac-specific protein connexin (Cx43) levels and improved electrophysiological maturation properties, which was determined by the increasing calcium transient propagation velocity [82]. Functional disorders after myocardial infarctions may cause cardiac arrhythmias. In order to successfully mimic the native heart, cardiomyocytes seeded on scaffold material can contract in synchronism with the applied electric field. PANI and PLGA have been used as a conductive scaffold to synchronize the beatings of the cultured cardiomyocyte cell clusters [28]. Similarly, cardiomyocytes seeded on electrospun PLA/PANI conductive nanofibrous sheet demonstrated synchronized beating and improved maturation. It can be interpreted that both conductive scaffold and applied electrical field have synergistic effect on the CX43 expression and synchronous cell beating (Wang et al. [92, 93]). In addition to in-vitro cardiac cell studies, in-vivo studies using conductive polymers for cardiac tissue healing have also been carried out. Mihic et al. have developed PPy-Cs hydrogels to determine its effect on cardiac regeneration after myocardial infarction in rats. In-vitro results showed improved Ca^{2+} signal conduction of the neonatal rat cardiomyocytes seeded on PPy-Cs hydrogels. Accordingly, in-vivo results demonstrated improvement in electrical conduction properties, determined by the decreased QRS interval, and an increase in transverse activation velocity in comparison to saline or Cs [56].

8.4.4 Bone Tissue

The application of electric field in the treatment of bone injuries is an approach that has existed for a long time [3, 17]. Electrical stimulaton can alter migration, proliferation, and osteogenic differentiation behaviours of MSCs and can lead to

an increase in the expression of osteoblast-specific markers, such as Runx2, Osteocalcin, Osteopontin and Type I Collagen [14, 58]. However, direct application of electrical stimulation may not be an effective method especially for in-vivo applications since it is not locationally specific, and could affect both the healthy and damaged tissues. Conductive scaffold-mediated electrical stimulation is an alternative method for bone tissue regeneration, which supports the proliferation and osteogenic differentiation of MSCs and provides better mechanical environment for regeneration. Chitosan incorporated PPy–Alg scaffold was fabricated to evaluate MG-63 cell attachment, distribution and in-vitro biomineralization of the scaffold. Results showed that PPy–Alg scaffold can be used as an interactive substrate between the seeded cells and external electric field [73].

8.5 Challenges and Future Directions

Conductive polymers possess unique electrical properties, while their biological properties are limited. In order to improve their biocompatibility and biodegradability, studies on a variety of blends and composite systems have been performed. Combination of conducting polymers with natural polymers, such as chitosan, collagen, alginate, etc. appears to be appropriate for developing useful composites for tissue engineering applications. However, most of the work is carried out on the in-vitro settings; thus careful evaluations should be performed with advanced animal models before clinical applications. Conductive hydrogels, electrospun nanofibers and polymeric films are often fabricated through chemical or electrochemical synthesis methods. Especially in the field of tissue engineering, there are many unknowns, and there is a need for further studies involving different components and synthesis methods to overcome the obstacles. Recent interest has focused on the development of 3-D printed polymers and their composites. Electroactive 3-D printed biomaterials containing conductive polymers and the design of conductive polymer-based microchip systems could be promising for a number of cellular applications, including the development of more realistic in-vitro model systems for drug discovery and tissue engineering.

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9 Materials and Applications of Smart Diagnostic Contact Lens Systems

Sijin Park and Dong Yun Lee

Abstract

Contact lenses were originally developed for the purpose of vision correction, but they have recently been used for various purposes. Because contact lenses are minimally invasive, they are used in diagnostic and drug delivery applications. In particular, interest in using contact lenses for the purpose of diagnosing diseases by fusing contact lenses with information technology (IT), nanotechnology (NT), and biotechnology (BT) is increasing. These contact lens-based platforms are getting more attention as Google and Novartis develop contact lenses for diabetes diagnosis. Therefore, this chapter introduces materials that can be used for contact lens materials and diagnostic contact lenses, and discusses future prospects.

Keywords

Contact lens · Smart · Diagnosis · Material

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9.1 Overview of Contac Lenses

Contact lenses were first proposed by Leonardo da Vinci over 500 years ago and most developed in the late 1900s. After the design of rigid contact lenses using poly (methyl methacylate) (PMMA) in the mid-twentieth century, more advanced models have been developed continuously. In addition, soft contact lenses using poly(2-hydroxyethyl methacylate) (PHEMA) were developed after rigid contact lenses were developed. In 1971, Food and Drug Administration (FDA) approved HEMA-based contact lenses as disposable lenses. In addition, in 1999, contact lenses with enhanced oxygen permeability were fabricated from silicone-based materials such as polydimethylsiloxane (PDMS). After the development of the contact lenses, various researches on mold and lens manufacturing methods have been made so that the contact lenses can be manufactured more efficiently and effectively [6].

As the manufacturing method has been diversified, the kinds of materials that can be used for contact lenses have also been diversified. In addition, the use of computer-assisted laser technology in lens fabrication has increased the accuracy of the production. With the development of technology and diversity of materials, it was possible to develop from disposable lens to long-term wearing lens while obtaining improved characteristics such as increased oxygen permeability and comfortable fit.

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In recent years, contact lenses have been used for various purposes such as treatment, diagnosis, and beauty as well as for correction of vision. In early 2014, Google has started to develop technologies to diagnose diseases by combining electrochemistry and information technology with contact lenses, and is collaborating with Novartis. Interest in diagnostic contact lenses has increased dramatically since then.

In this chapter, we discuss about the materials used in contact lenses and how they can be developed according to recently applied technologies.

9.2 Types of Contact Lenses

Although the initial contact lenses were only used for vision correction, current contact lenses are being developed for various purposes.

The contact lens for correcting vision corrects myopia, hyperopia, astigmatism, and the like. That is, corrects vision by correcting the refractive error by focusing the light coming into the eye. Recently, contact lenses for myopia correction have also been used to help people to live without a lens during the day by flattening the cornea by wearing a lens during sleep.

Therapeutic contact lenses are used for the treatment of the eye. It helps prevent cornea from being damaged by blinking in case of corneal damage. In addition, in order to overcome the disadvantage that eye drop has low bioavailability, the drug is loaded in the lens and is used to treat ocular disease by increasing the drug delivery efficiency by eye when the lens is worn. It is used to treat various ocular diseases such as watery keratosis, dry eye syndrome, corneal abrasion, corneal epithelial erosion, keratitis, corneal edema, corneal ulcer, and corneal hypertrophy [2].

Recently, beauty contact lenses are popular in young people. This is used for cosmetic purpose by changing the color of the iris part, and it is possible to correct the visual acuity at the same time when the user desires. It is also used for medical purposes to correct iris loss or the like by using the features of a cosmetic lens. However, it is possible to cause eye irritation, infection and the like due to the leakage of pigment existing in a cosmetic lens or due to a thickness and the like [14].

9.3 Materials Used in Contact Lenses

The materials used for fabricating contact lenses can be roughly classified into rigid lens materials and the soft lens material.

The rigid lens has been made of poly(methyl methacrylate) (PMMA) as a typical material in the past, but PMMA is not used at present because it has poor wettability and oxygen permeability and can cause corneal anomalies [9]. To overcome the disadvantages of PMMA, today's rigid lenses are the rigid-gas-permeable (RGP) lenses with good oxygen permeability made up of, for example silicone/acrylate (S/A) or fluoro-silicone/acrylate [12]. S/A has a high oxygen transmission rate by siloxane bonding, but this increases the hydrophobicity of the lens, so that a hydrophilic monomer should be used together. F-S/A is a material similar to S/A with fluorine added. It is chemically stable due to fluorine and can reduce the problem of S/A dryness. S/A is less dry and comfortable to wear, so they can be used by dry eye patients. Styrene is a material containing a benzene ring. It has a high refractive index and a low specific gravity. The lens of this material is more hydrophilic and stronger than other RGP lenses, so it is good for irregular astigmatism correction especially in the cornea [12]. Silicone Elastomer has good elasticity and its oxygen transmission rate is very good. Lenses made up of this material have a medium diameter and are good for children to wear. However, it is rarely used because of the hydrophobicity and adsorption of the surface, and it is used in aphakia patients [10]. The soft lens is more comfortable than the rigid lens because the lens material is soft, so there is less foreign body sensation of eye and less pressure. The diameter of the lens is larger than that of the

rigid lens. Monomers such as N-vinyl pyrrolidone (NVP) and HEMA which have good hydrophilicity are used as materials. HEMA is the most representative material of soft lenses, and has good hydrophilicity and flexibility. It has high water content and oxygen permeability, so it has good fitting property and good biocompatibility. However, since the mechanical stability of HEMA alone declines, it is synthesized with monomers such as Methacrylate to enhance durability. Methacrylic acid (MAA) is a monomer used to increase the water content of the lens. Since it always has charge at physiological pH, the lens in which this material is used has a negative charge [3]. N-vinyl pyrrolidone (NVP) is a hydrophilic monomer with a vinyl group and is mainly used to increase water content. However, the strength is low and the adsorbability is high, so it is used by copolymerizing with other monomers. Ethylene Glycol Dimethacrylate (EGDMA) is a material used as a crosslinking agent. This increases the stability of the polymer, but it makes the lens stiff and reduces the water content in excessive use. Divinylbenzene also has two vinyl groups and is used as a crosslinking agent [3].

9.4 Applications of Contact Lenses

Contact lenses are being developed for a variety of purposes, including vision correction, aesthetic needs, and treatment. Recently, new contact lenses converged with IT and BT have attracted great attention.

In 2014, Google, one of the world's largest IT companies, has started to develop contact lenses to measure the blood glucose level. Besides, interest in contact lenses with biosensor properties, is increasing. In particular, among these non-invasive and non-blood-drawn platforms, tear glucose measurement technology based on "PET (polyethylene terephthalate) contact lens" is being developed [16]. These techniques increase patient convenience and are very advantageous in terms of economy.

9.4.1 Smart Contact Lenses

Most of the 'smart contact lenses' currently being developed are equipped with sensor systems using electrochemical principles. Below are some of the most advanced contact lens platform technology-based disease factor measurement techniques.

Google released a prototype of 'Smart Contact Lenses' in early 2014, analyzing the tears of diabetic patients and analyzing blood glucose levels in the body [5]. In this prototype, between the two films made of soft lens material, it is possible to measure the change of tear glucose level by attaching a small size wireless chip, blood glucose measurement sensor, antenna and LED light. The sensor is designed to measure continuously once a second, and when the blood glucose is above or below the reference value, the LED light is used to show the LED signal in the user's field of view. Such readings can be wirelessly transmitted to the user's mobile device. You can also calibrate your vision in a similar way to an autofocus camera lens. The company has obtained patents related to the installation of multiple compact cameras, sensors, and communication devices on lenses of similar shape and thickness to conventional contact lenses. However, there is a need for extensive research and at least 5 years will be needed before actual implementation. However, this system seems to have great potential for fast and accurate measurement of trace amounts of glucose contained in tears [5].

SENSIMED has developed a smart contact lens called Triggerfish. It is a lens that can measure the intraocular pressure through an internally mounted sensor. Glaucoma is a serious disease in which the optic nerve pressure or the blood supply is obstructed by the rise of the intraocular pressure and the visual acuity is gradually lowered, eventually leading to blindness. Glaucoma patients need to be measured during the treatment of intraocular pressure is necessary, because the intraocular pressure of the patient changes several times a day, so the accurate measurement of intraocular pressure should be performed continually over a

long period of time, not a single measurement. The lens incorporates a thin strain gauge to measure the change in curvature of the cornea, and transmits this information to the patient's mobile device in real time using wireless communication. This sensor is designed to monitor the patient's condition for 24 h [11].

Korea Institute of Science and Technology (KIST) developed a "contact lens diabetes sensor". This system allows electronic circuits to be inserted into the contact lens to stably collect and analyze tears to determine diabetes and its progress [13]. The team developed the newly-constructed sensor which can measure a small amount of glucose contained in tears on a flexible platform. In addition, the team developed a micro-module capable of ultra-low-power operation and a microfluidic control system. In the sensor, the nanogenerator generates electric power whenever the eye blinks, and sends the collected tears through the microchannel to the sensor to analyze the glucose content in the tear film, and the film-type battery can store electricity. The researchers succeeded in using the tears of a diabetic patient to read the sugar components. It is expected that blood glucose will be continuously monitored by wearing a contact lens so that it can be diagnosed and managed more easily and accurately [13].

However, most of the 'smart contact lens' technology uses potentiometry or amperometry technology, so various control circuits, communication circuits, and antennas to implement these 'smart' functions must be miniaturized into a soft and transparent contact lens do. Therefore, more innovative technology development is required for commercialization.

9.4.2 Nanomaterials for Biosensors Applicable to Smart Contact Lens Platform

As mentioned above, most of the smart contact lens technologies have to be built in small contact lenses with miniaturization of various circuits. However, since there are technical limitations, a variety of nanomaterials for biosensors are being developed to overcome these problems. The present study suggests the possibility of diagnosing diseases by changing nanomaterials in contact lenses rather than electronic circuits by mounting nanomaterials in contact lenses (Fig. 9.1).

A "single-molecule electrical semiconductor", made by bonding titanium dioxide to carbon nanotubes, causes changes in electrical reactions when exposed to UV light or acetone. Acetone from respiration is known to be associated with diabetic ketoacidosis. At room temperature, the nanomaterial reacts quickly and very sensitively to acetone vapor (2–20 ppm) and reacts reversibly. This feature makes SWNTs-TiO_2 a suitable material for detecting acetone in the respiratory

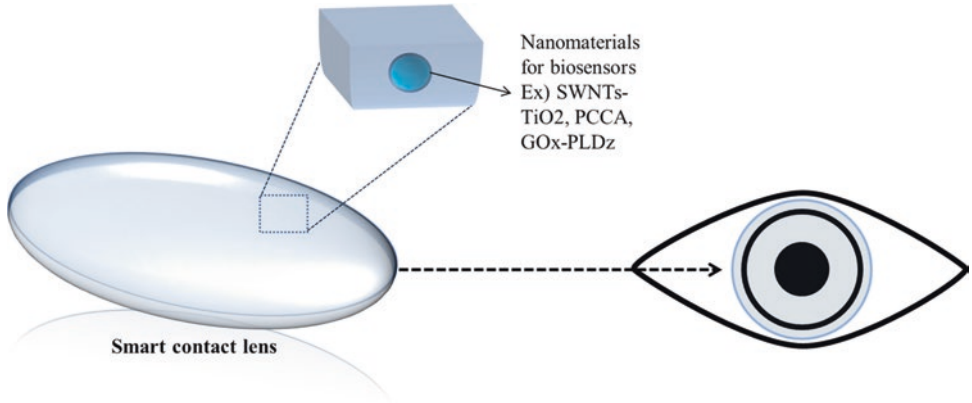

Fig. 9.1 Concept image of smart contact lens equipped with nanomaterial for diagnosis

tract. In particular, it has been proved that diabetes can be diagnosed by analyzing acetone content contained in patient respiration using this. Such an acetone detection sensor can be used as a personal measurement device for patients diagnosed with metabolic diseases or diabetes to conveniently diagnose diseases [4].

Materials using hydrogels, which are well known for their excellent biocompatibility, have also been studied. Hydrogels are characterized by the reversible reaction of contraction and relaxation by osmotic pressure when an analyte, such as glucose is present, and can be made to react specifically with the analyte. An example is the "photonic crystal polymerized crystalline colloidal array (PCCA)", which detects the change of glucose to the long wavelength by diffraction and scattering due to the increase in hydrogel volume with glucose concentration [7].

In addition, there is a technique of detecting a substance reacting with glucose by combining it with a fluorescent substance and applying it to a contact lens and then detecting it through the fluorescence resonance energy transfer (FRET) principle. The technique detects the concentration of glucose by measuring the emission light from an external light source [1].

We et al. developed a "poly(NIPAM-AAm-VPBA)-Ag hybrid microgel" that uses Ag ion as a molecular imprinting technology [15]. These microgels can be used as a "glucose indicator" by detecting glucose with high sensitivity and selectivity. As the concentration of glucose increases, the color changes from yellow to red, indicating that the glucose concentration rises. In addition, when glucose binds to the microgel and changes the binding of the polymer network, it causes a change in the refraction of the light beam, which can be detected by surface plasmon resonance (SPR), allowing glucose concentration to be measured [15].

Dazhi Jiang et al., on the other hand, developed a "glucose oxidase-coupled Pistol-like DNAzyme (GOx-PLDz)" for detecting glucose in tears and saliva [8]. This nanomaterial is a dual-enzyme biosensor consisting of DNAzyme and glucose oxidase. When glucose oxidase oxidizes glucose to form hydrogen peroxide, PLDz detects hydrogen peroxide and causes self-cleavage. This reaction is catalyzed by Mn^{2+}, Co^{2+} and Cu^{2+} cations. That is, glucose detection can be monitored through the degree to which PLDz is cleaved. This material can detect glucose at a minimum of 5 μM. However, there is a limit to overcome such that the detection time takes at least 1 h, the analysis is performed by electrophoresis, and the sample volume is as large as about 30 μl [8].

9.5 Future Outlook of Smart Contact Lens

The contact lens type sensor platform, which is currently being developed, is a non-invasive and continuous self-monitoring of blood glucose (SMBG). It is equipped with sensor systems mainly using electrochemical principles.

The important safety issues in the development of such a smart contact lens platform are: (1) It must be absolutely safe because it is a device that is directly attached to the eye. For example, AlGaAs (Aluminum Gallium Arsenide), one of the materials that make up LEDs, is toxic and must be completely encapsulated with biocompatible materials. (2) The heat generated in the circuit may also be a problem. In order not to damage the eyes, only a maximum of about 45 degrees of heat should be generated. (3) It is necessary to verify the possible adverse effects and toxicity on the eye for a long period.

In addition, tears are produced in very small quantities of 0.5–2.2 μl/min (0.72–3.2 ml/day) and contain about 20 components [1]. Therefore, it is expected that the material used for the smart contact lens platform should have very high sensitivity and specificity to the disease factor.

In order to solve the problem of stability, it will be necessary to mount a nanomaterial on such contact lenses. Since the particles detecting the movement of the eyelids may fall off when the contact lens is worn; thus the material should be safe and stably attached. It is also necessary to develop the technology in consideration of the fact that after the nanomaterial is combined with the contact lens, the physical properties of the lens itself must be maintained.

9.6 Summary

Contact lenses are being developed for a variety of new purposes in recent years, besides the originally developed purpose of visual acuity correction; in particular contact lenses for disease diagnosis are attracting attention. However, in order to commercialize diagnostic smart contact lenses, various materials that will overcome limitations such as sensitivity and specificity should be developed.

These new evolving smart contact lens systems will be able to detect/diagnose, through a tear, various disease agents in the future. This will lead to fast treatment measures established through the correct diagnosis. In addition, continuous noninvasive diagnostic systems will possibly reduce the test costs. This can eventually reduce the national medical burden of the patients. Such smart diagnostic systems will possibly improve the public awareness on disease prevention through easy self-diagnosis and continuous health management of patients. Creating prototypes of biosensors based on BT, IT and NT, will possibly help creation of new markets and jobs by introducing new models of new growth engines and convergence research and development.

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10 Advances in Protein-Based Materials: From Origin to Novel Biomaterials

Soon Mo Choi, Prerna Chaudhry, Sun Mi Zo, and Sung Soo Han

Abstract

Biomaterials play a very important role in biomedicine and tissue engineering where they directly affect the cellular activities and their microenvironment. Myriad of techniques have been employed to fabricate a vast number natural, artificial and recombinant polymers in order to harness these biomaterials in tissue regeneration, drug delivery and various other applications. Despite of tremendous efforts made in this field during last few decades, advanced and new generation biomaterials are still lacking. Protein based biomaterials have emerged as an attractive alternatives due to their intrinsic properties like cell to cell interaction, structural support and cellular communications. Several protein based biomaterials like, collagen, keratin, elastin, silk protein and more recently recombinant proteins are being utilized in a number of biomedical and biotechnological processes. These protein-based biomaterials have enormous capabilities, which can completely revolutionize the biomaterial world. In this review, we address an up-to date review on the novel, protein-based biomaterials used for biomedical field including tissue engineering, medical science, regenerative medicine as well as drug delivery. Further, we have also emphasized the novel fabrication techniques associated with protein-based materials and implication of these biomaterials in the domain of biomedical engineering.

Keywords

Protein · Biomaterials · Tissue engineering · Protein composite · Scaffolds · Biomedical · Protein materials

10.1 Introduction

In the last few decades, tissue engineering and regenerative medicines have grown with a very fast pace, where combination of materials science, engineering and medicine, have given a futuristic direction to these technologies. More recently, personalized tissue engineering has emerged a favorable option as they fulfill all the requirements according to the patient needs. With the advancement in the understanding of molecular organization and cellular functions of animal tissues, various biomimetic materials

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have been fabricated to replace damaged tissues and organs. These bioengineered products are synthesized in a way that they provide optimal environment for the cells to grow, proliferate and differentiate in a process of regeneration of damaged tissues [152, 153]. Biomaterials are the fundamental element of tissue engineering, drug delivery systems, immunotherapies, and implantology, where they are utilized as biocompatible supports, encapsulation agents to protect the delivery materials and as a replacement of damaged tissues. Due to these indispensable requirements, these materials should be in proper tuning with the *in situ* architecture of the tissues to improve their inherent biological potentials of regeneration [11, 133]. A number of materials, natural or synthetic, organic or inorganic, hybrid and composite have been investigated to use in different tissue engineering applications [41, 254]. Natural polymers like, protein, nucleic acid, and polysaccharides are extensively being used for the fabrication of scaffolds to regenerate tissue/organs, to generate 3D model mimicking the cellular environment and to study the mechanism of in vitro induced diseases [13, 25]. Among synthetic polymers, poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly(p-dioxanone), poly (caprolactone) and their copolymers are the most widely used in tissue engineering [99]. Synthetic polymers are associated with various drawbacks, which limits their universal use in biomedical applications. Natural source biomaterials present several advantages over synthetic polymers due to their enhanced biocompatibility, low immunogenicity, and higher degradability. Furthermore, natural polymers contain biomolecules that are natural to the cells, which can support and guide the cells to proliferate and differentiate at particular time interval and consequently can enhance the biological interaction with them [1, 15]. Among all natural biomaterials, use of protein-based biomaterials provides a uniquely powerful approach as these molecules are critically involved in biomechanical responses, and control macromolecular structure and function. The structural flexibility and biocompatibility of the proteins make them ideal candidates to use as building blocks for biomaterials engineering [87]. Besides, protein based biomaterials naturally possess binding sites for the cell adhesion and further modification for cell attachment and proliferation is not required [158]. Some of the traditional proteins that are used as biomaterials in biomedical engineering are collagen, elastin, fibrin, keratin, silk and gelatin [235]. Recently, novel recombinant proteins are introduced as biomaterials in the field of biomedical engineering where, they have come out to be superior to the currently used materials in terms of their fabrication and applicability [159, 289]. Many protein polymers such as collagen, elastin and silk fibers have been genetically engineered and are being used for the fabrication of tissue engineering scaffolds [53, 80].

In this review, various novel biomaterials will be discussed with a special emphasis on protein based biomaterials. According to the definition, biomaterials are the materials, which are either naturally originated or synthesized artificially and interact with biological system either as a drug delivery vehicle, or as a replacement for damaged and diseased human tissues. In addition, we also addressed the different strategies adopted for functionalization of protein-based biomaterials in order to enhance their performance, mechanical properties, biocompatibility as well as degradability with a target towards control of these process.

A number of biomaterials ranging from naturally occurring substances to artificially synthesized materials have already been employed in tissue engineering and regenerative medicines for drug delivery, mechanical support, implants and as building blocks for the regeneration of damaged tissues.

Over the other biomolecules, peptides and proteins have expedient properties for use as building blocks in the synthesis of biomaterials. These proteins are either naturally produced or can be synthesized artificially using the recombinant and genetically engineered technology.

10.1.1 Biomaterials in Tissue Engineering

The field of bioengineering, implies to understand the mechanism of cellular structure and functions as well as to utilize the techniques and materials for the designing of suitable environment for the damaged tissue or organ to grow [28]. Tissue microenvironment is a complex entity, which involves the interaction of cells, extracellular matrix, and the bioactive agents with each other as well as with outer environment. With the proper understanding of the tissue and cellular microenvironment like composition of ECM, cellular arrangements and their physical and chemical requirements, researchers can control the condition required for the morphogenesis of new tissues and organs. ECM not only provides the physical support to the cells but also creates an environment for the cells to grow and differentiate for morphogenesis and organogenesis. That is why providing only cells at damaged site is not sufficient for optimal tissue regeneration; a suitable matrix or support is also equally required. A scaffold or matrix assists the cells to proliferate and differentiate in the artificial environment and induces the cell based tissue regeneration. The support for the cells to grow should be biocompatible and should wash out with the passage of time from the body [254]. A number of biomaterials ranging from naturally occurring substances to artificially synthesized materials have already been employed in tissue engineering and regenerative medicines for drug delivery, mechanical support, implants and as building blocks for the regeneration of damaged tissues. The biomedical applications of these materials depends on their macromolecular structure and organization, their interaction with cells and their microenvironment and their compatibility with the cellular environment [133]. The biomaterials for use in tissue engineering should be dynamically responsive to the external stimuli that trigger the release of integrated chemicals such as drugs and ligands in a controlled manner for the repair or remodeling of the damaged tissue.

The biomaterials are the building blocks for the development of tissue engineering and regenerative medicine. Over the time a vast number of novel biomaterials, natural and synthetic have been developed dramatically to use in the field of biomedical engineering for the improvement of overall healthcare services. According to the definition, "biomaterials are the materials, which are either naturally originated or synthesized artificially and interact with biological system either as a drug delivery vehicle, or as a replacement for damaged and diseased human tissues". Biodegradability, porosity, poor immunogenicity, and inexpensiveness are the prerequisites for these biomaterials to be used in tissue engineering and regenerative medicines [47]. The restoration of damaged tissue depends on how the cells interact with their environment, which in turn is highly dependent on the biomaterial of the scaffolds. Natural biomaterials are able to mimic the structure and composition of the native extracellular matrix as a result, stimulate the cellular activities. If the biomaterials are biocompatible, they can enhance the growth and development of injured tissues and cells. With the help of these novel biomaterials, the devastating problem of tissue or organ loss can be solved proficiently.

Recently, extensive research is going on to develop tissue and organs outside of the body or to regenerate damaged tissues by using either patients own cells or from other sources. The cells can be grown on artificial environment made up of polymeric biomaterials in the form of mesh, fibers or scaffold. These degradable polymer matrices provide large surface area for cells to grow and differentiate into desired tissues and organs. The neo-morphogenesis of organs can give new hopes to the patients who are in dire need of replacement of lost organs.

Depending on their origin, biomaterials can be categories into: (1) Natural biomaterials, derived from animals or plants such as nucleic acid, protein and peptides, carbohydrate etc. (2) Synthetic biomaterials like polymers, ceramics, metals etc. (3) composite or hybrid biomaterials.

Over the other biomolecules, peptides and proteins have expedient properties for use as building blocks in the synthesis of biomaterials. These proteins are either naturally produced or

can be synthesized artificially using the recombinant and genetically engineered technology.

10.2 Proteins

Proteins were primordially delineated by Gerhardus Johannes Mulder of the Dutch chemist and Jöns Jakob Berzelius of the Swedish chemist in 1838. The protein firstly sequenced by Frederick Sanger who won the Nobel Prize in 1958 was insulin [132, 188]. The first structures of protein, hemoglobin and myoglobin, were solved by Max Perutz and Sir John Cowdery Kendrew, respectively in 1958 and then, they won the Nobel Prize in chemistry in 1962 for firstly investigating of the three dimensional structure of protein x-ray diffraction analysis. Proteins are purified from diverse cellular constituent using various techniques including ultracentrifugation, precipitation, electrophoresis, as well as chromatography. The techniques have been employed for conducting studies of protein structures and function.

10.2.1 Biochemistry

Protein known as polypeptides are organic compounds made of amino acids with linear chain and folded into globular form, which is critical macromolecules in biological systems. The amino acids are united in a polymer chain by the peptide bonds between carboxyl and amino groups of neighboring amino acid residues. Almost amino acids include a basic structure with α-carbon in a carboxyl group, an amino group, and varied side chains, however only proline is different with this common structure due to containing an unusual ring to the N-end amine group forcing the CONH amide moiety into a fixed conformation [195]. The side chains possess variable chemical structures and properties.

The amino acids of a polypeptide chain are connected though peptide bond, and the individual amino acid is called a residue when they are linked inside protein. The linked parts of carbon, nitrogen, and oxygen are known as the main protein backbone chain (Murray et al.). The peptide bond possess two resonance types attributing double bond character and restraining rotation within its axis, consequently the α-carbons are nearly coplanar. The end having a free amino group is named as the N-terminus (or amino terminus), while the end with a free carboxyl group is emerged as the C-terminus (or carboxy terminus). The word of protein can be semantically overlapped with polypeptide, and peptide. In general, protein is employed to refer to the consummate biological molecule in a stable conformation, while peptide is commonly used for a short amino acid oligomers lacking a stable three dimensional structure. The borderline between a protein and a peptide is actually not well defined.

10.2.2 Origin and Structure

Considering the fundamental constituents, proteins are averagely comprised of about 50–55% carbon, 6–7% hydrogen, 20–23% oxygen, 12–19% nitrogen and 0.2–3% Sulphur. The proteins made of variable amino acid, as shown in Fig. 10.1, could be sorted as follows; polar, nonpolar, aromatic, anionic, and cationic [102]. Almost proteins generally fold into unique three dimensional structures, and the structure is organized in four distinct aspects, as shown in Fig. 10.2 [23, 245, 308].

- Primary structure: amino acid sequence. The chain of amino acids which is identified as a polyamide is flexible due to the nature of the bonds holding the amino acids together.
- Secondary structure: Once the chain as mentioned above is long, hydrogen bonding can occur between amin and carbonyl groups within the peptide, leading to fold locally polypeptide chain into helices and sheets, known as α-helix and β-sheet. The helix is connected by hydrogen bonding between the oxygen of a carbonyl group within an amino acid and the hydrogen of an amino group. The pleated sheet are developed by hydrogen

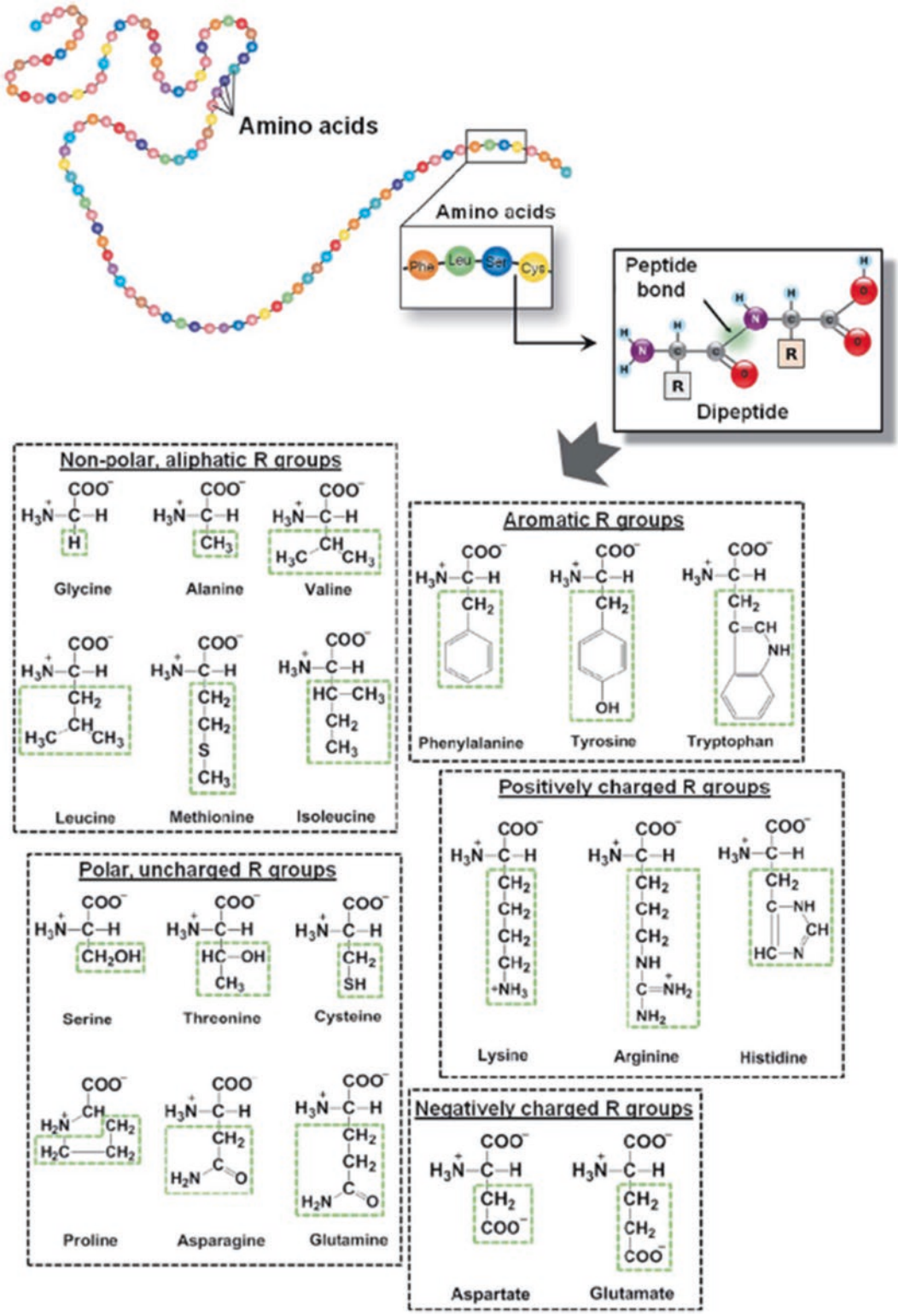

Fig. 10.1 Amino acid residues and peptide bonds. Adapted with permission from Silva et al. [245]. Copyright (2014) Royal Society of Chemistry

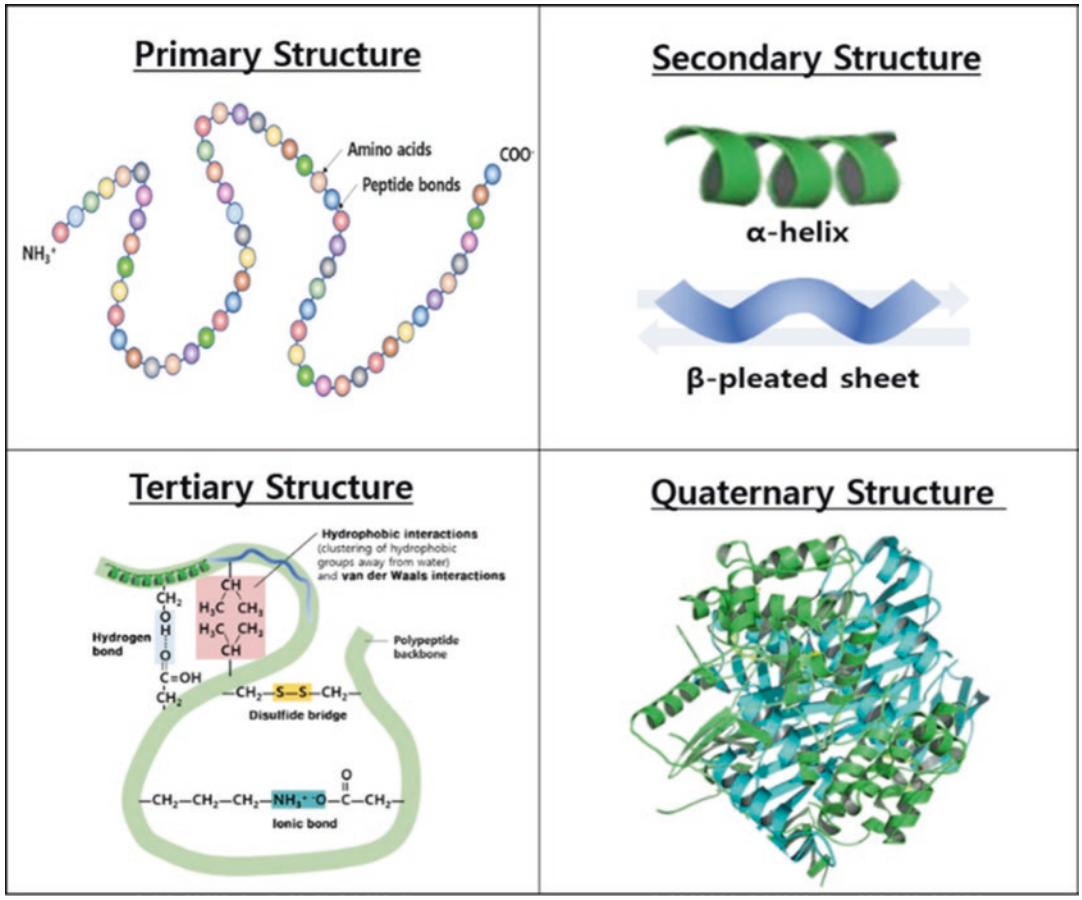

Fig. 10.2 Levels of protein organization

bonds between continuous sequences of carbonyl and amino groups which are separated on the backbone of the polypeptide chain.

– Tertiary structure: This structure is the large scale three dimensional type of a polypeptide chain, which is identified by interactions between amino acid residues that are distant in the chain. This tertiary structure is formed by various interactions such as disulfide bridges by the sulfhydryl functional groups on amino acid side groups playing an important role in the stabilization of protein tertiary structure, in addition to hydrogen bonds, ionic bonds, and hydrophobic interactions between nonpolar side chains.
– Quaternary structure: Some proteins are composed of several separated polypeptide, referred as protein subunits, arranging themselves to make a larger aggregate complex. The shape of the complex is again stabilized by diverse interaction such as hydrogen bonding, disulfide bridges, and ionic bonds.

Proteins can convert between several related structures during performing their functions. In this circumstances of functional rearrangements, the tertiary or quaternary structures are generally mentioned as conformations, and the transitions are named as conformational changes. These changes are usually caused by the binding of a substrate molecule to active site in an enzyme participating in chemical catalysis.

Proteins can be also categorized into three classes, according to their shape; globular, fibrous, and membrane proteins. Most of globular proteins are spherical or ellipsoidal as a result of rolling over themselves, e.g. hemoglobin, and

also soluble, existing as enzymes. Fibrous proteins are structural, stem-like shaped resulting from the rolling of linear peptide chains, e.g. collagen and keratin, which is the major constituent of connective tissue. Even though some globular proteins can play structural functions, e.g. actin, tubulin, however polymerize to form long like stiff fibers composing of the cytoskeleton which make the cells maintain its shape and size. Membrane proteins are provides as receptors, channels for polar, charged molecules to pass through the cellular membrane (van Holde and Mathews, pp. 165–85)

In addition, proteins can be divided into two classes, structural and functional types. The structural type is chargeable for conducting specific biological structural role in tissue or organ, involving retention of integrity, while the functional type is associated with specific amino acid domains for binding and catalysis in enzymes, ligands, receptors, antibodies, and adhesion proteins [23]

The complex structure of proteins related with holding together by non-covalent bonds, except disulfide bonds, is highly influenced by some factors including temperature, pH, organic solvents, and disulfide bond reducers. The changes of protein structure by exposing to such conditions are designated by denaturation. It is extremely influence the biological role of proteins, enzymes in particular. No primary structure, which is determined by a peptide bond, altered during the process. Although denaturation tends to be an irreversible process, the comprehension of structural changes that is occurred during denaturation can be manipulated in order to encourage protein extraction and processing.

The proteins are created by animals, plants, bacteria. Its treatment such as extraction, purification, and isolation dramatically depend on its source and properties. The first process for extraction of protein is cell lysis depending on the type employed for producing the protein. Cell lysis techniques involve freeze-thaw cycles, enzymatic digestion, chemical breakdown, and mechanical disruption methods such as sonication or grinding [245]. And then, soluble and insoluble components acquired by cell lysis are separated via centrifugation. These components are purified by precipitation or chromatographic techniques when the cellular lysate is regained. Though most processes are highly time consuming, it is rising rapidly.

10.3 Protein-Engineered Materials

Protein-engineered materials, consisted of genetically engineered protein domains, supply biopolymers with exact molecular level sequence specification (117–120). In addition, provide weighty merits to natural/artificial polymers. Design in molecules delineating the specification of a chain structure can be modified through altering the sequence of amino acids to create novel classes of engineered proteins with desirable mechanical integrity, self-assembly features, degradation behavior, and bioactivity.

The aim to use protein-engineered biomaterials in biomedical field such as tissue engineering, and regenerative medicine can be furthermore enlarged to involve unnatural amino acids. Incorporating of amino acid analogues including photoactive, fluorinated, as well as unsaturated amino acid into the materials design can cause novel chemical/physical functionality, design versatility, creativity and increasing the potential in applications, resulting in allowing photo-patterning, improved protein stability, and chemical tethering [57, 100, 174]. Guo et al. reported that α-hydroxy acid can be directly incorporated into proteins in *E. coli* with high fidelity and good efficiency in response to the amber codon TAG [100]. It was a novel approach towards the expansion of the genetic code of living organisms beyond the realm of l-amino acids. It was possible to accomplish systemic mutation of the protein in order to investigate its role in protein folding, due to the versatility, low cost, and high yield of recombinant protein expression techniques [76, 211], enzyme catalysis [302], ion-channel gating [163], and molecular recognition both in vitro and in vivo ([211]). In addition, they reported that α-hydroxy acid phydroxy-l-phenyllactic acid can be directly

incorporated into proteins in E. coli with good yields and high fidelity in response to an amber nonsense codon by means of an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase pair from archaebacteria (which does not crossreact with their endogenous counterparts in the host cell). The site-specific introduction of backbone ester mutations is a useful method to study the role of the polypeptide backbone in structure, folding, biomolecular recognition, and catalysis, as well as to cleave or derivatize the protein selectively [100]. Merkel and Budisa incorporated fluorinated non-canonical amino acids (NCAAs) to the existing repertoire of the 20 canonical amino acids prescribed by the genetic code. They could achieve high incorporation levels of fluorinated Phe derivatives into ansA5 and azurin, and thereby, characteristic absorption maxima in the UV/Vis spectra of the fluorinated amino acid analogs as well as the related protein congeners [174].

10.3.1 Design and Synthesis

Protein-engineered materials are generally prepared to be repeating sequences of peptides which can be derived from wild type or engineered sequences. Each peptide module is occupied in order to possess a specific functionality, and the modules are mixed and matched to produce a novel multifunctional biomaterial. The location and density of peptide module enable to be accurately controlled, consequently allowing tailoring of multiple material properties, due to the engineered materials synthesizing from a genetically encoded DNA sequence. In order to perfectly actualize the potential of molecular level design strategies, and exact DNA template should be originated for protein engineered biomaterials. Accordingly, ambidextrousness of the engineered materials is created by controlling DNA template design [236].

The design of a cloning strategy enables to modify easily DNA sequence, where DNA may be tied into or cut out of the recombinant gene, creating families of protein engineered biomaterials. A diversity of methods to template the synthesis of an engineered protein, and a selected DNA sequence can significantly influence protein yield due to the degeneracy of the genetic code. Even if the genetic code is the same for most organisms, the frequency with certain codons, which consist of three DNA bases, is different between each organism. Pursuantly, it is possible to optimize protein engineered materials by selecting the proper series of codons for the specific expression system according to applications. Though it is not enough to select the most common condon for each amino acid. There are a large number of codon usage to prevent DNA recombination once templating repetitive amino acid sequences [38]. Additionally, the formation of suitable or unsuitable DNA structure, similarity of the codons to any contiguous translational sites, and existence of relevant ribosome binding sites on the mRNA enable to support or hinder translation [78, 144, 193]. Though optimization of DNA codon based on the frequency of its usage for a specific organism usually interrelate with efficiency of its translation, it is significant that various codons require to be employed for recombinant proteins as resisted to highly expressed genomic genes [97, 249, 295]. Although it is not completely understood to process codon optimizing, it is fact that mRNA structure plays a crucial role in the gene expression [144].

DNA sequence optimized requires to be instituted into a proper organism for expression. *E. coli* is the most generally employed for expression system, since it is comparatively not expensive, and can be genetically modified to improve protein expression. Apart from commonly used *E. coli* expression strains, mutant strains have been explored to involve the incorporation of no canonical amino acids. However, it is impossible to incorporate posttranslational modifications, e.g. protein glycosylation into the recombinant protein without extensive organism engineering, because E. coli is a prokaryote with no Golgi apparatus or endoplasmic reticulum. Moreover, overexpression of the protein can result in inert, unfolded protein roducing aggregates, which are known as inclusion bodies. Yeast strains, including *Saccharomyces cerevisiae* and *Pichia*

pastoris, are extensively employed for recombinant protein expression, as yeast is a eukaryotic with a Golgi apparatus enabling for a degree of posttranslational modification in proteins [106]. Also, yeast is not costly, and grows quickly comparing with others, more complex expression systems. The glycosylation of yeast differ from mammalian glycosylation, and insect as well as mammalian expression systems have been employed to much more approximate native posttranslational conditions for recombinant proteins [10, 275, 296]. Higher organisms, however, can be utilized for protein expression, transfection and cell culture intend to be more complex and expensive. A diversity of protein engineered biomaterials have employ E. coli as expression system for modular proteins composing of functional peptides with simple domain folds [172, 262].

Therefore, the design as well as synthesis of protein-engineered biomaterials have faced with a specific cartridge of challenges. For each biomaterial, a pliable cloning strategy should be developed, in addition, a recombinant gene should be synthesized to accompany translational efficacy in order to create a novel engineered protein. Also, genetic engineering allows it enable to advance novel biopolymers with a complexity and multi (bio)-functionality mimicking natural polymers originated in nature. By adopting artificial DNA, it enable to hybridized diverse functional domains for a protein-engineered material, facing adherence and migration of cells, mechanical integrity towards multifunctional biomaterial systems. This strategy no need to employ chemical approaches for covalent bonding of bioactive motifs, resulting in denaturation of protein or toxic issue of residuals. Though genetic engineering has been dramatically progressed in biomedical field including tissue engineering, regenerative medicine, it still remains to be investigated for acquiring all merits of the excellent potential of genetic engineering used a tool in the advances of the next generation of engineered biomaterials for biomedical devices.

10.3.2 Techniques for Functionalization

The characters of protein-based biomaterials can be enhanced though hybridization with bioactive molecules to modify function in vitro or in vivo. The surface of engineered materials can be also modified via physical adsorption, physical encapsulation or physical/chemical modification. The techniques are generally employed in order to functionalize the engineered biomaterials with various biomolecules including growth factors and antibiotics.

The adsorption, which depends on the wettability, topography of surface, functional groups, pH, and electrical charge of the engineered materials, can be carried out through a simple immobilization procedure, and attachment of biomolecules including extracellular matrix (ECM) proteins, growth factors onto the surface of engineered biomaterials by dip coating [27]. Numerous engineered biomaterials show hydrophobicity, accordingly, it requires to improve wettability to modify more hydrophilic. In addition, physical methods related to modification including UV light, plasma are adopted in order to hinder chemical bonds between carbon and non-carbon atoms generating unsaturated bonds and radicals reacting with oxygen, causing increase of hydrophilicity and reactivity towards biological molecules [96]. From this point of view, natural polymers are more hydrophilic and easily interact with bioactive molecules, because they have enough reactive chemical functional groups such as hydroxyl, carboxyl, and amide. For example, Friess et al. bound recombinant human bone morphogenetic protein-2 (rhBMP-2) to the absorbable collagen sponge (ACS) for bone regeneration. the results indicated that the most hydrophilic double extended homodimer showed the least binding affinity to ACS. In addition, the amount of rhBMP-2 which could be expressed was decreased by heavier ACS material and allowed to a shorter waiting period, especially at lower rhBMP-2 concentration. It was found that higher product pH or anion concentra-

tion enabled to increase rhBMP-2 incorporation. In addition, the study of the in vivo release kinetics of I-rhBMP-2 from the collagen sponge demonstrated that the ACS/rhBMP-2 systems were significantly different with the in vivo retention of rhBMP-2. Accordingly, it is important to have variability in pH, anion, concentration, crosslinking, and ACS mass as possible to achieve consistent or maximum binding and avoid rhBMP-2 precipitation [86]. Karageorgiou et al. loaded bone morphogenetic protein-2 (BMP-2) in porous silk fibroin biomaterials derived from silkworm cocoons. The release profile of BMP-2 under dynamic culture conditions (spinner flasks) demonstrated that after one week in culture 25% of the initial BMP-2 was retained adsorbed to the silk fibroin biomaterials; up to 4 weeks no additional BMP-2 was released. In contrast, human bone marrow stromal cells (hMSCs) without BMP-2 of control biomaterials displayed limited osteogenesis [127]. It was reported that two types of silk fibroin scaffolds though the salt-leaching technique using two different solvent such as hexafluoroisopropanol (HGIP) and water by Wongpanit et al. [293]. Their in vitro release study proved that the opposite charge between the silk fibroin and basic fibroblast growth factor (bFGF) at physiological pH rendered them to form a complex, and the difference in the solvents used to produce the silk fibroin scaffold did not affect the affinity of silk fibroin to bFGF. From the in vivo studies, the employment of silk fibroin biomaterials as the carrier matrix enabled the control of the in vivo release of bFGF in a sustained fashion over two weeks, while the majority of the bFGF disappeared within one day after the injection of the bFGF in soluble form. A cytokine collagen complex in a circulation model was reported by Kleinheinz. With establishing a biological half-life for vascular endothelial growth factor (VEGF) of 90 min, the VEGF release showed a linear declining gradient, and the release kinetics were not depending on VEGF concentrations. After 12 h VEGF release reached a plateau, after 48 h VEGF was no longer detectable in the complexes charged with lower doses. The results demonstrated that VEGF linked to collagen fibrils displays its improved stability in direct electron microscopic imaging as well as in prolonged release from the matrix [140]. In most of the researches, the protein based biomaterials were soaked in a solution including the biomolecules. Because adsorption is grounded in electrostatic, van der Waals, hydrogen/hydrophobic interactions, and consequently it is comparatively weak. The stability of interactions depends intensely on environmental conditions.

To solve this limitations, covalent binding has been employed, which offers stable attachment of biomolecules to engineered scaffolds. It was proved that covalent immobilization is a very efficient approach to guide the release profile of the conjugate molecules at desirable site. Carbodiimide system is widely adopted in protein-based materials to react activated surface carboxylic acid groups with the amines existed on the peptide. Carboxylic groups are activated through 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) with either N-hydroxysuccinimide (NHS) or dicyclohexylcarboiimide (DCC) or carbonyl diimidazole (CDI) [39, 103]. Murphy and Kaplan reported that the surface modification of silk-based materials for the attachment of growth factors was conducted by using carbodiimide coupling [191]. These protein chemistry has been widely adopted in order to conjugate biomolecules including BMPs and RGD peptides onto silk and collagen biomaterials. One of the limitation of this binding strategy is to be difficult in characterization the new peptide-protein biomaterials, owing to the background noise itself, causing the difficulty to characterize the signal from the peptide conjugated on the surface of engineered materials for quantifying peptide immobilized [150, 191]. Another is a possibility of presence of reactive amine groups [103]. Glutaraldehyde has been also employed to bind insulin and lipase onto silk-based biomaterials as well as crosslink collagen/silk biomaterials, which has a drawback with potential release of toxic residual molecules formed during the crosslinking [44, 304]. In adopting the proper crosslinker, it is important to consider cytotoxicity as well as the potential for crosslinking to interfere with the bioactivity of other peptide modules. Diverse crosslinkers and

physical crosslinking mechanisms have been employed in protein-based materials for multiple cell types in both two-dimensional and three-dimensional cell culture,(Sengupta et al. 2010 [239; [63, 75, 108]).

One of the emerging strategies is to encapsulate biomolecules within protein-based biomaterials, resulting in control of release of the agents to desire site in the body. A minced collagen sponges was incorporated with gelatin microspheres containing 10 micrograms basic fibroblast growth factor (bFGF) in order to control release behavior of bFGF at a defect of rabbit fat tissues [2, 139]. The adipose tissue regeneration introduced by the administration of collagen sponge with microspheres with bFGF was significantly stronger than that of either collagen alone or microspheres with bFGF alone. This study was concluded that the repeated administration of collagen sponge and microspheres with bFGF is a promising approach to achieve adipose tissue regeneration inside inherent fat tissue [139]. Besides, it was reported that a novel crosslinked collagen microspheres loaded with bovine serum albumin (BSA) with excellent protein compatibility as well as injectable size for protein delivery, and demonstrated that photochemical crosslinking can be employed as a secondary retention mechanism in a photosensitizer dosage-dependent manner for protein in a collagen matrix [43]. The foregoing two researches are associated with crosslinking of microspheres to decrease the initial burst and accordingly, obtain much better controlled release behavior of protein. Silk microspheres were also adopted for the encapsulation of proteins by Wang et al. [282]. The study demonstrated that by exploiting the self-assembly behavior of silk fibroin protein, microsphere could be engineered as drug delivery systems.

A great number of formulations for delivery system have been advanced to accomplish functionalization as well as sustained release profile of diverse biomolecules without initial burst. Various factors involving structure and topology of protein in case of biomolecules incorporating into protein-based biomaterials should be considered for prohibiting denaturation of protein as well as aggregation of protein during releasing that cause the loss of bioactivity [294]. Numerous strategies used for functionalization of protein structures and sustained release of agents accompany drawbacks, therefore novel approaches have been required obviously.

10.3.3 Functional Peptide Modules

A wide range of peptide modules are possible to incorporate into protein-engineered materials, the modules are derived from natural wild type sequences, computationally derived sequences, sequences selected through high-throughput screening methods such as phage display [210, 243, 261]. In order to employ as biomaterials for biomedical devices, the module incorporating into the protein-engineered materials is usually adopted to allow specific tailoring of the structural, mechanical, biochemical, and biodegradation characters of the engineered matrix. In general, the design factor firstly adopted is the peptide module offering structural stability to the substituents as injured tissue. Among numerous studies, Urry et al. prepared elastic protein-based polymer prepared using recombinant DNA technology, designed as variations of six kinds of polymer as a subcutaneous implant in the guinea pig, a preclinical test used to evaluate materials for soft tissue augmentation [268]. Also, recombinant multi (bio)-functional elastin-like protein biomaterials with structural base of a elastin-derived repeating unit, containing fibronectin CS5 domains enclosing the well-known cell adhesion sequences REDV introducing to help in the bioabsorption of the polymer for tissue engineering. And the polymer was crosslinked by using glutaraldehyde yielding insoluble hydrogel matrices in aqueous condition [95].

The foregoing two biomaterials adopts motifs in nature providing structural integrity. The modules enable to be introduced to associate through physical crosslinks to create a biomaterial. Silk fibers exhibit a unique hierarchical structure with hydrogen bonded polypeptide strands which crystallize into β-sheet nanocrystals incorporated in a semi-amorphous protein domain [48]. The

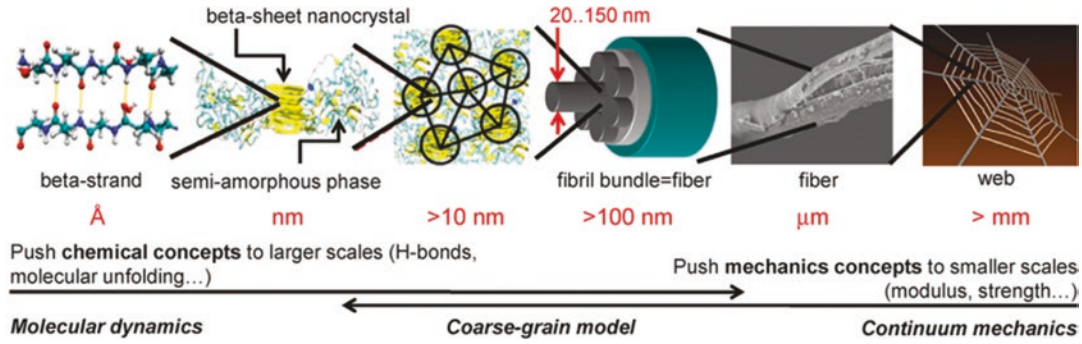

Fig. 10.3 Schematic representation of the hierarchical structure of spider silk including detailed views of the microscale structure and flaws in silk fibers. Adapted with permission from Giesa et al. [94]. Copyright (2011) American Chemical Society

embedded nanocrystals also assemble into fibrils, resulting in packing together to produce silk fibers, as shown in Fig. 10.3 [94].

The mechanical properties of the engineered biomaterial can be taken place due to a consequence of hierarchical self-assembly such as the formation of fibrillary structures. Accordingly, the resultant structural and mechanical properties can be controlled by altering primary amino acid sequence and assembly conditions [250].

10.4 Classes of Protein Materials

A goal in tissue regeneration is to obtain the potential to modulate specific interaction between cell and biomaterials which in turn control over cell functions. The tailor of protein-based materials allow independent controlling of a range of biomaterials functions and performances. Proteins are multi-block polymer playing an essential role in biological functions. In general, protein of fiber type involves elastin, collagen, silk from worm and spider, keratins from hairs, and zeins from corn [284]. It composes of nanocomponents in the hierarchically organized structures. In this part of the chapter, kinds of protein is paraded with the source, properties, biochemistry, structure, and applications in biomedical area.

10.4.1 Collagen

The word collagen has been derived from the Greek (Proteios) which means "of first rank" in that sense collagen completely fulfils the eligibility. Collagens are the most abundantly found protein in mammalian body, which accounts for approximately 30% of the total proteins. They provide the structural integrity and support to the tissues and organs in the body [176]. Collagens are abundantly present in the skin and are one of the main structural proteins in extracellular matrix, where they provide mechanical strength to the tissues. Although the main biological function of collagen is to provide mechanical stability, collagen is also responsible for cell migration, attachment and differentiation [287]. Collagen is being utilized in an array of applications like in skin tissue engineering, bone and cartilage tissue engineering, drug delivery, skin replacement, bone substitutes and for the development of artificial blood vessels and valves [151].

10.4.1.1 Biochemistry and Structure of Collagen

Majority of collagen is produced by fibroblasts, other cells like chondrocytes and osteoblasts can also synthesize them [96, 194]. Synthesis of collagen is a complex process and after many post-transcriptional regulations, final product are produced. Different collagens are characterized

by substantial complexity and diversity in their structure, their post-transcriptional mechanism and the presence of other non-helical domains. Depending on their tissue localization, all collagen molecules differ structurally inn reference of orientations of fibrils, their diameter and packing in the tissue [90]. To date, in collagen superfamily, approximately 28 different collagen types (I- XXVIII) have been identified which all display a typical α helix structure. Among all the family members, fibril-forming collagens are most abundant and share almost 90% of all the collagens [77]. In a typical collagen synthesis process, the building blocks of collagens assemble together in a triple helix to form the functional collagen network [242].

Biosynthesis of collagen takes place in endopalsmic reticulam, where a series of postranscriptional modifications takes place to synthesize mature collagen fibres from its precursor procollagen molecules. In this process various enzymes like hydroxylases, glycosyltransferases and peptidyl cis-trans isomerase and cheprons such as HSP47 are involved [126].

A typical triple helix of collagen is shown in Fig. 10.1, where, three parallel left-handed polypeptide chains coils to each other to form a right-handed triple helical structure. Despite of their structural differences, all collagen types have one common triple helix structure composed of three α-chains consisting of approximately 338 amino acid long Gly-Xaa-Yaa units where X and Y are predominantly proline and hydroxyl-proline. Two non-collagenous domains, the N- and the C-terminal pro-peptides (Fig. 10.4a.1) flank this region. The α-chains vary in size in different collagens and are assembled together either to form homotrimer or heterotrimers (Fig. 10.4a.2, 3). Finally, during the maturation, these N- and the C-terminal pro-peptides are cleaved off by specific proteases (Fig. 10.4a.4) [77, 90].

The Gly–X–Y repeats are common in all collagens but in non-fibrillar collagens (like IV, VI, VIII and X) the coli is interrupted at few loca-

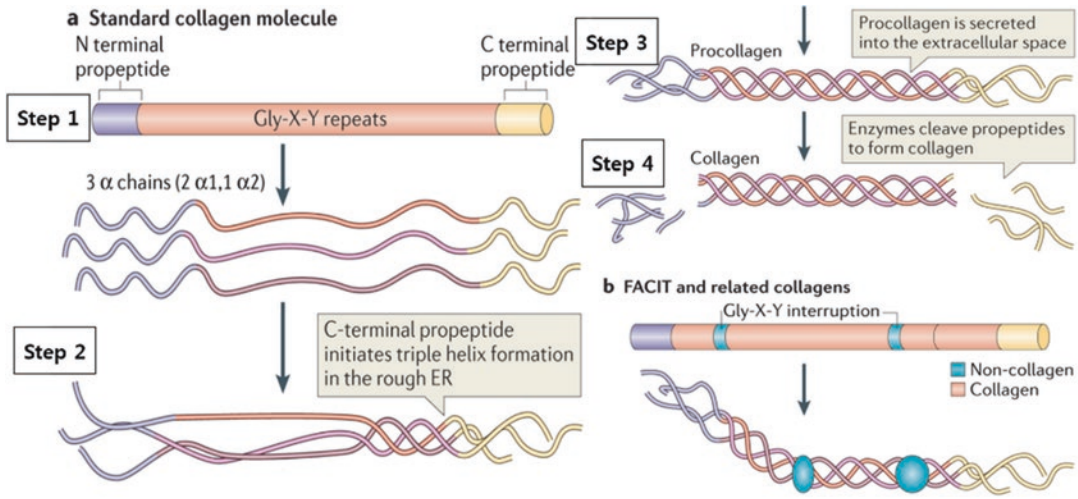

Fig. 10.4 Collagen structure (**a**) step1: The typical fibrillar collagen molecule is characterized by an N-terminal and C-terminal pro-peptide sequences, which flank a series of Gly-X-Y repeats (where X and Y represent any amino acids but are frequently proline and hydroxyproline). step2: These form the central triple helical structure of procollagen and collagen. Three α-chains (the illustration shows two α1-chains and one α2-chain, which is representative of type I collagen) are intracellularly assembled into the triple helix following initiation of this process by the C-terminal domain. step3: Procollagen is secreted by cells into the extracellular space and step4: then converted into collagen by the removal of the N- and C-propeptides by metalloproteinase enzymes. (**b**) Fibril-associated collagens with interrupted triple helices (FACIT) and related collagens have a different structure to standard fibrillar collagen; they contain non-collagenous regions — that is, non-triple helical sequences. These lead to kinks in the resulting macromolecular structure that straighten under small strains. Adapted with permission from [187]. Copyright (2014) Springer Nature.

tions (Fig. 10.4b). These interruptions in (Gly–X–Y) sequences are associated with the structural and functional role of collagen in the body [222, 258]. Glycine is prominently presented amino acid in collagen, and accounts for one third of the total amino acids in collagen which is essential in tight packaging of three α chains of collagen fibers [207]. Further, the triple helix structure of collagen is stabilized by predominantly presented amino acids proline and hydroxyproline, the inter-chain hydrogen bond and the electrostatic interactions [79].

Depending upon their structural organization and functions, collagens are divided into several groups like fibril-forming collagens, Fibril Associated Collagens with Interrupted Triple Helices (FACIT), network-forming collagens, beaded filament collagen, anchoring fibrils, transmembrane collagens, basement membrane collagens (Table 10.1) [90, 241]. In fibril forming collagens (I, II, III, V, XI, XXIV, XXVII), different collagen molecules are assembled together to form fibrils. In cartilage tissue, the collagen fibrils are made up of collagens II, XI, and IX, whereas in skin the fibrils are made up of collagen I and III and in cornea collagens I and V assembled together to form collagen fibril [222].

Among all the different types of collagen, type I and type V are the most prevalent and constitute 90% of the collagen in the body. Furthermore, type I collagen is the main component which determine the mechanical properties of the tissues [256]. Type I, a fibrillar collagen is predominantly present in dermis, bones, tendons and ligaments whereas Type V collagen is widely present in bone matrix, corneal stroma and the interstitial matrix of muscles, liver, lungs, and placenta [30, 257]. The fibrils of collagen are cross-linked to each other to provide mechanical strength and integrity to the extracellular matrix. Whereas, the degree of cross-linking highly affect the strength and elasticity of the tissues [9]. Although, the main function of collagen is to maintain biological and structural integrity of extracellular matrix and to support the connective tissues like tendon, skin, bone, cartilage, ligaments and blood vessels. However, with the structural variations in collagens, they perform diversae of functions like cell adhesion and migration, tissue repair and also have a role in tumor suppression [189]. Specifically, Collagen type IV is found to help in viability, migration, proliferation and differentiation of cells [151].

Table 10.1 Classification and distribution of collagens

Classes of Collagen	Collagen type	Tissue distribution
Fibril-forming	I, II, III, V, XI, XXIV, XXVII	Bone, dermis, tendon, ligaments, cornea, cartilage, skin, vessel wall, most tissues, lung, fetal membranes
Fibril Associated Collagens with Interrupted Triple Helices (FACIT)	IX, XII, XIV, XVI, XIX, XX, XXI, XXII	Cartilage, vitreous humor, cornea, perichondrium, ligaments, tendon, dermis, tendon, vessel wall, placenta, lungs, liver, human rhabdomyosarcoma, corneal epithelium, embryonic skin, sternal cartilage, tendon, blood vessel wall
Network-forming collagens or Basement membrane collagens	IV ,VIII, X	Endothelial cells, Descemet's membrane, hypertrophic cartilage, Basement membranes
Beaded filament or Micro-fibrillar collagen	VI	Dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils	VII	Skin, dermal–epidermal junctions, oral mucosa, cervix
Transmembrane collagens	XIII, XVII, XXIII	Epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver, dermal– epidermal junctions

FACIT (Fibril-Associated Collagens with Interrupted Triple-helices), the non-fbrilar collagen (IX, XII, XIV, XVI, XIX, XX, and XXI) are comparitively less abundent than fibrillar collagens but play equally important role in the maintenance of ECM. It is evident that these collagens works as the molecular bridges which prove structural stability to extracellular matrices [240]. The network forming collagen family include IV, VI, VIII, X, and dogfish egg case collagens [142].

Type IV, VIII and X collagens are the network-forming collagen and are the basic component of basement membrane. Basement membranes are involved in various cellular functions liken cell adhesion, growth and differentiation, tissue repair, molecular ultrafiltration, cancer cell invasion, and metastasis. In basememnt membrane, type IV collgen work for the molecular filtration. Type VIII collagen is resides in the Descemet's membrane which separates corneal endothelial cells from the stroma where itin the form of polygonal frameworks. Type X collagen is a short-chain collagen and can be found in the hypertrophic zone of growth plate cartilage and is thought to be structurally similar to that of collagen VIII (Stephan et al. 2004).

Collagen XIII and XXV are the member of transmebrane collagen family and have cell adhesion properties. These collagens have very important role in neural function and neural tube dorsalisation, eye development, and modulation of growth factor activity. Collagen VII is present in the anchoring fibrils beneath the lamina densa of epithelia (Maertens et al. 2007).

Collagen VI is a typical beaded-filament-forming collagen and is present in most of the tissues where it forms direct links with the cells. In collagen VI, monomers are crosslinked into tetramers to form long molecular chains known as microfibrils, which have a beaded repeat of 105 nm. The filaments also occur in bundles with beads laterally aligned to form long-spacing-type fibrils, which appear to be identical with many fibrous-long-spacing-type fibrils described [35].

Collagen fibrils constitute the main building block of many structural tissues, such as bone ligament, tendon and skin. The defects in collagen genes give rise to diseases like chondrodysplasias, osteogenesis imperfecta, Alport syndrome, Ehler's Danlos Syndrome [265].

10.4.1.2 Application of Collagen in Tissue Engineering and Regenerative Medicine

Due to their low immunogenicity and antigenicity, collagens have a wide range of applications. The extracted collagen fibers can be fabricated into hydrogels, sponges or scaffolds that can be further utilized in a number of biomedical applications like wound dressings, matrices for surgery and implants [9]. Although, majority of the collagen is extracted from the skin, tendons, cartilage and bones of bovine and porcine source however, recently, fish collagen has also received a lot of attention due to its ease of purification from fish skin and bones [190]. It also has been reported that collagen obtained from avian tissues are less immunogenic and non-allergic in compare to bovine collagen [221]. Collagen is biocompatible, biodegradable and safe material to use in biomedical tissue engineering applications. Due to its intrinsic properties like high tensile strength, biocompatibility and biodegradability make collagen a material of choice in tissue engineering [45]. Furthermore, collagen can be easily modified with other proteins like elastin, fibronectin or other materials such as hyaluronic acid, chondroitin sulfate, ceramic, hydroxyapatite-HA, to get better delivery system such as accurate release control, shape-control, increase tissue adhesion, and to promote proliferation and differentiation [71]. Some of the drawbacks of collagen to be used as biomaterial for cell delivery are its excessive swelling property, low mechanical strength, poor tolerance, unpredictable release profile, and high price [167]. Additionally, collagen may be associated with biological contaminants, which can cause severe allergic reactions limiting its biomedical applications. To overcome these drawbacks, collagen can be produced artificially by genetic engineering techniques.

Collagen and collagen-based biomaterials have been used in a number of applications such as a skin substitute, in would healing, dermal fillers, treating diabetic wounds [32, 109]. Collagen is a widely used material in wound dressings, as it is the main component involved in wound healing process. For decades, collagen based wound healing dressings are employed to treat burn injuries and ulcers [89]. The major advantage of collagen-based dressing is the structural and functional significance of collagen in wound healing. It has been reported that collagen facilitates the migration of fibroblasts and other microvascular cells at site of wound and enhance the

tissue regeneration. In addition, due to its hydrophilic nature, collagen-based dressings greatly absorb the wound exudates and provide a moist environment around the wounds [45].

Due to the inherent property of collagen in cell infiltration, and wound healing, many collagen based skin models have been developed. Cells growing on collagen matrix actively synthesize other biomolecules like glycosaminoglycan, which also participate in the rapid wound healing. Furthermore, these assemblies work as a dermal like matrix where keratinocytes can be grown to form a superior skin substitute. It also has been cited that full thickness wound can be treated purely by placing collagen scafoold at the wound site without seeding of fibroblast. Collagen works a chemo-attractant which attract the neighbouring fibrobalsts from the healthy tissues. These fibroblasts secrete glycosaminoglycan and other proteins required for wound healing leading to the construction of dermal tissue at the wound sites [119].

A large number of collagen based skin substitute are commercially available where fibroblast cells are seeded on to the acellular scaffold which leads to the depositoin ECM proteins on the scaffold and are used for the engineering of partial or full-thickness wounds. Dermagraft®, Orcel, Integra®, AlloDerm, Biobrane®, Apligraf®, Graftskin and PermaDerm® are the FDA approved skin substitutes for wound healing [279]. In many reports collagen derived from human dermis or porcine skin dermis or collagen- glycosaminoglycan matrix has been shown to regenerate dermis and epidermis in vivo [175]

Collagen is one of the major components of the bones. Bone is a complexed mineralized tissue of human body, which is made up of three components; bone cells (osteoblasts, bone lining cells, osteocytes, and osteoclasts), organic matrix and inorganic salts. The organic matrix is predominately consists of proteinaceous collagen (90%) which is secreted by osteoblast cells during the process of ossification, some other non-collagenous proteins like osteocalcin, osteonectin, osteopontin, fibronectin and bone sialoprotein II, bone morphogenetic proteins (BMPs), and growth factors are also secreted in the process. In bone tissue type I collagen nano-fibrils are paralleley assembled where HA is precipitated on their surface [3, 82].

A large number of biomaterials have been exploited for the engineering of damaged bone tissues. For this purpose, a scaffold should have porous structure for the migration and differentiation of progenitor cells and should provide mechanical support to the tissue. Furthermore, it should be biocompatible and non-toxic to the cells [81]. Due to the intrinsic part of the bone tissues, collagen have several applications in bone tissue engineering to treat several bones related complications. Generally, for the utilization in bone tissue engineering, collagen materials are hardened by mineralization with calcium phosphate and crosslinking with hydroxyapatite [313].

Collagen based material have also been utilized as a bone repairing and bone filling materials, in cartilage tissue engineering and for the treatment of the disease like osteoarthritis and Osteochondral defects [24]. Collagen have role in many other applications like vascular tissue engineering, neural tissue engineering and in the delivery of growth factors and drugs [173, 218, 247].

10.4.2 Fibrin

Fibrin, a fibrous matrix protein is one of the oldest biomaterials, which is widely used in a number of biomedical applications. It is a main component of extracellular matrix and is synthesized after the cleavage of fibrinogen by the activation of thrombin and the polymerization of its monomers [33]. Fibrinogen synthesized in liver and circulated in the blood, is the substrate of enzyme thrombin. Thrombin is a proteolytic enzyme, which is present in blood plasma and cleaves fibrinogen through a cascade of reactions into an insoluble fibrin matrix. The blood clots formed after the injury serves as a scaffold for the cell adhesion and migration. Fibrin gel obtained from the blood plasma is among the most com-

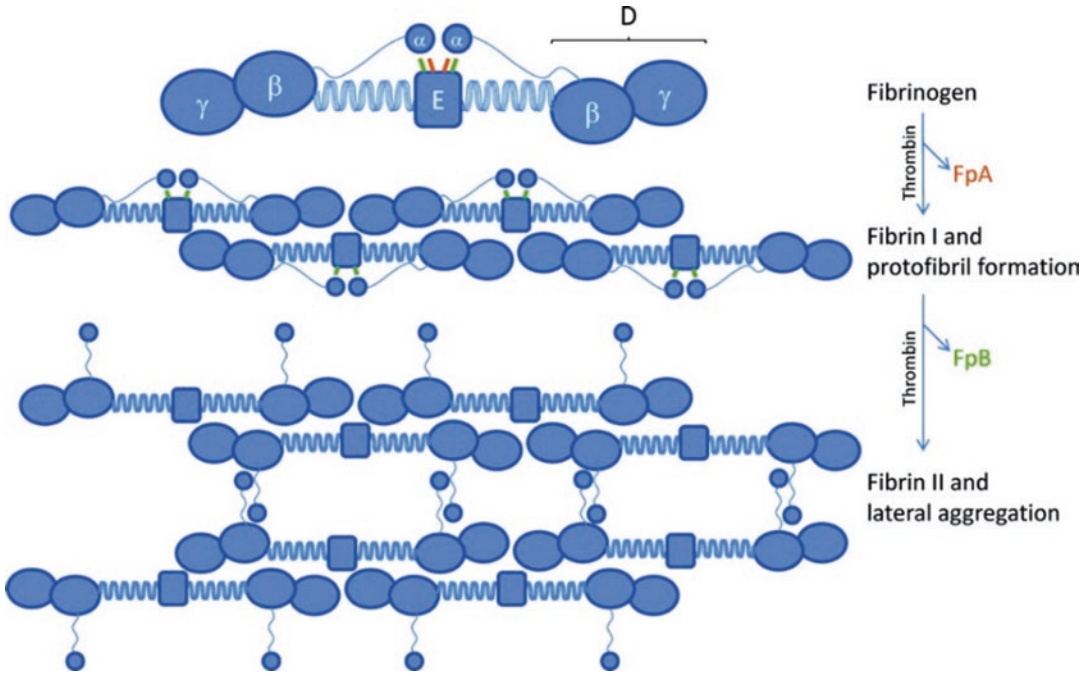

Fig. 10.5 Conversion of Fibrinogen to fibrin by enzyme Thrombin. Adapted from Li et al. [158]

monly used hydrogels [310]. Fibrin protein provides elasticity to the blot clots and stimulate the various cells, which are involved in wound healing process [160]. The network created by fibrin, entrap the cells like neutrophil, macrophage and fibroblast, which in turn produce fibronectin, collagen, and other components of extracellular matrix to reconstitute the damage tissue. Besides the structural support, fibrin also involve in the signaling by binding to the various proteins and growth factors [33]. Due to its intrinsic properties like biocompatibility, biodegradability and easily processability, it is widely used in tissue engineering applications.

10.4.2.1 Biochemistry and Structure of Fibrin

Fibrinogen, a 340-kDa molecular weight glycoprotein, which consists of three pairs of polypeptide chains namely Aα,Bβ, and γ. 29 disulfide bonds hold these pairs of proteins together. [158, 310]. Synthesis of fibrin from fibrinogen is shown in Fig. 10.5. Each polypeptide chain consists of two outer D domain, which are connected to central E domain by a coiled coil type fragment. The Bβ and γ C-termini resides in the D-region, whereas the E-region holds the N- termini of all 6 chains [266]. Polymerization of fibrin is divided into two steps; first is the proteolysis of fibrinogen to fibrin, which starts with the cleavage of fibrinopeptide A (FPA) sequence. The sequence is present at the N-terminal ends of Aα –chains of each fibrinogen protein by thrombin enzyme. After the initiation of the reaction, D and E domains associates to form double stranded fibrils, which further gets connected with later fibrils to form a clot. The second step is the stabilization of fibrin through the action of Factor XIIIa. These fibrin assemblies facilitates the alignment of antiparallel C-terminal chains, which are covalently cross-linked by factor XIII also known as plasma pro-transglutaminase or XIIIa to form γ –dimers. The conversion of fibrinogen to fibrin is highly dependent on concentration of calcium ions [186].

The fibrin matrix network works as a scaffold for the cells during the wound healing. Moreover, it works as a sealant and its primary role is to stop blood loss after the injury and to provide homeostasis [110]. Alteration in structure of fibrin can

lead to many diseases and complication such as acute or prior myocardial infarction, ischemic stroke, and venous thromboembolism [162].

Fibrin is known to interact with various proteins like fibronectin, vitronectin, plasminogen as well as tissue plasminogen activator and growth factors like FGF, VEGF, and insulin-like growth factor-1. This complex combination plays an essential role in the process of wound healing through the receptor mediated interactions with the cells. Fibrin complex binds to the cells mainly through αvβ3 integrin receptors on the cell surface by a tripeptide amino acid sequences (RGD) [200, 300]. Fibrin is a characterized as a viscoelastic polymer, which possess both elastic and viscous properties. Fibrin meets many requirements of biomaterial characteristics for tissue engineering. Fibrin-based biomaterials are highly adhere to many biological surfaces and are used in the repair of tissues in urinary tract, heart, liver and kidney [73]. In biomedical engineering, fibrin scaffolds have been used in bone tissue engineering, repair neurons, vascular tissue engineering and the surface of the eye [238].

Evaluation of fibrin biocompatibility was displayed through its non-toxic, non-allergenic and non-inflammatory nature [303]. In addition, its biodegradation and can be regulated by the presence of fibrinolysis inhibitors and fiber cross-linkers. One of the noticeable points is that fibrin is cheaper than collagen and it can be used without undesired immunogenic reactions [183]. Due its characteristic properties like tissue homeostasis, tissue regeneration, rapid polymerization and easier tunability makes fibrin gels are frequently used in surgeries for wound healing and as a sealant. Additionally, fibrin can work as a natural active scaffold, which can stimulate cell adhesion, migration and proliferation through acceleration in angiogenesis. The growth factors, which are naturally present in fibrin, stimulates the tissue regeneration [134, 158]. Advantage of using fibrin protein in tissue engineering is that it can be produced autologously in the body without any potential risk of contaminations. The ideal conditions for tissue engineering is that the rate of degradation of scaffold should be equal to the rate of tissue regeneration; one drawback of fibrin is its rapid degradation rate that can strongly affect some specific fibrin-based scaffold. The production of fibrin has been conducted from fibrinogen and thrombin materials. Ratio of fibrinogen and thrombin materials, ionic strength, pH, and calcium amount present causes variation in the production which can lead to variability in characteristics of fibrin molecule [120].

Being a product of blood plasma, fibrin may have the risk of transmission of blood borne diseases, however many products like Baxter's Tisseel, and Beriplast HS/Beriplast P™ has been widely for several years in surgical procedures without any re-occurrence of blood transmitted disease [129, 131].

10.4.2.2 Application of Fibrin Proteins in Biomedical Engineering

Fibrin based scaffolds are found to be more advantageous than synthetic polymers in being cheaper, less immunogenic and they can easily attach to many biological surfaces with high efficiency [273]. For more than 100 years, polymerized fibrin is being used as a sealant after surgeries. Currently, many different types of stabilized fibrin sealants, which are rich in fibrinogen, factor FXIII can be obtained commercially [5]. Furthermore, due to its higher biocompatible and biodegradable nature, fibrin is used as matrix for the stem cell differentiation and tissue regeneration. Fibrin gels are frequently used as drug delivery system to deliver various cells and growth factors to the damaged tissues. These gels worked as matrices to entrap the cells to maintain cell growth and viabilities in wound healing process [290]. The structure of fibrin such as thickness of fibers, number of branches, porosity of the matrix as well as molecular factors like binding sites on fibrinogen, pro-enzymes, clotting factors, enzyme inhibitors, and cell receptors are highly important in the process of wound healing. These characteristics can be manipulated by changing the pH, concentration of fibrin monomer, and ionic strength, according to the need of application [148]. The principle advantage of fibrin biopolymers is their biocompatibility, resorbability, and ability to incorporate both cel-

lular and non-cellular components to regenerate the specific microenvironment [20].

Fibrin glue, also known fibrin sealant is a biological adhesive, which contain fibrinogen, thrombin, fibronectin, calcium chloride and other component of plasma are used as a sealant in ophthalmology in various complications such as Conjunctival surgery, pterygium surgery, strabismus surgery, cataract surgery, and in the treatment of corneal ulcers [134, 205].

Fibrin gels have a caught a special attention in the bone tissue engineering and it has been reported that each component of fibrin contribute significantly in bone tissue engineering, where they are utilized to enhance the regeneration and repair rate of damaged bone tissues. Fibrin has excellent biocompatibility, biodegradability and poor immunogenicity, which makes it an ideal candidate for the synthesis of scaffold for engineering of damaged bone tissues [69]. Fibrin is a widely used candidate for the delivery of growth factors and cells. After wounding, a cascade of reaction starts which form a matrix where cells can be entrapped and release growth factors. The growth factor-cells combination initiates wound repair and tissue regeneration. Fibrin gels are found to be more advantageous in compare to local or systemic injections of cells and growth factors [290]. For the treatment of ischemic heart disease, fibrin has emerged as a potential biomaterial due its ability to binding and control release of the growth factors. Furthermore, fibrin is actively involved in receptor-mediated interaction with the blood vessels and cells resulting in the activation of ECM and stimulation of angiogenesis [34]. Despite of its role in various tissue engineering application, fibrin gels are frequently used for the delivery of drugs, genes and various proteins ([154]).

10.4.3 Elastin

Elastin, one of the most extensively studied is an extracellular matrix structural protein, which provides flexibility to the tissues and organs including blood vessels, arteries, skin, lung, ligaments and tendons. In addition to providing mechanical integrity to tissues, elastin also has critical role in the regulation and modulation of cellular behavior [291]. In human skin, elastin fibers are augmented in the dermis where they provide elasticity and extensibility to the skin. Generally, elastin is made up of two alternating domains, the hydrophobic domain provide elastic and self-aggregation properties to elastin where as another domain provide stability to elastin molecule by formation of covalent cross-linking [146]. Elastin is highly hydrophobic and is produced by a soluble precursor protein tropoelastin (TE) through a process called elastogenesis [237]. Tropoelastin, consisting of alternative hydrophobic and hydrophilic domains is secreted by elastogenic cells and is self-assembled in association with a fibrillin-rich microfibrillar structure to form elastin [4]. It is assumed that elastin consists of VPGXG repeats of amino acids which provide the characteristic rubber-like properties to the protein [229] (Li, Charati, and Kiick 2010).

Many diseases such as atherosclerosis, emphysema, hypertension, actinic elastosis, and aortic aneurysms are associated with the dysregulation of elastin synthesis [171, 267]. Several elastin protein based biomaterials are widely used as a scaffold for tissue engineering due to their long-term stability, self-assembly and tremendous biological properties. These proteins can either be obtained from animal sources or synthesized artificially through recombinant technology and can be fabricated to 3D scaffolds, films or hydrogels [12]. The main drawback of elastin is its high insolubility, which limits its wide applicability whereas its soluble derivatives have a wide range of applications.

10.4.3.1 Biochemistry and Structure of Elastin

Elastin is the protein of extracellular matrix of tissues, which undergoes frequent cycles of extension and recoil for their proper functioning including blood vessels, skin, bladder and lung. It is a fibrous, highly cross-linked, and insoluble protein and is present variably in different tissues depending on the functions [179]. The elastin polymers are composed of cross-linked tropoelastin monomers, which are interwoven around

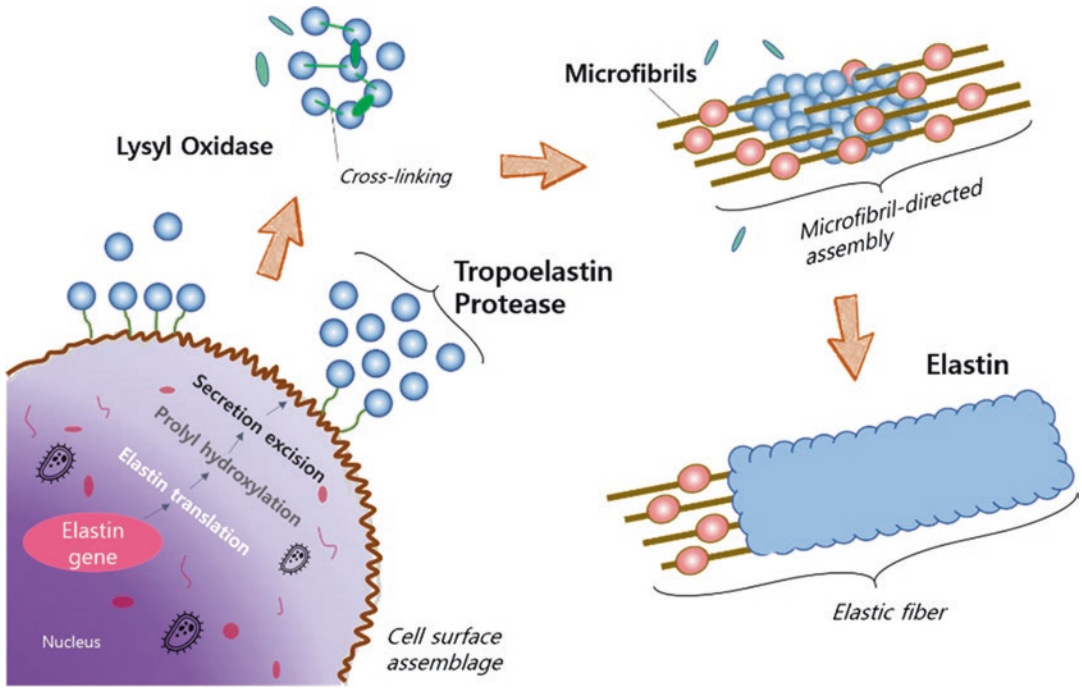

Fig. 10.6 Processing of Elastin fibers, 1) Tropoelastin is secreted by the cells, 2) Tropoelastin combines with microfibrillar proteins, 3) cross-linking to form mature Elastic fibers. Redrawing from Silva et al. [245]. Copyright (2014) Royal Society of Chemistry

a fibrillin-rich microfibrillar structure. The functional unit of elastin is elastin fibers, which are made up of multiple hierarchical components. The structure of mature elastin fibers have different morphologies according to the organization of the tissues, which reflects their specific function in different tissues. For example branched networks in lungs, skin and ligaments, concentric sheets in blood vessels and honeycomb like structure in cartilage [278].

Translation of Elastin from its precursor molecules is depicted in Fig. 10.6. Elastin, which is encoded by single copy gene in the mammalian genome, is translated to various tropoelastin forms by alternative splicing and post-transcriptional modifications [64]. Synthesis of elastin takes place through the deposition of tropoelastin molecules, the precursor of elastin on a fibrillin-rich microfibrillar template [135]. The expression of tropoelastin, a 72 kDa protein and synthesis of elastin takes place in fibroblasts, vascular smooth muscle cells, endothelial cells and chondrocytes [225]. The template and elastic microbrills are abundantly distributed in the tissues like blood vessels, skin and ligaments where they provide biomechanical strength to these tissues. However, very little is known about the shape and its structural compositions, many studies have been performed to elucidate the structure and function of these molecules. After a number of posttranscriptional modifications, tropoelastin molecules assembles to form elastin fibers. Elastic fibers are a complex structure, made of tropoelastin and microfibrils, which are further made up of acidic glycoproteins [278]. Fibrillins are the basic structural components of microfibrils. The three dimensional model of elastin shows that tropoelastin molecules are interwoven around the microfibrills as well as with themselves [16]. Cross-linking of tropoelstion to microfibrills is assisted by the enzyme lysyl oxidase which deaminates the lysine side chains of tropoelastin to allysine sidechains. The allysine side chains further cross-links with adjacent side chains to form complex elastic fibers (Lisa Nivison-Smith and Anthony Weiss 2011).

The hydrophilic lysine and alanine rich hydrophobic domain of tropoelastin are involved in cross-linking during the formation of elastin molecules and are remains unavailable for the cells to contact. On the other hand, the hydrophobic domains of elastin, rich in repeated sequences of proline, valine and glycine are supposed to interact with the cells and may change their behavior have also been identified. These hydrophilic and hydrophobic amino acids are essentially required for the assembly and elasticity of elastin fibers [135]. Elastin has found to possess chemotactic properties, which are essentially required for cell proliferation and differentiation to maintain the tissue homeostasis [6].

Elastin is a bioactive molecule and various cells interact with elastin through the receptors like, elastin binding protein, glycosaminoglycans (GAGs) and integrins on their surface. Among all these receptors, integrins are major protein receptors, which binds to the RGD motifs of other matrix components, and are involve in the cell adhesion, migration and proliferation of the cells. Tropoelastins are lacking in the RGD moieties and binds to integrins through their C-terminal ends as well as their central regions [202].

10.4.3.2 Application of Elastin in Tissue Engineering

Recently, a wide range of elastin-based biomaterials has been employed in the biomedical engineering. Due to its mechanical stability, elastic resilience, bioactivity and properties to form self-assemblies, makes elastin an ideal biomaterial candidate for the engineering of soft tissues. Elastin based biomaterials provide appropriate cellular interaction and signaling which enhance local elasticity of the tissues and help in the repairing of damaged elastic tissues [202]. Varied number of fabrication technologies such as heating, electrospinning, wet spinning, solvent casting, freeze-drying, and cross-linking have been employed for the fabrication of elastin and elastin based biomaterials to utilize in various biomedical and tissue engineering applications like drug delivery, nerve and vascular tissue regeneration, wound healing, and for the bone, cartilage and dental replacement. The physical, biological and chemical properties like elasticity, porosity, bioactivity, morphology and structure of the fabricated materials can be tailored according to their application [305].

Being an essential component of dermis of a healthy skin, a range of elastin based 3D scaffold have been generated for the treatment of burn wounds and dermal regeneration. It can be used as in various forms including autografts, allografts, xenografts, de-cellularised extracellular matrix, as well as purified elastin materials [6]. Elastin is an important component of vascular tissues where it provides mechanical strength and elasticity to the vessels. Owing to these properties, elastin based biomaterials could be utilized in the replacement of damaged or defective blood vessels. Though elastin is an insoluble polymer, it can be processed to form template for cellular remodeling and reorganization of damaged tissues [149]. Furthermore, elastin possess unique structural and cell signaling properties, which is exploited in coating of elastin to the surface of other polymers which are too hydrophobic to adhere the cells directly. In many reports, binding of elastin to various polymer surfaces to enhance their cell adhesion and other biological activities has been explored [6, 19].

In native form elastin is less advantageous, which is due to the insolubility of elastin polymers and difficulty in its purifications. Besides, elastin is associated with several glycoproteins, which confers a high susceptibility to calcify upon implantation. To overcome these limitations, various soluble form of elastin like tropoelastin, α-elastin and elastin-like polypeptides are regularly used for the fabrication of cross-linked hydrogels to use in different applications [146].

10.4.4 Silk

Silks are the fibrous proteins secreted by the specialized epithelial cells that lined the glands of arthropods like silkworm (*Bombyx mori, Anthereae pernyi*) and spiders (*Nephila clavipes, Araneus diadematus*) scorpions and some flies (Leal-Egana et al. 2010) [153]. These insects spin silk fibers during the process of metamorphosis.

Silk fibers obtained by the cocoon of silkmoths, Bombyx mori, which has been domesticated for thousands of years is used extensively in textile industry. In biomedical engineering, silk fibers has been fabricated into a number of materials like sponges, thin films, tubular architectures, fibers, hydrogels, and particles [274]. Silk produced by spiders are structurally different from the silkworm-derived silk and have outstanding mechanical properties. Silkworm silks are comprised mainly of fibroin, whereas the major protein of spider silks is spidroin [62, 72]. Recently, silk has attracted widespread attention due to its superlative material properties and promising applications.

10.4.4.1 Spider Silk

The use of spider silk can be dated back as thousands of years due its poor immunogenicity, antigenicity and strong mechanical properties. Spider synthesize silk for webbing, to catch their pray and to protect themselves from predators. Spider silks have very high tensile strength and possess toughness three times higher than the most natural and artificial fibers. Furthermore, spider silks are highly degradable, possess antibacterial, and hypo allergic properties [227]. Spider silk fibers are high performance, protein-based materials, which are synthesized by a very sophisticated hierarchical manner under ultra-mild conditions and have a much higher stiffness due to their greater extensibility [74].

Most studied silk is major ampullate silk (MAS) also known as dragline silk, which is produced in the major ampullate glands of spiders. Spider silks, called as spidroins, are larger proteins made up of monomers of 350-kDa molecular weight. Spider silk is considered to be made up of two type of proteins Spidroin I and II [271]. The amount of spidroin I is higher than the spidrion II and are present uniformly throughout the fiber core. Spidrion II is clustered in the core area and are distributed in homogeneously. Despite of their structural differences, all Spidroins share a common primary structure. Amino acid sequence analysis shows that these proteins are divided into three parts: an N-terminal non-repetitive domain; a highly repetitive central part composed of approximately 100 polyalanine/glycine-rich segments; and a C-terminal non-repetitive domain [227].

The mechanical properties of silk fibers are attributed to the presence of highly repeated backbone region composed of alternating blocks of a glycine-rich block followed by an alanine-rich block. Glycine rich regions are known to form different structures β-spirals and coil structures. Spidroin I provide tensile strength to the fibers whereas spidroin II are responsible for elastic like properties of fibers. It is assumed that the mixture of both proteins an exceptional combination of tensile strength and extensibility (Santos-Pinto et al. 2014). Due to their incredible properties, spider silk fibers have a high potential to use as a biomaterial in tissue engineering applications.

10.4.4.2 Silkworm Silk

Silkworm silk is a fiber of millennia, which is extensively used in the textile industry for more than 4000 years due to its soft, pearly luster and good mechanical properties. Silk is produced by the larvae of silkworm to protect them infection, injury, and environmental changes during their metamorphosis stage [168]. The silkworm fibers are made up of two proteins; silk fibroin a core protein, which provides mechanical strength and stability and an outer layer of sericin protein, which works as glue to hold the fibroin threads to each other. Silk fibroin is a naturally occurring, multi domain proteins, which encompass very high biocompatibility, biodegradability, superior mechanical properties and strength [51]. Silk fibroin is fibrous protein and has been found to be more biocompatible than many of commonly used polymers. Despite of its huge applicability in textile industry, silk fibroin equally studied in the fields of tissue engineering and biomedical engineering. Depending upon the application, silk fibers can be fabricated into fibers, porous matrix, hydrogels, and films to utilize in applications like controlled drug release, tissue regeneration and for the synthesis of scaffolds for tissue engineering [216]. Silk biomaterials provides appropriate support for cells adhesion proliferation, and dif-

ferentiation in cell culture and tissue regeneration. Silk biomaterials can be processed desired morphology with ease. Relatively high biocompatibility and enzymatic degradability makes it a material of choice for huge number of biomedical application [214].

10.4.4.3 Biochemistry and Structure of Silk Fibroin

Silk fibroin, isolated from silkworm (*Bombyx mori*) is composed of a single fiber, which has a length of about 700–1500 m long a diameter of 10–20 μm. Fibroin protein is encoded by a 17-kpb long fibroin gene present on the genomic DNA of Bombyx mori [314]. A typical silk fibroin molecule is composed of a heavy chain (H-chain) of molecular weight 350–390 kDa, and a light chain or L-chain of molecular weight 26 kDa, which are connected to each other by a by a single disulfide bridge. Along with them, there is an accessory glycoprotein of molecular weight 30 kDa. The H, L and accessory chain are assembled together in a ratio of 6:6:1 to form a high molecule weight elementary unit [113–115]. Silk fibroin is characterized by presence of block co-polymers made up of hydrophobic and hydrophilic blocks, which together provide elasticity and toughness to the fibers [128].

The primary structure of fibroin protein consists of layers of antiparallel-chain beta pleated sheets with amino acid sequence – (Gly-Ser-Gly-Ala-Gly-Ala)$_n$-. These repeats are the building block of the β-sheets, where Glycine is the most dominating amino acid (45.9%) throughout the structure. High glycine content allow the sheets to form more compact and tight packing, which contributes to silk high tensile strength and rigid structure. A combination of stiffness and toughness make it a material with applications in several areas, including biomedicine and textile manufacture [314]. Not only the primary structure, secondary structure and the organization of fibroin fibers also determines the biomaterials properties of silk fibers. Furthermore, the chain conformation in amorphous blocks is random coil, which in turn provides elasticity to silk [145].

10.4.4.4 Application of Silk Protein in Tissue Engineering

Silk proteins are highly biocompatible molecules and provide a suitable environment for cells to grow, adhere, and differentiate in course of tissue regeneration. For their utilization in various biomedical applications, silk fibroin has been fabricated to various forms like scaffolds, nano-fibers, gels, and films in various forms, depending on their application [150]. Several studies support the use of silk fibrin in many biomedical applications. electro-spun nanofibers made up of silk fibroin and cellular activities of keratinocytes and fibroblasts were assessed by Min et al. in 2014. The result obtained confirmed the cell adhesion and strong cyto-compatibility of silk nanofibers (Min et al. 2004).

The electro-spun matrices of silk fibers have also been utilized as a scaffold for the growth an attachment of human bone marrow stromal cells (BMSCs). The data revealed that these matrices were found to be biodegradable and biocompatible to the attached cells, which suggest that these matrices can be efficaciously used as scaffold for the tissue engineering [123, 124]. Silk fibers are efficiently used in the regeneration of musculoskeletal tissues. Silk fibers have high potential to repair defects in bones and cartilage due to their intrinsic properties of cell adherence. A vast number of literature have described the use of silk in bone tissue engineering. As mentioned above, silk can be fabricated appropriately for the treatment of various complications related to bones. Moreover, silk has active sites on their surface, which help in the binding of growth factors and bioactive molecules resulting in the biomineralization at the defected bone sites [29]. Being a protein polymer, silk fibrins are potentially utilized in the encapsulation and delivery of a large number of drugs and therapeutic agents. Owing to its superior biocompatibility, higher mechanical properties and slow degradation rate make it a desirable delivery vehicle. Furthermore, it also have been used proficiently for the delivery of sensitive molecules like protein and nucleic acids, growth factors and drugs at the desired destinations for the treatment of various ailments and tissue regeneration [264, 288, 312].

The animal model studies have also proved the efficacy of silk protein in the wound healing and tissue regeneration. Films fabricated by silk fibroin protein were used to investigate the ability of these films in skin repairing and to evaluate the safety and effectiveness of the films. The data reveled that these films effectively reduced the wound healing time and promote skin repair and regeneration. Which further suggest their potential role in tissue regeneration and engineering [309].

Although silk fibroin has been proved to be an model biomaterial for tissue engineering application but it have certain drawbacks which limits its wide range applicability. Presence of sericin in silk fibers are associated with some inherent limitations, which restrict their use in many applications. It is believed that these glue like proteins may reduce the biocompatibility of silk fibroin and are responsible for hypersensitivity of silk [7].

10.4.5 Keratin

Keratins are the insoluble and filament-forming proteins, which are naturally present in epithelial tissues in the form of soft keratins. On the other hand, epidermal appendages like hair, wool, horns, beaks, feathers, hooves and nails of the animals are characterized by the presence of hard keratins [105]. Keratins are high Sulphur containing, insoluble proteins, which are structurally different from other fibrous proteins like collagen and elastin. They are usually unreactive to outer environment and provide protection and mechanical support to the tissues [125]. They are considered the toughest biological material in human body and are involved in variety of functions. Keratins not only protects the epithelial cells from mechanical stress but also involve in cell growth, proliferation, apoptosis and organelle transports [31]. Industrially, keratins are extracted from several sources like hair, feathers, horns and a number of other animal sources to use as feed for livestock and fish [70].

Recently in the field of tissue engineering and medical biology, a wide range of biodegradable and biocompatible biomaterials is being experimented. Keratin also has been fabricated in the form of scaffolds, hydrogels and other forms to use as a novel biomaterial. In recent years, keratin based biomaterials have gained special attention as a naturally derived biomaterial owing to their ultra-structure, biodegradability, outstanding biocompatibility, and presence of cell interaction sites [152, 153]. Furthermore, simple, sustainable, and cheap extraction methods make it more assessable to use in the pharmaceutical technology for different applications [182].

10.4.5.1 Biochemistry and Structure of Keratin

In humans, as much as 54 keratin genes exits which have highly specific temporal and spatial expression mechanism depending on the stage of differentiation and type of the cell. There are different types of keratin present in the body depending on their function and tissue type. Keratins can be classified to soft and hard keratin depending on their occurrence in the body. Half of the all keratins are localized to various parts of hair follicle [166, 184]. Mutation in one or many keratin gene has been found to be involved in a number of diseases that affect epidermis and epithelial structures. Epidermolysis bullosa simplex (EBS) is a disease, which is the outcome of mutation in K5 and K14 keratin genes resulting in the skin fragility [42, 60].

Keratins are the members of intermediate filament family which consists of different structural pattern like α-pattern, β-pattern, feather-pattern (similar to β-pattern) and amorphous pattern depending on their localization. The primary structure of each keratin is a chain of amino acid, where number and sequence of amino acids vary in different types. The molecular weight of keratins in mammals ranges from 40 to 70 kDa [280].

According to morphology, keratin can be classified as α- and β-types. Both of them have striking dissimilarity in their molecular structure and pattern of filament formation but have a characteristic filament-matrix structure [285]. α- keratin are organized into coiled coils structure and all α-helices are in right-handed confirmation, whereas β-types have a beta-pleated sheet type of

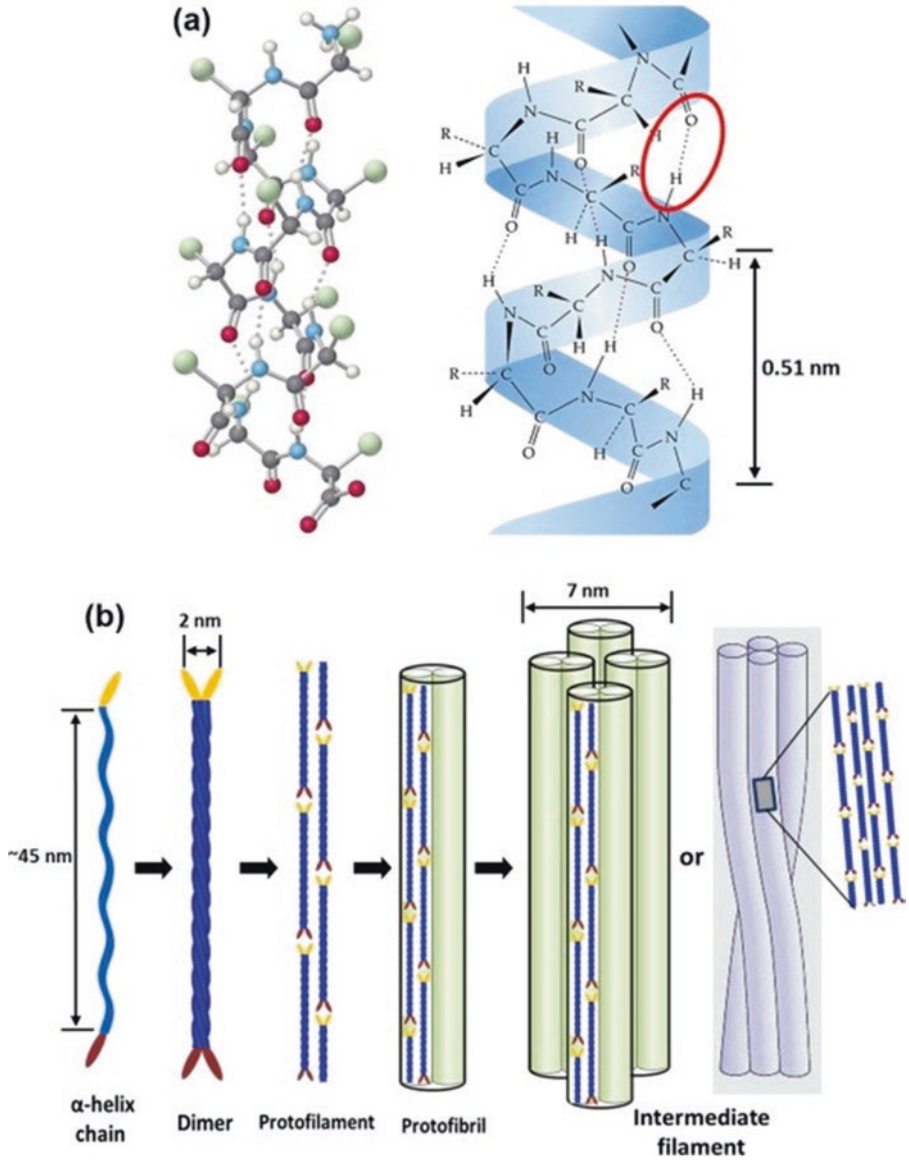

Fig. 10.7 Intermediate filament structure of α-keratin: (**a**) ball-and-stick model of the polypeptide chain, and α-helix showing the location of the hydrogen bonds (red ellipse) and the 0.51 nm pitch of the helix; (**b**) Schematic drawing of the intermediate filament formation: α-helix chains twist to form the dimers, which assemble to form the proto-filament. Four proto-filaments organize into the intermediate filament Adapted with permission from Wang et al. [285]. Copyright (2016) Elsevier

structure. β-types of keratins are usually found in birds and reptiles [269].

In α- type keratin monomers assembles into bundles to form intermediate filaments. The molecular structure of intermediate filament is shown in Fig. 10.7a. Internally hydrogen-bonded polypeptide helices, which are grouped into 3-stacks and each chain, is rotated to shift vertically at a distance equal to the pitch of helix which is 0.51 nm relatively two other chains [117]. Hydrogen bonds stabilizes the helix and provide the chain the helical form. Figure 10.7b, shows that two isolated right-handed a-helix chains coiled to each other in a left-handed coiled-coil

manner to form the dimers. Later, these dimers assemble end-to-end and stagger parallaly by disulfide bonds to form a pro-filament of diameter about 2 nm. Next step is the assembly of pro-filament to form protofibril. Kinetic studies have shown that this step is rate-limiting step, where four protofibrils aligned to form either a circular or a helical intermediate filament of 7 nm diameter (Norlen and Al-Amoudi 2004). Keratins isolated from various sources have been employed as a biomaterial in tissue engineering. Many factors like source of keratin, extraction and processing methods as well as the fabrication techniques highly affect the biocompatibility and biological properties of keratin (Shavandi et al. 2017).

10.4.5.2 Application of Keratin Based Biomaterials in Tissue Engineering

Keratin based biomaterials have been used for a variety of applications such as bone and skin tissue engineering, nerve tissue regeneration and wound healing in various forms including coatings, gels, films and scaffolds. Due the presence of cell attachment moieties, keratin is an ideal material for injured tissue regeneration. It has been observed that keratin filaments control the cell adhesion by regulating the properties of cadherin and cell adhering property of keratin is being utilized in many biomedical applications [277]. Recently it has been reported that keratin have the ability to promote alternatively activated macrophages which helps in tissue remodeling and repair [286]. Keratin extracted from wool and human hairs have been used to formulate into films by a procedure of solvent casting, to explore the structural and biological properties of keratin. Pure keratin are highly fragile due to that they are often used in combination with other biomaterials [182].

Saul and co-workers have exploit keratin in the form of hydrogels for the sustained release of antimicrobial drug ciprofloxacin. In their research, they loaded drug in the keratin hydrogel to treat the skin injury and to provide the antimicrobial environment during the wound healing. The results confirmed the controlled release of the drug over the period of time and the rate of release was in correlation with the degradation of keratin. The study confirmed that keratin have a high potential to use in regenerative medicine and tissue engineering [232].

In the treatment of peripheral nerve tissue engineering also, keratin has been used extensively. In a mouse model, keratin hydrogels have shown significant improvement in electrophysiological recovery at the time of regeneration of the tissue. The hydrogels were effective to produce long-term electrical and histological results, which were similar to the sensory nerve autografts [14].

Recently, it has been reported that keratin consists of large number of cell-attaching amino acids like, Leu-Asp-Val (LDV) as well as Arg-Gly-Asp), which in turn support cell attachment and proliferation and can be utilized as a cell culture coating material [152, 153]. Moreover, keratin sponges prepared from human hair have been studied for their role in cell attachment and proliferation and confirmed their utility in tissue engineering scaffold for better cell to cell interactions [276].

10.4.6 Plant Protein

Intense research work is going on to develop novel biomaterials for use in tissue engineering and regenerative medicine. Recently, for the production of biomaterials, an alternative technology other than mammalian system has been adopted. Plant produced biomaterials are coming up as the fastest growing class of pharmaceutical products. Plant materials like polysaccharides and proteins are frequently used in the fabrication of scaffolds used in an array of applications. Naturally, occurring proteins often exhibit a large number of superior properties over the synthetic materials (Scheller and Conrad 2005). Plant derived proteins have advantage over animal produced protein in being non-immunogenic and non-toxic [91]. Additionally, the molecular weight of plant-derived proteins are lesser than the animal proteins, they are more hydrophilic in nature and high cross-linking efficiencies. Due to

their effective cell attachment properties, they are frequently used as a biomaterial in biomedical engineering. The newly developed plant expression system can effectively produce materials with higher production rate, which is difficult to get in mammalian system [181].

Currently, a number of important plant proteins like Zein (derived from corn), soy-proteins and wheat proteins (gluten, gliadin and glutenin) have been presented as novel class of biomaterials, which are bioactive and have a potential to use in tissue engineering and drug delivery. Additionally, these proteins have intrinsic biocompatibility and shown to exhibit good mechanical properties (Reddy and Yang 2011). Among them all, soy protein has drawn lots of attention as a biomaterial due to advantages like low price, long storage time and high stability over other naturally produced materials.

10.4.6.1 Soy Protein Based Biomaterial

Legume proteins are the second most important class of vegetable proteins out of them soy proteins have highest quality. Soybeans are the rich and inexpensive source of protein and due to their high nutritional values they are the most widely used dietary protein in various forms. The excellent functional properties of soy proteins have been employed in a wide variety of applications [17]. In last few years, soy protein has emerged, as a novel biomaterial due to its easy availability, simple production methods and biocompatibility. Soy protein based hydrogels are fabricated for various biomedical applications.

Soy protein is a globular protein and can be isolated from the seed by removing oil contents. Depending on their sedimentation co-efficient, soy protein is made up of four fractions; these fractions include 2S, 7S, 11S, and 15S fractions and comprise 8%, 35%, 52%, and 5% of the total protein content, respectively [141]. The isolates consists of 20 different amino acids, including lysine, leucine, phenylalanine, tyrosine, aspartic and glutamic acid, etc. The isolated product of soy protein is called as soy protein isolate (SPI), which is a mixture of many different proteins where, β-conglycinin and glycinin are predominantly present and share more than 80% of total protein [56].

Isolated soy protein and their subunits can be modified and designed to various biocompatible structures. Soy proteins based novel scaffolds have been fabricated for their application in tissue engineering. Human mesenchymal stem cells (hMSC) were seeded on these scaffold and cultured in vitro for over a period. These results confirmed the fabricated scaffolds could support the cell proliferation and suggests the use of soy protein scaffolds have a potential to use in tissue engineering [55]. Additionally, soy protein isolates can be also be utilized to form membranes, nanoparticles, and thermoplastic systems which can be used as drug delivery systems.

Soy protein based bone fillers were proposed for the treatment of several bone related defects. It was noticed that these novel biomaterials have induced osteoblast differentiation, reduce the activity of immunogenic cells like monocytes/macrophages, and exhibit a potential in regeneration of bone tissue and many other biomedical application [61] Santin et al. 2007).

10.4.7 Recombinant Proteins

Genetically engineered protein based polymers are the new entries in the protein based polymers (PBP) groups. Currently, many attentions have been given to these molecules for being advantageous for many tissue engineering applications. With the advancement in genetic engineering and recombinant technology, several biological materials are being synthesized in prokaryotic as well as in eukaryotic expression systems. Currently, recombinant proteins are emerging as novel biomaterials in tissue engineering and biomedical sciences. Genetic engineering and molecular biology presents as invaluably safe and cost effective tools to produce desired proteins, where the monomer sequence and the length of the polymers can be controlled precisely according to the requirements [317]. With the help of these technologies, the production of desired proteins can be easily scaled up without compromising the quality of the proteins [84]. Although, recom-

binant technology is a very old discipline but the production of recombinant proteins for the fabrication of scaffolds is still in its infancy. Besides, these recombinant proteins are superior to their natural and synthetic counterparts in terms of structural specifications and biological properties. These recombinant proteins may be utilized as invariably safe and reliable sources of structural proteins for the synthesis of tissue engineering scaffolds [289]. Furthermore, the biological properties of these recombinant proteins can also be enhanced by adding other bioactive molecules, growth factors, and cell binding domains to them. Recently, a growing number of recombinant protein based biomaterials like collagen, elastin and silk are being used as a superior alternative to conventional polymers [137].

Additionally, recombinant technology proved to be useful in the higher production of proteins, which naturally have lower expression level. Genetic engineering has facilitated the modification of structure and functions of several proteins by incorporation of non-natural amino acids, which in turn can add multiple functionalities in a polypeptide chain. With the advancement in methods in genetic engineering, wide varieties of biomaterials with tunable mechanical and biological properties have been synthesized (Li et al. 162).

The era of protein and genetic engineering are the novel tools for the synthesis of macromolecular proteins with lots of diversity and precision, which are superior to synthetic chemical polymers in all aspects. To these recombinant proteins, further modifications can be made by adding metal chelating proteins motifs or other direct chemical modifications. These fully bioengineered biomaterials are advantageous over natural and synthetic counterparts in having both organic and inorganic constituents [87].

There are many successful reports of expression and purification of recombinant collagen [8, 36], elastin [12, 291] and silk [233]. Furthermore, the synthesis of fusion proteins where, the functional proteins are fused with the bioactive molecules further enhanced the applicability of these recombinant proteins [206]. Several reports have been published describing the development of novel fusion proteins with enhanced biological properties. In one example, silk fibrin has been genetically engineered to augment antibacterial activity where as in another report; silk proteins were fused with elastin like protein polymers to increase the mechanical strength of silk fibers [297, 317].

Collagen extracted from animal sources are associated with a number or drawbacks including inconsistency in the quality in each batch and very high chances of pathogenic contaminations. Due to these shortcoming, there is an urgent need of alternative options for the production of collagen fibers. Recombinant collagens, engineered in recombinant systems like yeast, bacterium, insect cells and plants, presents a better alternative to the naturally produced collagens. Collagens produced by recombination methods are devoid of all the limitations and have better biocompatibility and less antigenicity. Many recombinant collagen are commercially available are potentially less immunogenic than the collagens produced from animal sources (Berthod). In a similar way recombinant silk proteins were also produced and were shown to possess a high potential for biomedical applications (Spiess et al. 2010).

Spider silk protein spirodin are of superior quality, which have inherent properties like very high stability, superior flexibility and extreme toughness, as well as the ingenious amino acid sequence of spider silk makes it a special molecule [209]. Unfortunately, these spiders cannot be domesticated or farming due to their aggressive hostile behavior such as cannibalism. Genetic engineering overcame these problems and recombinant spirodin with similar amino acid sequences as their natural counterparts were synthesized in heterologous hosts like microbes and cell culture. Out of these expression systems, baculovirus system was found to be most favorable for the production of silk protein efficiently [234].

There are many examples where two different proteins with enhanced quality were synthesized together, outside of their natural host by genetically engineering. These chimeric proteins exhibit best qualities of both the proteins and presented the broader applicability. There are reports

of elastin-like polymers (ELPs) and silk-like polymers (SLPs), cell-bound growth factor-based recombinant fusion proteins synthesis. With these mechanism, modulation in mechanical properties, higher biodegradation and enhanced stimuli-sensitivity can be achieved through precise control of sequence, length, hydrophobicity and cell binding domain of proteins [192].

In another report, resilin-like polypeptides (RLPs) were produced by the mechanism of genetic engineering, which have all the incorporated qualities of native resilin. These engineered molecules presents attractive options for the modular designing of biomaterials for engineering mechanically active tissues. The polypeptides synthesized by genetic engineering were fabricated into various forms where they displayed a largely random-coil conformation, both in solution and in the cross-linked hydrogels. Furthermore, RLP hydrogels were checked for their mechanical integrity and cell encapsulation capacity and viability of primary human mesenchymal stem cells (MSCs). The results validated the encouraging properties of these RLP-based elastomeric biomaterials (Li et al. [157]) (Table 10.2).

Elastin based biopolymers are the potential biomaterials for the engineering of several elastic tissues due to their exceptional properties like, biocompatibility, biodegradability, non-immunogenicity and mechanical stiffness. However, elastin fibers are highly cross-linked and insoluble polymers, which limits their isolation process and their universal applicability. To overcome these limitations, several forms of soluble elastin such as animal-derived hydrolyzed soluble elastin, elastin-like polypeptides (ELPs) and recombinant tropoelastin can be synthesized by genetically engineered means using bacteria, yeast or plant as system [12]. Elastin mimetic recombinant proteins have been developed for their application in drug delivery, cellular and tissue engineering [121].

10.5 Protein-Based Composite Materials

Over the last some decade, a several of protein-based composite biomaterials have been investigated for numerous biomedical applications. In general, protein based composite materials which have been researched currently, involve film, sponge, nanofiber, microspheres, hydrogels, grafts, and patch with various ratios.

The composite biomaterials have a huge range of applications in tissue engineering, biomedical engineering and regenerative medicine. The composite mixture of biomaterials can be synthesized mixing different components, either natural polymers or synthetic polymers or combination of both. These composites can be blended and fabricated according to the need of the application and presents several advantages over their single counterparts. As it is evident that all biomaterials are associated with certain drawbacks, generally such as weak mechanical strength, biodegradability, elasticity, which limits their wider

Table 10.2 Recombinant proteins and their applications

Recombinat protein	System	Applications	References
Collagen	*Escherichia coli*, plants, yeast, insect cells	Several	Yaari et al. [299]
Elastomer (ELP, Tropoelstin)	*Escherichia coli* ,plants, yeast	Elastic tissue engineering	Annabi et al. [12] and Wise et al. [291]
Keratin	*Escherichia coli*	Wound healing, skin diseases	Paladini et al. [204] and Parker et al. [208]
Silk	*Escherichia coli*	Various	Nileback et al. [197] and Tomita [259]
Silk-Elastin	*Escherichia coli*	Tissue Engineering	Huang et al. [116]
Resilin	*Escherichia coli*	Regenrative medicine, Tissue engineering	Li et al. [157] and Li et al. [159]
Soy protein	Plants	Wound dressing	Rech et al. [220]

applicability, by mixing two compatible biopolymers these limitations can be overcome. Furthermore, most of the composites are synthesized to improve their overall properties as mentioned above and therapeutic importance can been modified. Natural biomaterials commonly tend to be skin, cartilage, bone, wood, and dentin as few examples in the nature which are present in the form of composite. With this approach biomaterials with superior physical and biological properties similar to native tissues can be synthesized. Proteins are the one of the important biological macromolecules which are involved in a number of cellular function in the body. They are associated with properties like mechanical strength, bioactivity, biodegradability and nontoxic, which provide them huge applicability. Novel biocomposites can be generated by combination of proteins and other essential natural or synthetic materials with enhanced desired properties, for example, proper mechanical/chemical properties, favorable electrical/optical features or other excellent characteristics. These protein-based composite biomaterials have a huge application area including injured tissue regeneration such as skin, bone, vascular as well as drug delivery [284]. In this part, we will discuss their mechanisms of interaction with other components through the phase diagram and glass transition temperature to comprehend their interactions in molecular level and stability.

10.5.1 Mechanism of Interaction Between Co-materials

Useful functions of biomaterials involve mechanical properties or elasticity, dielectric properties, biochemical signaling as well as processability. For instance, composites of silk as a tough, hard-wearing protein combining with tropoelastin as a highly elastic structural protein leads to novel multifunctional composite system with flexible mechanical properties to provide a wide platform of usefulness to the biomaterials for medical devices [112]. Tropoelastin allow to offer highly flexible structural properties with specific human cell signaling caused by specific peptide epitopes in the native sequence, whereas silk furnishes toughness, insolubility, as well as less degradation *in vitro* and *in vivo*. A range of mechanical properties, which is intermediate between the properties of two such materials as long as the ingredients stably interact each other, could be performed. Bone or cartilage as the stiffest tissue could be engineered via synthesizing of rigid protein-governed composites of a dense type. In contrast, skin, muscle, or liver as the elastic tissue could be mimicked through engineering of soft protein-governed composites of a hydrated type like gels. Consequently, one kind of protein can be suitably adopted in the combination in order to match targeted tissue biomechanics.

The interaction between two proteins can be regarded as interplay between a "solvent" and a "solute", based on the Flory-Huggins's lattice model [83, 104]. The interplay generally involved charge/charge (electrostatic), dipole, hydrogen bond, hydrophobic/hydrophilic, counter ion, solvent, as well as entropic effects between two components [113–115]. One of the major factor controlling the miscibility of the protein-mixture is free energy of mixing [104].

With consideration of two protein components blending, the miscibility of the mixture satisfies the following as shown in equation (10.1). The entropy of blending, ΔSm, is supposed to be purely combinatorial and is calculated by enumerating the number of arrangements of the molecules on a lattice, and the enthalpy, ΔHm, is van der Waals energy of contacting phase, and ΔGm is the Gibb's free energy of mixing at temperature T, respectively, are then given by

$$\Delta G_m = \Delta H_m - T\Delta S_m < o \qquad (10.1)$$

In binary systems, the Flory-Huggins could be expressed in the following as,

$$\Delta G_m = RT\left[\frac{\varphi_1 \ln \varphi_1}{r_1} + \frac{\varphi_2 \ln \varphi_2}{r_2} + x\varphi_1\varphi_2\right] \qquad (10.2)$$

where r_i is the number of protein segments and φ is the volume fraction of the species, R is the gas constant, and χ is the Flory-Huggins (FH) binary interaction parameter. The first two terms of right hand side in eq (10.2) are associated with the

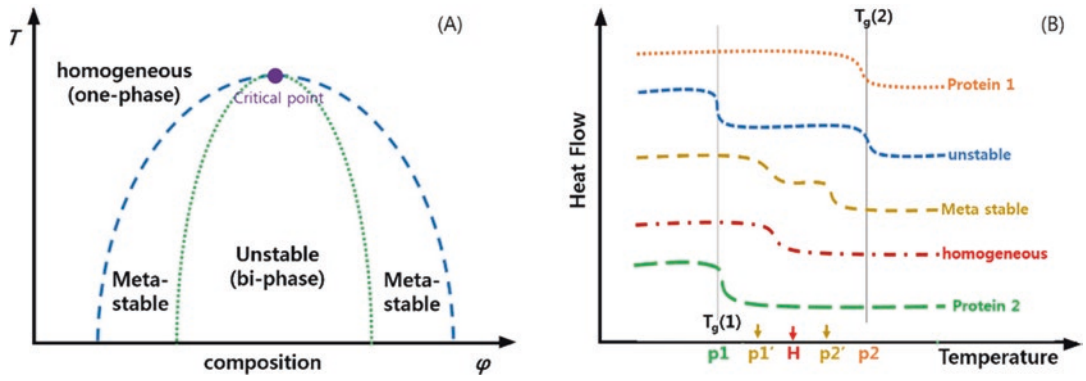

Fig. 10.8 Schematic phase diagram of bi-protein components. Adapted with permission from Hu et al. [113–115] Copyright (2012) Elsevier

entropy of mixing, while the third one attributes to the enthalpy of mixing.

Three regions with different phases is shown in Fig. 10.8a displaying schematic phase diagram of bi-protein blends, which include homogeneous one phase, metastable phase, and unstable bi-phase, in other words, it is immiscible. The homogeneous one phase and meta-stable are separated by the blue bimodal line, whereas the bi-phase and meta-stable state are divided by the green spinodal line [83, 215]. The protein system state can be proved by the glass transition temperature (T_g) by Differential Scanning Calorimetry (DSC). As shown in Fig. 10.8b, in case of homogeneous one-phase protein system, its T_g (H) is intermediate between the two individual protein, T_g (p1) and T_g(p2), while it can be determined by both T_g (p1) and T_g(p2) at their original positions, in case of bi-phase protein systems. In addition, in the condition of meta-stable, namely partial mixing, the component is altered depending on each location. Its interaction cause either broad T_g or two T_g which approach closer to each other (T_g(p1') and T_g (p2')), commonly mentioned as "semi-miscible" system.

This conception of two individual protein miscibility are significant in order to dynamic control protein-based composite materials with different properties, thereby matching with function of injured tissue. And to conclude homogeneous protein system as well as unstable (immiscible) system can be effectively employed for specific desirable biomedical applications.

10.5.2 Protein-Natural Polymer Composite Biomaterials

Current studies have attribute to the comprehending the interactions of different components at the molecular lever, analogous to the extracellular matrix (ECM) mode of assembly where the interaction is tuning by modulating interfaces in biopolymer constituents [93]. Multicomponent systems enable engineering of biomaterials with compatible characters. The hybridization of multi-components provides an effective optimized properties of the individual elements determining the structural and functional one in complex living tissues, such as skin, bone, cartilage, and etc. Protein-based composite biomaterials have been actively utilized in large number of biomedical and tissue engineering applications as the forms of hydrogels, tubes, sponges, fibers, microspheres, and films. Different natural proteinaceous biomaterials like, collagen, elastin, silk and fibrin have been mixed with each other or with synthetic polymers to use them in variety of biomedical applications. For instance, the combination of materials offering mechanically integrity with another materials with flexible may enable to control over biological conditions by mimicking the elastic tissue matrices. Various

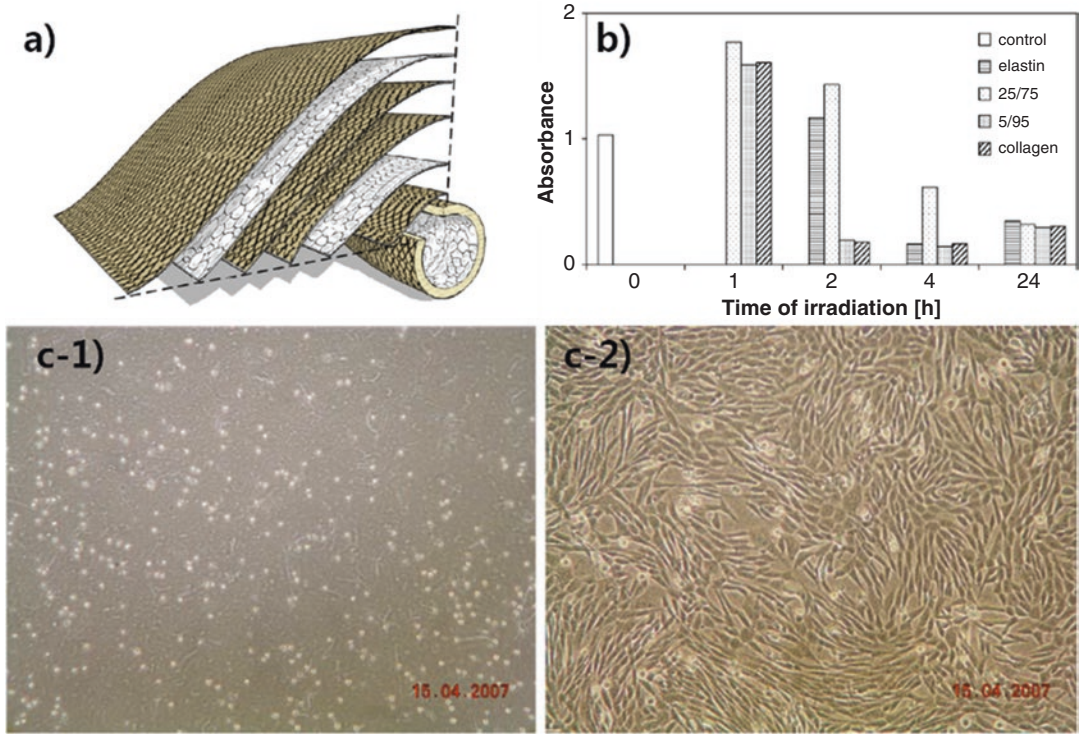

Fig. 10.9 (**a**) Multilamellar protein polymer microfiber composite vascular graft. The vessel wall consists of several layers (thickness 100 μm) of collagen microfiber embedded in protein polymer, with orientation and density established to attain mechanical design targets (**b**) The MTT assay testing 3T3 cells viability on elastin, collagen, and elastin/collagen films, and the images of 3T3 cells growing on c-1) non-irradiated collagen film and c-2) elastin/collagen film with the ratio of 5:95, respectively, after 1 h of irradiation with permission from [40, 248] Copyright (2010 and 2009) Elsevier

protein-based composite biomaterials have been currently progressed for regenerative tissue. Skopinska-Wisniewska et al. reported that the blending of the main components of ECM, both collagen and elastin, provided the challenges of obtaining novel composite materials for biomedical application such as tissue engineering and medicine. The collagen/elastin films with thickness of 0.015–0.030 mm obtained by solvent evaporation from solution poured onto glass plates covered. The higher portion of elastin in collagen/elastin biomaterials promotes the adherence of cells leading to their viability on the surface, as shown in Fig. 10.9b, c [248]. Caves et al.'s report described the generation of an acellular arterial substitute consisting of a multi-lamellar structure formulated from integrated synthetic collagen microfibers and a recombinant elastin-like protein as shown in Fig. 10.9a. They described a process that facilitated control over collagen microfiber orientation and density for the preparation of vascular grafts from a recombinant elastin-like protein reinforced with collagen microfiber and assess the structural and mechanical properties of a series of prototypes. The fiber architecture and processing of the elastin-like protein controlled the suture retention strength, burst strength, and compliance [40, 226].

Chen et al. developed knitted silk-collagen composite biomaterials in 2010. The study demonstrated that hESC-MSCs seeded onto the composite differentiate into the tenocyte-lineage, resulting in successful fabrication of a tissue engineered tendon that was evaluated by both in vitro and in vivo. The efficient of the engineered composite in promoting tendon repair was

positively demonstrated in a rat Achilles tendon injured model. Finally, the study supplied a challenge for the vast potential of using hESC in tendon regeneration, as well as proved that dynamic mechanical stimulation is beneficial to tissue engineered tendons, that could be an effective strategy in future biomedical application [50]. One of protein-based composite biomaterials is related to a three layered fibrous construct fabricated by plastic compression that was developed to be potentially used as a multilayered tissue substitute, such as the case of skin or duramater. Ghezzi et al. developed the layered meso-scaled fibrous dense collagen-silk fiber construct, which is able to maintain MSC growth and integration within the scaffold for 7 days. While the middle fibroin layer allows the structure and stability, as well as mechanical strength and toughness, the two dense collagen layers offer an ECM like structure in order to seed MSCs. The fabricated composite biomaterials possess excellent potential for multilayered tissue engineering such as skin, nervous system [92]. Silk fibroin-bone morphogenetic proteins (BMPs) composite biomaterials as a promising approach for the sustained delivery in tissue engineering applications were prepared in a semi aqueous system, avoiding the use of toxic organic solvents, by using dropwise addition of ethanol, exhibiting mean diameters of 2.7 ± 0.3 μm [26].

The hybridization of tropoelastin with a high elastic property and tough, durable silk can create novel multifunctional composite biomaterials. One of the most durable component of the ECM is the elastic fiber which are existed mostly during late fetal. The significant role of elastic fibers or elasticity in matrix for cell function is getting more and more obvious. The fibers are mainly composed of insoluble protein elastin formed by lysine mediated crosslinking of its soluble precursor tropoelastin [37, 180]. In contrast, silk is generally adopted in the biomedial field due to elaborate integration of mechanical integrity, slow degradation compared with other protein-based materials, and versatile processability into various applications. In spite of many advantages, elastin-based materials need crosslinking process in order to structural/mechanical strength. Accordingly, tropoelastins have been hybridized with silk –derived peptide by using genetic engineering [93]. The silk-troelastin protein blends based on silkworm silk fibroin and recombinant human tropoelastin were developed with the objective to explore the features of the blending to determine the invluence of interactions between the two different proteins on material properties. Semicrystalline silk protein presents mechanical strength to tropoelastin and eliminate the need for chemical crosslinking by varying β-sheet crystal content. In contrast, human tropoelastin provides elastic property and encourages cell interactions. Qiu et al. prepared recombinant silk-elastinlike protein nanofibrous tissue scaffolds. Their results of FTIR spectroscopy showed that chemical vapor treatment with MeOH, GTA, MeOH-GTA caused a structural transition from Silk I to Silk II, leading to the enhanced integrity and stability of the nanofibrous scaffolds, whereas, the samples without chemical treatment were not stale in PBS. The mechanical studies proved that the elastic modulus, tensile strength of the silk-elastinlike nanofibrous scaffolds exceed those of collagen- and fibrinogen-based scaffolds. In optical microscopic results established that 3T3 fibroblasts attached and spread onto the fabricated scaffolds. The cells showed excellent viability and proliferation rates on the scaffolds [217]. Both silk and elastin employed in the study are biocompatible and biodegradable, and the studies showed that the hydrophobic and hydrophilic interactions between silk and elastin chains are the main factor in order to modulate their composite materials properties. Moreover, the porous composite biomaterials with appropriate mechanical elasticity could help in adhering and proliferating of human mesenchymal stem cells. It was faced that domesticated silk fibroin lacked specific cell adhering sites such as arginine-glycine-aspartic acid (RGD) sequence, while it was found in other wild type silkworm silks [178, 284], and accordingly, wild-type silk composite materials should be prepared for guiding the differentiation of cells *in vivo* [185].

In addition, several investigation established that the combination of fibroin with carbohydrate polymer is an alternative strategy to opti-

mize the cell attachment and proliferation, as well as biodegradability and mechanical properties. Baran et al. developed novel chitosan based biomaterials preparing by the incorporation of fibroin and starch for the aim of enhanced cytocompatibility and controlled biodegradability, respectively. Fibroin is biocompatible, while it is very brittle owing to high crystalline structure causing unsuitable as biomaterials [155, 199]. They focused to functionalize chitosan matrix by the conjugation of readily biodegradable starch macromolecules hydrolyzed by α-amylase and improving degradation *in vitro* and *in vivo*. The study demonstrated that the integration of silk fibroin and starch into chitosan based biomaterials would increase both biodegradation and cell compatibilities, which are essential necessity of cell embedding scaffolds for tissue engineering [18]. In Zhou et al.'s study, silk fibroin-cellulose composite films were prepared through dissolving in BmimCl, a kind of ionic liquids as green solvent and coagulating in methanol for combining advantageous characters of silk fibroin and cellulose such as biocompatibility and mechanical performance, respectively. The studies exhibited that the cellulose allowed to enhance the mechanical strength of the composite films, as well as that the two constituents encouraged adhering and proliferating of L929 cells on the fabricated films. Consequently, it was reported that silk fibroin-cellulose blended films possess the excellent potential in the biomedical applications [316]. One of various studies was reported thermoplastic rice starch (TPRS) reinforced by silk fibers by Prachayawarakorn and Hwansanoet. Mechanical performance of the starch-silk composite matrices was improved by the incorporating silk fiber, due to the new hydrogen bonds formed between the starch matrix and silk fiber by the detection of IR peak shifts. The highest stress at maximum load and Young's modulus of the composites was acquired from the addition of 10% and 2 mm silk reinforcement, however, resulting in reduced its water absorption [212]. In addition, Prachayawarakorn et al. prepared thermoplastic rice starch-cellulose fiber. It was proved that the composite fiber displays much better mechanical performance and lower water uptake. Thermal stability of the composites also enhanced dignificantly with the addition of cellulose fiber [213]. Chitosan-silk fibroin (CHI-SF) multilayer films were assembled through layer-by-layer (LBL) deposition, which incorporated nanofibers aligned parallel to the dipping direction, by Nogueira et al.. It was possible to modulate fiber alignment on the film by controlling the substrate orientation. The composites allow to be an alternative for functionalization of the material's surface for biomedical applications [199]. In the study, silk could be assembled with chitosan components to create a layer-by-layer structure owing to the hydrogen bonds and electrostatic interactions, additionally, in Kozlovskaya et al.'s research [143, 199]. Collagen- chitosan biomaterials are produced by mixing two polymers together to enhance the properties of each macromolecules. Collagen has been widely used as a biomaterial in a range of different tissue engineering applications, including those related to bone and cartilage. Chitosan and collagen composites were developed to regenerate and mimic natural ECM like complex. [54]. A novel composite composed of keratin as a structural protein was synthesized as a wound dressing materials with immunological and antibacterial properties. The results of mechanical and antibacterial properties proved that the fabricated composite system have a great potential for effective wound healing [228]. And gelatin-pectin composites and soy protein with sodium caseinate composite were prepared for wound dressing and drug delivery [68].

In addition, there are numerous examples including composite films created by blending silk, cellulose, and wool fibers using a green solvents [244], silk-gelatin scaffolds influencing postexpansion redifferentiation of chondrocytes [66], the composite of silk with human collagen sequence [260], cellulose-silk fibroin blended fibers from solution in *N, N*-dimethylacetamide containing 7% LiCl (w/w) [169], silk fibroin-

hyaluronic acid composites [88], silk fibroin-chitosan sponges with appropriate mechanical property [67, 246], and etc.

10.5.3 Protein-Synthetic Polymer Composite Biomaterials

Amino acids are the building blocks of proteins that are organized in various structural forms. The side chains of peptides consists of several functional groups and the functional groups are highly reactive, thereby enabling to combination with synthetic polymer [101]. Varied researches have been described that combining protein with other synthetic materials is an efficacious strategy in other to overcome the disadvantages of protein-based materials [67, 255]. Numerous synthetic polymers possess unique advantageous properties, for example, thermal performance, easy tailoring degradability, light weight [165]. Accordingly, novel composite materials by the combination proteins with synthetic polymer have drawn attention in biomaterials area [21, 22].

Many composite materails have been employed for injured bone tissue regeneration. Brushite-collagen based composite materials have been synthesized for the bone regeneration. The composite biomaterial is proved to have high compressive strength, osteoconductivity, biodegradability which are essentilaly required for effective regeneration of bone tissues. Presence of collagen enhanced setting reaction with higher cellular adhesion. Several composite mixtures of natural polymers along with antibacterial components, have also been fabricated . 3 D scaffold prepared by gelatin and polyvinyl alcohol were fabricated to use in wound healing applications and found to have enhanced wound healing effects [46]. Choi et al. reported that gelatin-poly(vinyl alcohol) sponge scaffold, prepared by using freeze-drying approach, with ideal architecture and physico-chemical property could be obtained by optimal concentration of the component, and it demonstrates the effect of the component in three-dimensionality of the matrix [58, 59]. A collagen based composite was prepared by freeze drying method. The insulin molecules were encapsulated in poly(lactic-co-glycolic acid) beads and then added to collagen aqueus solution. The drug release profile revealed the release of insulin in a controlled manner [230]

Recently, waterborne polyurethane have been rapidly emerging due to many merits, such as low density, good elasticity, long storage life, excellent wear resistance, and shock absorption [161]. Therefore, it has been combined with natural polymer such as wool, cellulose, chitin in order to prepare composite materials. Hu et al. reported an injectable silk fibroin/polyurethane (SF/PU) composite hydrogel prepared in a liquid or semi-liquid form with barium sulphate added to enable monitoring of the hydrogel. The results of compression studies proved that the composite hydrogel displayed proper physical-mechanical performances to provide as prosthetic biomaterials for the replacement of the nucleus pulposus. In addition, the composites provided the clinically important advantage of visibility in CT and T2-weighted MRI that is important to real-time hydrogel monitoring both during and after surgery [113–115].

Also, osteogenesis is physiologically conducted by neovascularization, the growth of new blood vessels. It is essential to accomplish rapid development of a plentiful blood supply, regarding as the most important role in tissue regeneration. Limitation of oxygen and nutrient diffusion can cause the growth of cells. Therefore, major strategies have been faced in order to promote vascularization within constructs. Poly(lactic acid) is a perfectly biodegraded synthetic polymer obtained from grains. Stoppato et al. suggested the combination poly(D,L-lactic acid) scaffold with silk fibroin fiber for improving bioactivity prepared by a solvent casting and leaching technique that provided a certain three dimensional porous structure. Silk fibers was well integrated with the PLA matrix, resulting in the composite materials' elasticity and ductility. Silk fibroin fibers were adopted for supporting endothelial cell adherence and proliferation [251]. Silk fiber/poly(lactic acid) biocomposites were synthesized in order to advance the understanding of the mechanical and thermal perfor-

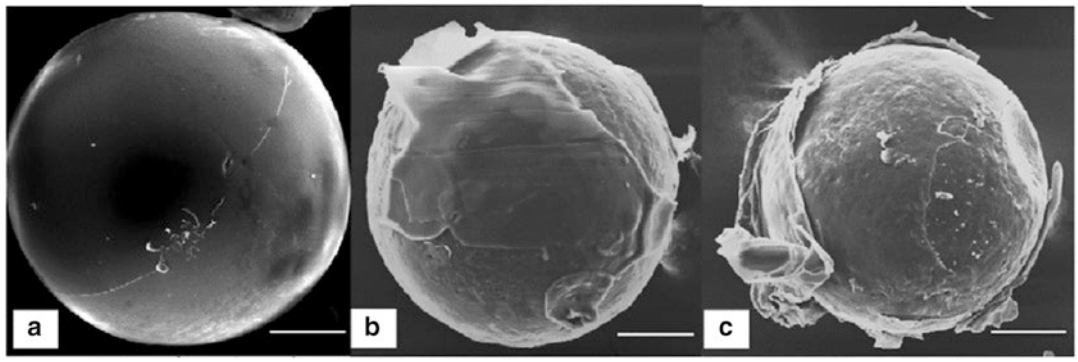

Fig. 10.10 The SEM photographs of microspheres coated with 0.1%, 0.5% and 1% silk fibroin. Microsphere coated with 0.1% SF showed a smooth surface without defects or cracks (**a**), Microspheres coated with 0.5% and 1% SF showed poor coating comprised of numerous surface defects and cracks in the film structure (**b**) and (**c**) (bar size 50 mm). Adapted with permission from [315] Copyright (2011) Taylor & Francis

mances. [52, 107]. Zhou et al. suggested that silk fibroin could be considered as a potential aqueous film coating agent for drug release system. The poly(ε-caprolactone) (PCL) microparticles coated with 0.1% silk fibroin displayed smooth surface without any cracks as shown in Fig. 10.10, and prolonged the drug release. Silk fibroin coating on vancomycin loaded PCL microspheres has a great potential for prolonging the drug release and improving the anti-infection effects. Chen et al. developed hybrid scaffolds composed of modifying poly(ε-caprolactone) (PCL) with silk fibroin in a porous structure by freeze-drying method. Methanol treatment enabled to ally the silk fibroin constituent when the composite scaffold was exposed to physiological like conditions. The study demonstrated that PCL modified by silk fibroin in a porous structure could improve the biocompatibility and has a potential for tissue engineering [49].

Besides, protein have been hybridized exclusively with poly (γ-glutamic acid) [298], poly(acrylamide) [65, 85], poly(methyl acrylate) [252], poly(acrylonitrile) [252], poly(ethylene oxide) [203], poly(vinyl alcohol) [301], and etc [122, 138, 198, 253, 263, 311] in order to enhancing mechanical performance, electrical properties, and other physical/chemical functions.

10.6 Conclusion

In biomedical fields, tissue engineering is one of the most attractive multidisciplinary research, which have achieved exponentially a rapid growth. Among the fields, biomaterials area has been progressing over the last decade, and protein engineering has emerged as a useful tool in order to investigate the interaction between cells and materials, thereby producing further understanding into regenerative medicine as well as tissue engineering. Proteins having various functions in living systems have, that is a momentous volunteer for biomedical materials been intensely investigated for the elaboration of novel biomaterials for medical devices, as the diverse research activities described in this review. This attractive research field is rising towards the employment of proteins engineered either alone or with others natural or synthetic polymers, in order to develop a collage of biomaterials with tunable properties for biomedical applications in the area of sophisticated products with a prominent added value.

The protein-based materials have been primarily advanced for biomedical including tissue engineering, regenerative medicine, drug delivery, because it is intrinsically biocompatible, biodegradable, bioactivity, and specific properties comparing with other natural polymers such as

chitosan, alginate, sugar, cellulose, etc. Both protein-based materials and their fabrication techniques have played an important role in biomedical field such as tissue engineering, and advanced radically. However, the problems with developed protein-based materials can be condensed. Firstly, there is still the issue of safety. It is necessary to remove hazard of disease contagion. And a second issue is mechanics. In spite of a wide use as biomaterials for biomedical area, proteins possess limited mechanical strength. Enhancing the mechanical properties is still a challenge for the replacement in injured tissue site. Both modification and hybridization of protein with other natural/synthetic materials are promising approaches to direct it. In addition, the poor neovascularization within engineered substitutes is one of the main obstruction for victorious applications of biomaterials, of course protein is no exception. As an alternative plan, it is possible that the active molecules including vascular endothelial growth factor are embedded into the protein-based scaffolds. Also, another is the engineered scaffolds cultured with diverse vascular cells in order to form the neovascularized tissue replacements.

In spite of numerous advantages as biomaterials, understanding and investigating in the progress of protein-based materials for biomedical fields should more conduct certainly for advances of novel biomaterials for other applications. Such advances will clearly be further promoted by integration with other innovative field of research, for example, the use of environment-friendly solvents such as ionic liquids in protein processing, and the hybrid of proteins with other materials either artificial or from a natural origin. All the research area have been widened as considering the a great potential, the engineered protein properties can be tailored for fabricating biomaterials with much better performance in terms of function.

Currently the greater employment of natural proteins for materials of medical devices, proteins are still a matter of research in this field. Scientists have come to grips with these challenges, the future of protein materials holds great promise. Progresses in biology, medical, chemistry have stood open the door to materials that can be exploited to understand and engineer novel products at the material science.

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Part III

Drug Delivery Systems for Regenerative Medicine

11 Crosslinking Biopolymers for Advanced Drug Delivery and Tissue Engineering Applications

Goutam Thakur, Fiona Concy Rodrigues, and Krizma Singh

Abstract

A popular approach to attaining controlled drug delivery from polymer based systems involves the use of cross-linkers. In order to improve the properties of polymers specific to their applications, they can be modified by either physical cross-linkers (high pressure, irradiation) or chemical cross-linkers (glutaraldehyde, genipin). This chapter provides an insight into the different types and mechanisms of cross-linking. It reviews the existing drug delivery systems to understand the effects of cross-linking in them. The recent applications of cross-linked polymeric drug delivery and tissue engineering systems are also discussed.

Keywords

Polymer · Cross-linker · Drug delivery · Tissue engineering

11.1 Introduction

Biopolymers i.e. polymer derived from natural sources, are increasingly gaining popularity for various biomedical applications. Of late, the properties of these polymeric materials, owing to their various favorable properties such as cytocompatibility, non-toxic degradation, etc., are being tuned for making suitable drug delivery matrices and tissue engineering constructs (TEC) [59, 62]. Biopolymeric scaffolds are engineered, natural or synthetic structures with defined porosity and physicochemical features [62]. A variety of matrices such as hydrogels, films, micro/nanoparticles, 2D and 3D structures, etc. composed of biopolymers, are being explored for their *in vitro* and *in vivo* applications [53, 59].

The majority of these polymeric materials that contribute to the development of scaffolds, generally deprive the matrix of its desired mechanical and degradation properties. Therefore, a molecules that are required to enhance these properties, are referred to as "crosslinking agents" or "cross-linkers". Crosslinking is a simple method in which the chemical or physical links between polymer chains are established to modify mechanical, biological and degradation properties of polymeric materials. Further, the selection of an appropriate biomaterial, tuning the scaffold microstructure, the biological efficacy of designed scaffold, and the type and physicochemical properties of crosslinking reagents, are important factors that should be considered in designing scaffold materials [62]. Among all crosslinking methods, chemical crosslinking seems to be the most popular and effective one. Here, we discuss the role of cross-linkers on the

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efficacy and biological properties of crosslinked bio-polymeric scaffolds. The entry will also discuss the recent applications of cross-linked polymeric drug delivery and tissue engineering systems.

11.2 Cross-Linking

In recent years, crosslinking technology is amongst the most promising research domains focusing on the development of new tissue engineered scaffolds and various drug delivery matrices. The main aim of the crosslinking is to enhance the biomechanical properties of the scaffolds, by forming a firm network in the polymeric matrix [62]. A crosslink is a physical or chemical bond that links the functional groups of a polymer chain to that of another one through covalent bonding or supramolecular interactions such as ionic bonding, hydrogen bonding, etc. [53, 97]. Crosslinkers, not only ameliorate the mechanical performance of the polymer network, but also demonstrate no cytotoxicity. Therefore, crosslinking of polymer chains can affect some physicochemical properties including bio mechanical parameters like tensile strength, stiffness, strain, cell-matrix interactions, performance at higher temperatures, resistance to enzymatic and chemical degradation, gas permeation reduction and shape memory retention of the products [11]. Furthermore, it has been reported that crosslinking can modify the antigenic sites of natural materials and reduce their antigenicity [74]. Cross-linking method insolubilizes matrix in water and improves the thermal and mechanical stability of the matrix under physiological conditions [5]. Moreover, cross linking can be tailored to modulate the release rate of the incorporated active agents [12, 16].

11.2.1 Methods of Crosslinking

Various crosslinking methods can be employed for fabricating scaffolds or designing drug delivery matrices, depending on the type and nature of the biopolymer. Through the crosslinking process, the active functional groups of polymer chains react chemically or physically with the crosslinking agents and form a three-dimensional network (Fig. 11.1). To reach the ideal biomechanical and degradation properties of the bone polymeric scaffolds, a variety of crosslinking techniques have been developed and classified into three large groups of physical, enzymatic and chemical crosslinking methods [53, 97]. The pros and cons of these methods are highlighted in Table 11.1.

Physical crosslinking includes different techniques such as UV irradiation, gamma radiation and dehydrothermal treatment. Chemical crosslinking is inclusive of various natural and synthetic crosslinkers like glutaraldehyde, PEG, genipin, etc. [48]. A setback of the physical crosslinking process is its inability to achieve the expected release properties in a matrix due to uncontrollable cross-linking density. The chemical crosslinking agents can be divided into two categories: non-zero-length cross-linkers and zero-length cross-linkers [41]. Non zero length crosslinkers (e.g., glutaraldehyde, polyepoxides and isocyanates) are bi-functional or multifunctional molecules, whose mechanism of action lies in their ability to bridge the groups between adjacent polymer molecules such as free carboxylic acid groups, amino groups and hydroxyl groups. Whereas, in the case of the zero length crosslinker, formation of a covalent bond occurs due to the reaction of the reactive groups such as carboxylic acid and amine groups which are present within the polymer network chains. Cross-linking agents in this category include acyl azides, transglutaminase and water soluble carbodiimides [41].

Dehydrothermal Method

Here, the process involves condensation reactions by removal of water molecules from desired materials under high temperature conditions to obtain polyesters, polyethers, polyamines, polyamides, etc. Principally, thermal stimulation is necessary to remove the water molecules through bonding two complementary functional groups (e.g. carboxyl

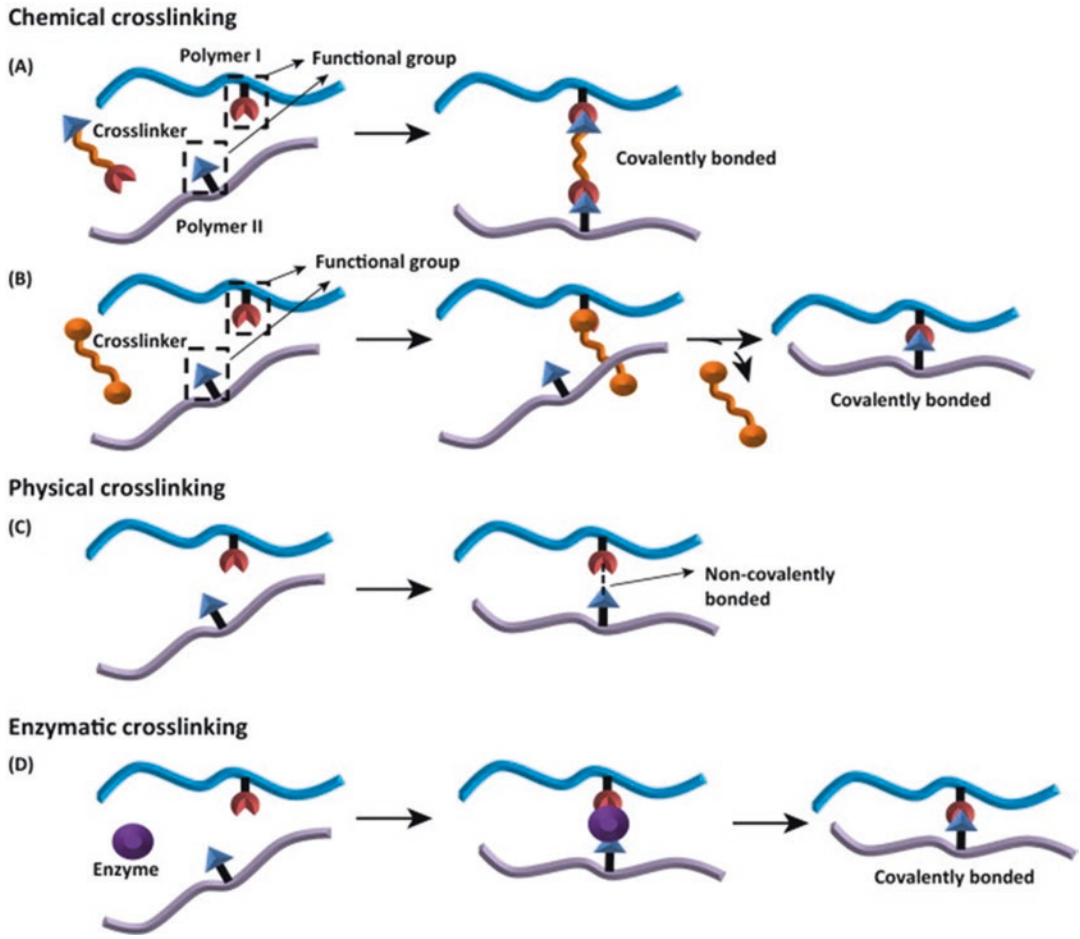

Fig. 11.1 Mechanisms of crosslinking. (**a**) Chemical cross-linker with the polymer chains incorporated into the bond, (**b**) Chemical cross-linker leaves the reaction after crosslinking process, (**c**) Physical cross-linkers form a non-covalent bond between the polymer chains, (**d**) Enzymatic crosslinking method

Table 11.1 Advantages and disadvantages of the physical, enzymatic and chemical crosslinking methods

Crosslinking method	Advantages	Disadvantages
Physical	1. Safe	1. Bonds are weaker than the chemical crosslinkers
	2. Less toxic for cells than chemical agents	2. May alter the properties of the materials
	3. Inexpensive	3. Needs more time for crosslinking
	4. Minimum tissue reaction after crosslinking process	4. Lack of control over the reaction kinetics of crosslinking
		5. Lower degrees of crosslinking
Enzymatic	1. Unlike many chemical agents, enzymes are most active under mild aqueous reaction conditions	1. The most expensive crosslinker
	2. Crosslinking process can often be controlled by modifying temperatures, pH, or ionic strength	2. Substrate specificity
Chemical	1. Forming very strong bonds	1. Almost toxic for the cells
		2. Needs washing to remove the residual crosslinker
		3. More expensive than physical crosslinker

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and amine or hydroxyl groups); therefore, this reaction is usually performed at elevated temperature and for longer duration [53]. This method has been repetitively used for crosslinking of the polymeric scaffolds. The DHT treatment can be theoretically performed at temperatures higher than 100 °C; however, Ma et al. found that the conditions of DHT reaction of collagenous materials can be optimized at 130–140 °C [53]. DHT treatment is a simple and safe method to improve the structural properties of polymeric scaffolds; however, in some cases, further modifications should be made, to obtain better properties [21].

Plasma Method

Surface modification using plasma treatment utilizes high-energy sources to generate discharge gas that provides energy to activate either initiators or sensitive functional groups of monomers and produce free radicals [57]. The resulting radicals can interact with functional groups of monomers and create covalent crosslinks between the surface of the material and desired monomer. As a matter of fact, the plasma cannot completely penetrate into the bulk of the material; therefore, this method is more acceptable for modification of materials surfaces [53].

11.2.1.1 Irradiation-Based Techniques

Crosslinking of scaffolds by irradiation-based techniques is performed using high-energy ionizing radiation or photoinitiator molecules. This method is widely used in crosslinking of the collagen scaffolds [18]. During irradiation by ultraviolet radiation (UV), there are two competitive and opposite processes namely, UV-induced denaturation and UV-induced crosslinking, maintaining a balance between the two acts as a critical factor during the preparation of scaffolds. This balance eventually influences the mechanical properties and degradation behavior of irradiated collagen-based scaffolds [18]. Some studies have used a modified UV irradiation technique called "riboflavin and ultraviolet-A (RFUVA)" method. In this method, photosensitive agents such as singlet oxygen are produced by UV irradiation and then they react with available collagen active groups [91]. In addition to the UV radiation, photocrosslinking can also be performed using the visible light. In this regard, Cao et al. used tris-bipyridyl ruthenium as a UV-sensitive photoinitiator to crosslink a photopolymerizable hydrogel containing bone morphogenetic protein-2 (BMP-2) factor and chitosan nanoparticles for bone regeneration [9, 53].

Enzymatic Crosslinking Agents

Modification and crosslinking of polymers using enzymes have been of great interest among scientists [34]. Transglutaminase is an enzyme present in many organisms including various vertebrates, invertebrates, plants and microorganisms. It is responsible for various biological events such as epidermal keratinization, blood clotting and regulation of erythrocyte membranes [52]. The microbial TGase (mTGase) which can be isolated from the culture medium of *Streptoverticillium* sp., catalyzes protein crosslinking by inducing the formation of an amide bond between the carboxylic acid group of glutamic acid and the amine group of lysine [3]. Unlike other sources, mTGase shows unique features including Ca^{2+} independence, which makes it ideal for various industrial and medical applications [51]. Involvement of TGase in a number of human diseases (e.g. Huntington's, Parkinson's, and celiac disease, as well as cancers and tissue fibrosis), has resulted in an increasing interest in TGase research [53]. A recent study emphasized on the usage of an enzymatic crosslinking strategy, using transglutaminases (TGase; proteinglutamine gamma-glutamyltransferase), Horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) have been increasingly used for fabrication of different types of biopolymeric scaffolds and hydrogels due to their crosslinking efficiency, mild reaction conditions and good cytocompatibility which has led to the wide applications of these enzymes in tissue engineering. Some of these enzymes are non-toxic and non-immunogenic crosslinkers which are commercially available and approved by the FDA [31]. Broderick et al. used mTGase to crosslink gelatin material with the aim of

improving its mechanical strength. Also, treatment of 3-Dhydroxyapatite/collagen (HA/Col) composites with mGTase has not only improved the mechanical strength but also the thermal stability of the scaffolds [7]. Moreover, it has been observed that cultures of the osteoblast-like cells and human umbilical vein endothelial cells have indicated various favorable properties such as good adhesion and proliferation, good viability, and good differentiation in such biopolymeric scaffolds [13]. With respect to drug delivery, Cui et al. reported the enzymatic crosslinking of casein fibers using TGase. The results not only indicated enhanced tensile strength but also of delayed and lower rate of drug release which demonstrates its suitability in controlled drug release [15]. Yin et al. also worked with casein hydrogels crosslinked with TGase for the controlled release of the model drug docetaxel [88]. Yang et al. fabricated hyaluronic acid (HA) nanogels by functionalizing HA using a methacrylation strategy. The nanogels were further loaded with Doxorubicin and tested for its *in vitro* and *in vivo* antitumor effect and the study indicated of superior activity of the nanogels [87].

11.2.1.2 Small Molecule Crosslinkers

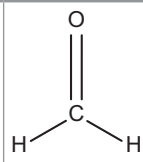
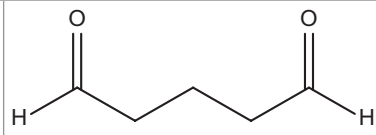
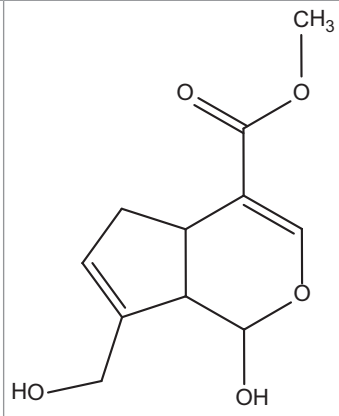
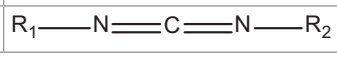
Glutaraldehyde

Glutaraldehyde (GA) has been extensively used as a chemical crosslinker to crosslink various types of biopolymeric tissue scaffolds, hydrogels and composites. It can significantly improve mechanical properties and durability of biomaterials in a fabricated matrix and scaffold [40]. Glutaraldehyde reacts with the amine or hydroxyl functional groups of proteins and polymers, respectively through a Schiff-base reaction and connects the biopolymeric chains *via* intra- or intermolecular interactions. Therefore, all the available free amine groups which are present in the chemical structure of protein molecules such as gelatin, react with GA, forming a more strongly crosslinked network. For many years, GA processing has been the gold-standard method in crosslinking tissue films and scaffolds that are used for transplantation procedures, such as heart valves replacement [46]. The bone tissue scaffolds that are fixed and crosslinked using GA demonstrated an enhanced tensile strength and a significant reduction in antigenicity. The functional groups of GA, aldehyde groups, are toxic for cells and cause severe inflammation in the body; thus, many detoxifying strategies have been proposed to increase the biocompatibility and durability of GA-crosslinked scaffolds. For instance, washing GA crosslinked scaffolds using solutions containing free amine groups or amino acid solutions such as glycine, led to the removal free aldehyde groups [38]. It has also been observed that GA makes more stable scaffolds as compared to crosslinking with other aldehydes such as glyceraldehyde and formaldehyde [71] (Table 11.2). The concentration of GA in polymeric solutions, from 0.01% to 2.5% W/V, is a determining factor in crosslinking processes [63]. In this regard, concentrations up to 8% of GA undergoing an appropriate detoxification have shown no cytotoxicity for crosslinking. Although different investigations have used different GA concentrations for this purpose, newer studies showed that the ideal crosslinking process can be performed when the GA concentration is determined based on polymer's amine groups [64]. According to the latter statement, desired GA-treated products should have a perfect crosslinking degree with the minimum residue of free aldehyde groups. Although GA is a widely-used chemical crosslinker, the fact that it may cause significant cell toxicity and biohazard problems, has limited its application in commercial products [33].

Carbodiimide Agents

A carbodiimide functional group is a chemical group consisting of C=N=C bond. 1-Ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC) is a water soluble carbodiimide that can react with a variety of chemical groups, such as carboxyl, hydroxyl and sulfhydryl functional groups (Fig. 11.2b). The optimum crosslinking activity of EDC is obtained under acidic conditions (pH of 4.5) and in a buffer devoid of extraneous carboxyl and amine groups such as 4- morpholinoethanesulfonic acid (MES) [72]. However,

Table 11.2 Comprehensive summary of cross-linker(s) with biomedical applications

Cross linkers	Structure	Biomedical applications
Formaldehyde		Tissue engineering scaffold, stabilizing matrix
Glutaraldehyde		Cell, enzyme immobilization, hydrogel synthesis, drug delivery, tissue engineering
Genipin		Stabilizing matrix, tissue engineering, drug delivery, stem cell behavior
Carbodiimide		Tissue engineering scaffold, stem cell behavior

neutral pH (like phosphate buffer, pH <7.2) is also compatible with this reaction [36]. When N-Hydroxysuccinimide (NHS) is used in combination with EDC, an activation of carboxylic acid groups occurs, which makes it less susceptible to hydrolysis and this results in an improvement in the efficiency of the crosslinking reaction [59]. An advantage associated with this process is that all of the residues are water soluble which means that they can be washed out of the scaffold construct easily by distilled water after the completion of the crosslinking reaction [55]. This crosslinker has been used in several studies on bone tissue regeneration due to its low cytotoxicity in comparison to GA. Based on the unpublished experiences of the authors, EDC-crosslinked scaffolds show softer surface compared to GA-crosslinked scaffolds. This property reduces their handling during *in vivo* implantation. In addition, it should be highlighted that EDC-crosslinked materials have poor biomechanical properties and more rapid degradation and biodegradation profiles, compared to GA-crosslinked ones.

Epoxy Compounds

Epoxy-based crosslinkers which are chemicals with a three-membered ring consisting of one oxygen atom and two carbon atoms, are organic compounds that can react with amine, carboxyl, and hydroxyl groups and are used in crosslinking bone tissue scaffolds [53]. These crosslinkers demonstrate considerable cytotoxicity which is comparable to that of formaldehyde and GA. Ethylene glycol diglycidyl ether (EGDE) is a recognized commercial epoxy-based material used in chemical industry and has a linear structure with bulky side chains.

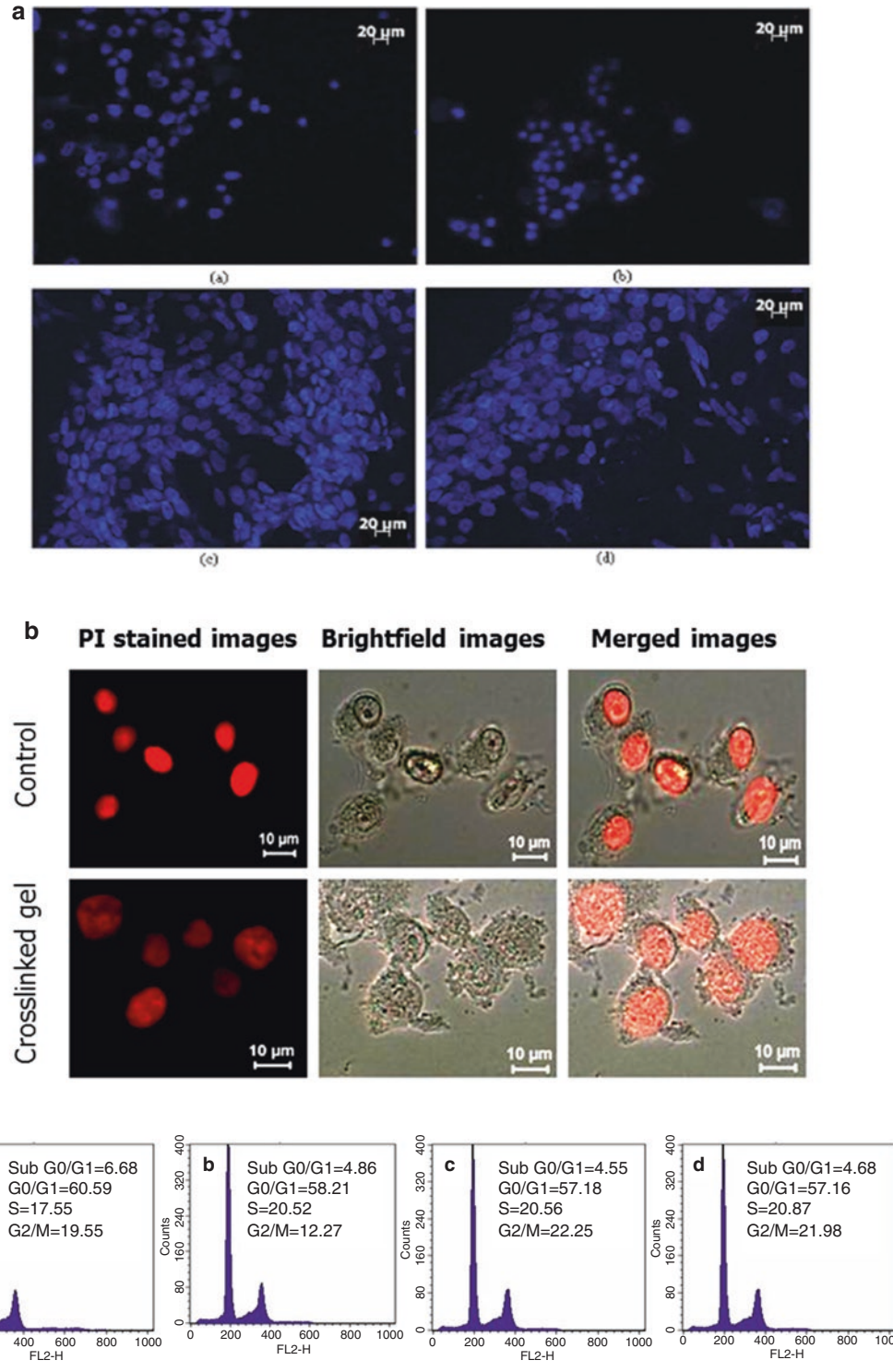


Fig. 11.2 (**a**) Fluorescent microscopy images of cell nuclei adhered to hydrogel surfaces visualized by means of Hoechst staining on(*a*) N methylene phosphonic chitosan (NMPC)-genipin hydrogel day 1, (*b*) NMPC glutaraldehyde hydrogel day 1, (*c*) NMPC genipinhydrogel day 5, and (*d*) NMPC glutaraldehyde hydrogel on day 5.16 × 12 mm (600 × 600 DPI). (**b**) PI stained cells treated with crosslinked gelatin gels observed under fluorescence microscope. Scale bar = 10 µm, PI: propidium iodide (**c**) Cell cycle analysis of AH-927 cells treated with different genipin-crosslinked gels. (*a–d*) represents 2 days' growth; (*a*) control, (*b–d*) gels crosslinked at 5, 15 and 25 °C, respectively. Different cell cycle stages are shown: Sub G0, G0/G1, S and G2/M. (Source: Reproduced from [17]© 2014 with permission from Taylor & Francis and [76] © 2011, with permission from Springer)

Genipin

Genipin (GP), the hydrolytic product of geniposide is extracted from the fruit of *Gardenia jasminoides* Ellis. It is an iridoid compound which possesses multiple active groups such as hydroxyl and carboxyl groups. Genipin is an active colorless material that spontaneously reacts with amino acids to form blue pigments [76]. Owing to its natural origin, biodegradability and low toxicity, GP has recently become a popular crosslinking agent in many biological applications ([49]; [76]). Many comparative *in vitro* studies indicate that GP possesses a significantly lower cytotoxicity than GA [77] For instance, the MTT assay results indicated that GP is about 10,000 times less cytotoxic than GA. Further, the colony forming assay (CFA) suggests that cell proliferation after exposure to GP has approximately been 5000 times greater than that observed following treatment with GA. It has been noted that live microorganisms and cells on the surfaces of GA-fixed tissues were not able to survive and because of this, GA is considered as a very good tissue fixative (Table 11.2). In contrast, the surfaces of GP-fixed tissues were found to be filled with 3T3 fibroblasts. The results showed that the cells were metabolically active and they produced new collagen materials [75]. It has also been suggested that there is a relationship between the dose- and time-dependent effects of GP-crosslinking, cell viability, and tissue scaffold mechanical properties [23]. Although GP is a very safe crosslinker, it is very expensive, especially when used for mass production of bioscaffolds. Today, GP is only used in experimental studies and there is no economic justification for its use for mass production.

11.2.2 Cross-Linking Index/Density

(a) ***Chemical process***

Under this section, cross-linking density can be determined by the use of the ninhydrin assay. Samples were weighed (~20 mg) and subsequently heated with a ninhydrin solution (2 wt % v/v) at 100 °C for 20 min. Afterwards, the optical absorbance of the solution was recorded with a spectrophotometer at a wavelength of 570 nm. Sample without crosslinker would be treated as control. After the sample was heated with ninhydrin, the number of free amino groups in the test sample was proportional to the optical absorbance of the solution. Cross-linking index (CI) of the samples were then determined as described [76].

(b) ***Crosslinking density from equilibrium swelling and Flory –Rehner theory***

Swelling dependent crosslinking of polymer gel can be determined by Flory and Rehner theory [24]. The theory implies that when a polymeric network swelling in a compatible solvent is allowed to attain equilibrium, there are only two forces at work – the force of thermodynamic mixing and the retractile force of the polymer. Crosslinked polymer gels when immersed in solvent, it swells and attains equilibrium. As a result of more solvent ingress into the polymer network, volume increases and this causes the network junction zones to expand. A modified theory was proposed which gives an insight into the polymer average molecular weight and crosslinking density of the gel prepared in water. The following equation was derived for the swelling of a hydrogel prepared in the presence of a solvent:

$$\frac{V_1}{4I}\left(\frac{v_{2,s}}{\bar{v}}\right)^2\left(\frac{K_b}{10^{pOH}-K_a}\right)^2=\left[\ln\left(1-v_{2,s}\right)+v_{2,s}+\chi_1 v_{2,s}^2\right]+v_{2,r}\left(\frac{V_1}{\bar{v}\bar{M}_c}\right)\left(1-\frac{2\bar{M}_c}{\bar{M}_n}\right)\left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3}-\left(\frac{v_{2,s}}{2v_{2,r}}\right)\right] \tag{11.1}$$

where $\bar{M}_n$ is the MW of the polymer without crosslinking, $\bar{M}_c$ is the number-average polymer MW between two adjacent crosslinks, $\bar{v}$ is the specific volume of the hydrogel prior to swelling, V_1 is the molar volume of the solvent water (18 mL mol^{-1}), $v_{2,s}$is the polymer volume fraction in the swollen state determined as roughly the inverse of the equilibrium swelling ratio, $v_{2,r}$ is

the polymer volume fraction in the relaxed state (the state of the polymer immediately after crosslinking but before swelling), I is the ionic strength, K_a and K_b are the dissociation constants for the acidic and basic moieties on the polymer, and χ_1 is the Flory-Huggins parameter describing the polymer-solvent interaction Using $\bar{M}_c$, the crosslink density, q can be determined from

$$q = \frac{\bar{M}_n}{\bar{M}_c} \tag{11.2}$$

The parameter $v_{2,s}$ is determined from the volume-swelling ratio, q_v:

$$v_{2,s} = \frac{1}{q_v} \tag{11.3}$$

The volume-swelling ratio is calculated as

$$q_v = 1 + \frac{(q_w - 1) \times \rho_2}{\rho_1} \tag{11.4}$$

Where, ρ_2 and ρ_1 are the densities of the polymer network and solvent, respectively. The weight-swelling ratio "q_w" is determined from

$$q_w = \frac{m_s}{m_o} \tag{11.5}$$

Where, m_0 and m_s are the mass of the unswollen gel and the mass of the swollen gel at equilibrium, respectively. Details can be seen from our reports [76]

11.2.3 Cyto/Biocompatibility of Cross-Linkers

Basically, good cyto- and biocompatibility are important factors for a crosslinking agent that is used for crosslinking of various scaffolds. Currently, it is clear that among all the available crosslinkers, some of them, especially the natural ones (GP), are more biocompatible and less cytotoxic. In this regard, extensive research is being carried out to determine the toxicity of different chemical crosslinkers and make a comparison [53]. It has been shown that GP at its maximum nontoxic concentration (0.5 μM), may inhibit osteoblast cytoskeleton reorganization and hinder the growth of osteoblast via stimulation of ROS-mediated cell apoptosis. Hence, it is recommended to keep GP concentration lower than 0.5 μM in tisssue engineering modalities [53].

In the literature, several drawbacks are reported for GA, particularly high cell toxicity. Classically, most of the *in vitro* studies have proven significant cytotoxicity for GA in different types of cells. Furthermore, *in vivo* studies have also shown that crosslinking of the scaffold by GA may contribute to inflammation. Such crosslinkers may be used for preparation of scaffolds by utilizing new protocols. Generally, the type and amount of biomaterials, concentration of GA and fabrication protocol affect the biocompatibility of GA-crosslinked scaffolds. For instance, GA may be added to collagen/chitosan gel before freeze-drying procedure to have a better crosslinking result; however, the cytotoxicity of GA in this manner may be higher than that observed when GA is added after freeze-drying. There are at least two major inflammatory behaviors in response to exogenous scaffold/matrix implantation. In type I, T helper type I (Th1) lymphocytes trigger macrophage type I cells (M1), resulting in an inflammatory reaction and consequently, graft/cell rejection. In contrast, in type II response, T helper type II (Th2) lymphocytes trigger macrophage type II (M2) activity, which is a remodeling reaction and results in graft/cell acceptance [53]. A recent study has shown that GP reduces the immunogenicity of xenogenic materials through regulation of immune cell proliferation and polarization[80]. In such circumstances, the M2 phenotype polarization is dominated in GP-crosslinked matrices, while the GA-crosslinked group is characterized by a shift from an M2-dominant phenotype to a mixed M1/M2 phenotype. Our study has also demonstated the cytocompatibility (highlighted in Fig. 11.2), swelling and drug release properties of gelatin gels crosslinked with Genipin [17, 76].

11.3 Applications for Cross-Linked Polymer Based Matrices in Drug Delivery and Tissue Engineering

Over the past few years, polymer based matrices have undergone great advancement. Also in terms of its applications, great progress has been made in terms of sustained drug delivery and designing scaffolds in tissue engineering. To improve its entrapment and encapsulation, different approaches to cross-linking are adopted and explored, catering to the specific applications of the polymer based matrix. Some of the recent reports have been discussed below.

11.3.1 Crosslinked Chitosan

The major constituent of the exoskeleton of invertebrates is chitin and on treating chitin with an alkaline substance to induce deacetylation, chitosan is obtained. Chitosan, an amino-polysaccharide, finds its applications in diverse fields. However, depending on the specific application, there is a need to utilize chitosan having low molecular weight or modifying it by grafting it with synthetic monomers to obtain copolymers which possess better and improved physicochemical properties. It was observed that during the grafting procedure, certain parts of chitosan undergo immense crosslinking which leads to obtaining a modified chitosan. The modified chitosan so obtained, demonstrates very different properties from native chitosan. It has also been observed that permanent covalent networks are formed due to polymer crosslinking, resulting in improved mechanical strength of the polymers which may be due to free diffusion of water/bioactive materials. The main applications of covalently crosslinked chitosan are in site specific sustained drug delivery through diffusion, and as permanent networks used in tissue engineering [30].

- Application in drug delivery

The utilization of crosslinked chitosan for sustained drug delivery is a common practice. Xu et al. explored the application of catechol–functionalized chitosan crosslinked with a non-toxic crosslinker genipin to formulate a buccal drug delivery system. The hydrogel was designed in such a way that the catechol functional group was covalently bonded to the backbone of chitosan, thereby crosslinking the polymer matrix with genipin. The system was tested for the release mechanism of lidocaine and further buccal patches were fabricated and their mucoadhesive property was tested in the rabbit buccal mucosa. The results indicated that the crosslinked chitosan and genipin would make an effective and biocompatible hydrogel system for drug delivery in buccal cavity [84]. Ju et al. developed a nanogel which indicated the property of reversible swelling-shrinking for the delivery of nanoparticles. The nanogel was designed in such a way that the crosslinked polyelectrolyte core consisted of N-lysinal-N′-succinyl chitosan and poly(N-isopropylacrylamide) which was encapsulated within a crosslinked bovine serum albumin shell. The swelling of this nanogel was controlled by changes in the pH of the environment which could be an effective carrier of chemotherapeutic drugs [35]. Wei et al. investigated the possibility of an "intelligent" controlled release system microcapsule which would be responsive to many stimuli such as pH, temperature etc. They would be composed of crosslinked chitosan with embedded magnetic nanoparticles for further rational drug administration[82]. Ganguly et al. prepared chitosan microspheres crosslinked with polyethylene glycol and loaded it with 5-fluorouracil to understand its *in vitro* cytotoxicity and *in vivo* efficacy for the treatment of colon cancer. The results showed a 24 h sustained release of 5-FU to the colon region and improvement in the antitumor activity [25].

- Application in tissue engineering

Naseri et al. developed fiber mats for the application of wound dressing by electrospinning chitosan/polyethylene oxide followed by reinforcement with chitin nanocrystals. The crosslinked nanocomposite mats demonstrated great tensile strength and biocompatibility to

adipose derived stem cells, making them ideal matrices for wound healing [50]. Zhang et al. also explored crosslinked chitosan for would healing by fabricating a novel hydrogel which was prepared by crosslinking carboxyl-modified poly (vinyl alcohol) with chitosan. These films indicated good biocompatibilty, no cytoxicity and great hemolytic potential. A drug release profile for Gentamicin sulfate was also carried out and the results indicated a controlled release profile and its improved ability to suppress infections [92]. Martinez et al. synthesized and characterized various chitosan/collagen scaffolds by making alterations to the composition and crosslinking and were able to obtain a wide range of scaffolds with different stability, degradation and swelling properties which could be tailored to the specific application in tissue engineering [47]. Zhang et al. prepared and characterized 3-D scaffolds of chitosan/gelatin which were crosslinked by glutaraldehyde, 1-(3-dimethylaminopropyl)-3-ethyl-carbodimide hydrochloride (EDC) or genipin for their application in liver tissue engineering. They were studied for their porosity, biocompatibility, morphology and their ability to maintain liver specific functionality when HepG2 cells were seeded on the scaffolds [94]. Chaochai et al. initiated crosslinking of gelatin and chitosan to obtain sponges. This was carried out by the freeze-drying process and glutaraldehyde/N-acetyl-D-glucosamine was used as cross-linkers. They were evaluated for their bone tissue engineering applications [10]. A similar approach was taken by Gomes et al. in 2017, where they prepared scaffolds of chitosan, gelatin and polycaprolactone which were crosslinked by glutaraldehyde for skin tissue engineering. These scaffolds demonstrated superior cell adhesion and growth properties[26].

11.3.2 Crosslinked Alginate

Alginate, a polymer of natural origin, has been extracted from brown seaweed. Its widespread usage is attributed to its reasonable cost, low toxicity, biocompatibility and mild gelation by addition of divalent cations such as Ca^{2+}. Alginate demonstrates a similarity in structure to the extracellular matrices of living tissues, thus making it ideal for biomedical applications such as delivery of bioactive agents, scaffolding material for wound healing as well as cell transplantation. Various cross-linking methods are used to prepare alginate matrices and depending on their cross-linker type and cross-linking ability, a sustained release of the drug can be achieved. Also due to its moist microenvironment, alginate wound dressings can inhibit infections at the site of the wound. Some of the recent reports regarding its biomedical applications have also been mentioned [43].

11.3.2.1 Application in Drug Delivery

Shtenberg et al. developed a hybrid crosslinked paste of alginate and liposomes as an oral mucoadhesive delivery system for oral cancer therapy. Three different compositions of alginate and liposomes were tested for sustained release of doxorubicin and polymer retention and the results demonstrated that the formulations could be effective in cancer cell death and can be further explored as a potential treatment for oral cancer [70]. Yan et al. synthesized crosslinked alginate-based gel scaffolds encapsulated with tetracycline hydrochloride (TH) which were further characterized for their drug release profile followed by their compatibility in bone tissue engineering [85]. Praveen et al. synthesized and optimized crosslinked alginate beads loaded with cefdinir to be delivered at the target site by a floating delivery system [58]. Cong et al. recently studied the fabrication of chitosan based micelle structure for the controlled delivery of Emodin [14].

Application in tissue engineeringAlginate has been incorporated in various scaffolds for different regeneration applications. Balakrishnan et al. have developed self crosslinked oxidized alginate/gelatin hydrogels for cartilage regeneration [6]. Kamoun et al. used physical crosslinking methods such as freeze-thawing technique to cross link polyvinyl alcohol and alginate for their application in wound healing. The PVA-SA membranes were examined for different properties such as gel fraction, water uptake,

swelling degree, surface morphology, etc. and also indicated improved hemocompatibility [37]. Sarker et al. reported preparation of alginate microcapsules crosslinked with gelatin and evaluated their various physico-chemical properties. They were further evaluated for their adhesive functionality by measuring the proliferation and spreading of the encapsulated MG-63 cells [65]. Desai et al. fabricated covalently crosslinked click hydrogels of alginate by adding tetrazine and norbornene groups to it. These hydrogels exhibited stability and the capacity to encapsulate cells without damaging them, which makes these type of hydrogels potent candidates for the delivery of bioactive molecules or cells in various applications of tissue engineering [19]. In the same year, Quraishi et al. identified a new technique to prepare aerogels of hybrid alginate-lignin with Ca-crosslinking by carbon dioxide induced gelation for similar applications[61].

11.3.3 Crosslinked Hyaluronic Acid

Hyaluronic acid (HA) is a mucopolysaccharide present in all living organisms as a part of their intracellular component of connective tissues such as the vitreous fluid of the eye, the scaffolding in the cartilage and the umbilical cord as well as the synovial fluid of joints. HA is known to serve multiple functions within the body such as its role in binding to water to ease lubrication for movement of joints as well as cell growth. Owing to its non-immunogenicity and biocompatibility, HA has shown a wide biomedical application. However, in order to facilitate enhanced mechanical properties, clearance and degradation rate, HA can be modified chemically or can be crosslinked to form hydrogel materials [66].

- Application in drug delivery

In 2015, Zhong et al. carried out an elaborate study of reversible crosslinked hyaluronic acid nanoparticles for the site specific delivery of the model drug – doxorubicin (DOX) to overcome drug resistance. The DOX loaded nanopaarticles indicated a great efficacy and biocompatibility both *in vitro* and *in vivo* and could prove to be a great anti-cancer therapeutic entity [96]. A similar study was carried out for the same drug model doxorubicin by Han et al. in which they developed a core-crosslinked polymeric micelle on hyaluronic acid and it proved to be highly effective with improved pharmacokinetic properties [28]. Also, due to hyaluronic acids' excellent biocompatibility and natural origin, Widjaja et al. have used crosslinked hyaluronic acid composite hydrogels incorporated with nano-carriers for ocular drug delivery. The *in vitro* study showed sustained release of latanoprost and also an effective degradation which could tackle many ocular diseases [83].

- Application in tissue engineering

In a study conducted by Kim et al., composite fillers of crosslinked hyaluronic acid and human collagen were developed in different ratios for soft tissue regeneration with an application as injectable dermal fillers for tissue augmentation. Shin et al. formed a hyaluronic acid and catechol hydrogel through oxidative crosslinking and carried out a comparative analysis of this with a regular HA hydrogel and the results indicated an improved viability and a reduced apoptosis in hepatocytes and adipose-derived stem cells [69]. Holloway et al. prepared matrix metalloprotease sensitive HA based hydrogels for the delivery of bone morphogenetic proteins. The modified hyaluronic macromers were crosslinked with the matrix metalloprotease peptides to allow the sustained release of the growth hormones [32]. Snyder et al. fabricated HA hydrogels crosslinked with chondrogenic fibrin seeded with bone marrow mesenchymal stem cells (MSC's). To improve the mechanical strength of HA, it was modified with methacrylic anhydride and further studied *in vitro*. The results indicated that the hydrogel was an effective matrix for bone marrow mesenchymal stem cells and could further be explored for its potential in the repair of cartilage for osteoarthritus treatment [73].

11.3.4 Crosslinked Collagen

Collagen belongs to a complex family of distinct proteins which are derived from the mammalian tissues (skin, tendons, cartilage and bone). It is made up of three polypeptide chains which assimilate to form a triple helix structure. The helical conformation is attributed to the presence of repetitive amino acid sequence (Gly-X-Y; X and Y are mostly proline and hydroxyproline). At the end of the helix, telopeptides help in forming cross-striated fibrils. Crosslinks occur between molecules to improve the stability of the fibrils. Soluble collagen which is starting material for collagen research, can be extracted by cleaving telopeptide region by adding proteolytic enzyme (such as pepsin)[4]. Collagen gels, which are physically formed, are thermoreversible in nature, but present poor physical and mechanical properties as compared to covalently crosslinked (glutaraldehyde or diphenylphosphorylazide) collagen gels. Gelation parameters such as temperature and pH have substantial impact on the gel ultrastructure. Collagen gels can be molded into different shapes by filling molds with a collagen solution followed by inducing gelation. In the recent years, 3D printing of collagen using layer-by-layer gelation technique by application of heat has been used. The applications of collagen gels have branched out to reconstructing organs such as skin, small intestines, liver, etc. [42].

- Application in drug delivery

Application of collagens in drug delivery is demonstrated by Quinlan et al. in which a collagen-hydroxyapatite scaffold was incorporated with PLGA and alginate microparticles for the sustained delivery of rhBMP-2 making it an effective controlled delivery vehicle of the pro-osteogenic factor [60]. Zhang et al. developed porous microsphere mats of collagen/polyvinyl alcohol which were crosslinked by UV-radiation and glutaraldehyde by electrospinning for the sustained delivery of salicylic acid [93]. Cibor and group designed crosslinked collagen based membranes loaded with gentamicin for the treatment as well as preventive measures for wound infections post surgery. Similarly, Tsekoura et al. fabricated collagen scaffolds crosslinked with hexamethylene diisocyanante containing the drug Cefaclor to fight bacterial infections (E K [79]) .

- Application in tissue engineering

Owing to excellent biocompatibility of collagen, Fagerholm et al. fabricated corneal implants developed by crosslinking carbodiimide with recombinant human collagen to enable stable regeneration of the cornea. These implants could further be used as alternatives to donor organ transplantations [22]. An ideal scaffold mimics the natural extracellular matrix. Carbodimide crosslinked type-II collagen-CH-based hydrogels are known to exhibit chondrocyte proliferation [56]. Similarly, Zhang et al. [90] have designed crosslinked genipin-HA hybrid hydrogels and investigated cell compatibility *in vitro* for chondrocyte transplantation in cartilage tissue engineering [90]. Grant et al. assessed the stability and biocompatibility of rat tail type I collagen crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and gold nanoparticles [90] and the results indicated an improved stability and biocompatibilty of the scaffolds [27].

11.3.5 Crosslinked Silk Protein

Silk is naturally harvested from the silk worms *Bombyxmori* or *Antheraeamylitta*. Natural silk is composed of two main components: fibroin, a filament component that maintains mechanical stability, and a gummy protein sericin, which is responsible for holding the fibers together [8]. It has been observed that both the above mentioned proteins are made up of the same 18 amino acids such as glycine, alanine and serine, however they are present in varying compositions [2]. The fibroin molecule's composition is such that two-thirds consists of the crystalline portion while the remaining one-third has an amorphous region. Repetitive amino acid sequences (-Gly-Ala-Gly-Ala-Gly-Ser-) are present along the crystalline

portion, forming an antiparallel β-sheet structure, this enhances the stability of the fiber and improves its mechanical properties ([1]; [2]). It consists of heavy and light chain of polypeptides which are connected by a disulfide link. Silk possesses good mechanical, thermal and chemical stability when compared with other protein based materials. In order to further enhance the tensile strength of SF, a variety of crosslinking methods have been adopted over the last few years.

- Application in drug delivery

In 2015, Seib et al. studied the drug release kinetics of Doxorubicin in physically crosslinked silk films which were further coated with gold nanocoating. A comparative study was done with doxorubicin-equivalent dose when administered intravenously and the results indicated a superior *in vitro* as well as *in vivo* activity of the crosslinked silk films. The group carried out similar studies with a different drug, crizotinib and the drug loaded films indicated a better activity [67]. Yan et al. fabricated core-shell silk fibroin hydrogels which were enzymatically crosslinked. The thickness of the shell was tunable depending on the methanol treatment time. It demonstrated sustainable release profiles when incorporated with albumin, thus making them ideal carriers for drug delivery [86].

- Application in tissue engineering

Due to remarkable strength and toughness of silk fibroins, they exhibit excellent promise in bone tissue engineering. Zeng et al., fabricated composite scaffolds of silk fibroin and chitosan of varying compositions and ultimately optimized a suitable scaffold for osteoblast growth. The different compositions were prepared using two different strategies viz.chemical crosslinking and freeze-drying methods. The scaffolds were further characterized and their internal structures were studied to deem them as stable and complete [89]. Shi et al. fabricated dually crosslinked silk fibroin hydrogels through photopolymerization of acrylamide groups. The scaffolds supported *in vitro* stem cell proliferation and improved bone regeneration and due to their self healing properties as well as injectability and fit-to-shape molding characteristics, they are ideal matrices for bone regeneration [68]. Wang et al. prepared a biomimetic scaffold using SF and sodium alginate by crosslinking with EDC and it possessed uniform pore structures. These scaffolds demonstrated improved swelling capacity and mimic the extracellular matrix of natural skin, making them ideal candidate for soft tissue engineering [81]. Pei et al. studied silk fibroin composite sponge to incorporate them in anti-bacterial wound dressing. They fabricated silk fibroin composite sponges by chemically crosslinking with carboxymethylchitosan (CMC) and further incorporated silver nanoparticles within the scaffold. The crosslinking improved various properties of the composite sponge making it an effective wound dressing material [54].

11.3.6 Crosslinked Keratin

Keratins fall under a broad class of insoluble proteins which are usually found as a part of intermediate filaments or epidermal appendage like structures (hair, wool, feathers, nails, hooves, etc.). Keratins possess the property of biocompatibility due to its natural origin, promotes cell interactions and do not degrade into toxic products; hence they have been given importance in various biomedical applications. Depending on its structure and regulation, two classes of keratins have been identified -soft keratin and hard keratin. One of the characteristic features of keratin is its high percentage of cysteine residues. By modifying the numerous covalent disulfide crosslinking in keratins, its properties such as viscoelasticity, stability and chemical reactivity can be altered.

Application in Drug Delivery

In a recent study conducted in 2015, Han et al. induced simple alkylation on kerateine (a form of keratin) which led to the regulation of the disulfide crosslinking in the keratin hydrogels. This modulation allowed controlling the rates of gel

erosion and drug release of rhBMP-2, rhIGF-1, and ciprofloxacin and also did not indicate of any toxicity in MC3T3-E1 pre-osteoblasts [29]. Similarly, Dou et al. fabricated feather keratin and polyvinyl alcohol blended films crosslinked with dialdehyde starch (DAS) and studied the release of Rhodamine B dye as model drug. The study indicated the improved compatibility and stability of the film on crosslinking with DAS and a decrease in 50% of the enthalpy value for its melting peak. The results indicate that the crosslinked feather keratin films can be explored further to be applied in drug delivery [20]. Li et al. formulated drug loaded (doxorubicin) keratin nanoparticles (KDNPs) by desolvation method and chemical crosslinking. The nanoparticles were further characterized for their size, size distribution, morphology and drug delivery profiles. The results revealed that the KDNPs exhibited dual-responsive characters, blood compatibity and also accumulated in the tumor region due to enhanced permeation and retention (EPR) effect. The results were conclusive of crosslinked keratin nanoparticles having potent applications in drug delivery and clinical medicine [44].

11.3.6.1 Application in tissue engineering

With appropriate tweaking in its structural framework by crosslinking, keratin is a potent candidate for tissue engineering applications and researchers are trying to incorporate it within various scaffolds. Zhao et al. explored the potential of crosslinked keratin scaffolds in bone tissue engineering in which keratin was first crosslinked with polycaprolactone composites to reduce its solubility and further uniformly coated it with calcium phosphate to obtain scaffolds. These composite scaffolds have improved the tensile strength and also facilitated interactions within the cell-matrix, which paves the way of these biocompatible scaffolds to be used in *in vitro* osteogenic differentiation studies [95]. Tomblyn and group conducted a comparative analysis of two types of human-hair derived keratin i.e. oxidatively extracted keratin and reductively extracted keratin, in which the former lacked covalent crosslinking and the latter was characterized by disulfide crosslinks. The results indicated that both the keratins had increased the number of multinucleated cells on the human skeletal muscle myoblast cultures. However they showed different degradation rates owing to the presence and absence of disulfide crosslinks [78]. Lin and group combined keratin with chitosan through photocrosslinking for enhancing the proliferation of adipose stem cells [45]. Kim et al. also fabricated keratin/chitosan scaffolds by crosslinking with glutaraldehyde for mimicking skin tissue to enable skin grafting [39].

11.4 Conclusion

Scaffolds fabricated from proteins and polysaccharides often lack the desired tensile strength and stability associated with aqueous environments which prove to be necessary for its proper functioning and medical applications. Crosslinking, as described in the section, improves the properties of the materials to a greater extent. Even though some cross-linkers meet the needs of enhancing the mechanical properties and stability, an adverse effect has been observed on cell attachment and proliferation. One such example is the commonly used crosslinking agent – glutaraldehyde which is known to be difficult to handle. Besides, many contradictory views have been reported debating about the cytotoxicity associated with glutaraldehyde crosslinked materials. Hence, instead of identifying different cross-linkers suiting the need of the independent study, a comprehensive evaluation of crosslinking different structure and biopolymers should be carried out extensively by *in vitro* and *in vivo* investigations to identify a universal cross-linker for bio-polymeric materials intended for medical applications. Research must be directed towards finding alternate crosslinking approaches and using green chemicals to derive bio-polymeric materials with properties desired for drug delivery and tissue engineering applications.

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12 Bone Tissue Engineering Strategies in Co-Delivery of Bone Morphogenetic Protein-2 and Biochemical Signaling Factors

Sungjun Kim, Sangmin Lee, and Kyobum Kim

Abstract

Administration of bone morphogenetic protein-2 (BMP-2), which is commercially approved by the food and drug administration to damaged bone sites has been investigated for the purpose of bone tissue regeneration. BMP-2 can promote osteoblastic differentiation of mesenchymal stem cells as well as regeneration of bone formation in early phase. This review highlights various factors such as vitamin D, dexamethasone, platelet-derived growth factor, placental growth factor, BMP-7, and NEL-like protein-1 that enhance and stimulate angiogenesis, cell differentiation, and bone regeneration. These biochemical signals and growth factors (GFs) accelerate bone repair and remodeling either synergistically or individually. Delivery systems and scaffolds are used for sustained release of these cargo molecules and support at damaged bone sites. Compared with direct administration of BMP-2, current studies have demonstrated that a combination of multiple GFs and/or therapeutic chemical factors with delivery platforms synergistically facilitates bone regeneration. Therefore, in the future, multiple combinations of various GFs, chemicals, and materials could provide patients and surgeons with non-invasive treatment options without secondary surgery and pain. To the end, this review summarizes the biological functions and synergistic effects of dual administration modalities involving BMP-2 as well as recent developments in bone tissue engineering applications.

Keywords

Bone morphogenetic Protein-2 · Co-delivery · Bone tissue engineering · Chemical supplements · Cytokines · Osteogenesis

12.1 Introduction

Bone is a complex tissue including mineral, extracellular matrices (ECM), blood vessels, and various cell types such as osteoblasts (bone forming cells), osteoclasts (bone resorbing cells), and osteocytes (functional mature bony cells). Although bone naturally regenerates in adulthood, natural bone healing is a complex and dynamic process involving a myriad of cellular, molecular, biochemical, and mechanical cues [11,

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28]. Currently, bone fractures are a major occurrence worldwide, and grafting is commonly used to treat the patients. However, bone grafting has limitations such as pain, high cost, limited supply, finite donor availability, potential donor site morbidity, disease transmission, and immunogenic response [53, 59]. Therefore, tissue engineering approaches utilizing biodegradable materials, stem cells, and exogenous growth factors (GFs) have been intensively investigated for the development of novel clinical applications [26, 27]. Biodegradable materials, especially synthetic biopolymers, have attracted great attention in the tissue engineering field since degradability avoids the common disadvantages of grafting and ceramic or metallic device implantation [55]. In addition, GFs and bioactive cytokines are often administered in a scaffold or delivery system to enhance osteoblastic differentiation of stem cell population and bone regeneration [44]. Among a series of GFs for exogenous delivery strategies in bone repair, bone morphogenetic protein-2 (BMP-2) is well known to promote osteogenesis of mesenchymal stem cells (MSCs). Additionally, BMP-2 is commercially available and has been approved by the US Food and Drug Administration [5, 57]. Although a recent investigation reported a minor limitation in exogenous BMP-2 administration such as a side effect of high dose of BMP-2 [23], BMP-2 mediated bone regeneration approach have been researched in decades. Recently, a co-delivery of BMP-2 and additional therapeutic GF has also been investigated for the development of more effective medical applications for bone repair [43].

In addition to the therapeutic efficacy of multiple GFs, a regenerative capacity of other biochemical supplanted factors also facilitate the activation of native and/or delivered stem cell population to differentiate into osteoblasts as well as a subsequent bone regeneration in physiological microenvironment. Various biochemical factors such as chemical compounds, bioactive cytokines, and drugs can synergistically promote osteogenesison the top of regenerative efficacy of BMP-2 [23]. For instance, vitamin D3 and dexamethasone (Dex) are important chemical compounds to maintain calcium and phosphorous balance in the body and to promote osteogenic differentiation of MSCs *in vitro*, respectively [14, 32, 50, 52]. Other bioactive cytokines including platelet-derived growth factor (PDGF) and placental growth factor (PLGF) involve in inducing angiogenesis and vascularization [2]. Moreover, BMP-7 can be also used as a substitute for BMP-2 [6, 9, 57] and NEL-like protein-1 (NELL-1), a secretory signal peptide sequence, is considered as co-delivery factor for BMP-2 mediated bone regeneration strategies [30, 60]. In order to maximize the therapeutic functionality and overcome the limitations of the current BMP-2 delivery, co-administration of these biochemical factors with BMP-2 could achieve an enhanced healing efficacy. To this end, a variety of interactions among biochemical factors, scaffolds, and delivery systems must be considered to ensure successful bone regeneration (Fig. 12.1) [49]. Therefore, this mini review summarizes recent tissue engineering developments in (1) co-delivery strategies using BMP-2 and multiple biochemical signals using various delivery applications and (2) the synergistic therapeutic effect of the co-delivery applications, especially for bone tissue repair.

12.2 Co-Delivery of BMP-2 and Chemical Supplements

Vitamin D3 is an effective chemical supplement to enhance BMP-2 mediated therapeutic effects on osteogenic differentiation of stem cells [19, 52, 56]. Song et al., reported a synergistic effect of BMP-2 and vitamin D3 on *in vitro* osteogenic differentiation of adipose derived stem cells (ADSCs) [52]. In the case of vitamin D3 single delivery, ALP expression and mineralization of ADSCs upregulated by increasing the dose of vitamin D3 up to 10^{-6} M per 30,000 cells in a well of 48 well plates. For a co-delivery, a combination of 50 ng/mL of BMP-2 and 10^{-7} M of vitamin D3 achieved an optimal dose for the differentiation of ADSCs over 14 days, and the synergistic effect (i.e., a higher osteoblastic phenotypes of ADSCs) was observed as compared with any single delivery. In addition, a

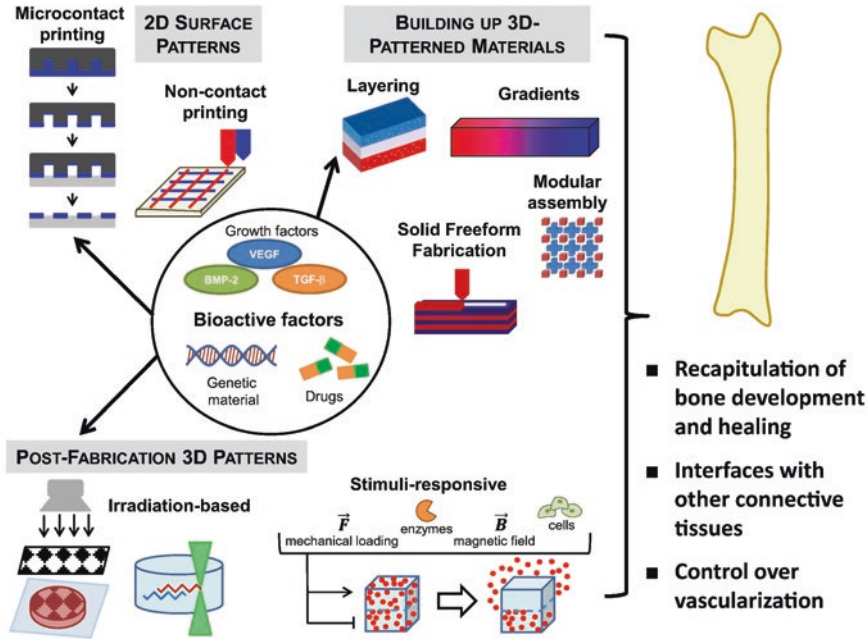

Fig. 12.1 Various and complex combination of bioactive factors with delivery systems for bone tissue engineering applications being developed recently. (Reproduced with permission from Ref. [49])

time-dependent therapeutic manner of BMP-2 and vitamin D3 also indicated the optimal administration timings. Based on the experimental results, it could be concluded that vitamin D3 treatment throughout the 14 days of culture period with the addition of BMP-2 in the later period is an effective and economical way for the induction of osteogenic differentiation of ADSCs.

In addition, Dex is another chemical compounds to induce bone regeneration and enhance the regenerative effect of BMP-2 [4]. Recently, Li et al., suggested a controlled co-delivery of BMP-2 and Dex using bovine serum albumin (BSA) nanoparticle-embedded electrospun poly(ε-caprolactone)-co-poly(ethylene glycol) (PCE) nanofibers [35]. Specifically, BMP-2 incorporated BSA nanoparticles were first synthesized by desolvation, and the final particles were stabilized by chitosan-mediated electrostatic self-assembly (Fig. 12.2a). Chitosan-stabilized nanoparticles and Dex were mixed with PCE polymers and electospun to result nanofibrous scaffold systems (Fig. 12.2b). The bioactivity of incorporated BMP-2 and Dex were maintained in an *in vitro* condition. A relatively faster release of Dex and more sustained release of BMP-2 were observed, and this nanofibrous scaffold released both cargos over 35 days. The synergistic effect of co-delivery was determined by ALP expression and Alizarin Red S staining of murine mesenchymal stem cells (MSCs) cultured onto the nanofibers. Moreover, a co-delivery of BMP-2 and Dex using nanofibrous composite scaffolds facilitated *in vivo* bone regeneration as well. Implanted scaffolds containing both BMP-2 and Dex in critical-sized rat calvarial defect exhibited the best repair efficacy over 12 weeks, determined by radiograms of the X-ray detection, Masson's trichrome staining, and osteocalcin(OCN) immunehistochemical staining. Although it is known that Dex generally promotes early calcified bone formation while BMP-2 is beneficial for a long-term new bone formation, this research suggested a controlled and sustained release of BMP-2 and Dex using engineered delivery platforms could activate bone regeneration processes. Furthermore, Fig. 12.3 indicates a possible mechanism of *in vivo* osteogenesis promoted by this dual-drug-loaded nanofibrous scaffold [20, 21, 35].

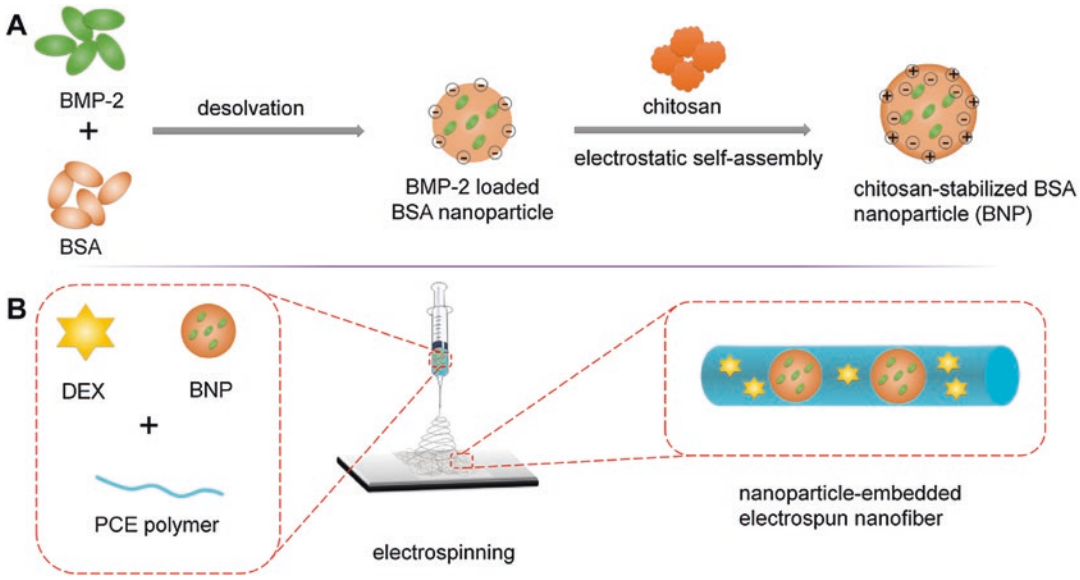

Fig. 12.2 Schematic diagram for preparation chitosan stabilized BMP-2 loaded BSA nanoparicle (A) and DEX/BNP embedded electrospun PCE nanofiber using electrospun for the repair of bone defects. (Reproduced with permission from Ref. [35])

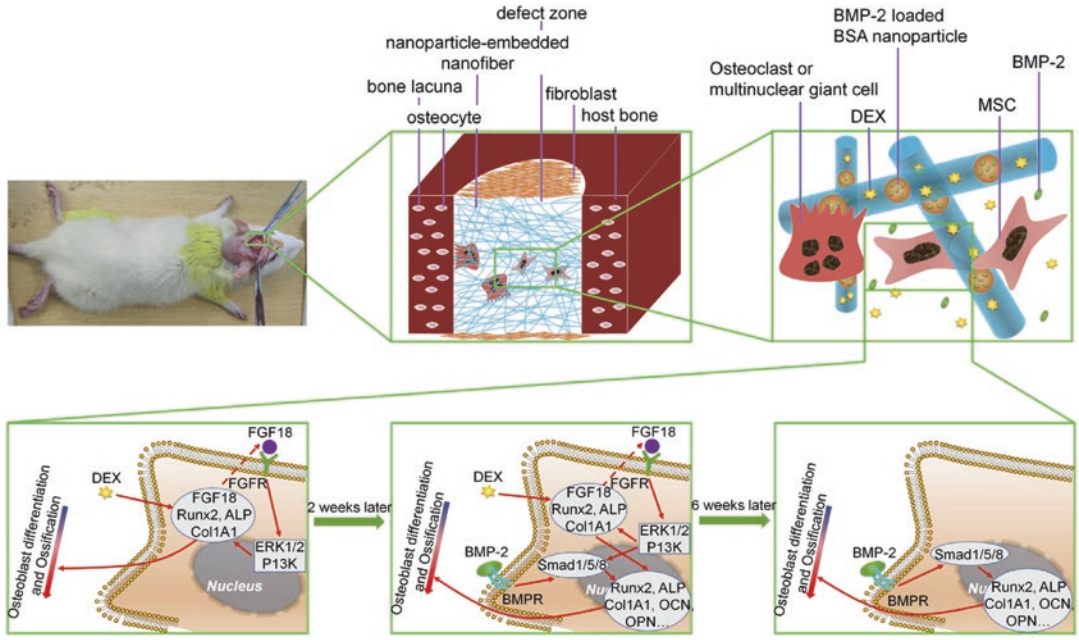

Fig. 12.3 Schematic illustration of signaling pathway that accelerated by DEX and BMP-2. In the early phase, DEX initially burst released and, it is promote osteogenesis for 2 weeks. From 2 to 6 weeks, DEX and BMP-2 simultaneously released, and enhance more new bone formation and mineralized bone formation. After 6 weeks, BMP-2 plays important role for bone regeneration. (Reproduced with permission from Ref. [35])

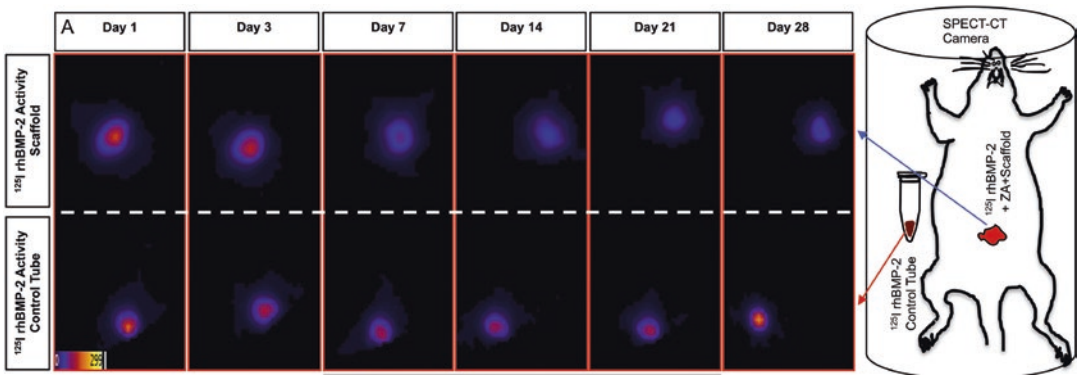

Fig. 12.4 In vivo ^{125}I-rhBMP-2 intensity from the scaffold that containing rhBMP-2 and ZA in the rat models during 4 weeks via SPECT. Control tube was located on the bottom side to compensate for tracer radiation decay. (Reproduced with permission from Ref. [46])

Similarly, Choi et al., developed core-shell microcapsules for a co-delivery of BMP-2 and Dex [7]. In this study, the size and shape of core-shell microcapsules could be modulated by controlling nozzle size, applied voltage, the volumetric feeding ratio of poly(L-lactide-co-glycolide) (PLGA) and alginate, feeding rate, and polymer concentrations via a coaxial electrodropping method. 100 μg of BMP-2 was encapsulated into the inner PLGA core while 10 mg of Dex was incorporated in the 0.5 wt.% alginate outer shells. A release profile exhibited a significantly different temporal pattern of both cargos. The result showed an initial burst of Dex over 5 days, followed by a sustained release pattern during the remaining time period (until 30 days) while a release of BMP-2 was more sustained. When rat bone marrow stromal cells were encapsulated with these core-shell microcapsules into alginate hydrogels, released BMP-2 and Dex induced osteogenic marker gene expression including ALP, OCN, and osteopontin after 4 weeks of *in vitro* culture. The results indicated that co-delivery of BMP-2 and Dex using core-shell type microcapsules could maintain the osteogenic potential of stem cells. Moreover, Raina et al., also reported a synergistic effect of co-administration of BMP-2 and zoledronic acid (ZA) for inducing ostoeclast cells death and osteogenesis [46]. In this study, a composite cryogel with gelatin/hydroxyapatite/calcium sulfate was fabricated for a delivery platform. *In vitro* assays demonstrated that cryogels with recombinant human BMP-2 (rhBMP-2) induced proliferation of MC3T3-E1 cells as well as expressions of osteoblastic gene markers including alkaline phosphate, osteric and osteocalcein. For evaluation of *in vivo* osteogenesis, rat abdominal muscle pouch model was utilized. *In vivo* X-ray and micro-CT results showed that significantly higher mineralization was observed in gelswith both 10 μg of rhBMP-2 and 10 μg of ZA as compared gels containing the same dosage of cargos in commercially available absorbable collagen sponge. Histological analysis also indicated a higher new bone formation in a dual delivery group of rhBMP-2 and ZA than a single delivery of rhBMP-2. In order to track the release of both rhBMP-2 and ZA in *in vivo* physiological environment, ^{125}I labeled BMP-2 was tracked via single photon emission computed tomography imaging (Fig. 12.4) while ^{14}C labeled ZA release was detected by a scintillation counting. 65.3 ± 15.2% of ^{125}I labeled BMP-2 was released for 28 days from abdominal muscle pouch while an initial burst release (i.e., 43.2 ± 3% release in the first day of implantation) of ^{14}C labeled ZA was observed in a dual delivery group. Taken together, the results demonstrated that a combination of BMP-2 and ZA into composite cryogels could promote both *in vitro* and *in vivo* osteogenesis than commercial collagen sponges.

12.3 Facilitated Osteogenesis by Co-Administration of BMP-2 and Various Cytokines

In addition to a dual delivery of BMP-2 and stimulus chemical compounds, bioactive cytokines have also been co-administrated with BMP-2 to achieve a facilitated osteogenic differentiation and bone regeneration. For example, angiogenic stimulants could support a physiological regenerative process during bone healing by upregulating angiogenesis and blood vessel formation in the bony defect sites. In addition to vascular endothelial growth factor (VEGF), a well-known angiogenic cytokine, placental growth factor-2 (PGF-2) is a multitasking cytokine to enhances angiogenesis, recruitment/activation of bone marrow-derived cells, and bone remodeling [17, 22, 38, 40].

Therefore, Liu et al., suggested a heparin-based nanocomplex for a sustained co-delivery of BMP-2 and PGF-2 [36, 37]. For a fabrication of this nanocomplex, a positively charged N-(2-hydroxyl)propyl-3-trimethyl ammonium chitosan chloride (HTCC) interacted with a mixture of heparin/BMP-2/PGF-2 via electrostatic complexation. The loading efficiency of both cargo molecules was over 97% and the size of the nanocomplex was around 485 nm. Cumulative % release of BMP-2 over 21 days was 9% and that of PGF-2 was 16%, respectively. The *in vitro* results indicated that proliferation and osteogenic differentiation of MC3T3-E1 preosteoblasts was effectively stimulated by co-delivery, as compared with any single administration. Moreover, PlGF-2 could enhance osteogenic differentiation at a lower loaded lose (i.e., 0.5 μg) as compared with 1.0 μg of BMP-2 in the case of a single delivery.

Another angiogenic cytokine for enhanced osteogenesis with the aid of BMP-2 is angiopoietin1 (Ang-1). This cytokine could promote vascularization and maturation by stimulating the Tie2 receptor/PIK3/AKT pathway [54]. Therefore, Choi et al., investigated the enhanced therapeutic effect of Ang-1 variant on BMP-2 induced cranial bone regeneration in calvarial defect models [8]. In this study, cartilage oligomeric matrix protein-angiopoietin 1 (COMP- Ang1), a synthetic and soluble variant of Ang-1, was administrated using absorbable collagen sponges in a critical sized defect (5 mm in diameter) of C57BL/6 mice. Dual delivery group using BMP-2 and COMP- Ang1 facilitated vascular formation, determined by the highest expression of CD31(an endothelial cell marker) and NG2 (a specific marker of pericyte) in immunofluorescence analysis. In addition, increased migration, osteogenic differentiation of pericytes, upregulated expression of osteoblast-specific genes including bone sialoprotein (BSP), OCN, osterix (OSX), and phosphorylation of Smad/1/5/8 were also observed. The same co-delivery group also significantly increased *in vivo* bone repair volume as compared to the control, presumably by angiogenesis via recruitment of MSC-like pericytes and osteoblastic differentiation of native stem cell population. Hence, this research suggested that the improvement of reconstruction of large craniofacial defects could be obtained by co-administration of BMP-2 and Ang-1.

Neural epidermal growth factor-like protein-1 (NELL-1) is a multi-functional cytokine involved in osteogenesis[24, 31, 61], stabilization of the intervented spine [13, 33], chonrogenic differentiation of perivascular stem cells [34], and odontoblastic differentiation of dental pulp cells [36, 37]. As a potent osteoinductive factor, this cytokine recruit immature cells and stimulate them to become preosteoblasts through the Wnt, Hedgehog, and MAPK pathway [42]. Binding of NELL-1 to its specific integrin-β1 receptor stimulates β-catenin nuclear localization and subsequent transcription of Runx2 and OSX, through intermediate intracellular ERK/JNK activation. Zhu et al., reported a rapid distraction osteogenesis of rabbit tibia by co-delivery of BMP-2 and NELL-1 [62]. Distraction osteogenesisis an effective medical technique to create new bone via the gradual separation of two bony fragments from each other after their surgical division, especially for longitudinal bone lengthening (Fig. 12.5). However, this technique still results in a considerable morbidity. Thus,

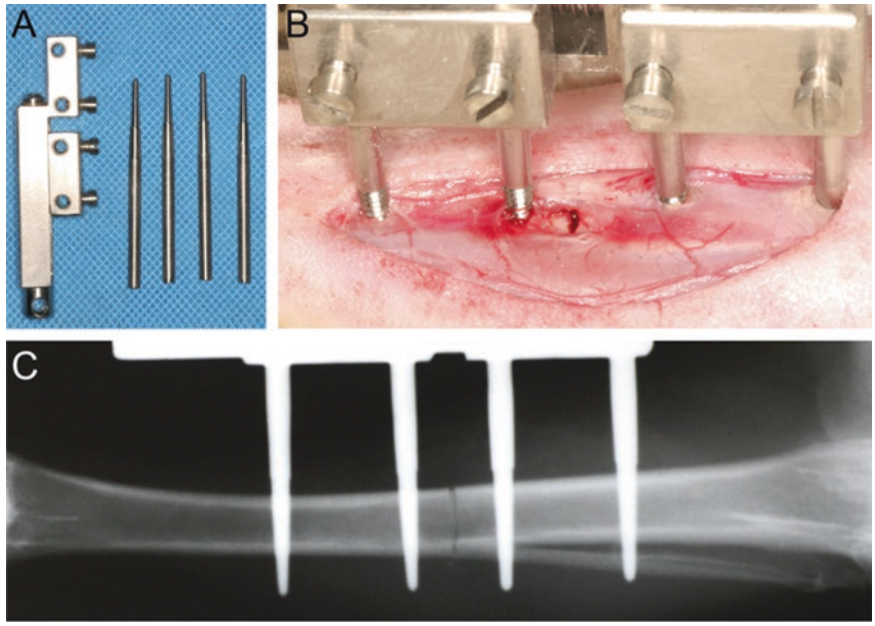

Fig. 12.5 Macroscopic description of a self-constructed external fixator with four self-taping screws (**a**). Macroscopic (**b**) and representative radiograph (**c**) of subperiosteal osteotomy to the fibula in rabbit model. (Reproduced with permission from Ref. [62])

this study emphasized a combinatorial effect of co-delivery of BMP-2 and NELL-1 on enhanced bone regeneration through improvement of callus structure/organization and mechanical strength, as evidenced by histology, mechanical testing, and μ-CT examination. The result demonstrates that BMP-2 and NELL-1 could enhance each other's functionality in tibia osteo-distraction as compared with either agent alone, and that the addition of NELL-1 might reduce the clinical dose of BMP-2 as well as unintended side effects. Although another recent study reported that the osteogenic potential of a single delivery of NELL-1 is less significant than BMP-2 administration [15], a co-administration of BMP-2 and NELL-1 could synergistically facilitate bone repair process.

Wnt1-inducible signaling pathway protein 1 (WSIP1, also known as CCN4) is a member of CCN protein family to enhance bone regeneration, as an osteoblastic signal regulator. In general, CCN family proteins participate in biological processes related to development, tissue repair, and tumor suppression. Wnt signaling pathway is closely involved in developmental process [10], and oncogenesis [45]. In the process of bone regeneration, noncanonical Wnt signaling regulates osteogenic differentiation, enhances bone development, and maintain bone homeostasis [3, 41]. Therefore, Kohara et al., investigated the effect of co-administration of BMP-2 and WSIP1 on ectopic osteoid formation [29]. Subcutaneous implantation of gelatin/β--tricalcium phosphate sponges incorporating both BMP-2 and WSIP1 exhibited homogeneous osteoid formation around the implant in middle-aged mice with decreased ability of bone formation (i.e., 38 weeks old).

In addition, platelet-rich plasma (PRP) could be also utilized to improve osteogenic differentiation of stem cells, treat bone defects, and support microfracture surgery in osteochondral lesions [18, 39, 47]. As a dual delivery application, Fernandes et al. reported that a controlled release of PRP in alginate beads along with BMP2 could significantly promote *in vitro* proliferation, osteogenic differentiation, and mineralization activity of MSCs [16]. As

PRP is a blood derivative containing a series of osteoinductive and angiogenic bioactive cytokines such as platelet derived growth factor (PDGF), transforming growth factor (TGF)-beta, platelet factor-4, interleukin-1, platelet-derived angiogenesis factor, VEGF, epithelial cell growth factor, insulin-like growth factor, fibrinogen, vitronectin, fibronectin, osteocalcin, and osteonectin, even a single delivery of PRP itself could promote ex-vivo bone regeneration using isolated femur bones of C57BL/6 mice and calvarial suture closure [16]. Similarly, PDGF, one of the components in PRP, has also used in a co-delivery with BMP-2 for critical-size calvarial bone reconstruction [51]. In this study, Shah et al. hypothesized that rapid bone formation in large, critical-sized defects could be induced by simultaneous delivery of BMP-2 and PDGF-BB. Both GFs were incorporated into polyelectrolye multilayer coating onto PLGA/alendronate membranes. Histological evaluation and μ-CT imaging demonstrated that mature bone formation with sufficient mechanical properties was observed in a co-administration group, with a greater number of vascular channels and higher cell density within regenerated bone area. This result indicated a mitogenic role of PDGF-BB in the bone formation process in craniomaxillofacial defects.

12.4 Combination of Other Bioactive Growth Factors for Enhanced Bone Repair

Other members of BMP superfamily such as BMP-6 and BMP-7 are also potentially used in clinical bone repair. These GFs improve bone mass or quality, repair damage to bone and joints, and treat diseases of skeletal overgrowth [48]. Recent studies investigated their potential bone tissue engineering applications along with a variety of delivery platforms. For example, Demirtas et al. evaluated osteoblastic differentiation of pre-osteoblastic cells by a co-administration of BMP-6 and PDGF-BB [12]. Similar to a combination of BMP-2 and PDGF-BB described in a previous section, a co-delivery of BMP-6 and PDGF-BB effectively stimulated *in vitro* proliferation and osteogenic differentiation of MC3T3-E1 cells via dual capacities of osteogenic and mitogenic activation. Moreover, a recent study comparatively evaluated osteoinductive bioactivity of BMPs combined with human plasma fibronectin (FN/BMP) delivered using titanium-hydroxyapatite (TiHA) [1]. This research demonstrated that each member of BMP superfamily possessed a different molecular binding strength with fibronectin and variant paracrine effect of BMPs around the FN/BMPs functionalized TiHA implant surfaces. Therefore, it could be emphasized that BMP-6 could be an alternative bioactive signal to develop a biomimetic microenvironment to induce osteogenic activity, especially when incorporated with a FN/TiHA engineered scaffold system.

In addition, BMP-7 has also been investigated to enhance the therapeutic capacity of BMP-2 via co-administration [25, 58]. In order to a sequential delivery of BMP-2 and BMP-7, Jo et al., suggested a heparinization of collagen membranes by a simple EDC/NHS chemistry (Fig. 12.6) [25]. Briefly, a collagen membrane surface was heparinzed by the generation of amine-reacitve NHS-ester. BMP-7 was first incorporated with heparin and subsequently BMP-2 was physically absorbed onto collagen surfaces. In this study, to evaluate *in vivo* performance of co-administration of BMP-2 and BMP-7, either 5 μg of any single GF or 2.5 μg of dual BMP-2/BMP-7 was applied into a 10 mm × 10 mm collagen membrane which covered a circular, transosseous, 8 mm defect in the middle of the cranium of Sprague-Dawley rats. Burst release of BMP-2 and sustained release of BMP-7 exhibited a significantly induced new bone formation after 8 weeks of transplantation, as compared with other single delivery formulations. A similar application was also developed using chitosan-based scaffolds for bone tissue engineering [58]. Yilgor et al., developed a sequential GF delivery system using synthetic polymer nanocapsules and chitosan-polyethyle oxide scaffolds. Here, BMP-2 and BMP-7 was incorporated into PLGA nanoscapsules while BMP-7 was loaded with

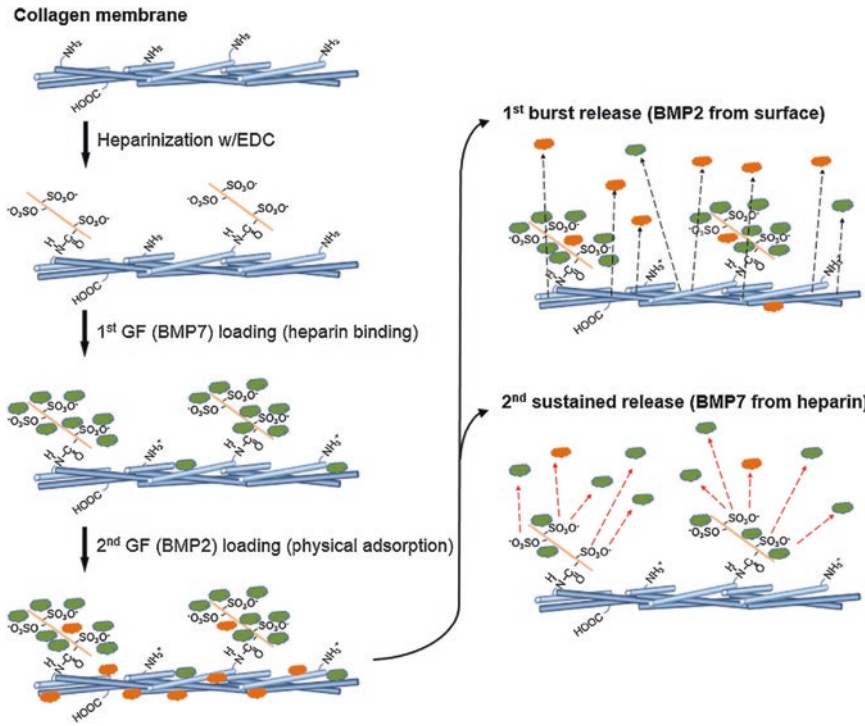

Fig. 12.6 Schematic illustration for the fabrication of heparinized collagen membranes containing BMP-2 on the surface and heparin bound BMP-7. (Reproduced with permission from Ref. [25])

poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanocapsules, respectively. This research also achieved a similar release profile of BMPs (i.e., the early release of BMP-2 and longer term release of BMP-7). While a simultaneous delivery of both BMPs was not particularly effective on *in vitro* proliferation and ALP activity of bone marrow MSCs, a sequential delivery of BMP-2 and BMP-7 exhibited to the highest normalized ALP activity indicating the synergistic effect of co-administration by modulating their release patterns.

12.5 Summary

Bone healing is a complex process involving cell interaction, ECM production, mineralization, and vascularization. Surgical procedures are limited and disadvantageous. Therefore, development of delivery systems that are injectable, biocompatible, and biodegradable is required. BMP-2 is the most proven and studied GF for bone regeneration. However, a biodegradable mechanism that provides mechanical strength must be developed. Administration of sufficient dosages of BMP-2 could have clinical side effects such as inflammation, radiculopathy, ectopic bone formation, osteolysis, urogenital events, and wound complications [23]. Thus, circumstantial proper treatment of BMP-2 could eliminate the need for complex bone grafting procedures and have long-term healing effects. Many recent studies have demonstrated the synergistic effect of a combination of BMP-2 with other GFs or other factors. BMP-2 promotes osteogenic differentiation in the early stage, and other GFs or factors accelerate and support later *in vivo* bone regeneration. ECM mimicking the structure of delivery materials for stable and sustained release of GFs must be considered. To this end, successful fabrication and manufacturing of injectable and/or transplantable biomaterials as a multiple GF delivery system could be a

reasonable biomedical approach. In the future, combinations of products including stem cells, GFs, biological cues, and gene therapy with delivery systems could provide minimal and non-invasive treatment for bone regeneration.

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13 Growth Factor Delivery Systems for Tissue Engineering and Regenerative Medicine

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Abstract

Growth factors (GFs) are often a key component in tissue engineering and regenerative medicine approaches. In order to fully exploit the therapeutic potential of GFs, GF delivery vehicles have to meet a number of key design criteria such as providing localized delivery and mimicking the dynamic native GF expression levels and patterns. The use of biomaterials as delivery systems is the most successful strategy for controlled delivery and has been translated into different commercially available systems. However, the risk of side effects remains an issue, which is mainly attributed to insufficient control over the release profile. This book chapter reviews the current strategies, chemistries, materials and delivery vehicles employed to overcome the current limitations associated with GF therapies.

Keywords

Growth factor delivery · Tissue engineering · Delivery vehicles · Scaffolds · Biomaterials

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13.1 Introduction

Since tissue and organ transplantation became a widespread medical procedure, there has been a tremendous disparity between the need and the availability of organs and tissue grafts. The inherent limitations associated with organ transplantation include immune rejection, risk of disease transmission and donor-site morbidity. The tissue engineering and regenerative medicine (TERM) field, which aims to regenerate or repair tissues or organs, has emerged as an attractive strategy to overcome these issues.

In order to successfully engineer tissues in the laboratory, it is vital to firstly understand the physiological regenerative and development processes. The main components in the developmental and regenerative microenvironments are cells, extracellular matrix (ECM) and soluble signalling molecules [105]. Cells are the central unit of the tissue as they proliferate, migrate and differentiate in response to certain environmental inputs. The ECM acts as a physical support to these cells, while also providing the necessary biophysical and biochemical cues for tissue homeostasis. On the other hand, soluble signalling molecules circulate through the bloodstream and/or diffuse through interstitial fluid to modulate cellular behaviour. Thus, controlling these signals holds the potential to control cellular fate, which includes triggering or enhancing regenerative/healing processes. Growth factors (GFs)

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have been identified as soluble signalling molecules that play critical roles in both the development and regenerative processes and have been a main focus in TERM strategies.

Levi-Montalcini and Cohen firstly discovered GFs by studying the effect of sarcomas on axonal growth from chicken embryos [33], where the signalling molecule that triggered nerve growth was identified as nerve growth factor (NGF) [34]. Since then, a number of GFs which modulate many physiological processes have been discovered and applied to different regenerative medicine applications [100]. Some examples include bone morphogenetic proteins (BMP-2 and BMP-7) for bone regeneration [119] or vascular endothelial growth factor (VEGF) [67] and platelet-derived growth factor (PDGF) [139] for diabetic foot ulcers.

GFs are defined as secreted, biologically active molecules that can affect the growth and differentiation of cells. GFs act on cells by binding transmembrane receptors in a highly specific manner, which triggers a transduction cascade that generally starts with phosphorylation of the cytosolic domain of the receptor. Each GF is unique with specific roles in cellular behaviour (Table 13.1). For example, BMP-2 is essential for the maintenance of bone density [208]. It was previously reported that adult mice lacking BMP-2 showed spontaneous fractures and impaired bone repair [200]. Due to these characteristics, BMP-2 has been used in clinical settings for spinal fusion procedures and for non-union fractures [39]. A comprehensive list of these GFs with their respective unique characteristics are tabulated in Table 13.1.

In the native microenvironment, GF concentrations are usually in the nanomolar to picomolar range, where their presence is continuous and can last up to several weeks or months [9]. Initial clinical trials, which involved injection [196] or spraying [16] of GFs directly to the wound site, showed limited therapeutic effects, mainly due to the short presence of the applied GFs at the wound site. In order to better mimic the natural spatio-temporal concentrations of GFs, continuous doses of the GF were administered, causing systemic overexposure that can result in an undesirable increase in cancer risk and other side effects [53]. These results led to the realization of the need for a suitable GF delivery system, which has been the focus of many research groups in the past decades. This chapter will cover the different GF delivery approaches reported in the literature that aim to mimic key aspects of the regenerative microenvironment by controlling the spatio-temporal presence of GFs.

13.2 Design Criteria for Growth Factor Delivery Systems

The selection of an acceptable GF will not only depend on the type of organ or tissue that we are trying to regenerate, but also on the desired cell function. Some GFs are required to trigger proliferation and differentiation of cells that are already present at the site. In other cases where the cells required for healing or regeneration are absent, chemotactic GFs are able to trigger migration of cells to the wound site [106, 204]. The ECM is another key factor that needs to be considered while designing any GF delivery system, since it can modulate the effects of GFs through different mechanisms. For example, heparan sulphate [4], decorin [201], betaglycan [205], versican [70], fibronectin [127], collagen [173], vitronectin [203], SPARC (Secreted protein acidic and rich in cysteine) [19] and tenascin C [41], are ECM components that can bind GFs and modulate their diffusion and localization, further influencing their availability at the cell surface and their receptor-binding kinetics. Due to the dynamic remodeling of the ECM during regenerative processes [54, 212], it is essential to understand the interaction between GFs and the ECM for the design of an optimized delivery strategy. The interactions between cell receptor, ECM and GFs are represented in Fig. 13.1.

In order to modulate these complex interactions, mimicking different dynamic aspects of the native GF such as its localization, expression levels and expression patterns has been identified as a key design criteria for GF delivery systems. These aspects are further discussed in the sections below.

Table 13.1 Usage of growth factors in TERM applications

Growth factor	Main receptors	Signal transduction pathways	Role in adult tissues	Application in regenerative medicine	Clinical trials and commercially available products
BDNF [25, 66, 78]	TrkB	Ras-MAPK	Sensory neuron function		–
	LNGFR	PI3K	Cortical circuitry function	Spinal cord injury	
		PLC-γ1	Synaptic strength and plasticity	Stroke	
BMP2/BMP7 [39, 208]	BMPR-1A		Bone homeostasis and function. BMP-2: indispensable for bone fracture healing, chondrocyte proliferation and maturation. BMP7: kidney function	Spinal fusion	INFUSE® and OP-1™ implant are commercially available
	BMPR-1B	Smad1/5/8		Non-union fractures	
	BMP2-R	Ras-MAPK			
	ActR-1A	PI3K			
	ActR-2A/2B				
EGF [12, 25, 216]	EGF-R	Ras-MAPK	Injury response, homeostasis and growth in mammary gland, GI tract, nervous system. Inhibits osteogenic and chondrogenic maturation.	Diabetic foot ulcers	Diabetic foot ulcers [52]
		PI3K		Stroke	
FGF1 [11, 47, 81]	FGFR1-4	Ras-MAPK	Maintenance of vascular tone, Adipogenesis.	Peripheral ischaemia Peripheral nerve lesions	Peripheral artery disease [142], Skin burns [16], Spinal cord injury [83]
		PI3K, STAT, PLC-γ			
FGF2 (bFGF) [11, 25, 47, 81, 82, 93, 96]	FGFR1-4		Heart homeostasis and repair, vascular tone, angiogenesis, cartilage homeostasis	Periodontal defects	Periodontal defects [118], Peripheral artery disease [190], Critical limb ischemia [101], Bone lesions [87], Tracheal defects [194], Coronary artery disease [182]
				Skin wounds	
		Ras-MAPK		Bone lesions	
		PI3K		Myocardial infarction	
		STAT		Stroke	
		PLC-γ		Ligament regeneration	
				Tracheal defect repair	
FGF7 (KGF) [11, 47, 81]	FGFR1-4	MAPK, PI3K, STAT, PLC-γ	Skin repair	Oral mucosa regeneration	Kepivance® is commercially available
				Skin wounds	
HGF [56, 159]	c-Met	Gab-1	Homeostasis and regeneration of liver, lung, stomach, pancreas, heart, brain and kidney	Cirrhosis	Critical limb ischemia [137, 158], Chronic vocal cord lesions [95]
		PLC-γ		Liver regeneration	
		Ras-MAPK		Critical limb ischemia	
		PI3K		Chronic vocal cord lesions	
NGF [78, 120]	TrkA	Ras-MAPK, PI3K, PLC-γ1	Sensory neuron function	Peripheral nerve lesions	Alzheimer's disease [50, 202]
	LNGFR		Cortical circuitry function		

(continued)

Table 13.1 (continued)

Growth factor	Main receptors	Signal transduction pathways	Role in adult tissues	Application in regenerative medicine	Clinical trials and commercially available products
PDGF [1, 22, 58, 84, 117]	PDGFRα PDGFFRβ	PI3K, PLC-γ, Ras-MAPK	Postnatal angiogenesis andvascularization. Smooth muscle cell and fibroblast homeostasis.	Periodontal defects Skin wounds Bone regeneration Tissue vascularization	Periodontal defects and gingival recession [118], Diabetic foot ulcers [139]. Regranex®, Augment™ and GEM 21S® are commercially available
SDF1-α [59, 64, 171, 177]	CXCR4 CXCR7	Ras-MAPK, PI3K, JAK-STAT	Angiogenesis, mesenchymal stem cell homing during tissue repair, heart homeostasis	Revascularization Wound healing Tendon regeneration Muscle fibrosis	–
TGF-β(1-3) [110, 124, 148–150]	TβR-II TβR-I	Smad 2/3	Wound healing and skin homeostasis, bone healing, heart regeneration.	Bone regeneration Cartilage regeneration Periodontal tissue regeneration	–
VEGF-A [25, 69, 75, 166, 220, 222]	VEGFR(1-2) Nrp (1-2)	PI3K PLC-γ MAPK	Angiogenesis. Brain, liver, bone and lung homeostasis and regeneration,	Diabetic foot ulcers Bone regeneration Brain and myocardial ischemia	Diabetic foot ulcers [67]

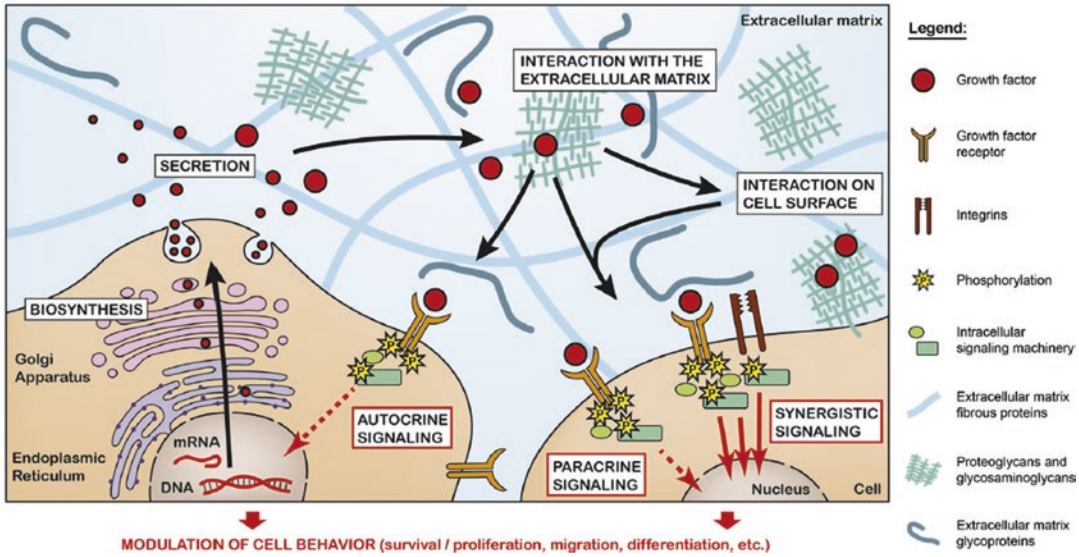

Fig. 13.1 From biosynthesis to cell receptor signalling, a growth factor's journey within the physiological ECM. After their biosynthesis, growth factors are secreted into the ECM, where they interact with ECM components before binding and activating their specific receptors. Growth factors mainly signal to cells in autocrine and paracrine fashion, to instruct their behaviour during morphogenetic processes. Complexes formed between growth factors, ECM components, and cell surface receptors may lead to additive or synergistic cell signalling events. (Reproduced from Ref. [134])

13.2.1 Localization of Delivered Growth Factors

GFs can have different effects in different tissues and cell types. For example, EGF promotes homeostasis in the GI tract [8] and mammary gland [90], but inhibits tissue maturation in the cartilage [24]. In order to achieve only the desired therapeutic outcome, the GF has to be delivered and contained spatially at the targeted tissue. Moreover, unnecessary presence of GFs in non-targeted tissues might also trigger cancer development and progression due to undesirable excessive cell proliferation [53, 68, 214]. In order to avoid these effects, major focus has been given to the study of different delivery systems that enable spatial containment of delivered GFs, such as nanoparticles or scaffolds.

13.2.2 Growth Factor Expression Levels

A major challenge in designing GF delivery approaches is optimizing the concentration required for the desired therapeutic effect. A recent meta-analysis on the use of FGF-2 for periodontal defects reported that insufficient amounts of GF failed to promote bone regeneration [118]. In the same study, it was also shown that excessive concentrations of FGF-2 resulted in insignificant promotion of bone growth. Furthermore, excessive concentration of BMP-2 has been reported to promote apoptosis in osteoblasts, mesenchymal stem cells (MSCs) [79] and periosteal cells [92]. Similarly, excessive VEGF concentrations promote the formation of aberrant and hyper-permeable blood vessels [146]. Therefore, the optimal concentration of GF to be delivered onsite has to be evaluated for each specific context and delivery system.

In developmental and regenerative processes, the culmination of spatio-temporal control over GFs is the formation of concentration gradients. GFs are generally secreted from a focal spot, which can be a cluster of cells with a specific phenotype or the defined space of a regenerative process. Cells at different distances from the spot will be exposed to different concentrations of the GF, and specific concentration thresholds strictly

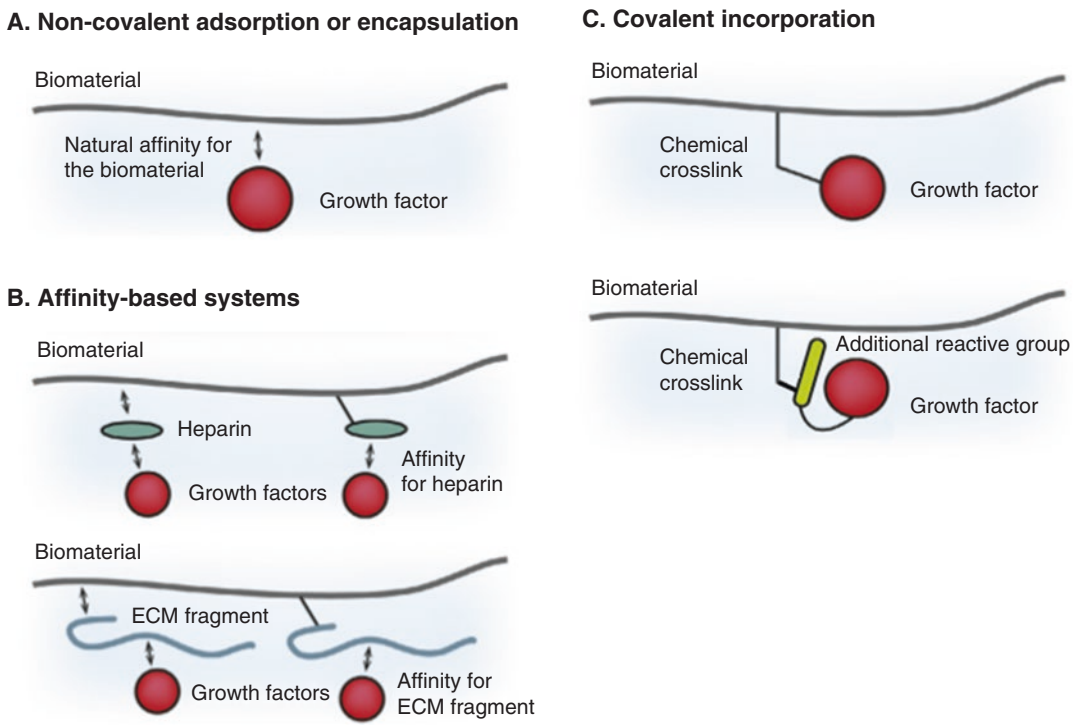

Fig. 13.2 Types of material-growth factor interaction. (**a**) Non-covalent interactions based on surface properties. (**b**) Affinity-based systems rely on natural interactions between growth factors and the extracellular matrix. (**c**) Covalent incorporation methods bind the growth to the material directly or through added functional groups or amino acids. (Adapted and modified from Ref. [134])

define spatial differentiation patterns in many stages of embryonic development [15, 154]. As individual cells are able to detect spatial differences in concentration, gradients of chemotactic GFs also represent a directional signal for cells to migrate to the wound site [6, 176] and for vascularization and innervation of tissues [78, 207]. The specific characteristics of these gradients are crucial for organized tissue formation. Thus, their adequate mimicry would be one of the pinnacles of controlled GF delivery.

13.2.3 Growth Factor Expression Patterns

The time period during which the GF is present on site is an essential parameter to achieve optimal therapeutic effects. The ordered presence and absence of specific factors corresponds to different stages of regeneration in natural processes [40, 126]. During bone regeneration, GFs that promote recruitment of MSCs and vascularization such as stromal derived factor 1 (SDF-1) and VEGF are firstly expressed. This stage is followed by the generation of a cartilaginous callus in which other GFs such as TGF-β3 are highly expressed, followed by a prolonged mineralization and remodeling phase in which expressions of TNF-α, IL-1 [126] and BMP-2 [125] are elevated. In an attempt to match these expression patterns, delaying the administration of a rhBMP-2-loaded calcium phosphate matrix for one week instead of 3 h post-surgery resulted in accelerated healing in a primate fibular osteotomy model [174]. It has also been reported that delaying the administration of an adenoviral BMP-2 vector by 5–10 days after surgery increases bone mineralization in a rat critical-size defect model [13]. It is also to be noted that the therapeutic effect of GFs is time-dependant. A 4 weeks sustained delivery of BMP-2 improves ectopic bone forma-

tion in comparison to just 5 days delivery [85], which agrees with the fact that the BMP-2 plays a major role in the long-term remodeling phase of bone regeneration [125]. These results indicate that matching specific GF expression patterns can result in improved tissue regeneration and should be taken into account in designing GF delivery systems.

Different biomaterial based systems with unique properties have been designed and employed for GF delivery in order to meet these design criteria. The following section reviews the different biomaterials and respective chemistries that have been used to deliver GFs for TERM applications.

13.3 Use of Biomaterials for Growth Factor Delivery

Low biochemical stability, short circulating half-life and rapid rate of cellular internalization are limitations of delivered GFs in TERM applications. In general, combining GFs with a biomaterial is an effective approach to overcome these drawbacks. However, no single material or strategy has yet allowed the required spatio-temporal control over the delivered GFs for optimal therapeutic effect. In recent years, the convergence of different materials, chemistries and fabrication techniques has brought the field one step closer to its goal by enabling more complex release patterns, including coordinated release of different GFs. This section will provide an overview of all these strategies, focusing on the advanced materials and procedures that enable control over the outlined design criteria.

13.3.1 Incorporation Methods

GFs can be incorporated into biomaterials through different strategies. The simplest procedure involves directly submerging a material in a GF solution to facilitate the adsorption of the GF to the material [94]. For example, GFs have been adsorbed on FDA approved polymers such as poly(lactic-co-glycolic) acid (PLGA) microspheres [44] and poly(caprolactone) (PCL) scaffolds [226]. Changes in material surface roughness [163] or the addition of nanostructured features [45] can increase the overall surface area, resulting in increased GF adsorption. Another prominent strategy that involves mixing the material with the GF in a liquid phase prior to scaffold fabrication allows the fabrication of scaffolds entrapped with GFs. Common scaffold fabrication techniques include freeze drying, phase separation, molding or *in situ* polymerization [105]. One issue in these strategies is the requirement to protect the GFs from harsh conditions during these scaffold fabrication processes in order to maintain their bioactivity. For example, melt molding can expose the GFs to high temperatures whereas radical based polymerization systems can facilitate GF oxidation/denaturation [114].

13.3.2 Interaction Between Growth Factor and Biomaterial

The interaction between GF and biomaterial plays a key role in all incorporation methods, affecting not only the release profile [94, 130], but also the biological effects of the GF [130]. The different types of material-GF interaction are summarized in Fig. 13.2.

Non-covalent interactions are weaker and can be mainly hydrogen bonds [43], Van der Waals forces, ionic forces or hydrophobic interactions [42]. Modifying the surface charges, charge density [3, 60] or available functional groups [61] of the material results in different GF binding affinities and release profiles. For example, increasing the surface hydrophobicity and decreasing the isoelectric point (pI) of PLGA microspheres resulted in an increase in the amount of rhBMP-2 adsorbed, whereas changes in molecular weight did not result in any significant change [172]. Functionalizing the material surface or the polymer chains with amino [45], alkyl [27, 45] or oxygen-terminated groups [185] can increase the adsorption of rhBMP-2 and result in a longer

Table 13.2 GF delivery vehicles fabricated using various biomaterials

Delivery vehicle or combined approach	Biomaterials	GFs
Particles	PLGA	IGF-1,VEGF, BMP-2 [31, 48, 211]
	PCL-PEG-PCL	bFGF [62, 63]
	PBCA	NGF [104]
	PEG-PLGA	bFGF [227]
	Tetronic®-PCL (Heparin)	bFGF [111, 112]
	PAMAM	EGF,VEGF [5, 197]
	Phosphatidylcholine liposomes with magnetite core	BMP-2, TGF-β1 [131, 193]
	DSPE-PEG-NHS	NGF [102, 217]
	Combined lipid SLN	NGF [103]
	Poloxamer 188/HSPC/cholesterol	bFGF [228]
	Silica (MSNs)	BMP-2,FGF [225]
	Iron oxide (SPION)	EGF, BDNF [156, 178]
	Semiconductor Qdot®	BDNF, NGF [162, 218]
Scaffolds	Collagen	BMP-2, BMP-7 [100]
	PLA	BDNF [151]
	PLGA	FGF, BMP-2 [55, 223]
	Chitosan-glycerophosphate	BMP-2, Insulin [29, 180]
	Fibrin	FGF-2, VEGF-A [230]
	PEGDA-Heparin	bFGF, TGF-β, KGF, Ang1, PDGF [153, 155]
	PEG	FGF-2, PIGF-2 [129]
	GelMA	BMP-2 [7, 170]
	PEG-PLLA-PEG	TGF-β1 [109]
	B-TCP	PDGF, GDF-5, BMP-2, hGH [65, 98, 99, 191]
	Bioglass	VEGF, BMP-2 [37, 215]
	CPC	BMP-7,VEGF [165]
	Titanium	TGF-β1, BMP-2, VEGF [168, 186, 192]
Particles incorporated in scaffolds or injectable systems	OPF	TGF-β1 [71]
	PLGA, Gelatin, PPF	BMP-2 [88]
	PLGA, PEG	CNTF, NT-2 [20]
	Gelatin, OPF	TGF-β1, IGF-1 [72, 73]
	PLA, alginate	BMP-2, VEGF [86]
	PLA, chitosan	IGF-1, BMP-2 [91]
	PHBV, chitosan	BMP-2,BMP-7 [175]
Core-shell	Gelatin, PPF	BMP-2, VEGF [89]
	PLLA,PLGA	BMP2-,FGF [209]
Layer by layer	Gelatin	BMP-2 IGF-1 [161]
	OPF	BMP-2,IGF-1 [123]
Biofabrication	GelMA	VEGF [21, 157]

release time. The interaction with the functional groups will also depend on the pI of the GF, and thus each GF will have specific release profiles when incorporated in the same material based on this approach [152].

A special case in non-covalent interactions is the use of GF-binding domains from ECM molecules. These strategies are generally classified as affinity-based, as the affinity of certain GFs for these domains is significantly higher and more specific than for single chemical groups or surface charges [136]. An example of these affinity-based domains are heparin or heparan sulphate, which have been extensively used for the delivery

of specific GFs such as NGF or BMP-2 [169, 221]. Fibronectin [128] and fibrinogen [129] GF-binding domains, which bind to several GFs from the PDGF, VEGF and FGF families and some from the TGF-β family, have also been used to functionalize scaffolds. The use of affinity-based systems has been extensively studied and reported in the literature [206], and the resulting release profile, which is significantly more sustained than for other non-covalent incorporation methods, positioned them as one of the most successful GF incorporation approaches to date. However, the strategies are limited to the release of GFs that display natural affinity for these domains, and the release profile differs between different GFs due to their distinct affinities with the system. In order to further improve the therapeutic effects, some engineered GFs containing additional ECM binding domains have been studied. Genetically engineered IGF-1 including the heparin-binding (HB) domain of HB-EGF was able to interact with specific GAGs in cartilage matrix after injection to the knee [133]. Through similar techniques, collagen-binding domains were added to NGF [188] or BDNF [66], promoting their interaction with collagen scaffolds [187] and the retention of the GF at the wound site.

The release profile for delivery systems that use non-covalent incorporation is generally characterized by an initial burst release [77]. The observed burst release profile has been suggested to have a role in early post-implantation complications [23, 199]. In order to reduce or eliminate burst release, protein immobilization to the matrix through covalent incorporation has been extensively studied [130]. It has been reported that the release of GFs conjugated to the biomaterial is then dependent on the materials' degradation profile. Moreover, it is possible to have further precise control over the GF release profile by adding features such as protease-cleavable sequences to the material [57] or to the GF-material linkage [46]. Aside from improved control over the GF release profile, presentation of covalently bound GFs to cells can result in a differentiated response in comparison to soluble GFs by inhibiting the internalization of the GF-receptor complex [80]. Covalent incorporation can also be used for patterning GF [116], including the formation of gradients in a material. Several GFs have been covalently incorporated in biomaterials for different applications, leading to improved functions such as endothelial cell proliferation [30], osteoblast adhesion to titanium implants [179], or even bone formation *in vivo* [219]. Common reactions for GF immobilization include carbodiimide coupling [121], photo-polymerization methods such as phenyl azide-based [219] or acrylate-based [116], and also click chemistry [115, 135]. One of the main limitations of these approaches is poor control over the exact reaction site of the GF, which can lead to disruption of the receptor-binding domain [130]. In order to improve the therapeutic effects of covalently incorporated GFs, some studies have engineered growth factors containing functional groups [144] or amino acids [189] at specific sites that do not overlap with the receptor-binding domain. Overall, covalent incorporation shows great potential for GF delivery as it offers higher control over the presentation and the release profile of GFs.

13.3.3 Delivery Vehicles for Growth Factor Administration

Biomaterials used for GF delivery can be fabricated into different types of vehicles, such as particles or scaffolds. Each delivery vehicle poses favourable characteristics and is adaptable to specific therapeutic strategies or administration procedures. A comprehensive list of different GF delivery vehicles is tabulated in Table 13.2 below.

13.3.3.1 Particle Systems

Particle systems, which can be in the range of <1 μm for nanoparticles or <1000 μm for microparticles, have been used to deliver GF for TERM applications [138]. The particle size affects the rate of GF release due to different surface-to-volume ratios and intracellular uptake [147].

Nanoparticles

Nanoparticles (NPs) can infiltrate deeper into tissue via capillaries and epithelial lining due to their small sizes, improving the transport properties and pharmacokinetic prolife of drugs *in vivo*. They are generally highly soluble and display low immunogenicity [107]. Targeted delivery of GFs to specific tissues can be achieved using surface functionalized NPs or using electromagnetic fields [224]. Surface functionalization can also enable NPs to cross the blood-brain barrier (BBB), which is not possible for other delivery systems without invasive procedures [51]. In general, NPs can be classified into polymeric, lipidic and inorganic depending on their composition.

Polymeric NPs can be fabricated as nanospheres, nanocapsules, micelles and dendrimers, all of which have been studied for GF delivery. PLGA is the most studied material to form nanospheres and nanocapsules, and it has been used for IGF-I [48], VEGF [31] and BMP-2 release [211]. Functionalization of PLGA nanoparticles with different concentrations of heparin has also been used to form an affinity-based system, where increasing the heparin concentration resulted in longer term release [31]. Low frequency ultrasound was combined with bFGF-loaded PLGA NPs to increase microvessel permeability for targeted skeletal muscle angiogenic therapy [26]. Apolipoprotein E (ApoE) was adsorbed to poly(butylcyanoacrylate) (PBCA) NPs in order to cross the BBB through an ApoE receptor-mediated response. NGF was adsorbed to the PBCA NP surface and then delivered to rats by intraperitoneal injection. Symptoms of scopolamine-induced amnesia were reduced after the administration, indicating targeted delivery to the brain [104]. Some polymeric NP systems have also been able to achieve long-term release: a heparin-conjugated Tetronic®-PCL micellar system was used for bFGF delivery, showing long-term delivery up to 2 months [111, 112].

Lipid based NPs that have been used for GF delivery are mostly liposomes and solid lipid nanoparticles (SLNs) [10]. Liposomes are closed vesicles formed by bilayers of hydrated phospholipids which enclose an aqueous core [35]. The main advantages of these formulations are their inherent low toxicity and scalable production methods [17]. Phosphatidylcholine liposomes loaded with magnetite particles were used for bone and cartilage regeneration after loading with BMP-2 [131] or TGF-β1 [193] respectively. Both tissues were targeted by magnetic induction [131, 193]. Despite their flexibility, liposome nanoparticles display low GF loading capacity and low stability due to enzymatic degradation, leading to a short release [224]. Other types of lipid NPs with different conformations have been used in order to overcome these issues. For example, a lecithin anionic nanolipid core was loaded with VEGF and covered by a Pluronic F-127 shell. The system showed increased stability in comparison to liposome systems, and a sustained release of VEGF for more than 30 days. The release period was extended by increasing the lecithin/Pluronic F-127 ratio, presumably due to changes in the ionic charge that enabled stronger interactions with VEGF [145]. Recently, an SLN system has been conjugated with heparin and loaded with NGF for neuronal differentiation. The release could be tuned by changing the composition of the solid core, where using stearylamine resulted in a faster release than using esterquat, and increasing the amount of cholesterol resulted in slower release [103].

Inorganic nanoparticles such as mesoporous silica NPs (MSNs), quantum dots (QDs) or metallic NPs have also been applied to GF delivery. In general terms, inorganic nanoparticles excel due to their easy handling and their physical properties. MSNs are used due to their high surface area and porosity [210]. For example, BMP-2 has been covalently grafted to the MSNs surface through an aminosaline linker, while dexamethasone was loaded in the nanopores to form a dual delivery system. The combination resulted in synergistic induction of bone formation in an *in vivo* ectopic model [229]. It has also been shown that the release kinetics can be tuned by controlling the porosity of the nanoparticles, where increased porosity leads to faster release [74], or by coating them with PEG, resulting in increased release time [14]. Magnetite NPs have been combined with other types of NPs, includ-

ing MSNs [160] and liposomes [131, 193], in order to provide them with magnetic properties that enable guided targeting using an external magnetic field. Other magnetic NPs can also be directly incorporated with GFs, such as superparamagnetic iron oxide nanoparticles (SPIONs), which enabled targeting specific areas of the brain using a magnetic field after adsorption of BDNF to their surface [156]. The main drawbacks of metal-based nanoparticles are their poor degradability and their tissue accumulation. Thus, their long-term toxicology should be further evaluated [210]. QDs have fluorescent properties that can be used to track conjugated molecules. Conjugation of BDNF [218] and NGF [162] with QDs enabled tracking of the GF after internalization by neurons [218] and PC12 cells [162], which was used to monitor its receptor internalization dynamics. In the field of bone regeneration, calcium phosphate nanoparticles have also been studied due to their high biocompatibility and bioactivity [18].

Overall, NP delivery systems represent a promising approach for GF delivery. One of the most important advantages of NP systems is the possibility of intravenous administration, which positions them as the least invasive GF delivery method. The specific properties of different NPs provide great advantages such as targeted delivery, enhanced MRI contrast or tracking of the NPs. Other systems such as MSNs can be used as a sequential delivery system, and complex NPs can be synthesized in order to combine the advantages of different nanostructured materials. On the other hand, aspects such as long-term toxicity and tissue accumulation of NPs should be further investigated before advancing to the clinical field.

Microparticles

The use of microparticles (MPs) generally results in a lower cellular uptake and tissue penetration in comparison to NPs due to their larger sizes [147]. On the other hand, their increased volume results in higher drug loading capacity, slower release and ease of production. These characteristics enable a longer-term release, which can be extended by increasing the particle size [28]. The materials used to generate MPs for GF release include naturally derived polymers such as gelatin [149, 150], alginate [122], and chitosan [164], as well as synthetic polymers such as PLGA [167]. As MPs adaptability to intravenous administration is low in comparison to NPs, most of the applications require the formation of a scaffold through microsphere fusion [143] or being incorporated in a solid scaffold [49] or an injectable hydrogel [38].

13.3.3.2 Scaffold Systems

Biomaterial scaffolds can be incorporated with GFs and implanted at the damaged area to achieve local release [114]. Scaffold systems can be classified as solid scaffolds or hydrogels depending on their composition.

Solid scaffolds are typically porous matrices fabricated by techniques such as solvent casting, gas foaming, particulate leaching, electrospinning or rapid prototyping [105]. These systems can be classified as organic or inorganic. Due to their mechanical properties and inherent tissue compatibility, inorganic scaffolds such as ceramic, bioglass or titanium play an important role in regenerative medicine [108]. Calcium phosphate-based systems excel due to their compositional similarities to the native bone ECM, and thus they have been extensively studied for GF delivery [18]. Most commonly used calcium phosphate materials include hydroxyapatite and TCP scaffolds with different porosities, which have been used for BMP-2 delivery resulting in positive effects ([65, 99, 191]. The incorporation of GFs within TCP to treat bone defects has resulted in different commercially available products. Therapeutic Goods Administration (TGA, Australia) and Health Canada have approved the safety of utilization of tricalcium phosphate (TCP) as scaffold to deliver PDGF (Augment™ Bone Graft; BioMimetic Therapeutics, Franklin, TN). Different clinical trials have concluded that PDGF-BB [2, 132, 140, 184, 195] and FGF-2 [32] loaded in β-TCP resulted in improved bone regeneration in periodontal osseous defects [118]. Clinical trials using β-TCP as scaffold to deliver GDF-5 for sinus lift augmentation in 2010 [98] and for periodontal defects in 2012 [213] also yielded posi-

tive results. Other calcium phosphates have also been studied for GF delivery. Mesoporous bioglass scaffolds have been fabricated to load VEGF, and the addition of pores resulted in more than 90% loading efficacy and extended release profile while retaining VEGF bioactivity [215]. Sumner et al employed a titanium scaffold to deliver TGF-β1 and BMP-2 in a dog humerus model, resulting in improved integration [186]. In another study, VEGF and antibacterial peptides were bound to titanium scaffolds, resulting in increased cell attachment and reduced bacterial growth [192].

Polymeric solid scaffolds have also been extensively studied for GF delivery. Homo- and copolymers of lactide and glycolide (like PLGA or PLLA) have been widely used due to their degradation into lactide and glycolide, which can enter into metabolic pathways [76]. The physical properties of these polymers can be altered by varying the ratio of lactide/glycolide, molecular weight or crystallinity [183], which directly influence the release profile of GFs. For example, PLA scaffolds have been loaded with BDNF by entrapment for spinal cord injury applications [151] while PLGA has been loaded with BMP-2 for bone regeneration [55]. Affinity-based systems have also been generated by conjugating heparin to the surface of PLGA scaffolds. FGF was incorporated in the scaffolds, resulting prolonged release and stimulation of vascularization *in vivo* [223].

Hydrogel scaffolds are one of the most successful and versatile GF delivery approaches, and the major proof of that are the commercially available products [100]. A collagen hydrogel loaded with BMP-2 (INFUSE®-BMP-2; Medtronic, Minneapolis, MN) has been approved by the FDA for treatment of degenerative disc disease. Another similar design using type I collagen matrix to encapsulate BMP-7 (OP-1™ Putty; Olympus Biotech Corporation, Hopkinton, MA) is also approved for fractures of long bones and lumbar fusion procedures. Furthermore, PDGF impregnated in a hydrogel (REGRANEX®, BioMimetic) has been approved for diabetic ulcer treatment. However, an increased rate of mortality secondary to malignancy was detected in patients treated with high amounts of REGRANEX® [53], which clearly shows the need for optimized controlled delivery systems.

Synthetic hydrogels such as poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG) are biologically inert, but have well-controlled and reproducible physical and chemical properties and no risk of disease transmission. These characteristics are of special interest for clinical translation and mass production. As an example, PEG has been crosslinked using thiol-ene chemistry [198]. This combination enabled high control over the mesh size and the degradation time, where decreased mesh size and increased degradation time led to longer-term release for up to 60 days. Naturally-derived hydrogels have higher batch-to-batch variation, but they hold the potential to interact with cells and undergo cell-mediated degradation. Most widely used naturally-derived hydrogels include fibrin, collagen, gelatin, chitosan, alginate and hyaluronic acid. As an example, tyraminated hyaluronic acid crosslinked using horseradish peroxidase (HRP) has been studied as an injectable system for protein delivery, showing increased release time by increasing the crosslinking density through changes in HRP concentration [113]. Fibrin sealants have been used for controlled release of FGF-2 and VEGF-A, enhancing blood reperfusion after myocardium infarction or limb ischemia [230].

Different strategies have been designed in order to obtain the benefits of synthetic and natural polymers in the same scaffold. In a comprehensive study, gelatin or heparin were crosslinked to PEG diacrylate (PEGDA) and the composites were used for incorporation of bFGF, TGFβ, KGF, angiopoietin-1 (Ang1) and PDGF. In general, the heparin conjugated PEGDA resulted in a longer GF release profile, which was different for each GFs due to differences in their interaction with heparin [153, 155]. In another study, the GF-binding domain of fibrin was incorporated in a PEG hydrogel. Co-delivery of FGF-2 and

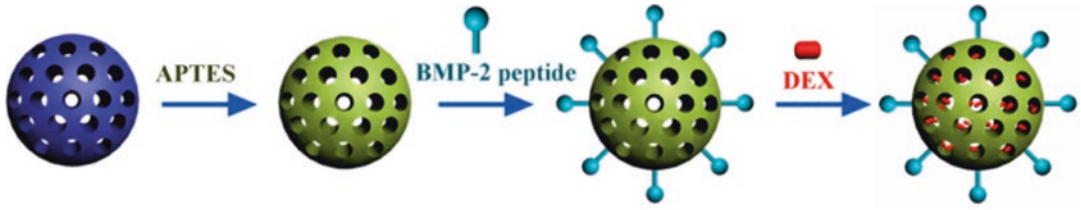

Fig. 13.3 Mesoporous silica nanoparticles were incorporated with two different bioactive components. Firstly, MSNs were functionalized with an amino group by treatment with APTES. BMP-2 was covalently linked to the amino groups through carbodiimide chemistry, and Dexamethasone was incorporated into the MSN pores by surface adsorption. (Reproduced from Ref. [229])

PlGF-2 using these gels enhanced skin wound healing [129]. On the other hand, modification of naturally-derived polymers with functional groups that enable controlled crosslinking is also a generalized strategy. These modifications enable tailoring the crosslinking density and mesh size, providing higher control over the release profile. As an example, gelatin undergoes gelation at temperatures under 35 °C. This process is not adequate for applications requiring high control over the network characteristics. Thus, gelatin functionalization with methacryloyl (GelMA) has been used for different applications that demand high control over the crosslinking density [97], including the generation of scaffolds for BMP-2 encapsulation [7, 170]. Increasing the degree of functionalization of GelMA results in decreased mesh sizes, increasing the release time of GFs such as BMP-2 [141].

Both hydrogels and solid scaffolds are the most successful platforms for GF delivery, as shown by the amount of commercially available products and clinical trials performed to date. The ability to spatially deliver GFs at the wound site by implantation of the scaffold or injection followed by *in situ* crosslinking is the most important advantage of these platforms.

13.3.3.3 Combined Approaches in GF Delivery

The combination of different materials and platforms allows several advantages in GF delivery applications. Firstly, it enables coordinated delivery of GFs by incorporating them in different materials or through different methods [9]. Secondly, combining different materials that can be independently modified increases the tailorability of the release profile.

Multiple incorporation strategies can be used in the same material in order to deliver different GFs with independent release profiles. For example, encapsulation of PDGF in PLGA microspheres, followed by surface adsorption of VEGF and generation of a scaffold by gas foaming-particulate leaching, resulted in a burst release of VEGF and a prolonged PDGF release [181]. In a different study, BMP-2 was covalently grafted to the surface of MSNs through an aminosaline linker while dexamethasone (DEX) was incorporated in the nanopores, obtaining short-term DEX release profile and a longer-term BMP-2 release profile (Fig. 13.3) [229].

MPs or NPs can be further incorporated into a scaffold (Fig. 13.4). TGF-β1 loaded gelatin particles have been immobilized in oligo poly(ethylene glycol) fumarate (OPF) and resulted in a reduction of the burst release. The release time could be further increased by increasing the molecular weight and the crosslinking time of the OPF hydrogel [71]. Encapsulation of NT-3 in PLGA MPs and inclusion of these MPs in a ciliary-neurotrophic factor (CNTF) loaded hybrid hydrogel resulted in a rapid CNTF release and a more sustained NT-3 release. Increasing the crosslinking density of the hydrogel phase resulted in increased release time from weeks to months [20]. Also, gelatin MPs encapsulated in OPF have been used for coordinated and tailorable delivery of TGF-β1 and IGF-1 with the aim of cartilage regeneration [72, 73]. Further examples include BMP-2-loaded

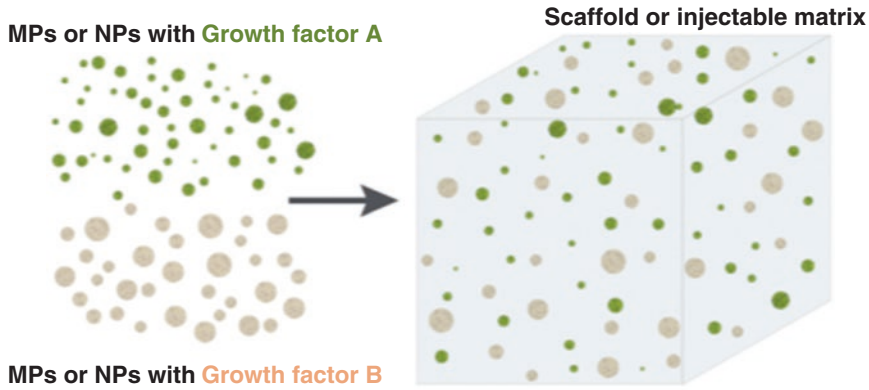

Fig. 13.4 Materials with different release characteristics are used to generate MPs or NPs, enabling high control over the release profile of one or more growth factors. These particles can be incorporated into a matrix in order to generate a scaffold or an injectable composite material. (Adapted from Ref. [9])

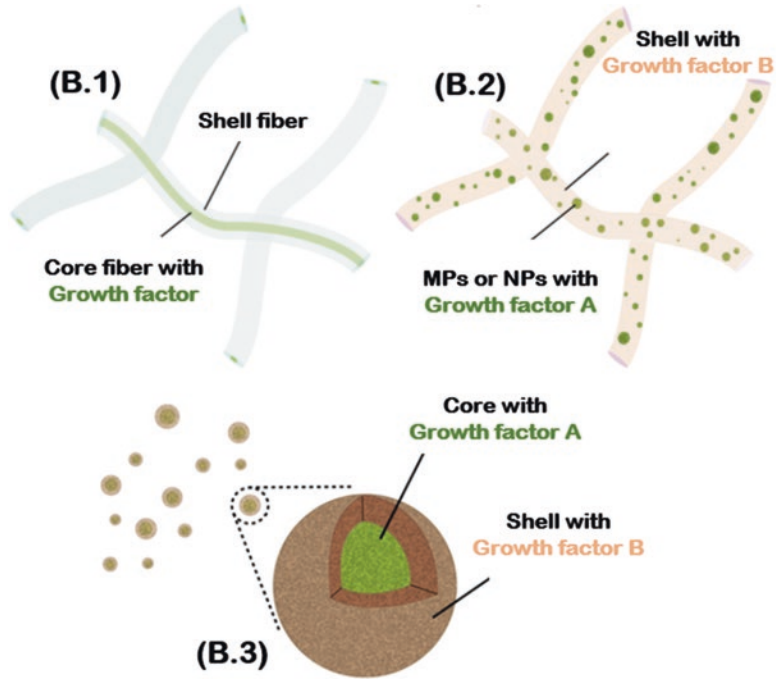

Fig. 13.5 Representation of core-shell approaches. Materials with different release characteristics are used to generate scaffolds with an internal core and an external shell, enabling high control over the release profile of one or more growth factors. Core-shell approaches can take the form of fibres, particles or scaffolds. (Adapted from Ref. [9])

PLA microspheres incorporated in VEGF-loaded alginate hydrogels [86] and IGF-1 encapsulated in gelatin microspheres loaded into chitosan scaffolds containing BMP-2 [91], both resulting in enhanced bone regeneration. Other strategies used to combine different materials for coordinated GF delivery include Layer-by-layer and core-shell approaches (Fig. 13.5 and 13.6).

In the past decade, emerging biofabrication approaches that generate complex scaffolds following a layer-by-layer automated deposition technique have also been studied for GF delivery. This automated high resolution approach offers a superior level of control over the spatial distribution of the materials in each single layer, dictating the scaffold architecture. Byambaa et al. bioprinted a scaffold with similar architectural features as bone using bioinks consisting of VEGF covalently conjugated to GelMA through carbodiimide chemistry. The GelMA-VEGF

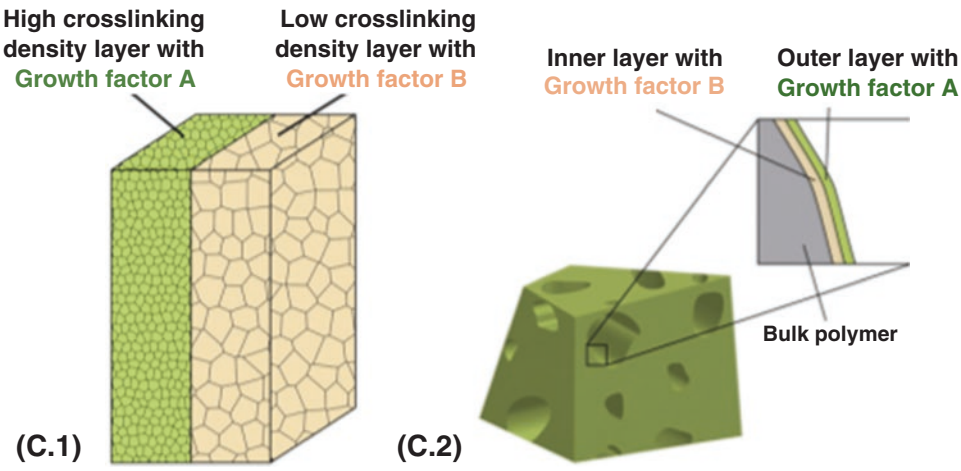

Fig. 13.6 Representation of Layer-by-layer approaches. Materials with different release characteristics are used to generate layered scaffolds or coatings, enabling higher control over the release profile of one or more growth factors. (Adapted from Ref. [9])

regions of the scaffold resulted in increased endothelial cell proliferation and tubulogenesis [21]. In a different study, VEGF-loaded GelMA MPs with different crosslinking densities were bioprinted in a Matrigel®/alginate bioink, showing increased release time by increasing the crosslinking density of GelMA. GelMA MPs with a more sustained delivery resulted in increased bioactivity in *in vitro* 3D cultures, and the presence of VEGF-releasing particles resulted in increased vascularization *in vivo* [157]. The possibilities of fine tuning the release profile expand if a coaxial systems is used for rapid prototyping [36], enabling the combination of both core-shell and biofabrication approaches. These examples showcase the potential of biofabrication to generate complex biomimetic scaffolds that include spatially- and temporally-controlled GF release systems for both tissue regeneration and *in vitro* modelling.

13.4 Conclusions and Future Perspectives

Although several GFs have been identified as signalling molecules that play important roles in developmental and regenerative processes, the use of GFs as therapeutics agents has yet made significant progress in the clinic. One major issue that still persists is the lack of suitable GF delivery systems that achieve optimal therapeutic effect while avoiding side effects. It was identified that the ideal GF delivery system should meet key design criteria such as being able to deliver the GF to a localized site, as well as mimicking the native GF expression levels and patterns during a typical tissue regenerative process.

A number of commercially available GF products exist in the market, but showed limited clinical success with potential side effects, further highlighting the need for development of more advanced delivery systems. The spatial and temporal control over the GF release profile from these delivery systems is highly desired. Various biomaterials, incorporation methods and fabrication techniques have been developed and employed for GF delivery. Although these delivery platforms often pose desirable characteristics, they are usually only adapted to the release of one specific GF. In the native regenerative microenvironment, several GFs work concurrently with different expression levels and profiles, synergistically facilitating the desired cellular behaviour. With our current understanding of this basic biological phenomena and the limitations of the current GFs delivery vehicles, it

is recommended that the field moves forward with combinatorial approaches that enable orchestrated release of multiple GFs.

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New Combination/Application of Polymer-Based Nanoparticles for Biomedical Engineering

14

Ray Chang, Peng-Yuan Wang, and Ching-Li Tseng

Abstract

Polymer-based nanoparticles (PNPs) are attractive in part due to their ultra-small size, versatility and target specificity. Therefore, PNPs have been increasingly used in a variety of biomedical applications including diagnoses and therapeutic treatment. In this chapter, we focus on the recent studies (within 5 years) with some new ideas/agent's application in biomedical field and roughly divide applications of PNPs into four categories: (1) Delivery, (2) *In vivo* imaging, (3) Therapies, and (4) Other applications. First, we introduce how PNPs can enhance the treatment and delivery efficiency of therapeutic agent. Second, how PNPs can be used to help *in vivo* imaging system for disease tracking and monitor. Then, we reveal some novel PNPs which is able to function as an agent in photodynamic, photothermal, sonodynamic and neuron capture therapy. Furthermore, we also mention some interesting applications of PNPs for biomedical field in individual form or cluster employment, such as immunoswitch particles, surface fabrication. Finally, the challenges and future development of PNPs are also discussed. In delivery section, we focus on how polymer "can be used" as vehicles in delivery application. But, in the section of imaging and therapies, we carried on how polymer as an "adjuvant" for functional enhancement. The biodegradable property of PNPs is the feature that they can be controllable for itself degradation and drug release as a chief actor. Besides, in imaging and therapies application, PNPs can be the support role for helping contrast agent or photo/sonosensitizer to enlarge their imaging or therapeutic effect.

Keywords

Polymer-based nanoparticles (PNPs) · Delivery · Imaging · Therapeutics · PNPs cluster

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14.1 Introduction

During the past decades, the development of nanotechnology makes the generation process of polymer-based nanoparticles (PNPs) more efficient. In recent years, PNPs have been increasingly used in a variety of biomedical applications including diagnoses and therapeutic treatment.

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Nanoparticles were first developed by Speiser and co-workers [1] around 1970 and are frequently defined as particles with diameters below the micron dimension (i.e., in the range 10–1000 nm) [2]. The concept of nanoscale devices has led to the development of nanoparticles. Two types of systems with different inner structures: (1) a reservoir-type system, consisting of an oily core surrounded by a polymer wall, defined here as a nanocapsule; (2) a matrix-type system composed of an entanglement of polymer units, defined here as a nanoparticle or nanosphere. The term nanoparticle can be used to refer to both systems including nanocapsules as well as nanoparticles.

Due to their ultra-small size, the PNPs have much larger surface area to volume ratio compared with bigger particles, enabling them to have unique biological behaviors. Their large functional surface area allow PNPs to interact with substances such as nucleic acids, proteins, lipids, ions, carbohydrates, probes or small molecule drugs and deliver these substances into the desired biophase via endocytosis. Furthermore, PNPs can be fabricated by different method. Their properties, such as size, shape, stability and particle composition are tunable, allowing them can be prepared to meet the requirements specific to biomedical application. For example, several pathways were involved in the endocytosis procedure of PNPs. This cellular uptake procedure can be controlled effectively by the properties of nanoparticles, such as nanoparticle size, shape, and surface chemistry [3–5]. After entering the target cell, PNPs can be made to respond to a different local stimulus, which will trigger PNPs to execute therapeutic functions, such as hyperthermia, imaging and drug release. Theses stimulus not only include biological endogenous signals like pH, glucose, enzyme, etc., but include external stimulus like alternating magnetic field (AMF), near infrared (NIR), temperature, and etc. [6, 7]. Due to their versatility and target specificity, PNPs have received much of the attention in various aspects of medicine. There are several excellent reviews on PNPs have been published, like their fabrication, characterization and strategies in the application [8–10].

In this article, we focus on the recent literature (past 5 years) and roughly divide the biomedical application of PNPs into four categories: (1) Delivery, (2) *In vivo* imaging, (3) Therapies, (4) Other applications, and summarized in Table 14.1.

Table 14.1 Summary of current PNPs in biomedical application

Application	Indication	References
Delivery		
Dendrimers	Dendrimer with instantaneous size switching ability for tumor penetration	Li et al. [11]
Lipid Materials	PNPs as a delivery platform for CRISPR/Cas9 system	Yin et al. [12]
Polymeric materials	Combination of PNPs with CAR T-cell therapy	Smith et al. [13]
	Preventing obesity by using PNPs	Xue et al. [14]
***In vivo* imaging**		
Magnetic	pH-activatable PNPs for non-invasive MRI of tumor	Mi et al. [15]
Nuclear	Quantification of the inflammation in atherosclerotic plaque using PET signal	Keliher et al. [16]
Optical	Finding a relationship between nanoparticle shapes and the ability of nanoparticle access into the nucleus	Hinde et al. [17]
	Examining the radiation effect for tumoral therapy	Miller et al. [18]
	SERS for high-precision cancer imaging	Harmsen et al. [19]
	Detection of endogenous H_2S within living cells via FRET	Zhao et al. [20]
	Semiconducting polymer based PNPs as probes for photoacoustic imaging	Pu et al. [21]
Therapies		
PDT	Performing PDT for deep tissue penetration	Punjabi et al. [22]
PTT	Combination of PTT and checkpoint-blockade immunotherapy to eliminate primary tumors	Chen et al. [23]
SDT	Combination of SDT and MRI for SDT monitoring	Huang et al. [24]
NCT	Incorporating Gd for MRI-guided gadolinium NCT	Mi et al. [25]

(continued)

Table 14.1 (continued)

Application	Indication	References
Other applications		
Individual application	Separation the protein biomarkers	Nehilla et al. [101]
	Simulates the immune system to inhibit tumor growth	Kosmides et al. [102]
	PNPs for neutralization of venomous biomacromolecules	O'Brien et al. [104]
	PNPs as a protein affinity reagent for inhibition of angiogenesis	Koide et al. [105]
	Predict the therapeutic efficiency of anticancer drug in a personalized manner	Yaari et al. [106]
	Identification the ideal targeted therapeutics by DNA barcoded PNPs	Dahlman et al. [107]
Cluster employment	Self-assembled nanoparticle monolayers as a cell culture tool	Wang et al. [113, 114]

Abbreviations: MRI, magnetic resonance imaging; PET, positron emission tomography; SERS, surface-enhanced Raman spectroscopy; FRET, fluorescence resonance energy transfer; PDT, photodynamic therapy; PTT, photothermal therapy; SDT, sonodynamic therapy; NCT, neutron capture therapy; Gd, gadolinium

14.2 Delivery

The ultra-small size of PNPs lead it enter cells more easily via endocytosis. Therefore, the application of PNPs in drug delivery, especially for cancer treatment, is particularly welcome. In order to grow quickly, tumor tissues tend to possess higher vascular permeability rather than normal tissues. Rapid vascularization to serve fast-growing cancerous tissue leads vessels to a leaky and defective architecture. The enhanced permeability and retention phenomenon (EPR) is based on two factors: (a) the capillary endothelium in malignant tissue is more disorderly and thus more permeable towards macromolecules than the capillary endothelium in normal tissues. This allows extravasation of circulating PNPs within the tumor interstitium, and (b) the lack of tumor lymphatic drainage in the tumor bed results in particles' accumulation. Thus, the accumulation of PNPs in tumor tissue is much higher than normal tissues. PEGylation, coating the surface of nanocarriers with poly(ethylene glycol) (PEG), is a commonly used strategy to prolong circulation time of PNPs [26]. The EPR effect provide the PEGylation PNPs with increased opportunity to access tumors site. Due to the EPR effect, PNPs have been widely used as a vehicle to deliver the therapeutic agent, such as drug, nucleotides and protein, into tumor tissue.

Many recent studies, however, have described the limitations of EPR effect. The EPR effect is not suitable for metastatic liver cancers and less vascularized cancers, such as prostate cancer [27]. On the other hand, it has been reported that a phenomenon known as "accelerated blood clearance (ABC)" which repeated injections of PEGylated nanocarriers may cause of an unexpected immunogenic response, leading to lose their long-circulating characteristic [28]. Therefore, it is necessary to develop actively-targeted PNPs to specific recognize the specific cells.

To perform active targeting, specific ligands able to interact with the specific receptors localized on cell membranes can be coupled to the surface of PNPs. Several studies have summarized the various kind of ligand, such as small molecules, carbohydrates, peptides, enzymes or antibodies, have been used for active targeting [29–31]. In addition to ligation strategies, recently, a novel method called "cell membrane coating" has been developed for designing of PNPs. Cell membrane coating method is a technique, which the whole membrane will translocate from a cell to the surface of a nanoparticle, make the nanoparticle potentially perform some cell-specific functionalities, such as, immune evasion and targeting abilities. Zhang et al. [32, 33] have used this technique to address the issue of ABC phenomenon and improve PNPs functionality to achieve longer circulation time, higher tumor specificity and lower exocytosis of drug (Fig. 14.1) [34, 35]. The PNPs can be roughly classified nto three types; dendrimer, lipid based PNPs and biopolymeric based PNPs.

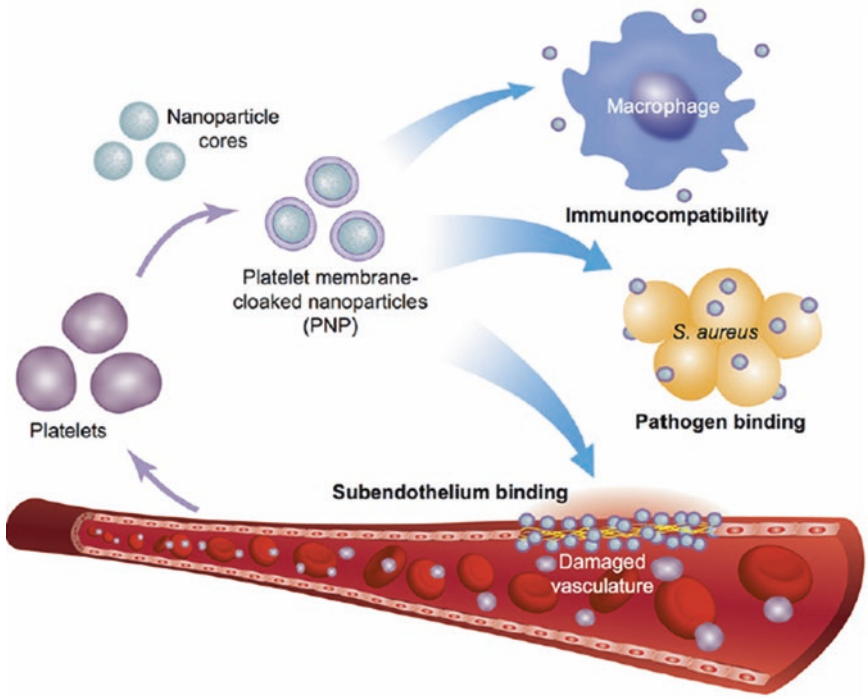

Fig. 14.1 Schematic preparation of PNPs. poly(lactic-co-glycolic acid) (PLGA) nanoparticles are enclosed entirely in plasma membrane derived from human platelets. The resulting particles possess platelet mimicking properties for immunocompatibility, subendothelium binding, and pathogen adhesion. (Image adapted from Zhang et al. [33] and reprinted with permission from Springer Nature)

14.2.1 Dendrimers

Dendrimers, a large number of branching points, including precisely-defined molecular structure, numerous functional groups on the surface and cavities in the interior, have been widely used in the field of drug delivery in a three-dimensional spheral shape, nanometric size and well monodispersity [36]. With appropriate branching units and surface groups modification, dendrimers can provide the targeted delivery of gene or drugs. Kesharwani et al. [37, 38] reviewed the recent advancements in dendrimer-based PNPs for tumor-targeted delivery. They summerized the different kinds of ligands, including the biotin, folate, amino acids, peptides, aptamers and antibodies, which have been successfully conjugated to dendrimer [37]. Also, they have reviewd the different routes, such as parenteral, transdermal, oral, plumonary, ocular and colon, can delivery the various dendrimers [38]. In the following, several publications within these years will be discussed.

Dendrimers can be used for gene delivery. For example, Cheng et al. [39] systematically summarized the functional ligands can modifided to the dendrimers to improve the DNA- and membrane-binding affinity, transfection efficacy and biocompatibility of dendrimers. Also, dendrimers can be applied in drug delivery, including doxorubicin, 5-Fluorouracil, paclitaxel and other types of chemotherapy drugs. [40]. For example, Li et al. [11] preapred the size switching PNPs construcring from assembly of platinum-prodrug conjugated polyamidoamine dendrimers. At neutral pH, this PNPs have initial size of ~80 nm. Under acidic tumor microenvironment, however, the PNPs rapidly dissociate into the dendrimer building blocks and penerate into tumor cell. Wei et al. [41] synthesize the amphiphilic dendrimer, which can form the supramolecular micelles to enclose the anticancer drug doxorubicin with high loading

capacity. Not only for gene transfection and chemodrug delivery, dendrimeric nanoparticles also have advantages for ocular drug delery: nanoparticle platform of hybrid dendrimer hydrogel/poly (lactic-co-glycolic acid) with anti-glaucoma drug loading was evaluated. The hybrid dendrimer nanoparticle platform (HDNP) consists of three domains: the polyamidoamine (PAMAM) dendrimer core to encapsulate hydrophobic drugs, the poly (lactic-co-glycolic acid) (PLGA) nanoparticles to deliver either hydrophobic or hydrophilic drugs, and the PEG network to load hydrophilic drugs. The major advance of this novel dendrimer is its ability to deliver simultaneously multiple drugs in the same dosages and release them in a slow manner with sustained efficacy [42]. These platforms are very promising tools in ocular nanomedicine.

14.2.2 Lipid Based PNPs

Lipid based PNPs are delivery system mainly prepared from natural and/or synthetic phospho- and sphingo-lipids. Because they are absorbed more easily in human body and producing fewer toxic degradation products, therefore, lipid materials based PNPs are more suitable for clinical trial rather than other delivery system. In addition, some of them, particularly liposomes, due to their bilayer structure, can enclose and tranport the both hydrophobic and hydrophilic drugs. Several researchers have comprehensive review the current state of lipid based PNPs, including development process of them, strategies of them for tumor targeting, and commerical applications of them [43–45]. Zhang et al. [46] reported that upconversion nanoparticles encapsulated Azobenzene (Azo) liposome (UCNP@Azo-Lipo) could convert near infrared (NIR) light into the UV/vis region; then UV/vis light was absorbed by the Azo molecules in the liposome. When stimulated by UV/vis light, the synthesized Azo amphiphilic derivatives created continuous rotation-inversion movement for the liposome membrane then driving drug released. The results show that they can precisely control the drug release amount via tuning the intensity and duration of light irradiation. The combination of NIR liger trigger system and liposome leads an on-off switch controllable liposome was created. Recently, lipid based PNPs and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) systems have been combined in a new method to increase genome editing efficiencies. Yin et al. [12] use the lipid based PNPs to deliver the sgRNAs (e-sgRNA) and mRNA encoding Cas9, which can significantly decrease the specific genes such as Pcsk9 in liver (>80% editing), and serum to undetectable levels by a single intravenous injection in mice. These lipid-Cas9 based PNPs provide a non-viral genome editing vehicles for liver genome correcting in clinical setting. The lipid based PNPs, called the liposome protamine/DNA lipoplex (LPD), was electrostatically assembled from cationic liposomes and an anionic protamine-DNA complex, it promoted efficient delivery of the retinal pigment epithelium protein 65 (Rpe65) gene in mice in a long-term expression and cell specific-mode leading to *in vivo* correction of blindness [47]. The efficacy of this method of restoring vision is comparable to AAV and lentiviral gene transfer of the Rpe65 gene to Rpe65 knockout mice [48].

14.2.3 Biopolymeric Based PNPs

Biopolymeric based PNPs, compare to other PNPs, have better storage stability and diversity of designs [49]. They can roughly divide into nanocapsules and nanospheres, based on their structure. Both of them have spherical structure comprising polymer, however, the cargo is dispersed within a matrix for nanoshpere and encapsulated in central cavity for nanocapsule, respectively [50]. Nanogels are common nanosphere for delivery. Li et al. [8] summarize the method to fabricate the nanogels, based on natural and synthetic polymers, for drug and nucleic acid controlled release. Gaitzsch et al. [51] systematically review papers about how to prepare the "smart" nanocapsules for delivery system based on different techniques like self-assembly, emulsion polymerization, microfluidics, or Pickering emulsion, etc. Therefore, due to their diversity of designs, various release strategies,

including heat, light, ultrasound stimulation; pH change, oxidation process, or enzyme participation, these ways have been employed to improve the efficiency of biopolymeric based PNPs to sustained drug release within the target site [52]. In the following, we introduce some newly publications within these years.

Satchi-Fainaro et al. [53] designed a biodegradable amphiphilic polyglutamate amine PNPs (APA) PNPs that can deliver the small interfering RNA (siRNA) and microRNA (miRNA) into the tumor. After arrive the tumor site, PNPs will be degraded by an enzyme highly expressed in tumor tissues leading to release the siRNA or miRNA to silence or dysregulate the gene associated with cancer, respectively. PNPs can be used to enhance the efficiency of chimeric antigen receptor T-cell immune therapy (CAR-T). Stephan et al. [13] prepare the PNPs, based on poly(beta-amino ester) (PBAE) polymer functionalized with the microtubule-associated-nuclear localization (MTAS-NLS) peptide, can transport the leukaemia-targeting CAR genes into T-cell nuclei to rapidly program T cells with abilities of tumour-recognizing, resulting in simplifying the time consuming traditional method. Also, PNPs can be used to address obesity. Langer et al. [14] demonstrate the PNPs self-assembled from biodegradable triblock polymer composed of adipose vasculature targeted peptides conjugated poly(lactic-coglycolic acid)-b-poly(ethylene glycol) (PLGA-b-PEG) can increase the accumulation of drugs in white adipose tissue (WAT), which accelerate the transformation of WAT into brown adipose tissue (BAT) and then cause the weight loss.

Although novel polymeric based NPs for drug/gene delivery are designed/developed for biomedical application popularly; traditional biopolymer such as chitosan or gelatin also plays an important role in drug delivery system. For example, gelatin, the biodegradable polymer, exhibits excellent biocompatibility, plasticity, and adhesiveness [54]. Its degradation rate can be regulated by the degree of cross-linking. The functional groups on gelatin NPs, such as carboxyl, hydroxyl, and amino groups, are available for conjugation with ligands to bring about surface modifications. Variant gelatin nanoparticles (GPs) were synthesized by Tseng et al. [55–57] for different application such as inhalation delivery of cisplatin loaded GPs with EGF modification for lung cancer treatment [55]; or as an efficient and safe drug carriers for ocular drug delivery in an eye-drops formula [56]; and even be a gene delivery carriers with pEGFP-C1 loading (plasmid encoded enhanced green fluorescence protein) for transgene sic chicken manipulation [57].

Nonmatter novel one or old one, biopolymeric based PNPs is the major components as delivery system for drug/gene. Beyond this, another role such as a protector for contrast agent for imaging or other therapeutic chemical application, PNPs can also participate as a supporting role for reaching final clinical requirements. These are adders thereafter.

14.3 *In vivo* Imaging

Medical imaging techniques are powerful tool allowing researchers to look inside a cell or to find difference between the normal and abnormal biological processes. However, most imaging agents, such as organic molecules and inorganic nanomaterials, do not have ability to recognize and target specific cells hindering their applications to cellular studies and biological imaging. To deal with this problem, PNPs have been introduced into the biomedical imaging field as a helper.

Currently, most studies using PNPs for molecular imaging focus on designing of nanoparticles with a structure of combination of imaging agent core and polymer shell. This designing has some advantages. First, it can decrease the cytotoxicity of imaging agent. Second, various targeting ligands can be modified to the polymer shell allowing imaging agent can active bind to cellular target moieties. In addition, it will promote imaging agent accumulation in the tumor, increasing the signal-to-noise ratio, highlighting tumor tissue within the body. Third, compare to imaging agent alone, polymer shell can make it with larger size, allowing them to have longer circulation time, decreasing in the time to agent's administration. Zheng et al. [58] discuss the clearance pathways and tumor targeting of imaging nanoparticles, including ultra-small inorganic core modified

with protein-adsorption-resistant zwitterionic or PEGylated surface. Lammers et al. [59] summarize the advantages and limitations of imaging modalities, such as such as magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT) and optical imaging. Also, they provide principles for the rational use of PNPs in noninvasive imaging. Smith et al. [60] divide the imaginable nanomaterials based on the fundamental physicochemical properties. In addition, carrier, such as micelle, liposome, dendrimer and polymeric materials, for delivery of these nanomaterials are discussed.

In this section, we discuss the recent applications of PNPs in bioimaging. Based on imaging modalities, we have roughly divided imaging into three parts: magnetic, nuclear, and optical. The research we discussed here will mainly focus on how polymers are utilized to coat on the inorganic nanoparticles for *in vivo* distribution/biocompatibility. However, several studies, which utilize as polymers, like conjugated polymer, as co-component of imaging agents, will also be examined.

14.3.1 Magnetic

Magnetic resonance imaging (MRI), rather than use of damaging radiation, is based on the tiny magnetic moments produced by the spin of certain atomic nuclei within the body. MRI is a valuable technique for the clinical diagnosis that producing the non-invasive three-dimensional detailed anatomical images of soft tissues. Because soft tissue is present all over the human body, therefore, in medical diagnosis, MRI has a wide range of applications, such as detection of peripheral neuropathy, systemic cancer and cardiovascular disease. Gadolinium (Gd) and iron oxide based nanoparticles is common MRI contrast agent [61]. Recently, MRI agent made by Gd hexanedione nanoparticle has been developed to label and track the stem cell with low toxicity but higher image enhancement capacity [62].

Zhang et al. [63] describes the way to improve the biocompatibility and reflexivity of Gd-based contrast agents by conjugation of natural or synthetic polymers to it. Bakhtiary et al. [64] summarize the work of using PNPs with super paramagnetic iron oxide nanoparticles (SPION)-based contrast agents to early detect and image different major cancer types, including liver, prostate, brain, breast and cervical.

In addition to Gd and SPION, manganese (Mn)-based materials have been used for MRI contrast agent in recent years. Mi et al. [15] prepared pH-activatable PNPs for non-invasive imaging of tumor. They confined Mn^{2+} within calcium phosphate (CaP) core enveloping by a PEG shell, which can avoid aggregation of core. After arrive the tumor, the acidic tumor environment make pH-sensitive CaP core to release the Mn^{2+} ion, which bind to proteins increasing the MRI contrast (Fig. 14.2).

14.3.2 Nuclear

Nuclear imaging is a technique using radioactive agents to observe metabolic processes in the body, which can be used to diagnose abnormalities in the bodily functions, especially effective in identification of cancerous tumors. The most common used nuclear imaging techniques are positron emission tomography (PET) and single photon emission computed tomography (SPECT). Both of them can provide accurate three-dimensional images, but the main difference between their imaging is the type of radioactive agents used. The decay of the radioactive agents used with PET measure positron emission, whereas the SPECT detect gamma ray emission. Radioactive agents can be used for imaging have been discussed earlier [60, 65, 66].

However, some clinical radioactive agents, such as ^{18}F-fluorodeoxyglucose, do not have the ability to conduct themselves to target site. Moreover, due to low molecular weight, their circulation time is short. Therefore, to overcome these problems, several studies have incorporated the radioactive agents into PNPs [67]. For example, Phillips et al. [68] prepared the silica particles labeling with ^{124}I for PET and coating the nanoparticle with cRGDY peptide and PEG to improve the accuracy of metastatic melanoma diagnostic. Chen et al. [69] encapsulate vana-

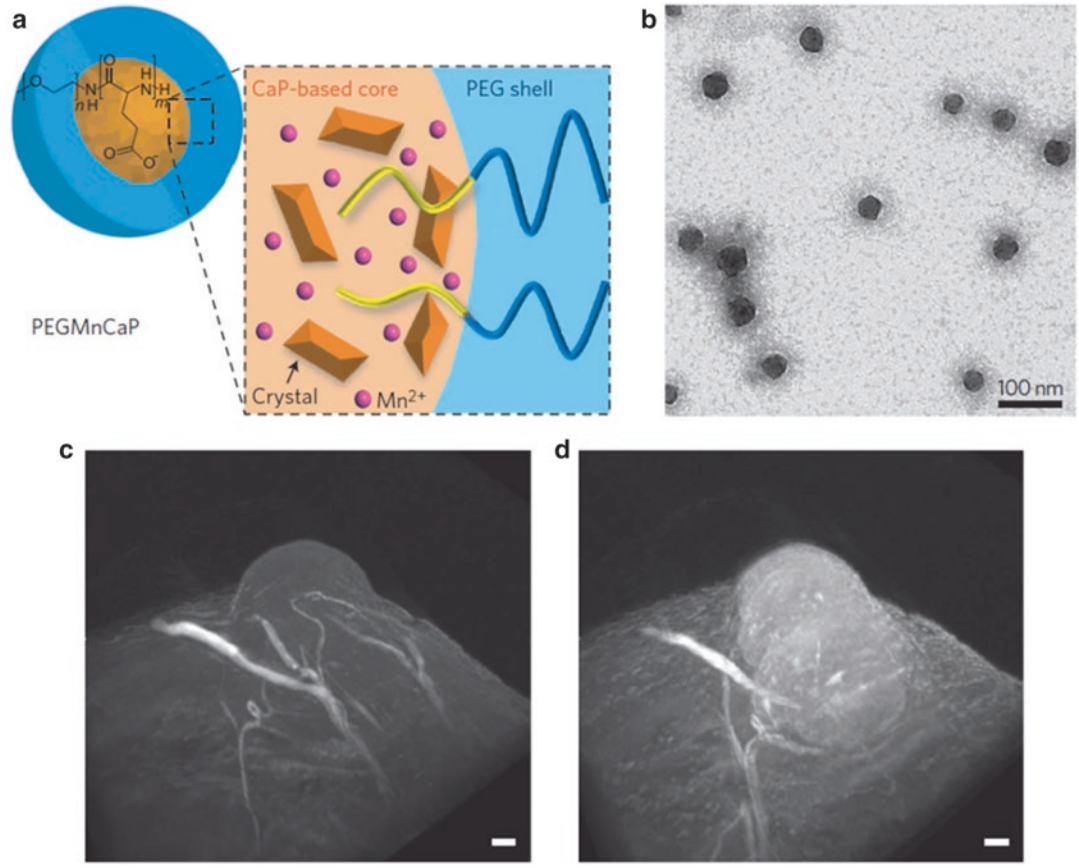

Fig. 14.2 (**a**) Illustration of the PEGMnCaP structure. (**b**) TEM image of PEGMnCaP. (**c** and **d**) 3D MRI of C26 tumors before (**c**) and 1 h after (**d**) the intravenous injection of PEGMnCaP measured with 7T MRI. Scale bars, 50 μm. (Image adapted from Kataoka et al. [15] and reprinted with permission from Springer Nature)

dium disulfide (VS_2) nanodots, a MRI agent, labeling with $^{99m}Tc^{4+}$, a SPECT agent, inside PEG modified lipid micelle leading to fabricate the VS_2@lipid-PEG PNPs. In addition to MRI and SPECT, the high NIR absorbance of VS_2@lipid-PEG PNPs provides the PNPs with strong photoacoustic (PA) contrasts, resulting in multiple imaging functions. Keliher et al. [16] synthesize the ^{18}F labeled polyglucose nanoparticles with size around 5 nm, can be excreted out of kidney and used to quantify the inflammation in atherosclerotic plaque using PET signal. Combination of PNPs with variant nuclear signaling agent enhances its image quality in cancer diagnosis and expands its application in other filed such as atherosclerotic detection.

14.3.3 Optical

Optical imaging technique is typically faster and cheaper than magnetic and nuclear imaging. In addition, it can be used in high-throughput analysis in numerous cells which's interested Though the limited penetration deep of optical light hinders its clinical deployment, optical imaging can achieve highest spatial resolution in all of imaging modalities [60]. Therefore, currently, optical imaging has been widely used to observe molecular processes or detailed features of physiologic structures.

The most commonly used optical imaging agents are inorganic nanomaterials, such as semiconductors, metals and metal oxides. To provide

these inorganic agents biocompatible and enhance the solubility/suspension in buffer media, several strategies have been studied to functionalize the surface of them with various amphiphilic block copolymers and to specific interact with target biomolecules [70].

In addition to inorganic nanomaterials, organic nanomaterials, such as conjugated polymer and fluorescent dye, can be also applied to bioimaging. Unlike other imaging modality, the unique opto-electronic properties of conjugated polymer can provide PNPs with intrinsic imaging ability without incorporation of imaging agent. Therefore, conjugated PNPs have widely been studied both *in vitro* and *in vivo* imaging [71].

On the other hand, current developments in the organic dyes design provide the new strategy in tuning the imaging properties of PNPs. Traditionally, the way to achieve high brightness in dye loaded PNPs depend on confining a large amount of dyes within the PNPs matrix. However, the aromatic structure of dye prefer to aggregate leading to fluorescence quenching. Recently, several studies have shown that PNPs can be used to increase the quantum yields of certain organic dyes, called aggregation-induced emission dyes (AIE dyes), by restricting the intramolecular rotation of these dyes within PNPs [72]. Klymchenko et al. [73] have reported that the brightness enhancement of AIE dyes-based PNPs are higher than quantum dots (QDs) for a comparable size, and have nearly reaching level of the brightest conjugated polymer-based nanoparticles.

Due to improve the brightness, biocompatibility, and selective targeting capability of these optical imaging agents, the development of PNPs for biomedical imaging have drawn attention [74, 75]. For example, Kim et al. [76] used the PEGylated Cornell dots (C dots) to observe the difference of propagation from cell to cell between the ferroptosis and other types of death. Hinde et al. [17] synthesize the fluorescein-labelled PNPs, based on poly(oligoethylene glycol methacrylate)- block-poly(styrene-co-vinylbenzaldehyde) P(OEGMA)-b-P (ST-co-PVBA) block copolymer, with different shapes but identical surface chemistries to understand how nanoparticle shapes will affect itself to access into the nucleus. Weissleder et al. [18] used the fluorescein-labelled dextran coated nanoparticles to examine the relationship between radiation effects and tumoral therapeutic nanoparticles concentration. Moreover, recently, several techniques have been used to enhance the accuracy and efficiency of imaging, including the surface-enhanced Raman spectroscopy (SERS) [19, 77], fluorescence resonance energy transfer (FRET) [20, 78], and photoacoustic effect [21, 79]. AIE dye, SERS or FRET etc. all these methodologies are developed for getting high image quality from optical signals. Combination of PNPs provides a way to enhance signal or improve biocompatibility of dye/metal/metal oxides contents to reach the goal.

14.4 Therapies

Incorporation of metal nanoparticles or organic molecule into PNPs not only can ensure a precise assessment of imaging for biomedical purposes, but also use in treating cancer, such as inhibition of tumor promotion or induction of cell death. Phototherapies based on nanotechnologies have attracted tremendous attention for their potential in biomedical application. Systematic classification of nanomaterials for phototherapies has been studied by Liu et al. [80]. Also, they review the different types of PNPs to deliver these nanomaterials to cancer cells. Zheng et al. [81] discuss about the photophysical relaxation pathways of organic molecules for phototheranostic techniques including the radiative emission, intersystem crossing/triplet-state relaxation and vibrational relaxation.

To monitor the treatment efficiency of therapy, most of researches will combine therapeutic PNPs with imaging agents enhancing the ability to visualize the targeted tissue. Elsabahy et al. [82] review the way to use PNPs to overcome challenges in imaging and therapy. The principle to design PNPs, such as components, type of structure and cross-linking, for delivery of diagnostic and therapeutic agents will be discussed.

In the following, we will focus on the application of PNPs in photodynamic therapy (PDT) and photothermal therapy (PTT). In addition, we will also discuss the sonodynamic therapy (SDT) and neutron capture therapy (NCT), the promising strategies combining PNPs with low-intensity ultrasound or epithermal neutrons.

14.4.1 Photodynamic Therapy (PDT)

A promising biomedical application of PNPs based therapy is photodynamic therapy (PDT). PDT kill the cells via singlet oxygen or reactive oxygen species (ROS) generated from light-activated chemical called a photosensitizer (PS). PS can transfer the absorbed light energy to either oxygen molecules to produce singlet oxygen or to surrounding molecules to form free radicals, leading to generation of different radical oxidizing agents, such as superoxide, hydrogen peroxide, and the hydroxyl radical [83]. Though, singlet oxygen only causes a destruction in nanometers, they will lead to activation of significant and complex cascade, resulting in local, regional, and systemic alteration of both cell and immune response [84]. Therefore, PDT has been extensively studied in the treatment of various disease, especially in cancers [10].

The development of PS has been comprehensive review by Zhang et al. [85]. Many photosensitizers used in PDT for cancer treatment are based on the tetrapyrrole backbone, a hydrophobic structure which is only slightly soluble and exhibits certain tendency to aggregate in aqueous resulting in a low therapeutic efficiency [86]. The combination of PS with PNPs not only decrease cytotoxicity of PS, but improve the solubility and stability of PS in water, which increase tumor accumulation of PS, which enhance the effect of PDT. Thus, many studies focused on design/synthesis different PNPs for effectively transport PS into specific tumor regions and then conduct the PDT by external light irradiation [80, 85]. Near-Infra red (NIR), due to relatively low, compared to visible light, absorption coefficient resulting the deep penetration in tissue, has been widely used in PDT treatment [87].

To increase the depth of PDT, recently, PS combined with upconversion nanoparticles (UCNPs) has attracted great attention. UCPNs is a materials converting low energy NIR light into high energy UV/visible light, which can activate the PS creating a photodynamic reaction [88]. Punjabi et al. [22] prepared the PNPs containing the PS (aminolevulinic acid) to convert it into protoporphyrin IX in the UCNPs treated by a biocompatible laser, which perform PDT for deep tissue penetration (>1.2 cm) than others with <1.0 cm depth).

To further penetrate into the deeper layer, scintillating nanoparticles (SCNP) have emerged as promising candidates for PDT application. After exposure to ionizing radiation, SCNP absorb the radiation and emit energy as visible light, which can trigger the PDT more efficiency. Cai et al. [89] summarized recent developments of SCNP. They also discuss about the strategies to loading SCNP, including the way to wrap it with polymer. Although the incorporation of SCNP within PNPs can overcome the penetration limit, the application of scintillating nanoparticles is still in infant stage. The relevant techniques for this strategy need to find ways to improve further.

14.4.2 Photothermal Therapy (PTT)

Another type for phototherapy is photothermal therapy (PTT). Different from PDT killing the cell by ROS, PTT use the heat to treat tumors. After light irradiation, the PTT agents will produce the photothermal effects, which can transform the energy of light into local heat. The heat not only can be used to cause thermal ablation of tumor cell, leading to cell membrane disruption and protein denaturation, but also can be helpful to address some of the limitations of nanodelivery systems, such as endosomal escape or cargo release. Therefore, to improve the therapeutic efficiency, PTT therapy has been combined with other therapeutic approaches. Kim et al. [90] revealed synergistic therapeutic systems combining gene and PTT ablation, they focus on how PTT effect enhanced cellular uptake, facilitate endosomal escape and induce

gene release for transfection. Zou et al. [91] showed when PTT combined with PNPs, it can be used alone or synergize with the imaging, radiotherapy, chemotherapy and immunotherapy to improve the efficiency of cancer treatment. For example, El-Sayed et al. [92] prepared the gold nanoparticles containing PNPs that can induce PPT cell death and molecular changes of single cell through real-time surface enhanced Raman spectroscopy (SERS). Chen et al. [23] design the PNPs, which combine the PTT with checkpoint-blockade immunotherapy, could eliminate primary tumors and inhibit metastases.

Different types of agents for PTT, including inorganic and organic nanomaterials, have been reported in various works [80, 93]. Inorganic nonmaterial, such as metallic nanomaterials, carbon nanostructures, quantum dots, and heavy metal nanocrystals and porous silicon nanomaterials, have shown the ability to efficiently convert light into heat. Shao et al. produced the biodegradable poly (lactic-co-glycolic acid) (PLGA) nanoparticle loaded with black phosphorus quantum dots (BPQDs), these hydrophobic PLGA polymer shell not only increased the photothermal stability via separation the BPQDs from oxygen and water, but mediated the BPQDs degradation [94]. On the other hand, organic nanomaterials, including organic compound and conjugated polymer based PNPs, have considered as potential agents in PTT due to their biodegradability. Among organic agents, conjugated polymer based PNPs have drawn great attention arising for their photothermal efficiencies, which is similar to gold nanoparticles [93]. Zhou et al. [95] prepare the conjugated polymer based PNPs, which can release the heat shock protein inhibitor. This inhibitor could reduce the cellular tolerance to heat resulted in better PTT effect.

14.4.3 Sonodynamic Therapy (SDT)

Different to the phototherapies conducting by light, sonodynamic therapy (SDT) executes the remedy by "sound". After activated by low-intensity ultrasound stimulation, specialized chemical agents, sonosensitizers, can produce ROS leading to cell damage. Due to ultrasound can non-invasive penetrate deeper to internal organs, compared to traditional phototherapies, SDT has attracted more attention in recent years.

The sonosensitizer, however, suffer the same problems as photosensitizer, such as low biological stability, tumor-accumulation. Therefore, to increase the SDT efficiency, several researchers have explored ways to combine PNPs with sonosensitizer, such as inorganic nanoparticles or organic compound. Xu et al. [96] confirmed the design of PNPs as carrier for sonosensitizer nanoparticles, including gold, Fe_3O_4, silver, porous silicon, and carbon fluoroxide, they introduce different methods to encapsulate the sonosensitizer into PNPs. Qian et al. [97] provides systematic description about the development of amplified SDT performance assisted by PNPs. A recent study demonstrated that, with proper design, MRI imaging can be displayed by sonosensitizers. Huang et al. [24] chelate MRI agents, Mn ion, to the sonosensitizers, protoporphyrinc (PpIx), and anchored the Mn chelateing sonosensitizers, Mn-protoporphyrin (MNPpIx), in to the inner mesoporous organosilica nanoparticles (HMONs-MnPpIX); then, the surface of HMONs-MnPpIX was also covalently modified with PEG, these PNPs made by HMONs-MnPpIX not only increased the SDT efficiency, but also augmented the Mn^{2+} ions chelating on protoporphyrin, these make this composite sonosensitizers with good MRI performance combined with SDT monitoring.

14.4.4 Neutron Capture Therapy (NCT)

Neutron capture therapy (NCT) is a treatment based on the nuclear reaction. Unlike aforementioned therapies which destroy the cells by ROS or heat, NCT kill the cell by gamma ray. After penetrate into the tissue, the epithermal neutrons would slow down and be captured by NCT agents, causing lethal radiation to injury in tumor cells. Boron (B) has been extensively

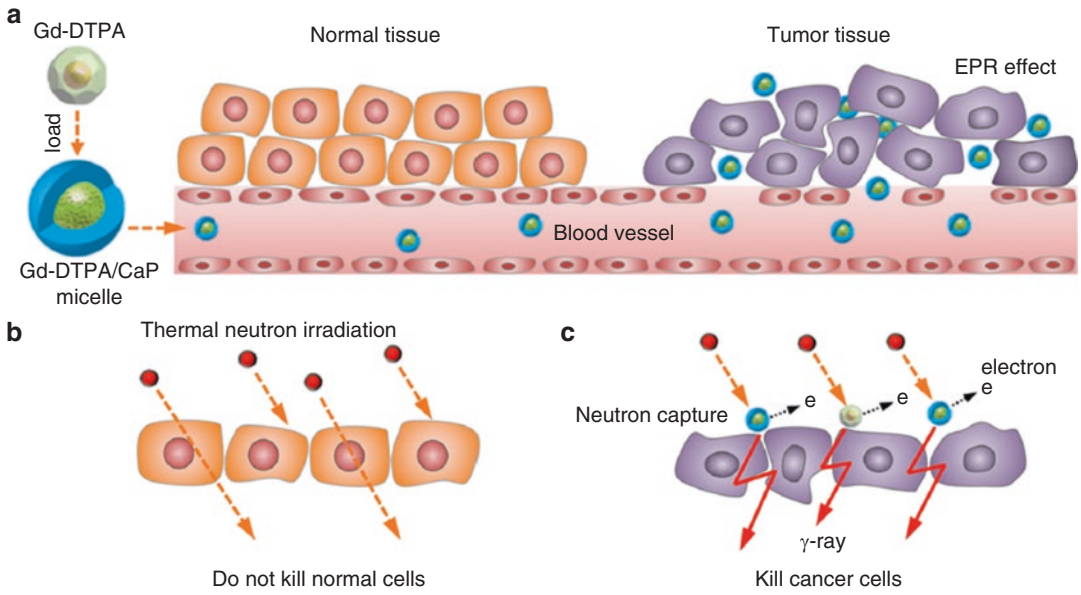

Fig. 14.3 Scheme of Gd-DTPA/CaP hybrid micelles targeting tumors for gadolinium neutron capture therapy (GdNCT). (**a**) The accumulation of Gd-DTPA delivered by Gd-DTPA/CaP in tumors through the EPR effect. (**b**) Low energy thermal neutron irradiation does not kill normal cells without NCT agents. (**c**) Thermal neutron irradiation could kill or cause hazardous damage to cancer cells by the γ-rays emitted from the Gd nuclides after nuclear reaction with captured thermal neutrons. (Image adapted from Mi et al. [25] and reprinted with permission from ACS publish)

studied as NCT agent. Subsequent to the capture of neutron, the nuclear fission of B could produce high energy alpha particles and lithium-7 nuclei [98].

To achieve sufficient deposition of NCT agents in a tumor site, NCT agents could be delivered by PNPs. For example, Huang et al. [99] synthesize the amphiphilic carborane-conjugated polycarbonates, which can self-assembly into different sizes in water, after irradiation of thermal neutron, the boron content of the carboranes conducts the NCT and suppresses tumor growth. In Kataoka's study [25], they replace the B with Gd, and loaded Gd into calcium phosphate core with hybridizing of PEG-polyanion block copolymers. There's two reasons to use Gd in their study: first, Gd has the largest capture cross-section, among NCT agents for thermal neutron absorption (larger than B) resulting in the emission of high energy gamma rays; the other reason is Gd can helpful for guiding the NCT under MRI (Fig. 14.3).

14.5 Other Applications

The development of nanotechnology has been successfully selected as a alternative way to design, synthesize, and apply to present materials in biomedical filed. In addition to the above-mentioned application, such as diagnosis and therapy, PNPs have been addressed in various applications. In the following, we roughly divide the application of PNPs into two categories by its condition; individual application and cluster employment.

14.5.1 PNPs Individual Application

In the preceding sections, we described how metal nanoparticles can be incorporated into PNPs to adhere to cells for imaging or treatment of them. However, the use of PNPs is not only limited to these. Recent studies have shown excellent performance of PNPs in mediation of

cell behavior, biomolecular isolation, immunotherapy, protein affinity reagents and personalized medicine. In the following, we will introduce these applications.

PNPs with magnetic nanoparticle can control the mechanical stimulation of single-cell behavior. Tseng et al. [100] manipulate the dextran-magnetic nanoparticles, via magnetic fields, within HeLa cells. They apply localized nanoparticle-mediated forces producing the tension on the cortex of cells and observe the responses in cellular behavior. This technique offer a tool to analysis of molecular, such as proteins or nucleic acids, localization and its functional role in cell.

In addition to mediation of cell behavior, magnetic PNPs can be employed for separation the protein biomarkers. Nehilla et al. [101] use the classical temperature-responsive polymer poly (N-isopropylacrylamide), or pNIPAAM, to create the stimuli- responsive binary reagent system, including polymer coating magnetic nanoparticles (PNPs) and polymer−antibody conjugates (Ab), to capture the antigen. When the stimulus is applied, the PNPs and Ab with captured antigen will aggregate to form magnetically separable species. After removal of a stimulus, the captured antigen will release from PNPs. This system can customize for types of affinity reagents, providing a potential platform for biomarker discovery and diagnostics.

By combining immunotherapy with PNPs, nanoparticle platform can simulates the immune system to inhibit tumor growth is developed Kosmides et al. [102] prepare the "immunoswitch particle" by coating dextran-magnetic nanoparticles with two different antibodies that switch off the inhibitory checkpoint PD-L1 pathway on tumor cells and switch on CD8+T cells via the 4-1BB co-stimulatory pathway. These immunoswitch PNPs can increase effectiveness of immunotherapy over soluble antibody, resulting in reduction in cost and complexity of therapeutic.

PNPs are used for protein affinity reagent in biomedical application. Because the biological affinity reagents, such as antibody, have some disadvantage, including high cost, difficulties in production and storage. Several researchers have tried to try to find out alternatives. Hoshino et al. [103] prepare the PNPs, which interact strongly and weakly with denatured and native lysozyme respectively, to refold the aggregated lysozyme. Shea et al. [104] develop a PNPs can neutralize venomous biomacromolecules, phospholipase A_2 (PLA_2), inhibiting the hemolytic reaction. Koide et al. [105] synthesis the PNPs with affinity to a key vascular endothelial growth factor, $VEGF_{165}$, leading to inhibit VEGF-mediated angiogenesis without exhibition off-target activity.

PNPs can apply to personalized medicine to match specific patient with the most effective treatment. Schroeder et al. [106] load the different cancer drug and corresponding DNA barcodes into PNPs. Through the analysis of DNA barcodes, the correlation between the drug and cell viability is revealed finding out the most efficient drug. Similar to Schroeder et al., Dahlman et al. [107] use DNA barcoding to analyze the biodistribution of PNPs with varying PEG characteristics. The DNA barcoding can facilitate the researcher to understand the relationships between treatment and disease.

14.5.2 PNPs Cluster Employment

PNPs, especially spherical particles, have been used as building blocks to form close-packed two-dimensional (2D) or 3D structures so called self-assembled colloidal crystals [108, 109]. Various methods have been proposed such as evaporation induced colloidal self-assembly or self-assembly of colloids at air-water interfaces [109, 110]. The chosen method is often dependent on the particle material and size. The method and particle used also often determines the ultimate quality of crystal structure (i.e. the size of defect). High quality of 2D or 3D crystals is a common goal in various applications.

Colloidal crystal (CC) monolayers provide unique property which can be utilized in different fields such as photonics, sensors, and biology [111]. Recently, CC monolayers or CC-derived substrates have been utilized as a cell culture tool for modulating cell behavior [112, 113]. CC monolayers can be composed of single, binary,

and even more types of particles. For example, binary colloidal crystal (BCC) monolayers have been fabricated on glass substrate in large area using large silicon particles and small polymer particles. Both 2D single and binary CCs have been used in cell culture. BCCs provide higher complexity on the surface than single CC such as heterogeneous chemistry. The ability of present more chemical or physical cues on cell adhering surface such as ternary CCs may elicit useful bioinformation.

Wang et al. [109] first used BCCs as a substrate for stem cell culture. BCCs were fabricated using evaporation-induced colloidal self-assembly. The detailed mechanism was described using this method. Depending on the BCC combinations, long ranged BCC can form on a 2D surface. Mammalian cells including MG63 osteoblasts, L929 fibroblasts, and human adipose stem cells (hASCs) were cultured on those long ranged BCCs, and cell spreading area was found to be inhibited on BCCs compared to flat controls. However, once cell adhesive protein, fibronectin, was pre-adsorbed onto BCCs, a synergic effect of BCCs and cell adhesive protein was found that cell spreading was significantly increased. Based on this result, hASCs were expanded on BCCs for longer term [113]. Interestingly, osteo- (BSP, RUNX2, and OPN) and chondro-genes (AGG, SOX9, and COL2) were upregulated on BCCs, but not adipogenes (PPARγ and adiponetin). Thus, it has been demonstrated that BCCs can induce early osteochondral differentiation during stem cell expansion.

In another study, BCCs have been demonstrated that can replace the cell adhesive protein, vitronectin, during cell reprogramming of fibroblasts into induced pluripotent stem cells (iPSCs) (Fig. 14.4) [114]. Currently, the standard protocol for *in vitro* cell reprogramming needs pre-coating of extracellular proteins which support not only cell attachment but also provides abundant biosignals during cell growth. The result showed that human iPSC colonies formed on BCCs without vitronectin coating with high percentage while the colony was difficult to form on flat sur-

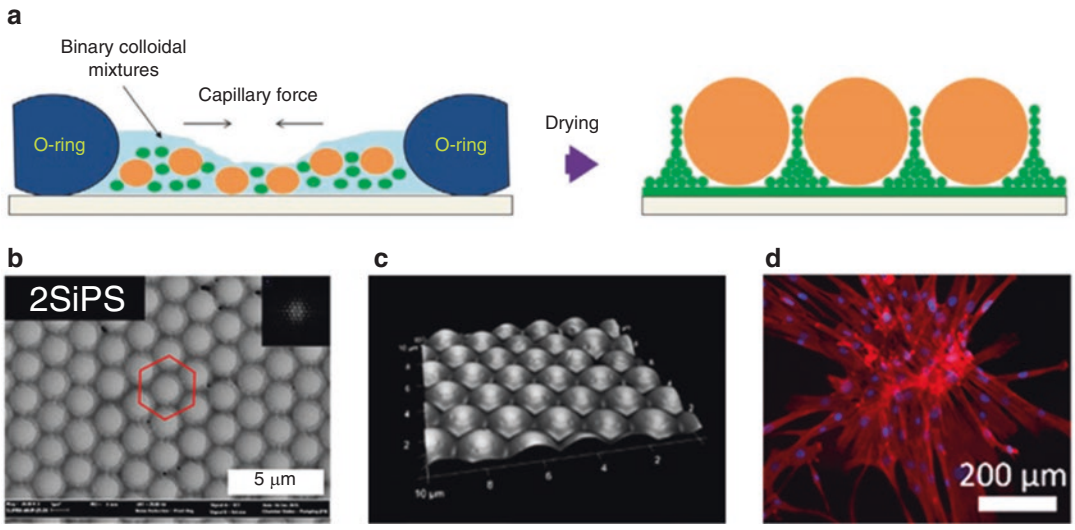

Fig. 14.4 PNPs self-assembly into monolayers as cell culture substrates. (**a**) Binary colloidal mixture self-assembled into crystal monolayer. (**b**) Scanning electron micrograph of one example, 2 micron silica and 0.1 micron polystyrene (2SiPS). (**c**) Atomic force microscopy of 2SiPS. (**d**) Human adipose stem cells (hASCs) grew on 2SiPS for 4 days and formed clumps on the surface. (Image adapted from Wang et al. [114] and reprinted with permission from Springer Nature)

face without vitronectin. This result implies that BCCs regulates the cytoskeleton of fibroblasts and/or promotes extracellular matrix synthesis by fibroblasts which in turns facilitate cell reprogramming process. Overall, PNPs can be self-assembled into monolayer as a mask to producing biochemical patterns or substrate for *in vitro* cell expansion.

14.6 Conclusions

Although negative impacts of nanoparticle on patient tissue, such as liver [115], lung [116] and cardiovascular [117] were reported, due to some PNPs have capacity to cause inflammatory condition of lung, or accumulated lung to weaken the immune system, then activate a portion of latent virus in body, therefore, the proper design of PNPs can help decrease the risk of problems we mention.

Due to their good biocompatibility, large surface area to volume ratio, and versatile design, PNPs are considered competitive potential system for biomedical application. As a delivery system, PNPs not only can protect and transport of cargo, such as drug, plasmid or protein, more specific into the desired target site but can "smart" control of cargo release rate from PNPs. Also, via entrapment of metallic nanoparticles or fluorescent organic compounds, PNPs could serve as imaging or therapy platform to observe or destroy the specific tissue. Moreover, PNPs could be used to control of cell behavior, biomolecule isolation, protein affinity reagents and surface fabrication. Recently, by associating specific PNPs with photoreactvie agents as therapeutics, PNPs become a powerful tool for the application of personalized medicine to treat and improve cancer care.

In this chapter, we introduce some recent publications, including original and review articles with new agent or nanoparticles combination. All of them show that PNPs offer the new possibilities to develop both new diagnostic and therapeutic ways for biomedical application. We believe that PNPs could create more medical breakthroughs in the future.

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15 Reactive Oxygen Species Responsive Naturally Occurring Phenolic-Based Polymeric Prodrug

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Abstract

Reactive Oxygen Species (ROS) play a vital role in the biological system. Exaggerated, ROS have devastating effects on the human body leading to the pathophysiological condition including the transformation of a normal cell into a cancer phenotype. Nature has blessed us with various biomolecules that we use along with our dietary supplements. Using such therapeutic small molecules covalently incorporated into biodegradable polyoxalate polymer backbone with a responsive group forms an efficient drug delivery vehicle. This chapter "Reactive oxygen species responsive naturally occurring phenolic-based polymeric prodrug" will be focusing on redox-responsive polymers incorporated with naturally occurring phenolics and clinical application.

Keywords

Reactive oxygen species · Prodrug · Polyoxalate polymers · Redox responsive polymers

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15.1 Introduction

In a biological system, Reactive Oxygen Species (ROS) play a vital role and has prominently influenced the metabolic pathways such as regulating cell signaling, eliminating bacteria, mediating inflammation and protein function modulation. The intracellular modulatory effects depend upon its concentration, secretion site and exposure time to cells. In excess, ROS have devastating effects on the human body leading to autoimmune disorders, cardiovascular disease, and neurodegenerative disorder and so on. In addition, higher ROS level also

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enhances the risk for DNA mutation and could cause malignant transformation of a normal cell into a cancer phenotype. Nature has blessed us with various biomolecules that we use in our daily day to day diet. Due to the influence of food in the human physiology, a wise man like Hippocrates inferred "Let food be thy medicine and medicine be thy food". This chapter "Reactive oxygen species responsive naturally occurring phenolic-based polymeric prodrug will be focusing on redox-responsive polymers incorporated with naturally occurring phenolic's and their drug delivery application.

15.2 Reactive Oxygen Species

Living cells would cease to exist without oxygen; mitochondria, the powerhouse of the cell, reduce the molecular oxygen to provide adenosine triphosphate (ATP) and a byproduct H_2O_2. This mitochondrial transformation also partially reduces oxygen leading to the generation of highly reactive molecules which is altogether named as Reactive Oxygen Species (ROS) [1]. In human body, ROS are generated during enzymatic reaction every day. Prolonged exposure to high ROS concentrations and its intracellular accumulation may lead to capricious damage to the physiological system. Some of the ROS compounds include H_2O_2, $O_2^{\cdot-}$, $HO^{\cdot}$, hypochlorous acid (HOCl) and $^1O_2$ [2].

Oxygen-centered small molecules contain unpaired electron in the valence-shell which is unstable and highly reactive. Intra-cellular components such as nucleic acids, lipids, and proteins are potential susceptible sites for this lone pair electron (Fig. 15.1). This lone pair, if it interacts with the biomolecules it can inactivate target molecules irreversibly [4]. If there is a redox imbalance due to the accumulation of ROS, major cellular antioxidants such as glutathione and thioredoxin are depleted [5]. In the event of imbalance of excessive ROS generation due to physical or chemical factors inability to detoxify corresponding reactive intermediates results in oxidative stress [6].

15.3 H_2O_2 in Human Physiology

H_2O_2 is one of the ROS that plays an essential role in living organisms and it has several clinical implications from a molecular to a cellular level. Some beneficial effects of moderate and low levels ROS on physiological processes are eliminating pathogenic invasion, stimulating wound healing and tissue repair processes. [7–9]. To terminating, microorganisms and fungal spore H2O2 is employed as a clinically accepted biocide [10]. The pathophysiological outcome of intracellular H_2O_2 is determined by the molar concentration secreted at the cellular subspaces [11].

Vascular endothelial growth factor activation, the secondary messenger that involves cell signaling, epidermal growth factor, keratinocyte growth factor and are activated by the moderate level of H_2O_2 concentration [12, 13]. The chemical reactivity of H_2O_2 is poorly reactive and oxidizes mildly. Unless the bio-molecules have hyper-reactive thiol groups or methionine residues, it cannot readily oxidize. Conversion of the reactive hydroxyl radical ($OH^{\cdot}$) from H_2O_2 makes it more dangerous. Henceforth, nature has put enzyme based detoxifiers, checkpoints and balances to regulate H_2O_2/ROS surge [14]. However, the abnormally high level of H_2O_2 could affect the viability of cells, impairing cellular process and result in the serious breakdown of cell function [15, 16].

15.3.1 Prodrug

A prodrug is an inactive form of a drug molecule that metabolizes inside the body into an active molecule. Ingestion of inactive form of drug molecules i.e. prodrug improves absorption, distribution, metabolism, and excretion (ADME) of the drug molecule. For example, 4-hydroxycyclophosphamide is an active anticancer agent (metabolite) which is a potent alkylator, is given as cyclophosphamide a prodrug form of 4-hydroxycyclophosphamide. Cyclophosphamide is activated by CYP450 enzymes to forms 4-hydroxycyclophosphamide which reduces the adverse effects of the orally ingested drug molecule.

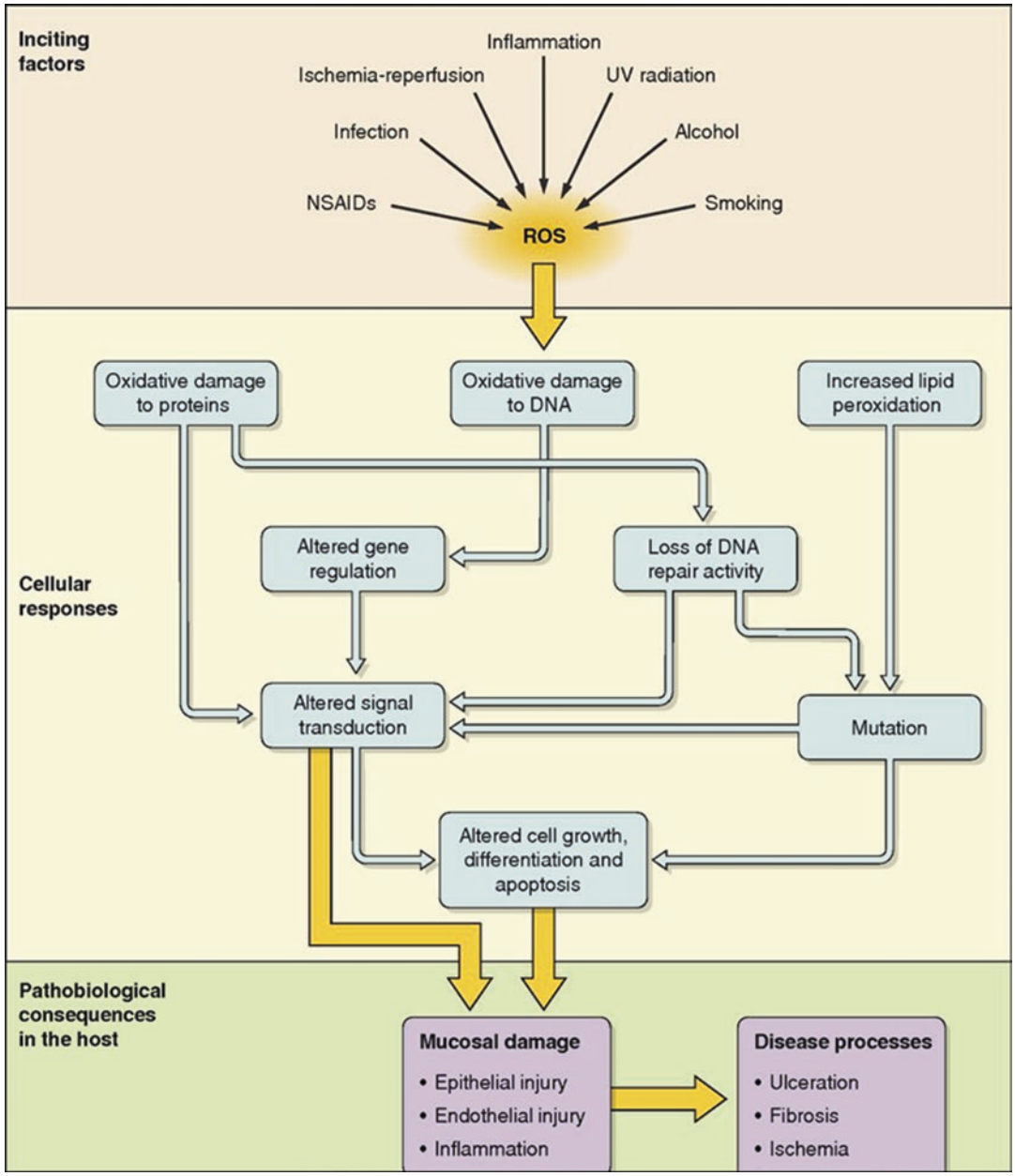

Fig. 15.1 Pathophysiological effects of Reactive Oxygen Species (ROS) [3]

15.3.2 Polymeric Prodrug

Polymeric Prodrug (PPD) is a subclass of a prodrug which includes drug delivery systems that release drug-facilitated by cleavage of a chemical bond. PPD technology is very promising owing to its easy tunability and bio-engineerable capability. It ensures the incorporation of pharmacologically less active drug into the polymer chain, thereby enhancing the release of bioactive drug molecule at the site of action. Some of the pharmaceutically approved PPD is listed in Table 15.1.

Table 15.1 Polymer-drug conjugates available in market [17]

S. No.	Conjugate	Indication	Marketing year	Company
1	PEG–adenosine deaminase (Adagen)	SCID syndrome	1990	Enzon
2	PEG–asparaginase (Oncaspar)	Acute lymphoblastic leukaemia	1994	Enzon
3	SMANCS (Zinostatin, Stimalamer)	Hepatocellular carcinoma	1993	Yamanouchi pharmaceutical
4	Linear PEG-interferon α2b (PEG-intron)	Hepatitis C, clinical evaluation of cancer, multiple sclerosis and HIV/AIDS	2000	Schering plough/Enzon
5	Branched PEG-interferon a2a (Pegasys)	Hepatitis C	2002	Roche/Nektar
6	PEG–growth hormone receptor antagonist	Acromegaly	2002	Pfizer (Pharmacia)
7	Branched PEG–anti-VEGF aptamer	Age-related macular degeneration	2004	EyeTech pharmaceuticals (now OSI pharmaceutical)/Pfizer
8	PEG–anti-TNF Fab	Rheumatoid arthritis and Crohn's disease	2008	UCB (formerly Celltech)

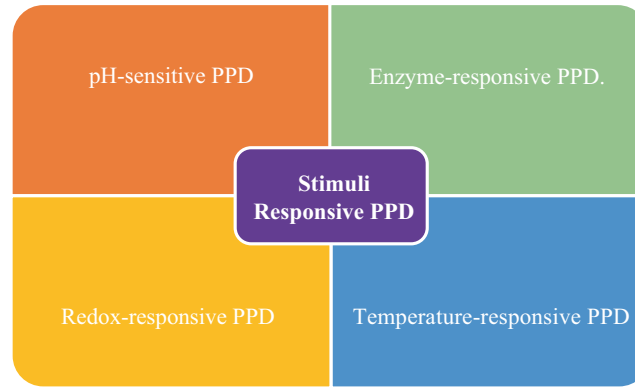

Fig. 15.2 Classification of Polymeric Prodrug (PPD) systems

The advantage of PPD system:

1. Alteration of ADME
2. Reduction in drug toxicity
3. Increase in drug loading
4. Increase bioactivity
5. Increased specificity

15.3.3 Classification of PPD

PPD could be classified as follows based on the responsive groups present in the polymer i.e. pH-sensitive, enzyme-responsive, redox-responsive and temperature-responsive, etc. In this chapter, we have highlighted the redox-responsive PPD incorporated with a naturally occurring small molecule, shown in Fig. 15.2.

15.3.4 Naturally Occurring Molecules as Drug

Most clinical drugs currently in use are from natural or semisynthetic (naturally existing molecules modified chemically) sources. Discovering a new drug is a complex process that requires huge investment, yet, has a very low success rate. Due to the risk involved in discovering a potent new chemical entity, scientist chooses to improvise the naturally existing bio-active drug molecule [18]. Known molecules that could modulate

a specific biological target *in vitro* are good candidates to start with [3]. For example, Penicillin, the first antibacterial agent that was used to treat infection, was modified by mutation at 6-aminopenicillanic acid leading to the production of O-acetylated penicillin. Modification of O-acetyl group of penicillin produces a semisynthetic penicillin that was effective against clinically resistant staphylococcal infections [19]. Koehn et al., have discussed the usage of natural products, in discovering new chemical entity and further backed their statement based on Derwent World Patents Index, which stated that 'round the world, there have been a global increase of patents granted for pharmaceutical products from a natural source' [20]. This clearly implies that improving the existing small molecules involves less financial and medical evaluation based risk. More importantly, the chemical modified preexisting drug or increasing bioavailability of the formulations could have a better activity [21].

Drug delivery systems (DDS) is a means of engineering technologies to target delivery and/or release therapeutic agents in a controlled manner (a definition according to National Institute of health, from the United States). Significant efforts were devoted to designing efficient micro- and nano-formulations for delivering pharmaceutical agents. Nano-formulations are a subclass in drug delivery system that the polymer is designed in such a way as to release a drug molecule when it is exposed to the stimuli. These systems possess a variety of interesting applications for encapsulating small molecules in the polymer backbone, such that a polymeric prodrug could be synthesized [22]. Controlled DDS can be formulated by encapsulating small molecule or an anticancer agent into a smart polymer core which releases drug molecules in response to molecular triggering (redox agent such as H_2O_2) [23].

15.4 Hydrogen Peroxide Responsive Smart Polymers

15.4.1 History of Peroxyoxalates and Copolyoxalate

M.M. Rauhut et al., first reported peroxyoxalate by 1967 [24]. In the presence of H_2O_2 with oxalyl chloride or oxalate diesters react to form esters known as peroxyoxalate. These peroxyoxalates are intermediates that will rapidly transform into high-energy intermediate 1,2-dioxetanedione. The mode of a mechanism for reactions between aromatic peroxalate ester and hydrogen peroxide is illustrated in Fig. 15.3 bis(2,4-dinitrophenyl), bis(2,4,6-trichlorophenyl), bis(2,4,5-trichlorophenyl-6-carbopentoxyphenyl) oxalate are more prevalently used oxalates. Peroxyoxalate chemiluminescence (POCL) is a reaction in which the highly reactive 1,2-dioxetanedione excites the dye molecule present around it. This is a typical example of sensitized (indirect) chemiluminescence, 1,2-dioxetanedione has the capacity to produce energy in an excited state and transfer it to a suitable fluorescent molecule. This process enables photons to be released to the ground state especially when dye molecules are relaxed. Decomposition of 1,2-dioxetanedione produces a chemical reaction of an annihilation of chemical fluoresce radical cation and carbon

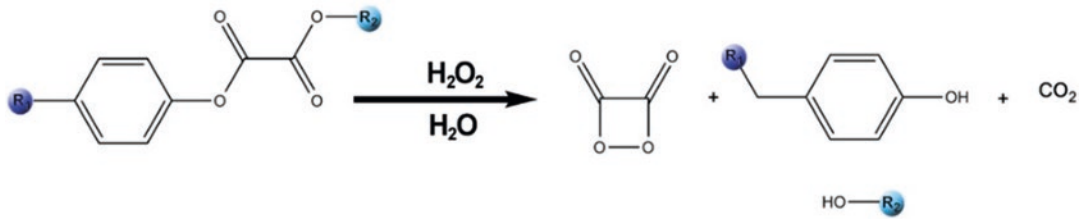

Fig. 15.3 Reaction mechanism between aromatic peroxalate ester and hydrogen peroxide

Table 15.2 Examples of Nano formulations that are currently used in clinics [25]

Trade name	Drug	Use
Myocet	Doxorubicin	Metastatic breast cancer
Doxil	Doxorubicin	Leukaemia, lymphoma, carcinoma and sarcomas
DaunoXome	Daunorubicin	Kaposi sarcoma, leukaemia and non-Hodgkin lymphoma
DepoCyt	Cytarabine	Lymphomatous meningitis
Marqibo	Vincristine sulfate	Lymphoblastic leukemia
Visudyne	Verteporfin	Macular degeneration
Abraxane	Paclitaxel	Breast cancer and metastatic adenocarcinoma of the pancreas
Adagen	ADA	Enzyme replacement therapy for severe combined immunodeficiency
Copaxone	Glatiramer acetate	Multiple sclerosis

dioxide radical anion. This process is called chemically induced electron exchange luminescence. Due to their biocompatibility and biodegradability have several advantages over the conventional drug delivery methodologies. Several drug delivery systems have been accepted by USA Food Drug Administration (FDA) to treat disease etiologies (Table 15.2) [25]. There are several nanocarriers that have been synthesized from organic polymers, inorganic materials, and organic-inorganic hybrid. Due to the tunable nature, stimuli responsive nanocarriers have gained a considerable level of attention in the field of drug delivery [26]. The stimuli that could potentially be used in drug delivery are temperature, pH, light, electric field, ultrasound, magnetic field, and bio-molecules (such as enzymes and redox state of the intracellular microenvironment) [27–31].

Stimulus-responsive polymers are class of smart, molecule sensitive polymers that perform desired functions in response to various stimuli. The stimuli could be a physical or chemical means to get a sustained drug delivery (Table 15.3) [33]. Physical parameters that could

Table 15.3 Polymers with stimuli-responsive capabilities [32]

Environmental stimulus	Responsive material
Temperature	Poloxamers
	Poly(*N*-alkylacrylamide)s
	Poly(*N*-vinylcaprolactam)s
	Cellulose, xyloglucan
	Chitosan
pH	Poly(methacrylicacid)s
	Poly(vinylpyridine)s
	Poly(vinylimidazole)s
Light	Modified poly(acrylamide)s
Electric field	Sulfonated polystyrenes
	Poly(thiophene)s
	Poly(ethyloxazoline)
Ultrasound	Ethylenevinylacetate

be used for DDS are temperature, light, radiation, etc. [22] Chemical stimuli that could be used for DDS are bio-molecular in origin, E.g. pH, ions, matrix metalloprotease, redox, etc. [34, 35] Responsive nature of DDS (polymer) are tailored to the polymer backbone depending upon the final application [36]. For the DDS, polyoxalate, and copolyoxalate were introduced for the use of biomedical applications. Initially, it was used for suture coating purposes [37]. They degrade into nontoxic diols and oxalic acid which could be excreted by the biological system.

15.4.2 Polymeric Prodrug from Polyanhydride Polymers

An alternative method of delivering a therapeutic small molecule involves polymeric prodrug, in which therapeutic compounds are covalently incorporated into its backbone and not in a side chain. As the polymer backbone degrades, therapeutic compounds get released to the environment. The pioneering example of polymeric prodrugs was demonstrated by Uhrich et al., who developed polyaspirin, in which salicylic acid is covalently incorporated into the backbone of biodegradable polyanhydride [38]. This strategy allowed a high content (62 wt %) of deliverable drugs that could be released during the hydrolytic degradation of the polymer backbone.

15.4.3 Biodegradable Polyoxalate Nanoparticles

For hydrogen peroxide imaging, Lee et al. were the first to prepare polyoxalate nanoparticles loaded with fluorescent dye in his work. Afterward, he prepared several polyoxalate based drug delivery system that could be activated by ROS for treating various inflammatory diseases (Fig. 15.4) [39].

Polymeric nanoparticles prepared from biocompatible and biodegradable materials from natural source would be efficient drug delivery vehicle. Polyoxalate polymers could also serve in the synthesis in one step which makes its synthesis easily and due to its biodegradability; it could form nontoxic components that could be metabolized in the human body. These polymers were around MW of ~11,000 Da. Polyoxalate nanoparticles could be engulfed by RAW 264.7 macrophage cells and HEK 293 cells and exhibited good biocompatibility [40].

15.4.4 Small Molecules Incorporated Polyoxalate Polymers

Polyoxalate polymers p-Hydroxybenzyl alcohol (HBA) are phenolic compounds predominantly found in herbal agents. These phenolic compounds exhibit an inflammatory effect which is protective against oxidative damaged related diseases. The HBA-incorporated copolyoxalate (HPOX) were designed to release pharmaceutically active p-HBA during hydrolytic degradation.

The enzyme nitric oxide synthase found in Lipopolysaccharide (LPS)-activated RAW 264.7 macrophage cells can be suppressed by nanoparticles synthesized from HPOX. This inhibits the production of nitric oxide (NO). Examination of HPOX's angiogenic effects in a mouse model of

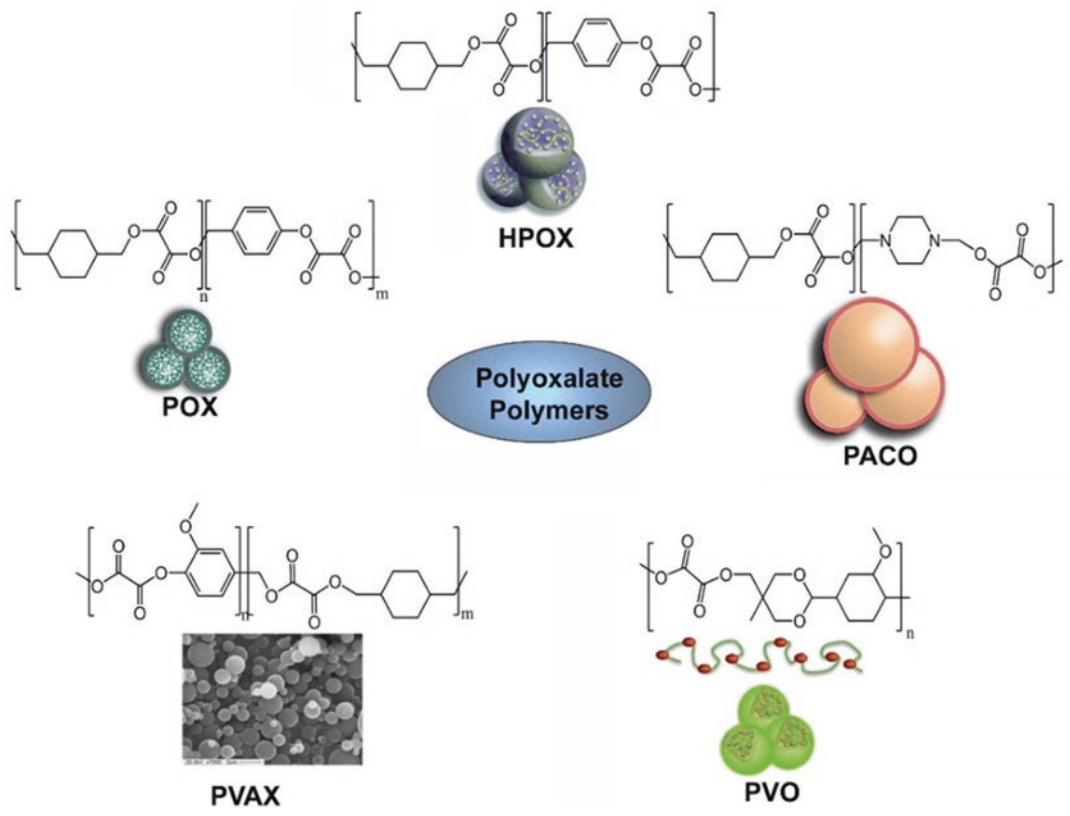

Fig. 15.4 Polyoxalate polymers proven to work in vivo

hindlimb ischemia proved a dose-dependent up-regulation of angiogenic proteins such as vascular endothelial cell growth factor (VEGF), Akt/AMPK/endothelial nitric oxide synthase (eNOS) were observed in ischemic hindlimb muscle of the mouse [41–44]. Park et al., described a polymer formulated from copolyoxalate incorporating vanillyl alcohol (VA) named as PVAX to treat an inflammatory condition. VA was incorporated along with H_2O_2-responsive peroxalate ester linkages covalently in its backbone. To validate or determine the efficacy of the PVAX nanoparticles, specified concentrations were injected in hind-limb I/R and liver I/R models in mice. The results of the experiment showed a specified reaction between overproduced H_2O_2 and the inoculated nanoparticles which resulted in a highly potent anti-inflammatory, anti-apoptotic and wound healing activities which reduced cellular damages to a minimal level. [45–49].

15.4.5 Boronic Ester Containing (*p*-Hydroxybenzyl Alcohol) (BRAP)

Lee et al. had described H_2O_2-activatable antioxidant prodrug (BRAP) which contains boronic ester that could scavenge H_2O_2 and release HBA (Fig. 15.5). In a mouse model with hepatic I/R and cardiac I/R, boronic esters and HBA exert antioxidant, anti-inflammatory and anti-apoptotic activity [50].

One of the upsides of the polymeric prodrug is their tunable biodegradability and other physiochemical parameters.

Compared to commercial biodegradable polymers, poly(lactide-co-glycolide) and poly(caprolactone) polyoxalate-based polymers have rapid hydrolytic-degradation kinetics and more tunable nature [51].

15.4.6 Highlights on Multimodal Prodrug Delivery

Multimodal prodrug delivery systems are used for delivery of drug cargo or a smart polymeric. Nano-formulation to a target simultaneously track the homing capability of the particle in real-time (Fig. 15.6) [52]. Benefits of the nanoparticle's in comparison with molecular images are:

(a) The surface of the particle could be decorated with antibodies, small molecules or cell targeting/penetrating peptides and the core could be tweaked to accommodate fluorescent shell;
(b) A wide array of imaging capability (e.g., near-infrared (NIR), optical, magnetic resonance, ultrasound, fluoroscopy etc.) facilities multimodal cross-evaluation of the site of interest using various imaging;
(c) Theranostic abilities- therapeutics could be intertwined with imaging capability i.e. imaging and simultaneously release drug cargo on the site of tissue impairment.
(d) Micro size particles that are incorporated with materials that could generate a bubble in the presence of a trigger signal could be used as an imaging with improvised stability/long term imaging; and.
(e) It could accommodate from a wide range of imaging from a Petri dish to a live animal for disease model. The application of these multicable drug delivery systems are endless and can be tailored depending upon the final application.

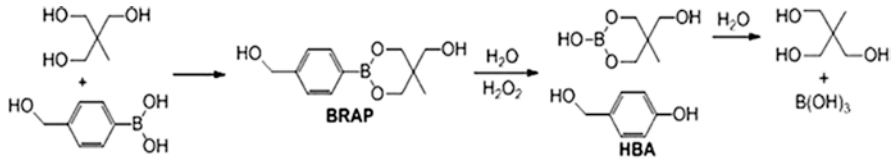

Fig. 15.5 H_2O_2-activatable BRAP antioxidant prodrug synthetic and degradation

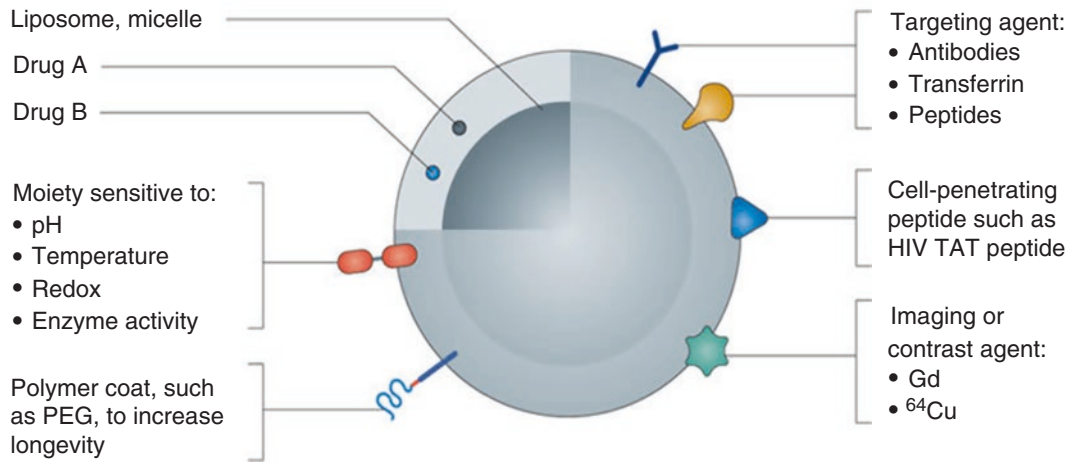

Fig. 15.6 Multimodal Prodrug delivery system. Reprinted with permission from Ref [52]. Copyright (2014) with permission from the Nature Publishing Group

15.5 Conclusion

Polymeric prodrug has been intensively investigated over the past few decades. Due to the advantages possessed by the naturally occurring phenolic compounds, its incorporation expands its biomedical applications.

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16 Biodegradable Polymeric Nanocarrier-Based Immunotherapy in Hepatitis Vaccination

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Abstract

Various commercial vaccines are used for immunization against hepatitis B. However, these immunotherapeutic vaccines require invasive administration, which can induce side effects, and require multiple shots to elicit an immune response, limiting their efficacy. Compared to traditional hepatitis B vaccines, polymer nanoparticles have more advantageous inherent properties as vaccine delivery carriers, providing increased stability of encapsulated antigen, the possibility of single-shot immunotherapy, and the capability of mucosal administration, which allows various routes of vaccination. In this review, we present up-to-date information on the potential of a biodegradable nanoparticle-based delivery system in treating hepatitis B. We also discuss the application of nanoparticles in various vaccines and highlighted strategies for eliciting an appropriate immune response.

Keywords

Nanoparticles · Vaccine delivery · Hepatitis B vaccine · Immunotherapy

16.1 Introduction

Hepatitis B is the most common liver disease worldwide and is a major cause of cirrhosis and hepatocellular carcinoma, which account for 600,000 deaths per year. Estimates based on serology indicate that more than 2 billion people have been exposed to hepatitis virus. Among them, approximately 350–450 million may have chronic infections [1, 2]. Hepatitis B is 50–100 times more infectious than human immunodeficiency virus (HIV), which is transmitted by direct contact with skin wounds or mucous membranes. In addition to this route, hepatitis B can be passed from one person to another via contaminated needles, transfusions, and sexual contact. The global prevalence of hepatitis B prompted the implementation of a global immunization

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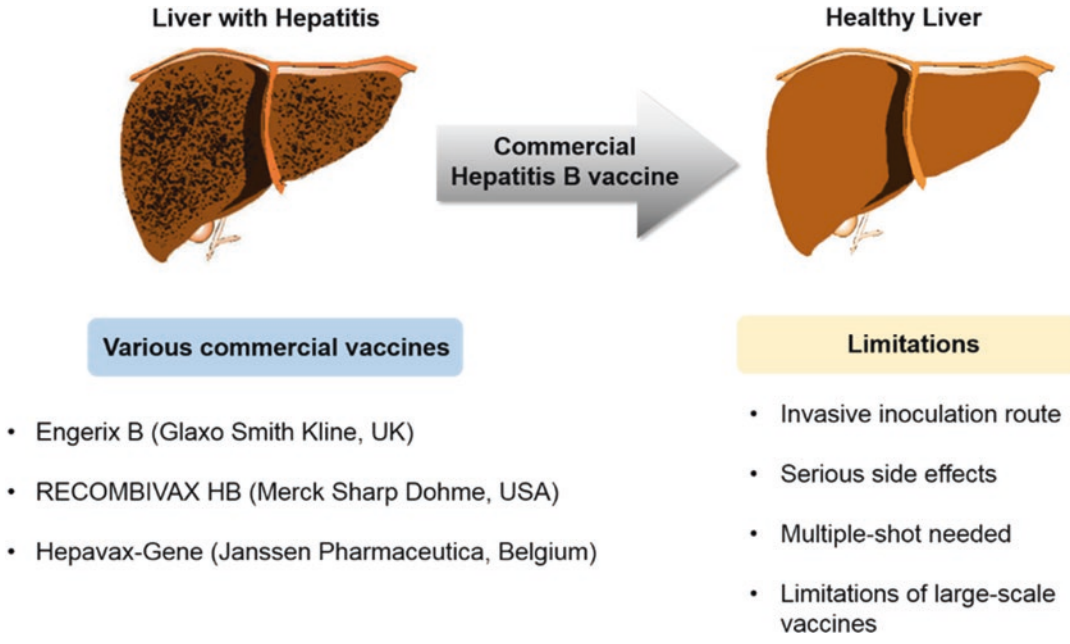


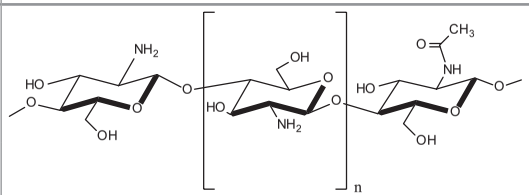
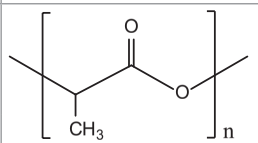
Fig. 16.1 Limitations of commercialized vaccines using aluminum adjuvant

program in the 1990s. However, the program could not be conducted in developing countries, where extensive vaccination was a burden for health authorities. Thus, it is imperative to develop an inexpensive hepatitis B vaccine and ease access to vaccines while reducing the occurrence of side effects and chronic liver disease [3].

Various types of vaccine adjuvants have been used in conjunction with an antigen to boost antibody production and immunization *in vivo*. Among these, alumninum is often used in commercial hepatitis B vaccines, significantly increasing the immune response to toxins. Aluminum adjuvants generally stimulate T cells, activating the antigen and improving lymphocyte retention. They also induce the release of water-soluble enhancing factors by stimulating the production of lymphokines. However, aluminum adjuvants can cause serious side effects, such as eosinophilia and myofibrosis, and there is growing concern over reports that the use of aluminum adjuvants can induce severe neurodegenerative diseases, such as Alzheimer's disease [4, 5]. Furthermore, there is the drawback that multiple booster doses are needed because these adjuvants do not lead to high, sustained immune activity (Fig. 16.1). Several attempts have been made to overcome the shortcomings of traditional vaccines that use aluminum as an adjuvant. Then, it may not come as a surprise that nanoparticles, with low cytotoxicity and increased immune stimulation, have emerged as promising hepatitis B vaccine delivery systems.

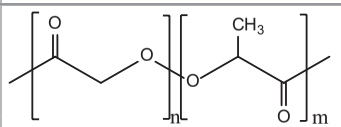
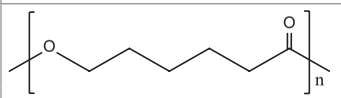
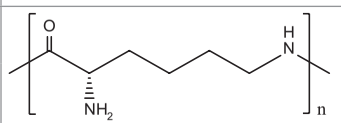
Synthetic and natural biodegradable nanoparticles may be used for various applications, such as tissue engineering, drug delivery, and gene carriers (Table 16.1). Owing to their controlled and specific targeted release properties, nanoparticles have also been used in different types of drug and vaccine delivery systems, to induce a desired local immune response within a short amount of time. In addition, nanoparticles play a role in protecting encapsulated antigens from enzymatic degradation *in vivo* [30]. Unlike conventional vaccines, nanoparticles may be used for mucosal vaccination, thereby improving antigen uptake by antigen-presenting cells (APCs), and can induce both humoral and cellular immune responses. [31] (Fig. 16.2).

Table 16.1 Biomedical Applications of Polymetric Nanocarriers

Nanoparticles	Structure	Main features	Characteristics as vaccine delivery system	Application in pharmaceutical industry
Chitosan		Composed of glucosamine and N-acetylglucosamine with β (1–4) glycosidic linkage.	Advantage: Biocompatible, biodegradation, nontoxicity, mucoadhesive, and acting better adjuvant for HBsAg than PLGA and PLA.	Vaccine delivery [6]
		Biologically compatibile, biodegradable, non-toxic, and non-immunogenic.	Disadvantage: insoluble in water and forming sticky solution in dilute acid.	Antibacterial agent [7]
		Suitable porosity for transporting signal molecules such as cells, nanopolymers, metabolites, nutrients.		Tissue engineering [8]
				Drug delivery [9]
				Food additive [10]
Poly (lactic acid) (PLA)		Produced by the bacterial fermentation of polysaccharides.	Advantage: biocompatible, biodegradation, and having sustained antigen release properties.	Drug delivery [11]
		High coupling properties and low unit cost.	Disadvantage: showing slow degradation rate in the human body and having strong hydrophobic properties.	Tissue engineering [12]
		Exists in three isoforms: D-L-lactide, L-L-lactide, and D-D-lactide.		Vaccine delivery [13]
		Decomposed by hydrolysis in the liquid phase.		Cosmetic formulation [14]
				Gene delivery [15]

(continued)

Table 16.1 (continued)

Nanoparticles	Structure	Main features	Characteristics as vaccine delivery system	Application in pharmaceutical industry
Poly (Lactide-co-Glycolide) (PLGA)		Aliphatic polymer that is biodegradable via ester linkage.	Advantage: FDA approved, biodegradation, and having controlled antigen release properties.	Tissue engineering [16]
		Degree of decomposition can be controlled according to the bonding ratio of the glycolic acid and the lactic acid.	Disadvantage: showing slow degradation rate in the human body and having strong hydrophobic properties.	Drug delivery [17]
		The most commonly used nanoparticle in drug delivery.	Disadvantage: poor long-term stability due to its degradation by-products	Vaccine delivery [18]
				Cancer diagnosis [19]
				Gene delivery [20]
Poly (ε-caprolactone) (PCL)		Excreted from the body.	Advantage: easy to manufacture and useful for long-term antigen delivery system.	Drug delivery [21]
		Controlled drug release due to slow decomposition.	Disadvantage: showing slow degradation rate *in vivo*.	Tissue engineering [22]
		Most widely used in tissue engineering.		Vaccine delivery [23]
		Hydrophobic property and polyester group with carboxylic acid.		Gene delivery [24]
		Lower limits of thermal and mechanical stress.		
Poly (L-lysine) (PLL)		Highly biocompatibile and completely biodegradable.	Advantage: non-immunogenic.	Gene delivery [25]
		Antibacterial, produced by microorganisms.	Disadvantage: a few studies have been reported in its usage as vaccine applications.	Antibacterial agent [26]
		Positive charge makes possible transfer of negatively charged molecules, like DNA and protein, in soluble state.		Vaccine delivery [27]
				Food additive [28]
				Tissue engineering [29]

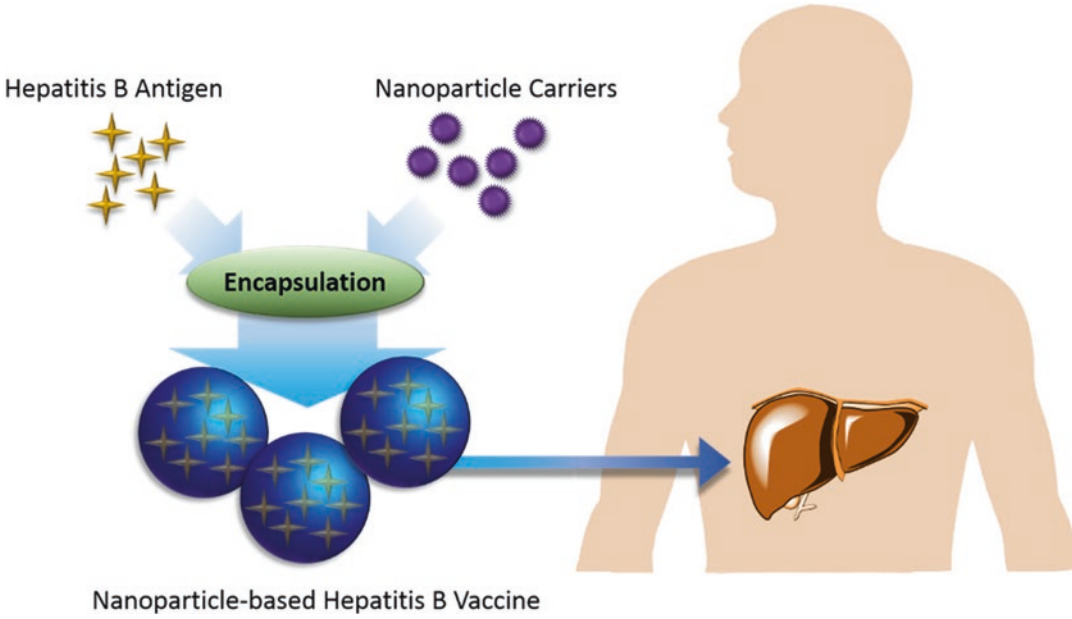


Fig. 16.2 Nanoparticle-based hepatitis B vaccine by encapsulation

In this review, we summarize state-of-the-art biodegradable nanoparticles that are used as vaccine delivery systems for the hepatitis B antigen. We also present the application of nanoparticles in several vaccine delivery routes. Nanoparticles with diverse properties such as biocompatible and biodegradable have the potential to be a next-generation vaccine delivery system.

16.2 Nanoparticle–Based Hepatitis B Vaccine Delivery Systems

16.2.1 Potential of Chitosan–Based Nanopolymers in Hepatitis B Antigen Delivery

Chitosan is a biodegradable polysaccharide that exists in nature and can be obtained by deacetylating chitin extracted from crustacean shells. It is non-toxic, and is approved by the US Food and Drug Administration (FDA) [32, 33]. Moreover, chitosan is biodegradable, biocompatible, and bioavailable, capable of mucosal adhesion, incorporates large amounts of antigen, and increases the absorption of hydrophilic polymers by opening the tight junction, all of which are critical for applications in immunology [34]. Furthermore, the potential for controlled release when using a chitosan-based polymer can vary the dose of the antigen.

There are several advantages to using chitosan as a vaccine delivery carrier. Firstly, chitosan is a natural adjuvant. Lugade et al. [35] reported that non-adjuvant vaccines using chitosan were more efficient that those using the conventional aluminum adjuvant. The aluminum adjuvant only stimulates reactions from T helper 2 cells (Th2). On the other hand, chitosan can upregulate T helper 1 cells (Th1) and simultaneously downregulate Th2 reactions due to the inherent property of chitin as an adjuvant agent of Th1 [36]. Consequently, chitosan-based vaccines are as effective in eliciting an immune response with one shot of vaccine as several shots of the conventional vaccines. Furthermore, it has been reported that chitosan-based vaccines can effectively control antigen release and deliver it to dendritic cells without an adjuvant [37].

Secondly, chitosan is positively charged in normal conditions, allowing for the encapsulation or

binding of antigens. Lebre et al. [38] conducted a vaccine delivery study using cationic chitosan. It forms a polymer through electrostatic interactions with negatively charged hepatitis B viral DNA. In general, DNA vaccines have the disadvantage of low transfection efficiency. However, transfection efficiency has been successfully increased through balanced interactions between chitosan and DNA. Fluorescein isothiocyanate (FITC)-labeled chitosan/DNA complexes were incubated on A549 cell lines and examined using a confocal microscope to confirm the cellular uptake and biodistribution of the complexes. In the cytoplasm, fluorescent chitosan/DNA complexes were observed 4 h from incubation. On the other hand, fluorescent pDNA was not observed in either the cytoplasm or nuclei of A549 cells. Confocal microscopy studies conducted to evaluate the DNA release efficacy of chitosan nanocomplexes revealed that a large amount of DNA was released from the complex after 8 h incubation time. This demonstrates that chitosan can potentially be used as a DNA vaccine delivery system overcoming the low immunity of conventional DNA vaccines.

Thirdly, encapsulation of antigens in chitosan is highly stable. Increasing antigen stability has been a significant motivator for further development of traditional vaccines to maintain their efficacy during storage and transport. Prego et al. [39] reported that antigens positioned in chitosan-based polymers have maintained stability for about 3 months at room temperature. Similar results were obtained by Premaletha et al. [40], who demonstrated that using biodegradable chitosan as a vaccine carrier that internalizes the hepatitis B surface antigen provided high stability at room temperature for about 4 months and indicated signs of overcoming the incompleteness of vaccine application such as storage cold chain.

Finally, chitosan's bioadhesive properties make it possible for delivery to occur via the nasal or oral mucosa, which has many advantages over the conventional routes of injection. Oral inoculation overcomes the disadvantages such as use of disposable products, necessity of professional manpower and safety issues of the classical invasive vaccination method. Oral administration of vaccines resulted in higher patient acceptance, compliance, and manageability. Moreover, oral vaccination makes mass inoculation possible, having a significant impact on vaccination in developing countries [41]. Valero et al. [42] performed oral immunization with chitosan to protect European sea bass (*Dicentrarchus labrax*) from nodavirus. A DNA vaccine against nodavirus was encapsulated in chitosan and delivered to the gut by the oral route. The European sea bass vaccinated in this manner had a lower mortality rate and nodavirus incidence.

Mishra et al. [43] reported that oral administration of *Lotus tetragonolobus* (LTA)-anchored chitosan nanoparticles had high mucosal immunization efficiency. The researchers chose LTA to conjugate chitosan due to its ability to target microfold cells (M cells) of the Peyer's patches. According to their data, LTA-anchored chitosan can accurately deliver antigen specifically to M cells. Moreover, while LTA-conjugated chitosan nanopolymers showed clear binding to M cells in mice, no binding was observed in the case of conventional nanoparticles. Cytokine assay experiments confirmed that unlike conventional vaccines, oral immunization evoked both cellular and humoral immune responses. *In vivo* immunization studies have also demonstrated that LTA-anchored chitosan nanoparticles have significantly higher antibody titers than conventional nanoparticles.

In addition to oral immunization, nasal administration is possible with chitosan because of its mucosal adhesion properties and its inherent ability to open tight junctions. Nasal passages provides an effective route for the vaccines, offering a more suitable environment than the acidic and enzyme-rich one of the stomach. However, nasal administration requires increased retention time of the antigen or protein in the nasal cavity. The natural properties of chitosan to enhance the immune response and open tight junctions in the nasal cavity have been widely leveraged to overcome this challenge [44]. A number of studies have examined the advantageous inherent properties of chitosan as a vaccine carrier. Additional studies are listed in Table 16.2.

Table 16.2 Current development and potential strategies of using chitosan as a vaccine delivery system

Modification of chitosan	Pathogen	Route of immunization	Detected immune response	References
Poly(vinyl alcohol)-coated chitosan	Recombinant hepatitis B surface antigen	Wister rats fed via oral route	IgG	[45]
Mannosylated phenylalanine grafted chitosan	Plasmid-coded hepatitis B antigen	BALB/c mice injected via intradermal route	Th1, Th2	[46]
N,N,N-Trimethyl chitosan (TMC)	Hepatitis B virus surface antigen (HBsAg)	BALB mice vaccinated via intranasal route	IgA, IgG	[47]
TMC	Whole inactivated influenza virus (WIV)	C57-BL/6 mice vaccinated via intranasal route	IgG	[48]
N-phosphonium chitosan	Ovalbumin (OVA)	BALB/c mice injected via intramuscular route	IgG1, IgG2a (Th1, Th2)	[49]
Modified porcine circovirus type-2 (PCV2) vaccine with chitosan oligosacchrides	PCV2 antigen	C57BL/6 mice injected via intramuscular route	Th1, Th2	[50]
Mannosylated chitosan (MCS)	Multi-epitope *Mycobacterium tuberculosis* (TB) DNA vaccine	C57BL/6 mice vaccinated via intranasal route	Secretory IgA (sIgA), IgG	[51]
Phosphorylated chitosan (PCS)	OVA	BALB/c mice injected via intramuscular route	IgG, Th1, Th2	[52]
N-(2-hydroxy) propyl-3-trimethylammonium chitosan chloride	Hepatitis E virus (HEV) recombinant polypeptide	BALB/c mice injected via intramuscular route	IgG, Th1, CD4+, CD8- T cell proliferation	[53]
N-2-hydroxypropyl trimethyl ammonium chloride chitosan	Newcastle disease viruses (NDV)	Specific pathogen-free (SPF) chickens vaccinated via intranasal and oral route	IgG, IgA	[54]
Quaternized chitosan microgels	H5N1 (A/AnHui/1/2005(H5N1)) split vaccine	BALB/c mice injected via intramuscular route	Th1, Th2	[55]
Glycol chitosan (GC)	Hepatitis B surface antigen	New Zealand white rabbits and BALB/c mice vaccinated via intranasal route	IgG, IgA, Th1, Th2	[56]
Methylglycol chitosan	Detergent-split influenza (flu) viruses	BALB/c mice vaccinated via sublingual route	IgG, IgA, Th1, Th2	[57]
Chitosan coated with sodium alginate	Measles antigen	BALB/c mice fed via oral route	IgG, IgA	[58]
Glucomannosylated chitosan	Tetanus toxoid	BALB/c mice vaccinated via intramuscular and oral route	IgG, sIgA, Th1, Th2	[59]
Chitosan-coated poly(lactic-co-glycolic)	The Newcastle disease virus (NDV) F plasmid DNA	SPF chickens vaccinated via intramuscular and intranasal route	IgG, IgA	[60]
MCS	Foot and mouth disease (FMD) virus full length-VP1 gene and outer membrane protein A (Omp A) gene of *Salmonella typhimurium*	Guinea pigs vaccinated via intramuscular and intranasal route	IgG1, IgG2a, IgA	[61]
Glucomannosylated chitosan	Bovine serum albumin (BSA)	BALB/c mice vaccinated via intramuscular and oral route	IgG, sIgA	[62]
Chitosan-coated liposomes	Plasmid pRc/CMV-HBs encoding the small protein of hepatitis B virus	BALB/c mice fed via oral route	sIgA	[63]
TMC	WIV	C57-BL/6 mice vaccinated via intranasal route	IgG1, IgG2a	[64]

16.2.2 Recent Advances in PLA-Based Nanopolymers Against Hepatitis B Infection

PLA is biodegradable, and may be hydrolyzed by enzymatic activity in a physiological environment. Moreover, PLA is biocompatible, approved by the FDA, and can be adapted for various applications such as for medical implants, dermatology, cosmetics, drug delivery system, and vaccine carriers [65].

However, PLA elicits a relatively weak immune response due to the instability of the internalized antigen [66]. Strategies reported to overcome the hydrophobicity of PLA and improve the stability of the enclosed antigen include: wrapping liposomes loaded with the DNA of the hepatitis B virus with PLA [67]; stabilizing PLA-based vaccine carriers by crosslinking with chitosan [68]; and coating PLA with cationic polymers such as chitosan, polylysine, and polyethylenimine to enhanced hepatitis B antigen adhesion capability [69].

The immune responses caused by nanoparticles are influenced by several physiochemical characteristics, including size [70], surface charge [71], and shape [72]. Kanchan and Panda [73] reported that differential interactions between macrophages and PLA-encapsulated hepatitis B antigen were dependent on particle size. They also demonstrated that bigger PLA carriers (2~8 μm) elicited higher antibody titers than smaller vaccine carrier (less than 1 um), but other studies have reported the opposite, where the use of large particles reduced cellular uptake in macrophages. To clarify the relationship between the particle size and cellular uptake, fluorescence-quenching studies were performed in the presence or absence of an uptake inhibitor (cytochalasin D). The results showed that nanoparticles ranging from 200 to 600 nm were internalized into macrophages. Smaller microparticles (2~8 μm) were not internalized, but instead attached to the surface of macrophages. Cytokine assays have demonstrated the effect of PLA particle size on the regulation of the immune response. These data indicate that using nanoparticles elicits both Th1 and Th2 immune responses with increased particle size. Taken together, these results demonstrate that the inherent properties of PLA, especially its size, play an important role in its efficacy as a vaccine carrier.

PLA has been used as a vaccine delivery carrier along multiple routes, such as oral [74], mucosal [75], nasal [76], and injection [77]. Recently, novel vaccine delivery pathways have been proposed for PLA as a hepatitis B vaccine carrier. Jain et al. [78] developed a PLA vaccine system with delivery to the gastrointestinal tract (GI tract) via oral immunization. Unlike the conventional invasive vaccine delivery, oral vaccination produces secretory IgA (sIgA), which plays a role in protecting mucosal surfaces from invasion by pathogens and neutralizing toxic byproducts from pathogens [75]. However, oral immunization was necessary to stabilize the antigen and to avoid its degradation in the harsh conditions of the GI tract. To circumvent these problems, the researchers enhanced the stability of PLA nanoparticles by PEGylation.

Another vaccination approach is to use the pulmonary process. Thomas et al. [79] were the first to apply an aerosolized PLA hepatitis B vaccine. A pulmonary vaccine is less infectious than traditional vaccines administered through needles. Moreover, current invasive hepatitis B vaccines can only produce a humoral immune response, while aerosolized pulmonary vaccines elicit both humoral and mucosal immune responses. Antibody production can also be increased with lower amounts of antigen. Pulmonary route has a stronger immune response because the antigen is transmitted through the mucous.

Several studies have been conducted to supplement the method of injection vaccination. While a traditional vaccine requires several injections, a PLA-based hepatitis B vaccine can trigger an immune response with only one shot. A single inoculation can minimize possible complications stemming from needle usage during inoculation. Also, single-shot vaccination only needs a small amount of antigen [80].

Current reports offer various strategies for modification of PLA-based carrier systems to boost the immune response and to increase the

stability of their cargo. In addition, many studies have demonstrated that the route of immunization when using PLA can influence the therapeutic response. However, thus far, few clinical trials have evaluated the use of PLA nanoparticles as a vaccine delivery system to treat hepatitis. Therefore, more clinical studies are needed before PLA is proved a useful vaccine carrier and employed in practice.

16.2.3 Recent Developments of PLGA-Based Nanocarrier Hepatitis B Vaccine

Poly (Lactide-co-Glycolide) (PLGA) is synthesized by a ring-opening copolymerization of two monomers: glycolic acid and lactic acid [81]. PLGA can be degraded via hydrolysis of the polyester bond and can be cleared from the body through renal filtration. Furthermore, PLGA shows low cytotoxicity *in vivo*, which allows for various biomedical applications such as tissue engineering, diagnosis, immunology, and drug delivery [82].

Generally, DNA vaccine carriers are formed by the electrostatic interaction between positively charged carriers and negatively charge DNA. Since the surface charge of PLGA is negative, the emulsion solvent evaporation method has been used to transmit antigens. Eratalay et al. [83] developed three DNA vaccine carriers by using two different methods: water-in-oil-in-water (w/o/w) and oil-in-water (o/w). The w/o/w method was used to contain pDNA in an inner water-PLGA emulsion. In the o/w method, pDNA was positioned in two different formulations: in the outer phase of PLGA, and on the surface of PLGA. In order to confirm the loading efficiency of the nanoparticles, experiments were conducted to compare the amount of DNA extracted from the nanoparticles with the amount of initial DNA. The loading efficiency analysis revealed that loading pDNA on the surface of PLGA was most efficient (45%). In addition to loading efficiency, the release of pDNA was studied *in vitro* at pH 7.4 and 37 °C for 45 days. The release studies showed that carrying pDNA on the surface of PLGA through o/w method was threefold higher than that of the other two carriers. *In vivo* immunization using nanoparticles was performed to examine the titers of hepatitis B antibodies in the serum; the lowest level of immune response was observed from pDNA internalized in PLGA, due to the limited encapsulation and releasing efficiency of this carrier form. The highest immune response was elicited by the formulation in which pDNA was located on the surface of PLGA by the o/w method, in which a large amount of antigen was loaded.

However, the emulsion solvent evaporation method adversely affects the stability and aggregation of the antigen from synthesis to lyophilization. To overcome this challenge and increase antigen stability, much attention was given to the modification of PLGA. Paolicelli et al. [84] synthesized PLGA coated with chitosan to enhance antigen stability. Chitosan-coated PLGA nanoparticles become positively charged, but did not differ significantly in terms of particle size. The *in vitro* stability study established that, even after 14 days at 4 °C, 90% of the antigen could be activated. Moreover, chitosan-coated antigen was found to be slightly more stable than freeze-dried antigen. Xu et al. [85] reported that the stability of the antigen was enhanced by co-encapsulating PLGA with the hepatitis B antigen and human serum albumin (HSA). *In vitro* release studies revealed that polymer nanoparticles without HSA showed 60% antigen stability, whereas the co-encapsulated polymer nanoparticles exhibited 90% stability. Furthermore, Xu demonstrated that hepatitis B antigens were released from co-encapsulated polymer nanoparticles for more than 70 days.

Injectable PLGA polymers are promising candidates for vaccine delivery, as they can simplify immunization and increase antigenicity of encapsulated antigens. The ability to encapsulate an antigen in PLGA can trigger an immune response in a single dose. On the other hand, some of the traditional carriers require additional immunization. Feng et al. [86] demonstrated that the size of PLGA nanoparticles with different molecular weights is correlated with efficacy of single-dose vaccination. Large particles with higher

molecular weights (>30 μm) remained at the injection site because they were too large to undergo phagocytosis. Thus, they continuously stimulate the immune system and require boosting dose at the injection site. In case of the smaller particles of low molecular weights (<10 μm), phagocytosis occurs easily and can rapidly induce reaction to the antigen through the lymph node. The rapid migration of antigens accelerates the production of antibodies. The combined use of both large and small PLGA nanoparticles can allow for single-dose vaccination. To demonstrate this, a single-dose immunization was performed, and the results were monitored for 3 months, a length of time comparable to the 3-dose aluminum-B hepatitis vaccine. Similar results have been reported by other groups also. Saini et al. [87] examined the effects of a single-inoculation vaccine containing PLGA of two different molecular weights. *In vitro* observations revealed that the antigen was continuously released from the nanoparticles for 28 days.

Multiple routes are used in vaccinations using PLGA. The PLGA-based hepatitis B system, established through oral vaccines, offers high safety, low cost, and better patient suitability. Mishra et al. [88] reported a hepatitis B vaccine in which PLGA was supplemented with lectin to target M cells in the GI tract and used to deliver the vaccine to the mucosa via oral administration. The lectin-supplemented PLGA enhanced the efficiency of vaccine delivery transporting the encapsulated antigen to the desired position in the intestinal Peyer's patches. To confirm the targeting ability of these nanoparticles, confocal microscopy was used to examine M cells in the Peyer's patches of mice that had been orally vaccinated. FITC-labeled LTA-PLGA polymer nanoparticles were, in fact, observed in M cells, proving the targeting capabilities of LTA. Another study evaluated the M cell targeting efficiency of PLGA when supplemented with different lectins. Of these, PLGA nanoparticles bound to *Ulex europaeus* agglutinin 1 (UEA-1) were most effective at targeting M cells eliciting high immunological activity [89].

Pulmonary administration of vaccine was found to abrogate drawbacks such as high infection risk and disposable discards associated with traditional injections vaccine. Mutti et al. [90] demonstrated the potential of pulmonary inoculation with PLGA containing dried and powdered hepatitis B antigen. A vaccine in liquid form has a shelf life determined by the length of stable antigen presence, but this limitation does not apply when the vaccine is in powdered form. Guinea pigs were administered vaccinations either by the pulmonary route or by injection in order to compare the amounts of hepatitis B antibody elicited by both methods. Pulmonary vaccination was found to elicit fivefold higher IgA antibody titers than intramuscular injection. PLGA has been frequently studied and is the most popular material to use for vaccine delivery. It has been modified in various ways for more efficient use, and corresponding studies are summarized in Table 16.3.

16.2.4 Nanocarrier Vaccines Based on Other Polymeric Nanoparticles

Recently, a number of studies have demonstrated the efficacy of chitosan, PLGA, and PLA as vaccine carriers. These nanoparticles are also frequently used as hepatitis vaccine carriers due to their potent bio-functional activities. Similar properties can be found in other polyester and polyamide nanoparticles, however, these have not been widely used in vaccine delivery due to low accessibility and inefficiency in antigen transport. Therefore, to overcome these limitations, researchers modified their structures by cross-linking with other efficient working molecules to boost immunization [111].

Poly (ε-caprolactone) (PCL) is a potential injectable polyester biomaterial that has been shown to be absorbed into tissues *in vivo*. It can be absorbed through the mucosal membrane, has low toxicity, and is readily available and hydrophobic. Due to these properties, PCL is widely used in not only drug delivery, but also vaccine delivery systems [112]. In addition, PCL is biodegradable, but degrades more slowly than PLGA. Jesus et al. [113] synthesized PCL-coated

Table 16.3 Current development and potential strategies of PLGA as a vaccine delivery system

Modification of PLGA	Pathogen	Route of immunization	Detected immune response	References
PLGA coated with chitosan conjugated with M cell homing peptide (CKS9)	Membrane protein B of *Brachyspira hyodysenteriae* (BmpB)	BALB/c mice fed via oral route	IgG, IgA, Th1, Th2	[91]
(3β-[N-(N′, N′-Dimethylaminoethane)-carbamoyl]cholesterol hydrochloride-PLGA	OVA	C57BL/6 mice injected via intramuscular route	IgG1, IgG2a (Th1, Th2)	[92]
Chitosan-coated PLGA	Plasmid DNA-encoding surface immunogenic protein (sip) of tilapia *S. agalactiae*	Tilapias vaccinated via intranasal route	Th1, Th2	[93]
PLGA modified with polyethylenimine (PEI)	BSA	BALB/c mice injected via intradermal route	IgG, Th1	[94]
PLGA-PEG-PLGA	OVA	BALB/c mice injected via intramuscular route	IgG, Th1, Th2	[95]
PLGA-poly-L-lysine/poly-γ-glutamic acid (PLL/γPGA)	Ebola virus DNA	Mice administered via microneedle through skin route	IgG	[96]
Chitosan-coated PLGA and glycol chitosan coated PLGA	HBsAg	BALB/c mice vaccinated via intranasal route	IgG, sIgA, Th1, Th2	[97]
1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)- hyaluronic acid (HA)-PLGA	OVA	C57BL/6 mice injected via intramuscular route	IgG, Th1, Th2	[98]
PLGA-TMC (N-trimethyl chitosan) and tri-polyphosphate (TMC-TPP)	OVA	BALB/c mice injected via intramuscular route	IgG, sIgA	[99]
UEA-1 conjugated PLGA-lipid	OVA	BALB/c mice injected via intramuscular route	IgG, IgA	[100]
Lipid-coated PLGA	OVA	BALB/c mice injected via intravenous route	IgG1, IgG2a	[101]
Dimethyldioctadecylammonium (DDA) bromide-PLGA	Major outer-membrane protein (MOMP) of *Chlamydia trachomatis*	B6C3F1 mice injected via subcutaneous route	IgG1, IgG2a, IgA	[102]
Cellobiose-coated PLGA	Non-toxic recombinant subunit B (SbB) of diphtheria toxin	BALB/c mice fed via oral route	IgG, IgA	[103]
PEG-PLGA and poly (ε-caprolactone-b-ethylene glycol) (PEG-b-PCL)	Melanoma associated antigens	C57BL/6 mice injected via subcutaneous route	IgG1, IgG2a (Th1, Th2)	[104]
Mono-phosphoryl lipid A (MPLA)-PLGA	OVA	BALB/c mice injected via intradermal route	CD4+, CD8+ T cell	[105]
MPLA-PLGA	Hepatitis B virus core antigen (HBcAg)	C57BL/6J mice injected via subcutaneous route	IgG, IgM, Th1, Th2	[106]
MPLA-PLGA	HBcAG	C57BL/6 mice injected via subcutaneous route	IgG, Th1, Th2	[107]
7-Acyl lipid A-PLGA	Melanoma antigen tyrosinase-related protein 2 (TRP2)	C57BL/6 mice injected via subcutaneous route	CD 8+ T cell	[108]
MPLA-PLGA	TRP2, hgp100	C57BL/6 mice injected via intradermal route	CD 8+ T cell	[109]
CpG ODN-chitosan-PLGA	OVA	BALB/c mice injected via intradermal route	IgG, Th1, Th2	[110]

chitosan nanoparticles to encapsulate the hepatitis B antigen and nasally administered them in order to assess the immune effect elicited by delivery to the mucosa. The mucin uptake of both uncoated and PCL-coated chitosan nanoparticles was measured to evaluate mucosal delivery. High levels of mucin uptake were detected for both types of nanoparticle, but were slightly lower for the PCL-coated chitosan nanoparticles. These results can be attributed to different interactions of the nanoparticles with mucin. Mucin absorption with chitosan nanoparticles is caused by electrostatic interactions between the sialic acid of mucin and the amino groups of chitosan. The cell monolayer near the mucosa was evaluated using Z-stack confocal microscopy, with cross-sections captured every 32 μm. The Z-stack revealed that the nanoparticles were not observed in the mucosa but were found in the cells. It also revealed that most of the nanoparticles were present in the upper part of the cells, while there was a significantly reduced amount of nanoparticles in the deepest part of the mucosa. This proved that the nanoparticles diffused into the cell monolayer near the mucosa. In addition, researchers measured the levels of anti-hepatitis B sIgA and total sIgA 4 days after immunization to compare the vaginal or nasal routes of mucosal administration. Nasal administration elicited tenfold higher IgA levels than vaginal administration. These results indicated that nasal administration may lead to the production of extra-specific antibodies.

Dinda et al. [114] assessed whether oral administration with a PCL-based vaccine carrier could be used for mucosal delivery and mass immunization against hepatitis B. To determine the influence of PCL nanoparticles in an oral vaccine, fluorescence microscopy was used on samples collected from mice after 6 h of inoculation. Nanoparticles were found in the lymph nodes, indicating that the vaccine could quickly draw out an immune response. Moreover, even 2 months after administration, the nanoparticles remained in the lymph nodes, spleen, and small intestine. Due to the persistency of its presence at the administration site and the slow degradation of PCL, the nanoparticle fluorescence intensity after 2 months was approximately half of that observed 6 h after vaccination. In addition, to confirm the sustained release of antigen, the levels of anti-hepatitis B antibody elicited by different inoculation methods were compared for 2 months. The results confirmed that antibody levels were 1.5 times higher with oral vaccination than with intramuscular and intradermal injection. Furthermore, because PCL nanoparticles can boost immunization to necessary levels by continuously releasing antigen, additional inoculation was not required. Based on these results, PCL can be considered a potential nanoparticle for single-dose vaccination in mass immunization attempts.

PLL is a kind of polyamide nanoparticle that has high antigenic effect. It is an antibacterial substance secreted by *Streptomyces* microorganisms, and can be obtained from extra-cellular materials [115]. It is also biodegradable and biocompatible [116]. Non-toxic PLLs have a variety of applications, including in cosmetics, medical care, and biomaterials [117]. The backbone of PLL is positively charged, which makes it easy to transport negatively charged drugs or DNA. Therefore, as a carrier, PLL is useful for both gene and drug delivery [118]. To confirm the enhancement of immune efficacy, Machluf et al. [119] developed an alginate-PLL hydrogel with phosphatidylcholine and cholesterol, which encapsulated the hepatitis B antigen. In order to investigate the antigen release of PLL-coated nanoparticles with different molecular weights (25 kDa and 214 kDa), the researchers compared the amounts of hepatitis B antigen *in vitro* over time. The 214 kDa PLL nanoparticle released 3.5-fold more antigen at the injection site than the 25 kDa PLL. In addition, the 241 kDa PLL nanoparticle layer was demonstrated to exhibit higher antigen permeability than the 25 kDa PLL nanoparticle layer, and antigen release was found to be controlled by molecular weight interactions. Furthermore, levels of anti-hepatitis B antibodies were measured to compare immunoreactivity with other carriers. These comparisons revealed that the overall reactivity was low for liposome and aluminum vaccine carriers, but was much higher for PLL-coated

nanoparticles. In addition, PLL-coated nanoparticles could elicit booster-dose levels of anti-hepatitis B antibodies up to 16 weeks after inoculation. This suggests that PLL-coated nanoparticles may function as antigen reservoirs at the injection site. The consequent sustained release can lead to stronger immune activation at the vaccination site, contributing to the recruitment of macrophages and APCs. In comparison, most liposome carriers were degraded by phospholipase at the site of inoculation, leaving only naked antigen. Naked antigen has low immunological activity because it induces a low humoral immune response. On the other hand, PLL-coated nanoparticles can elicit a significant immune response comprising both humoral and cellular immune responses. When comparing antibody levels measured 4 weeks from the first inoculation to those measured 12 weeks after secondary inoculation, PLL-coated nanoparticles led to a tenfold increase in antigen levels from the first inoculation to the secondary, but only a threefold increase compared to aluminum and liposome vaccine carriers. In summary, PLL nanoparticles could protect the antigen by serving as a reservoir and providing a means of sustained antigen release, offering improved vaccine quality.

16.3 Conclusion and Future Perspectives

Nanoparticle-based delivery systems have the potential to be used as vaccine carriers for a variety of diseases. In recent years, our knowledge of the influence nanoparticle-based hepatitis B vaccines on the immune system has expanded. Nanoparticle-based vaccination offer several advantages over the conventional immunization, including selective and controlled release of entrapped antigens to dendritic cells, reduced toxicity via hydrolysis its functional linkages under the physiological conditions, and decreased the serious side effects of parenteral vaccination through needle-free oral vaccination. Moreover, highly versatile and easily modified nanoparticles are suitable and efficient platforms for delivering large amounts of therapeutic payloads to the desired sites. However, further research is still necessary to determine the most promising vaccine carrier that can be utilized in clinics. In addition, current nanoparticles require modifications to improve the stability of bound antigen. While these limitations are yet to be solved, nanoparticle-based vaccine delivery systems are set to make impressive progress in hepatitis therapy.

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Part IV

Future Enabling Technologies for Regenerative Medicine

Biomaterials Developments for Brain Tissue Engineering

17

Eduarda P. Oliveira, Joana Silva-Correia, Rui L. Reis, and Joaquim M. Oliveira

Abstract

The Central Nervous System (CNS) is a highly complex organ that works as the control centre of the body, managing vital and non-vital functions. Neuro-diseases can lead to the degeneration of neural tissue, breakage of the neuronal networks which can affect vital functions and originate cognitive deficits. The complexity of the neural networks, their components and the low regenerative capacity of the CNS are on the basis for the lack of recovery, having the need for therapies that can promote tissue repair and recovery. Most brain processes are mediated through molecules (e.g. cytokines, neurotransmitters) and cells response accordingly and to surrounding cues, either biological or physical, which offers molecule administration and/or cell transplantation a great potential for use in brain recovery. Biomaterials and in particular, of natural-origin are attractive candidates owed to their intrinsic biological cues and biocompatibility and degradability. Through the use of biomaterials, it is possible to protect the cells/molecules from body clearance, enzymatic degradation while maintaining the components in a place of interest. Moreover, by means of combining several components, it is possible to obtain a more targeted and controlled delivery, to image the biomaterial implantation and its degradation over time and tackling simultaneously occurring events (cell death and inflammation) in brain diseases. In this chapter, it is reviewed some brain-affecting diseases and the current developments on tissue engineering approaches for a functional recovery of the brain from those diseases.

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Keywords

Brain · Biomaterials · Cells · Molecules · Tissue engineering

17.1 Introduction

The Central Nervous System (CNS) is a highly complex organ that controls vital and non-vital function of the body [57, 69]. Insults or diseases of the CNS lead to partial (e.g. stroke) [25], selective (e.g. Parkinson) [19] or broad degeneration of the neural tissue (e.g. ALS) [61], breaking the neuronal networks, affecting vital functions (e.g. breathing) and originating cognitive deficits (learning, memory) [69]. These diseases affect the patients' the quality of life (or even leads to their death), burdens the families and have a big socio-economic impact in the health systems, worldwide [75]. The complexity of the networks and the low regenerative capacity of the nervous tissue are the basis for the lack of recovery, having the necessity of therapies that promote the tissue's repair and recovery [35]. After an insult/cancer removal the brain tissue is injured, neurons are damaged or degenerating, inflammatory response exacerbates the damage, the extracellular matrix (ECM) is disrupted, and overall the tightly controlled brain homeostasis becomes compromised. Neurons are the control centre of the brain, receiving and transmitting information through small tracts for short distances (e.g. transfer within the brain) and long tracks for distant cells/tissues (e.g. transfer to the PNS) [57]. The neurons can be damaged physically by trauma or laceration (brain injury, stroke), or chemically/biologically, for example by genetic mutations, cancer or toxic compounds. After injury, the affected neuron will start a cell death process (apoptosis), but if the cell body is intact and functional (able to express growth-promoting genes) the neuron might survive, while atrophied (shorter axon) [6]. More rarely, axons distal to the damage degenerate and the proximal parts might recover by undergoing a growth cone mode (through intrinsic remodelling) and start sprouting from the damaged axon [73]. Nevertheless, new sprouts are unable to extend over long distances and/or to re-populate degenerated areas [6]. Moreover, is difficult to connect new axons with the correct target (e.g. motor neurons connecting to other motor neurons) and to form active neuronal networks that will allow the synaptic transfer of information [68]. Thus, the repair of the surviving cells and/or replacement with new functional cells is in great need [71]. Molecules administration and/or cell transplantation hold great potential for brain recovery, since the injured CNS has lower levels of growth-promoting molecules and the injury affects all interconnected cells [4, 14, 18, 31]. Biomaterials aid cell/molecule administration by protecting from body clearance, enzymatic degradation and hostile site environment [23, 65]. Biomaterials can also provide an ECM-like microenvironment to which transplanted and host cells can adhere and proliferate/differentiate, thus improving the changes of engraftment and survival [23, 71]. Natural-based materials are attractive candidates since they are present or contain molecules of the ECM, which increases their cytocompatibility and decreases the possibility of immune rejection [76]. Moreover, by combining several components, it is possible to obtain a more targeted delivery (e.g. targeting only cancer cells), to allow molecules to overcome the blood brain barrier (BBB) that otherwise would not pass, to image the biomaterial implantation and its degradation/fate over time or to tackle simultaneously occurring events (e.g. cell death and inflammation) in brain diseases [5, 23]. Herein, brain-affecting diseases and the current developments on tissue engineering approaches for brain recovery are overviewed and discussed.

17.2 Structure and Function of the Brain

The nervous system is composed by the CNS and peripheral nervous system (PNS). The CNS is divided into the brain and spinal cord and they allow the brain, the most complex organ in the human body, to control from the most basic and unconscious functions (visceral function, breath-

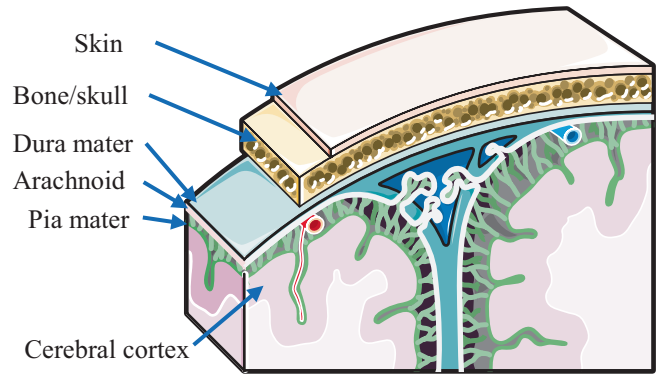

Fig. 17.1 Skull and meninges that protect the brain. (Images were adopted from Servier Medical Art by Servier (https://smart.servier.com) and modified by the authors under the following terms: CREATIVE COMMONS Attribution 3.0 Unported (CC BY 3.0))

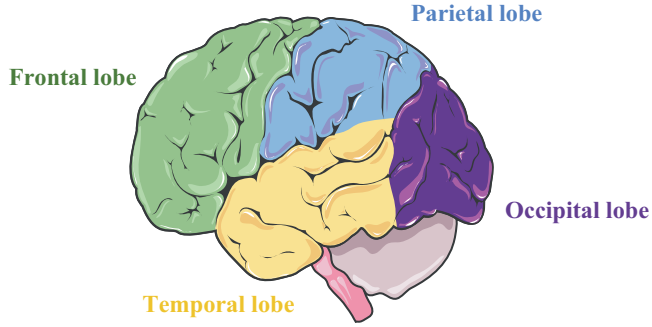

Fig. 17.2 The four lobes of the cerebral cortex. (Images were adopted from Servier Medical Art by Servier (https://smart.servier.com) and modified by the authors under the following terms: CREATIVE COMMONS Attribution 3.0 Unported (CC BY 3.0))

ing) to the most complex cognitive capabilities (short and long term-memory, and learning) [52]. This sensitive structure is protected by the skull and by membranes of connective tissue (meninges), termed the dura, the arachnoid and the pia mater (Fig. 17.1). Further protection is offered by the cerebrospinal fluid (CSF) that works as a shock absorber of external blunts/forces and also hydrates and nourishes the brain.

The current classification of the brain subdivisions are: (1) the cerebrum (bigger area of the brain), (2) the cerebellum ("little brain"), and (3) the brainstem [57]. The structure connecting the brainstem with the cerebrum is called the interbrain (diencephalon) and is composed by the thalamus, hypothalamus and subthalamus. The brainstem is divided into the midbrain, the pons and the medulla oblongata [53] and the cerebrum into the left and right hemispheres (the largest compartments of the brain), which are joined by the corpus callosum. These compartments are composed by grey matter (outer tissue), often termed "cortex", and white matter (inner tissue) [57]. The cerebral cortex is divided into four zones or lobes, the frontal lobe, the parietal lobe, the temporal lobe and the occipital lobe, shown in Fig. 17.2 [53]. The wrinkles present in the brain surface are called *gyri* and the space that separates them are the *sulci*. The main gyri are the precentral gyrus, which contains the motor cortex (motor information) and the postcentral gyri that includes the somatosensory cortex (sensory information) [57]. Underneath those structures there are another important components, namely the basal ganglia, the amygdala and the olfactory bulbs, and between all the structures there are fluid-filled cavities called ventricles. Near the hippocampus there is a region termed substantia nigra, which is one of the production "stations" of the neurotransmitter dopamine and neurons in this area are the most affected in Parkinson's disease [24].

Regarding the function of these structures, briefly, the cerebrum functions as the control centre for the brain, performing advanced functions has interpreting touch, hearing and vision and managing/transmitting information to form speech, reasoning, learning, emotion and also controls movement. The brainstem controls the breathing, heart rate, digestion and blood pressure and the cerebellum has a crucial role in learning motor tasks, planning and coordinating movements, balance and maintaining posture (involved in motor memory) [69]. The basal ganglia collectively collects, controls and sends motor and cognitive information to other regions of the brain. The hippocampus and amygdala have a role in memory and emotional behaviour. The olfactory bulbs, as the name suggests, receive, control and process the chemosensory information transmitted from the nasal cavity. The thalamus has a vital role on managing and transmitting sensory and motor input and the hypothalamus is responsible for the homeostasis of the body (controls body temperature, circadian rhythms and the autonomous action of drinking and eating) [57].

From the cellular point of view, the brain tissue is composed by neurons, vascular cells, neuroglial cells that are neuron-supportive cells (glia means glue, "neuroglue") and choroid plexus cells (CSF-secreting cells). The neuroglia is divided into microglia and macroglia. Microglia are cells of undetermined characterization related to the lineage of macrophages/monocytes, which act as the immune system of the brain. The macroglia is composed by astrocytes and oligodendrocytes. Astrocytes have a supportive role on the brain metabolism and synapsing, deliver energy intermediates to neurons and maintain the tissue "clean" from excessive extracellular neurotransmitters and other molecules. Oligodendrocytes are called myelin-producing cells because they wrap their compacted membranes around a neuron and form multiple layers, termed myelin, which insulates the long axons of the neuron, accelerating the velocity of the action potential, making the synaptic (information) transmission quicker and more efficient [69]. Neurons pass information to other neurons through synapses, where a pre-synaptic axon releases neurotransmitters that are collected by the post-synaptic dendrite or cell body of the target neuron. The neurotransmitters are released into the synaptic cleft and transported by synaptic vesicles [69]. The grey matter of brain is formed by neurons' cell bodies, axon terminals (nerve fibres), dendrites, glial cells and vessels, while the white matter has few vessels and is composed by bundles of axons (tracts), most of them myelinated (myelin gives the tissue its white colour) and oligodendrocytes [52]. Neurons are all interconnected between them and with the neuroglia, making zillions of synaptic connections that maintain the homeostasis of the body, and can allow performing day-to-day tasks [63] (Fig. 17.3).

17.3 Considerations for Brain Repair/Regeneration

When considering neurorepair/neuroregeneration, we should take into account the brain's low endogenous capacity of self-regeneration. Moreover, neurogenesis - production of neurons from neural stem cells in the brain is scarce, decreasing as age increases, i.e. mature neurons are unable to divide as other cells [57]. Another important consideration is that surviving cells are hindered/limited by the hostile and non-regenerative environment that is created on the post-damaged brain. Some steps need to be achieved in order to establish an adequate repair/regeneration [68]. The most relevant are described in Fig. 17.4.

17.4 Different Brain Pathologies

17.4.1 Traumatic Brain Injury

TBI can be caused by a blunt, laceration or penetrating force (with or without skull fracture) as a consequence of vehicle accidents, falls and sports or military related activities. TBI affects more than 57 million people, worldwide [36]. The statistics reveal that 52,000 deaths and 124,000 disabilities were caused by this pathology. TBI can be categorized as mild, moderate or

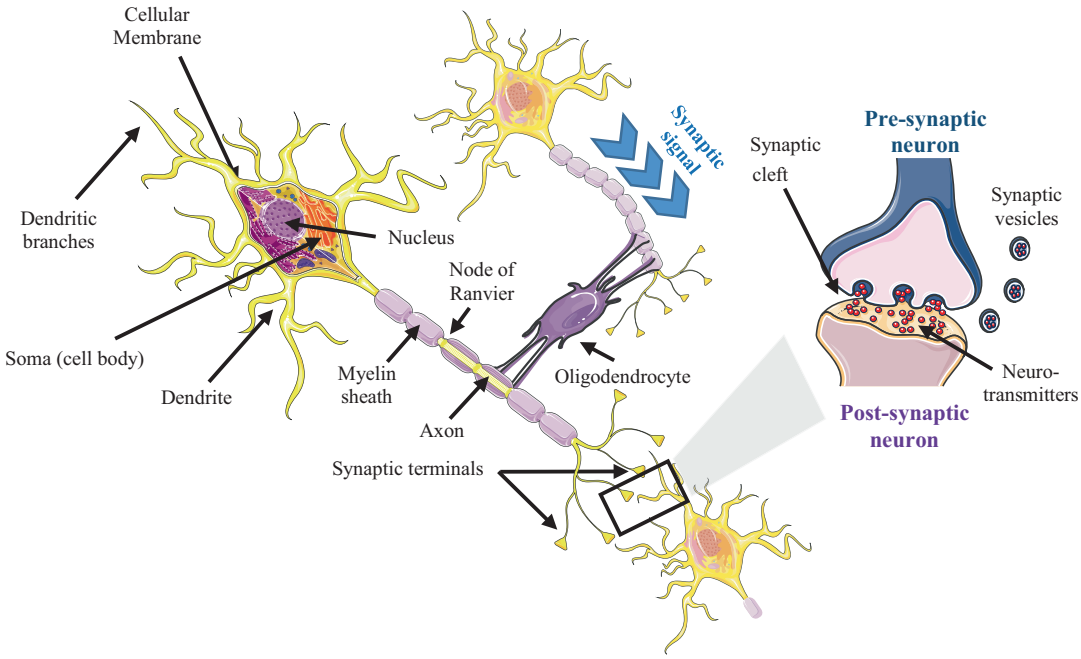

Fig. 17.3 Anatomical view of a neuron and its synaptic processes. (Images were adopted from Servier Medical Art by Servier (https://smart.servier.com) and modified by the authors under the following terms: CREATIVE COMMONS Attribution 3.0 Unported (CC BY 3.0))

severe. Depending on the extent of the lesion [21] that can be focal (localized injury), diffused (spread injury) or a mix of both, brain compression (increased intracranial pressure), neuronal and vascular damage, tissue swelling and hypoxic damage can occur. These are the first events that appear in TBI and are called the "primary injury" (neuronal damage, haemorrhage, tissue laceration). The downstream events are entitled "secondary injury" as they are a consequence of the primary events, and can occur from hours up to 14 days after the initial insult to the brain, exacerbating the damage [36]. The secondary injury is typically characterized by excitotoxicity (excessed production of neurotransmitters), oxidative stress, inflammatory response, astrogliosis (activation of inflammatory astrocytes), edema and hypoxia. More severe cases can present extensive loss of the brain parenchyma originating cavities on the lesion site. Often, the formation of a glial scar can be observed, which is a mechanism to protect the remaining tissue from the damage, but at the same time hampers regeneration [15]. Patients with TBI that survive will experience prolong or permanent loss of motor and sensory function and/or cognitive deficits. There are no available treatments for TBI besides controlling the intracranial pressure, maintaining the cerebral perfusion and palliative care (rehabilitation, physiotherapy), which can give the patients a better quality of life [77].

17.4.2 Stroke

Stroke is a leading cause of death, worldwide. The most common type of stroke is the ischemic stroke that is characterized by an obstruction of brain's vascular structures, by a formed clot or a thinned vein, resulting in lack of blood supply to the affected area. Simultaneously, an inflammatory response initiates and increases the generalised cell death until a cavity of dead tissue (termed penumbra) surrounded by a glial scar is created. The second type of stroke, which causes the highest impairment and mortality rate among patients, is the haemorrhagic stroke (accounts for 15% of all strokes). This is caused by the rupture or degeneration of veins, small arteries or arteri-

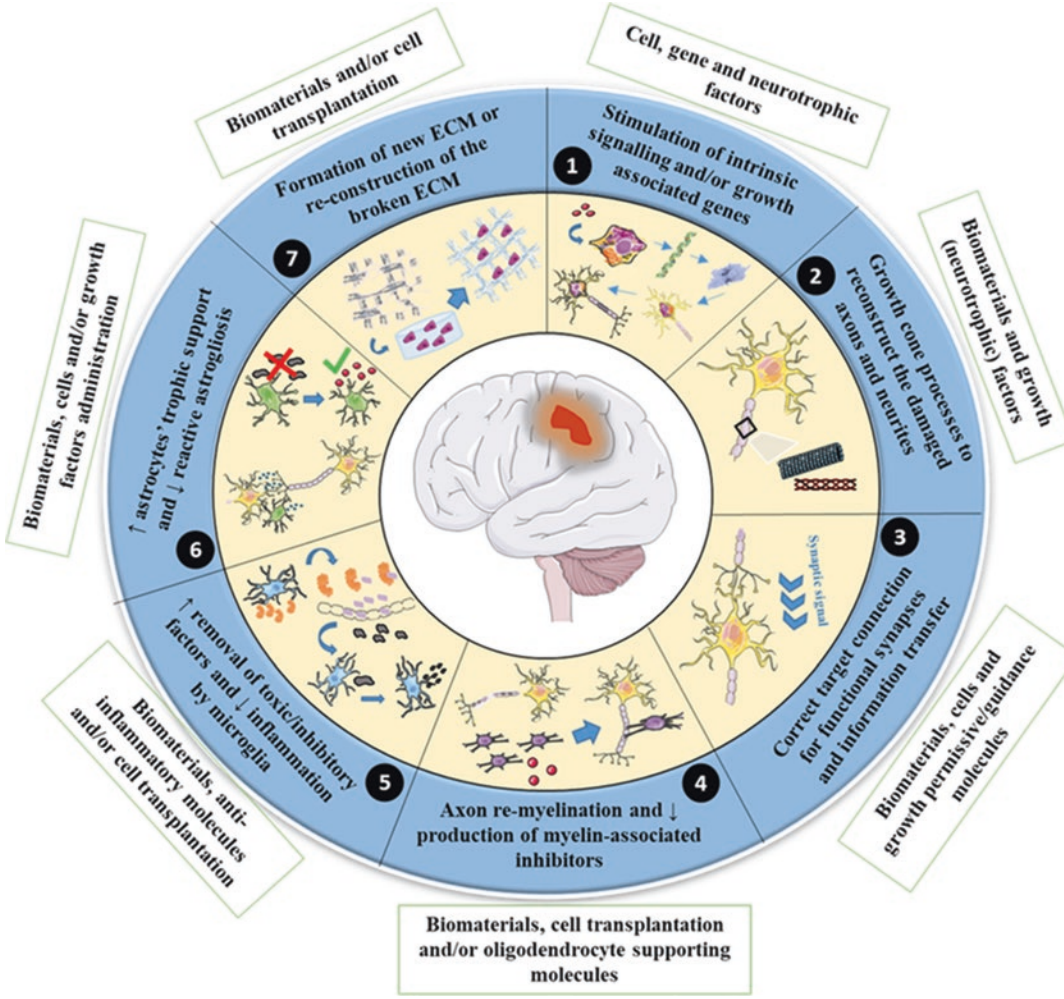

Fig. 17.4 Processes needed for neuronal regeneration/repair are described in the circle and corresponding strategies are mentioned in the green boxes. (**1**) After damage the neurons that survived (undamaged nucleus) can still express signalling and/or growth associated genes but to some extent, requiring trophic support by means of gene, cell and growth factor therapies to further increase this capacity (e.g. increase in cAMP levels encourages intrinsic repair processes) [7, 31]. (**2**) The trophic support is also needed for growth cone processes, to re-construct the damaged neurons' actin/intermediate filaments and microtubules [6]. Through the use of biomaterials is possible to give support to newly forming axons/neurites [71]. (**3**) Another pivotal event is the re-connection of neurons to correct targets, for the creation of functional synapses [12]. Biomaterials with cells and/or growth/guidance cues are appealing strategies because the biomaterials can aid the extension/elongation of axons and neurites by the creation of a permissive environment and molecules/cells might provide a guided growth [50]. (**4**) For the efficient transfer of synaptic information is essential the re-myelination of damaged axons by oligodendrocytes [66], which can be assisted by cell transplantation (e.g. glial-restricted progenitor cells) and/or biomaterials. Upon neurons' injury the damaged myelin accumulates in debris and produces myelin-associated proteins, which are known inhibitors for axonal growth (e.g. myelin-associated glycoprotein-MAG, Nogo-A) [80]. Some approaches to decrease the levels of myelin inhibitors include the use of biomaterials with cells that secrete anti-myelin factors or biomaterials coupled with myelin antagonists' proteins, for example [43]. (**5**) Microglia protects against toxic molecules and removes debris from damaged/dying neurons [62]. However in injury scenarios the exacerbated inflammatory response causes further damage and needs to be controlled [41], with for example anti-inflammatory molecules or through transplanted cells paracrine actions, which can be further combined with biomaterials to potentiate their effect [17]. (**6**) Astrocytes have an important role in trophic and metabolic support to neurons (e.g. synapses maintenance), they are permissive to newborn axons and can provide a guided growth [79].

oles (mostly due to sustained hypertension or age related, but can also be by trauma or malformed vascular system), originating a fast loss of blood into the brain parenchyma, decreasing the intravascular pressure, and increasing the intracranial pressure [11]. It is called a progressive stroke because has the brain bleeds out, the patient health rapidly, and progressively deteriorates. Depending on the type of affected vein, stroke's violence and time of treatment, patients can partly recover from stroke, whilst having neuronal impairments for life. Current treatments for ischemic stroke includes reperfusion via intravenous thrombolysis for acute ischemic stroke, followed by patient rehabilitation [8]. However, reperfusion is often ineffective and results in haemorrhagic complications, besides being limited by a narrow therapeutic time window, where only a small percentage of affected patients benefit from such treatment strategy [51]. Regarding the haemorrhagic stroke, patients with mild stroke undergo conservative treatment (e.g. vital signs monitoring and support). For advanced cases, surgery is the only option (e.g. blood drainage and vein/artery repair) [13].

17.4.3 Progressive Neurodegenerative Diseases

17.4.3.1 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS), a fatal progressive disease, is estimated to affect around 2 persons in each 100,000 a year in Europe [37]. Around 90% of the affected population has the sporadic form of ALS. The other 10% suffers from hereditary ALS (familial ALS), being 20% of which caused by mutations in the SOD1 gene, the first ALS-related gene to be discovered [60]. ALS is characterized by the degeneration and/or loss of upper motor neurons in the motor cortex and lower motor neurons in the brainstem and spinal cord. ALS patients display symptoms of muscle weakness and/or atrophy, cramps, dysarthria and dysphagia (caused by bulbar weakness). Over the years they suffer from progressive paralysis and ultimately die by respiratory failure (3–5 years after the first symptoms start to manifest) [27]. The diagnosis is performed by a neurological examination to assess the patients' motor function state, namely the presence of hyperreflexia (overexcited or hyper-responsive reflexes) of motor segments and muscular weakness or atrophy. Is also measured their response to plantar stimulation (Babinski response), which in ALS patients causes the fingers to arch up directing to the foot (positive Babinski response) [2]. The scope on ALS pathological events is still misunderstood. The mechanisms contributing to the disease include oxidative stress, mitochondrial dysfunction, protein aggregation, excitotoxicity, glial cell dysfunction [27] and gene defects [49]. In familial ALS, gene mutations lead to the creation of toxic free radicals and accumulations of intracellular deposits, which will obstruct the proteasome and/or chaperone activity. This mutated proteins get misfolded, leading to the inability to clear or exclude them from the cell [60]. A molecular event characteristic of sporadic ALS is the loss of normal TDP-43 (primarily a nuclear protein) in the motor neurons' nucleus and presence of cytoplasmic ubiquitinated inclusions of abnormal TDP-43-positive (pathological TDP-43) [81].

Fig. 17.4 (continued) However, upon damage astrocytes undergo reactive phenotype (astrogliosis) and start releasing growth-inhibitory factors (e.g. chondroitin sulfate proteoglycans) [84] and sometimes a glial scar is formed [28], which physically and chemically restricts the growth and extension of axons across the affected area [42]. Biomaterials might help neurons cross the scared tissue [38] and growth factors/cell administration can decrease the reactivity of the astrocytes and/or might even reverse their phenotype to unreactive/repairing astrocytes [29]. (**7**) Another important consideration is that in pathological events the brain natural ECM is damaged or lost, which hampers the regeneration (no physical and chemical support by the ECM) and also the accumulation of broken ECM debris (e.g. laminin) can be detrimental for axonal growth [67]. Biomaterials are promising approaches for the formation and/or re-structuration of the damaged or broken ECM and in combination with cells (e.g. Neural Stem Cell transplantation) this beneficial effect is even more pronounced [22]. (Images were adopted from Servier Medical Art by Servier (https://smart.servier.com) and modified by the authors under the following terms: CREATIVE COMMONS Attribution 3.0 Unported (CC BY 3.0))

TDP-43 (DNA and RNA binding protein) has a role in the regulation of transcription and splicing, in the processing of microRNA and stability of mRNA. In normal situations of trauma, this protein goes from the nucleus to the cytoplasm and originates granules that get expelled after injury recovery, indicating also its role in the healing process. However, in the cortex of ALS patients this protein gets phosphorylated, ubiquitinated and cleaved differently, forming an insoluble C-terminal fragment that aggregates inside the cytoplasm. This originates a lack of normal TDP-43 in the cells' nucleus, altering the neurons' normal functioning and therefore starting the death and degeneration process of the affected neuron [2]. Besides gene mutations, glial cell malfunction also has a big impact in this disease. It has been reported that the presence of ALS patients derived-astrocytes in co-cultures with neurons cause healthy motor neurons to die [27], indicating a severe negative influence of these dysfunctional cells in the disease onset/progression [59]. The only FDA approved drug for this pathology is the Riluzole [49]. This drug is a glutamate antagonist [61] that blocks the sodium channels. It reduces glutamate toxicity by impeding the glutamate release in the pre-synaptic terminal and inhibiting (non-competitive inhibition) the excitatory amino acids' action in the post-synaptic terminal. However, Riluzole provides only a slight protection that mildly extends the patient's lives (2–4 months), without improving their quality of life or causing an actual cessation of the disease and/or a regeneration effect on the surviving cells [49].

17.4.3.2 Alzheimer's Disease

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that causes the decay of brain function. AD is associated with the loss of cognitive capacity, loss of memory and inability to retain memories, and ultimately dementia [75]. AD can be genetically passed (familial AD) or sporadically occurring (sporadic AD) [3]. Approximately 10% of the global population is estimated to suffer from this pathology, which is considered to be the most common neurodegenerative disease affecting the elderly/aging individuals (mostly occurs in patients over 60 years old) [33]. The exact molecular cause for this pathology is still unknown but some events typically occurring in AD might be related or be the actual causer of the disease [75]. Brains with AD show the presence of insoluble extracellular fibrillar β-amyloid plaques (also termed senile or neuritic plaques), formed by the aggregation of amyloid-β (Aβ) peptides, which are the proteolytic derivatives of the transmembrane protein that cleaves them, the amyloid precursor protein (APP) [63]. Another characteristic of AD is the deposition and aggregation of intraneuronal hyperphosphorylated TAU protein (microtubule-associated protein) that forms neurofibrillary tangles, frequently in neuronal dendrites of cell bodies [75]. These aggregations impair the normal neuronal function by accumulating and impeding the synaptic transfer of information. Eventually, leading to the neuron death as the TAU protein gives structural support to the microtubules, which are structures that also allow the exchange of molecules and nutrients within the neuron internal circuit. As neurons' homeostasis gets deregulated, their processes' slow down or completely stop, ceasing the transfer of information, causing the typical symptoms of loss of memory and cognitive decline [75]. The progressive neuronal death and consequent neuro-inflammation impairs the neuronal circuit and can lead to a generalized atrophy of the brain tissue (especially the grey matter). The widening of the sulci, a narrowing of the gyri, and the enlargement of the ventricle, are characteristics of advanced AD [63]. Treatment options for mild or moderate AD consists of cholinesterase inhibitors such as donepezil, rivastigmine and galantamine and for severe cases it is used memantine, a NMDA receptor antagonist. Nevertheless, these drugs only induce temporary benefits for symptom amelioration [65].

17.4.3.3 Parkinson's Disease

Parkinson's Disease (PD) is the second most common neurodegenerative disease in the elderly population (over 60 year old) [33]. It affects 10% of people aged between 20 and 50 year old (young onset PD). PD predominantly occurs in

men, which is thought to be related with the protective effects of estrogen in women [19]. Annually, it is estimated that 5–20 in 100,000 individuals are suffering from this pathology. The symptoms include resting tremor (involuntary and accentuated movements), bradykinesia (slowed movement) caused by muscle rigidity, abnormal posture and dysfunction of the autonomic nervous system (controls breathing and heartbeat) [65]. As the disease progresses, besides the previously mentioned motor symptoms, patients experience "non-motor symptoms" such as depression, sensory anomalies, sleep pattern disturbance and cognitive deterioration [19]. AD symptoms are caused by the loss/degeneration of dopaminergic neurons in the substantia nigra (in the pars compacta), where the normal functioning of the nigrostriatal pathway is affected. These neurons are responsible for the production of dopamine and their degeneration results in a decreased production of this neurotransmitter, which controls the motor and neurogenic activity of the brain. Another disease feature comprises the presence of intracytoplasmic aggregates of proteins within the neurons, known as Lewy Bodies [9]. The cause is still unknown but reports shows that Lewy Bodies and their neurites have in their composition large quantities of misfolded a-synuclein. As the dopaminergic degeneration advances, the related pathways (e.g. the hippocampus, basal ganglia, amygdala, and striatum) start to slowly die and disintegrate. Curiously, all degenerating brain regions have the presence of Lewy Bodies in their neurons, further connecting misfolded a-synuclein to this pathology, often termed synucleinopathy [19]. Nevertheless, besides the nigrostriatal pathway many other pathways (and neurotransmitters) are affected by PD, which hardens the task of finding a regenerative treatment for this pathology. Levodopa (L-dopa, LDA) administration, an isomer of dihydroxyphenylalanine (metabolic precursor of dopamine), as proven to be the most efficient drug on the market for PD motor symptoms amelioration. Dopamine alone is unable to cross the BBB, owed to its hydrophobic property and absence of a specific transporter, while L-dopa is able to pass the BBB and be converted to dopamine in the basal ganglia. However, L-dopa can cause deleterious side effects such as syskinesia, sleepiness and nausea [9], which lead to alternative drugs to treat this disease, such as amantadine, monoamine oxidase type B inhibitors (MAOBIs), b-blockers, and dopamine agonists. Depending on the patient's reaction the treatment, the medication can be adjusted (l-dopa dosing regimen adjustment) or combined with other drugs (catechol-O-methyl-transferase inhibitors, MAOBIs or dopamine agonists). All the aforementioned treatments only ameliorate the symptoms, without actually stopping the degeneration process [65].

17.4.4 Brain Cancers

Cancer is estimated to affect over 11 million persons each year and by 2020 this number is predicted to rise to the 16 million worldwide. Cancer is a highly heterogeneous disease, which is one of the reasons is so difficult to discover a single and efficient treatment. Thus, researchers are now focused on patient-centred approaches that ideally would be adapted to each individual patient [34]. Tumours can be termed as: (i) primary tumours, which are tumours that start in the brain, and (ii) secondary/metastatic tumours caused by a malignant tumour that started in another organ (lungs or breast cancer) and metastasized to the brain. Brain tumours can also be categorised as: (i) children tumours, such as the medulloblastoma and neuroblastoma that occur more often before the birth or in infants/children, and (ii) adult tumours, most common in young/adult individuals [47]. The World Health Organization (WHO) classifies the tumours according to: (i) the cell type of the tumour (e.g. astrocytes, oligodendrocytes, etc.), and (ii) the cells' behaviour, that is, the grade of aggressiveness of the tumour (least aggressive are grade I and most aggressive are grade IV) [48]. Among the existent primary adult cancers, the most common and aggressive is the malignant glioma, this is a term used to describe cancers that arise from glial cells ("gli" + "oma"). Gliomas are normally associated with a bad prognosis, as patients pres-

ent only a 5% survival rate 5 years after diagnosis and a high chance of relapse/recurrence of the cancer [83]. This aggressiveness is owed to the capacity to penetrate rapidly the healthy brain tissue and easiness in forming satellite tumours, which are small neoplasms caused by malign cells that leave the primary tumour and spread further into the surrounding tissue [86]. Gliomas include oligodendrogliomas (grade II), ependymomas (from grade I to II), astrocytomas (from grade I to II), glioblastomas that is an advanced form of astrocytoma (grade IV) and oligoastrocytomas (grade II), among others. The terminology of the cancers relates to the cell type that is mainly affected, for example, ependymomas are cancers occurring in the ependymal tissue, astrocytomas are cancer arising from astrocytes and oligoastrocytomas have mixed population of oligodendrocytes and astrocytes [34]. Glioblastomas are the most aggressive and concerning tumours, accounting for 82% of malign gliomas, affecting more the males than the females and is more common on white populations than black populations. This type of tumor presents extensive and rapid cellular activity (high proliferation rate), high vascular recruitment, strong invasion capacity and presence of necrosis, making it an heterogeneous tumour (cells with different sizes, shapes, types and aggressiveness), sometimes termed glioblastoma multiform (GBM) [55]. The treatment for brain cancers depends on their grade and invasiveness. A grade I-II tumour with clear margins can be surgically removed (if accessible) as first option, trying to resect all the tumour to avoid leaving some cells that will lead to the recurrence of the cancer and improving the patient's chances of survival. However, if the tumour is highly aggressive (big tumour and/or with large area invaded) with unclear margins (unable to distinguish tumour from brain tissue) the treatment gets difficult and each case/tumour has different needs [86]. Some tumours require to start chemo or radio-therapy first to reduce the tumour's size and allow a safe removal, while others perform this treatment after chirurgical removal to kill any remaining cells. Even so, brain surgeries are always risky, some more than others depending on the brain region affected by the tumour, and achieving a complete resection that does not cause neurological deficits is still a goal, besides the fact that the resected area will never regenerate, which will also affect the patient's quality of life [72].

17.5 Biomaterials in CNS

The emergent need to repair/regenerate the injured tissues/organs or target the cause of the disease, instead of just ameliorating the symptoms, is one of the pillars of tissue engineering (TE). TE focus on the development of biologic substitutes with the aim of maintaining, repairing/restoring or improving the function of a tissue and/or organ that have been affected by disease or injury, through the combined use of life sciences and engineering. This constantly expanding field can use alone or combination of cells, biomaterials, genes, among others to attain that goal. In the particular case of brain associated pathological events, neuronal and non-neuronal cells are compromised and/or die, inflammatory response occurs, but also the natural ECM is compromised or damaged. Previously this fact was overlooked since the ECM's only function was supposedly structural support, but now researchers have unravel the influence that the ECM has not only structurally but also functionally and biologically [46]. This finding gave a new vision on the use of biomaterials for biomedical applications, namely their use as carriers for molecules, genes or cells, where it will support or transport the implanted components, acting as an artificial ECM or personalized shield for a more accurate delivery. Moreover, natural based biomaterials have inherent biological effect that can be used to obtain a specific cellular response, are biodegradable and possess low cytotoxicity. Biomaterials can be the modified in terms of their physicochemical and mechanical properties by altering the "recipe" of their production, adjusting the material to the purpose and to the tissue on which its implantation is intended [76]. Therefore, one biomaterial can be used for treating several pathologies, but not necessarily in the same physical form (e.g. hydrogel for cell

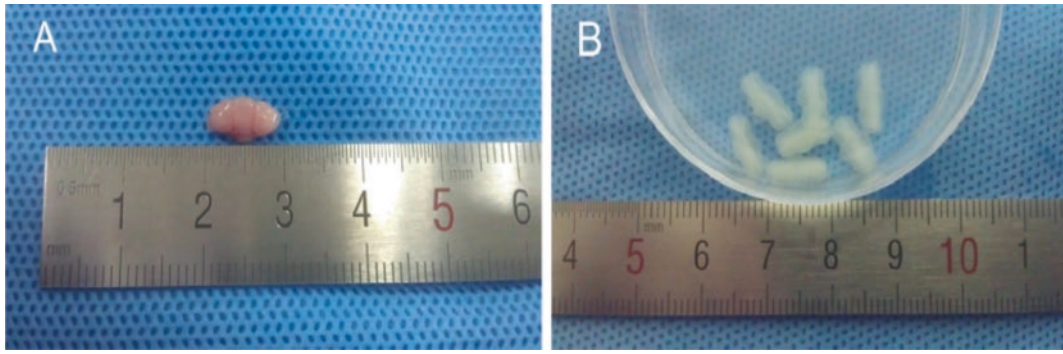

Fig. 17.5 (**a**) Isolated mouse cerebellum to be used for scaffold production. (**b**) Final scaffold (decellularized cerebellums). (Figure reprinted from [85])

support or nanoparticles' for cancer cell recognition). Different reports demonstrated that cells response accordingly to the surrounding environment cues, either physical, chemical or biological, that is, if the topography, the consistency of the material is different or the polymer suffered alteration, the cells' response might also be different [54, 64]. Indeed, Ulrich [74] and co-workers cultured a glioma cell line in fibronectin-coated ECMs substrates with different degrees of rigidity and discovered that extremely rigid ECMs provide the conditions for these cells to spread extensively, mature focal adhesions and migrate quickly. Interestingly, as the rigidity of the structures decreased (similar to brain tissue consistency) the cells demonstrated an untypical round-shape and were unable to productively migrate and spread. This plasticity of biomaterials allows researchers to use biomaterials in different forms, such as scaffolds/structures, hydrogels, and micro- and nano-particles, which will be further discussed herein.

17.5.1 Scaffolds

The malleability of the biomaterials allows the production of a material with defined physical-chemical properties while taking advantage of their natural characteristics. For example Zhu and co-workers [85] produced a cerebellum scaffold (Fig. 17.5), obtained by a decellularization protocol, to remove the cellular components and maintain its structure and intrinsic neurotrophic proteins and growth factors. The decellularized scaffold had higher growth factor content (BDNF and NGF) when compared to urinary bladder ECM, a commonly used material (Fig. 17.6). The cerebellar scaffold demonstrated cytocompatibility by supporting the survival, proliferation and differentiation of Neural Stem Cells (NSCs) *in vitro*. Moreover, a higher neural differentiation was obtained on the cerebellar scaffolds in comparison with the urinary bladder (Fig. 17.7). This suggests an advantage of using CNS-derived molecules for brain applications, which increases the probabilities of scaffold integration.

A study performed by Clark et al. [16] developed a biocompatible and biodegradable cylindrical scaffold containing fibrin, neurotrophins (NGF and VEGF) and ECM molecules that was implanted to intersect the migratory path of endogenous neural progenitor cells (NPCs) and the non-neurogenic striatum. The authors intended to promote the recruitment and guidance of the endogenous NPCs by providing a new migratory path (by the enriched environment of the scaffold) from the neurogenic niche into the non-neurogenic region on interest (in this case the striatum). The scaffold was implanted into hemi-Parkinson rats and vigorously recruited neuroblasts that had long and complexed neurites, after its complete degradation the region containing new neural cells and the animal's Parkinsonian behaviour was ameliorated. Moreover, the animal did not suffer any inflammatory, tumorigenic or behavioral sequelae from the scaffold insertion.

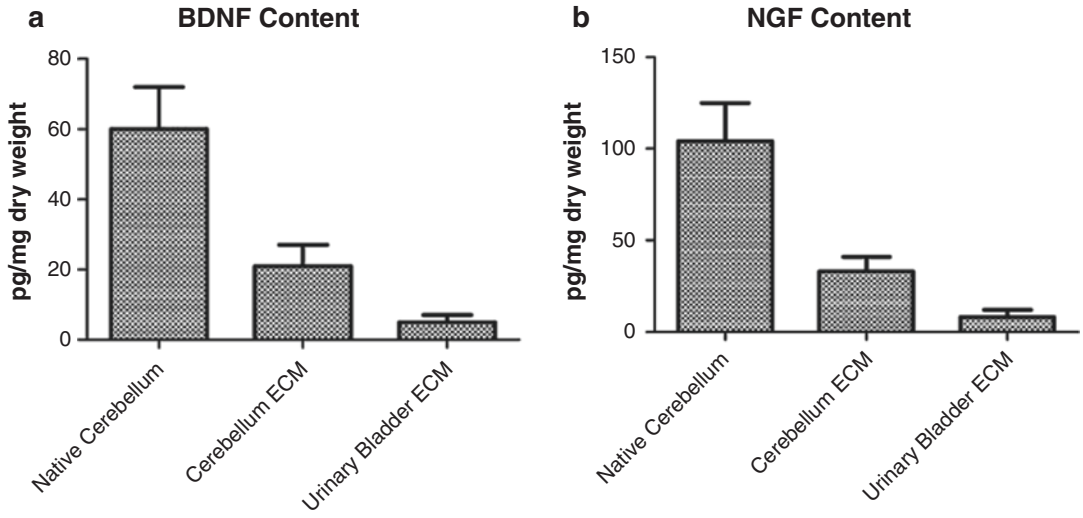

Fig. 17.6 Amount of growth factors, namely (**a**) GDNF and (**b**) NGF, present before and after the decellularization protocol and in comparison with the Urinary bladder ECM. (Figure reprinted from [85])

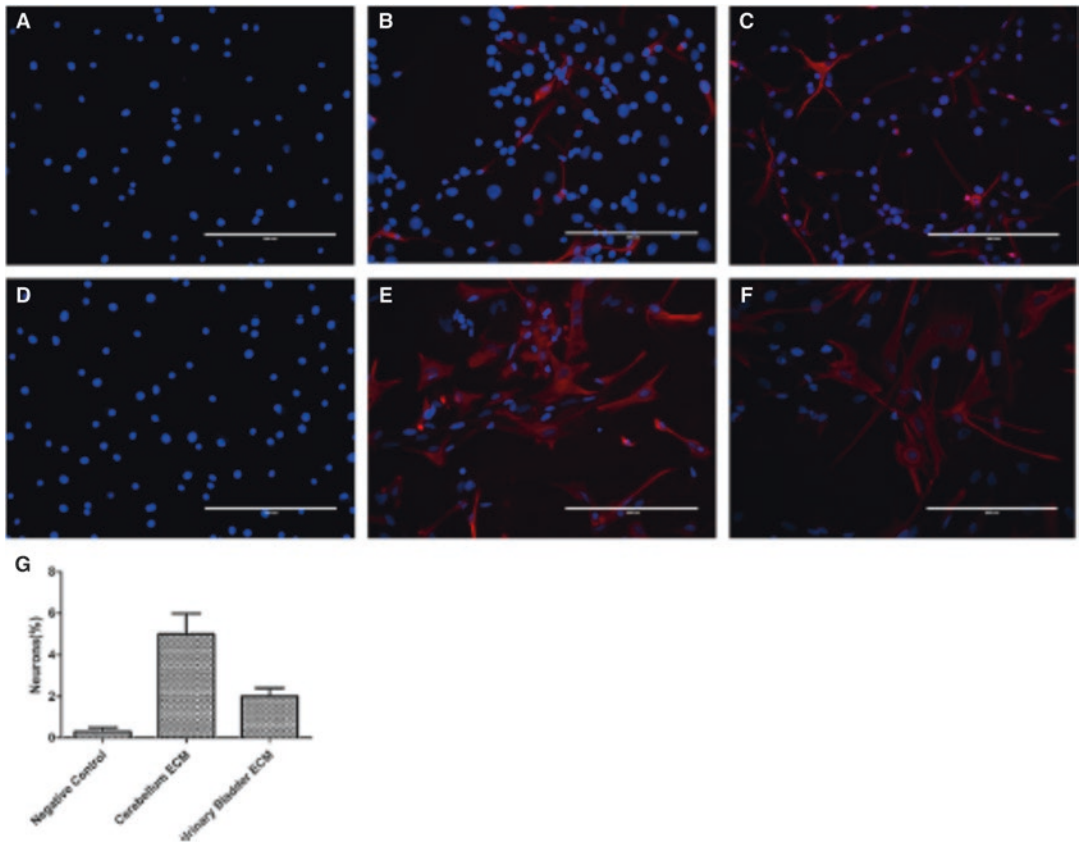

Fig. 17.7 Human NSCs differentiation into neurons, confirmed by the expression of βIII-tubulin (**a**, **b** and **c**) and astrocytes, by the expression of GFAP (**d**, **e** and **f**) in vitro. **a** and **d** are images of the control with no differentiation, **b** and **e** images represent the differentiation obtained by Cerebellar scaffold and images **c** and **f** are from the Urinary bladder ECM. (**g**) Percentage of hNSCs differentiated into neurons on the different scaffolds, showing higher number of neurons on the Cerebellar scaffold. Scale bar: 200 μm. (Figure reprinted from [85])

Winter et al. [79] also developed micro-columns, containing tubular agarose hydrogels with bioactive collagenous matrix inside, taking advantage of this recruitment capacity to create a neural guidance pathway. They evaluated the effects of different scaffold concentrations, compositions and geometries and the influence of seeded cell density, to obtain the optimum cable-like 3D networks (bundles) suitable for directed axonal regeneration. The group seeded astrocytes on the system, which induced their alignment along the cable like micro-columns of 180 μm or 300–350 μm, but the systems with 1.0 mm did not produce this effect, suggesting that radius of curvature had an influence on the occurrence and extension of the alignment. In micro-columns with small inner diameter the seeded astrocytes assumed bi-polar morphology while in large diameter structures this only happened to 10% of the population. Moreover, high cell densities induced astrocytic contraction but the low cell density did not produce an effect, indicating cell-cell interaction also plays a role in the formation of dense 3D aligned neural bundles. Interestingly, the neurons co-seeded in this system were able to extend their neurites along the aligned bundles, indicating that the system provides permissive cues for neurite growth and extension, which makes this scaffold an appealing strategy for repopulation of large areas of neurodegeneration (e.g. stroke).

Harris [30] and co-workers developed a more complex system of micro-tissue engineered neural networks, containing mature primary cortical neurons and long axonal tracts entrapped on hydrogel micro-columns, to mimic the grey and white matter, so the scaffold successfully integrates the brain. The authors further optimized the material by adding a thin layer of low viscosity carboxymethylcellulose to obtain a softer material similar to the brain consistency and also to allow its needle-free administration. The scaffold supported the neuronal viability and long-reaching axonal cytoarchitecture for 22 days and by adding the carboxymethylcellulose the stiffness increased, allowing the minimally invasion needle-free implantation into the rats' brains.

Cancer-fighting drugs have the limitation of affecting all cells instead of selectively target the cancer cells and also the administration route is a challenge, as the drug has to be able to pass the BBB or allow its direct implantation into the brain.

Zhu et al. [86] produced poly(ε-caprolactone) (PCL)/gelatin (GT) scaffolds, through electrospinning, in order to allow the direct brain insertion and promote a sustained release of 7-ethyl-10- hydroxy camptothecin (SN-38), a chemotherapeutic drug that induces cell death. They also added acetic acid for a more homogenous and transparent polymer solution. The authors tested PCL/gelatin with different concentration of SN-38 since the physical-mechanical properties of the scaffold varied depending on those formulations. They observed that as the SN-38 concentration increased, the fibbers' swelling and surface roughness increased and the degradation rate and crystallinity decreased, suggesting SN-38 acts as a plasticizer in this scaffold. Additionally, the scaffold's optimum concentration was able to exert a cytotoxic effect on the 251 treated cancer cells by killing almost 50% of the population without causing any adverse effect, making them a possible preventive measurement for locoregional cancer recurrence after surgical resection.

In another study Ward et al. [78] focused on the malignant properties of cancer cells, namely their capacity to grow anchorage-free and consequent invasiveness, which depends on the cancer cells' interaction with surrounding cells and ECM. Having this in mind, the group manipulated the interactions between endogenous Hyaluronan (HA) present in the brain ECM and the cancer cells by two different routes to elucidate the role that this cross-talk has in cancer malignancy. One approach consisted on administrating small HA oligosaccharides to compete with the HA-cancer cell interactions. In the second strategy soluble HA-binding proteins were overexpressed to competitively sequestrate endogenous HA, both avoiding the polysaccharide connection to the cells to diminish HA-induced signalling. They discovered that both routes decreased anchorage-independent

growth (assessed by colony formation in agar matrix) and invasiveness of glioma cells (measured by the cells' capacity to penetrate reconstituted basement membranes). This showed that interaction between endogenous HA and cancer cells may be a prerequisite for these hallmarks (e.g. invasiveness and anchorange-free growth) of malignant glioma cells. This information further accentuated the important role that ECM has, not only physiologically but also in pathological context.

17.5.2 Hydrogels

Biomaterials in the form of hydrogels have appealing characteristics for brain tissue engineering approaches due to their consistency (owed to their hydrophilic networks) similar to the brain's soft tissue and high permeability allowing oxygen and nutrients exchange [44]. Also, like previously said, they can be adjusted to numerous purposes and pathologies exploiting each polymers' intrinsic biological influence to better suit the materials with the combined/implanted components. For example, cells have different degrees of affinity with cytokines, receptors, cells and molecules of the ECM, and these should be beared in mind when considering the development of a hydrogel for transplantation purposes. For example, Gwon [26] developed a 3D stem-cell responsive heparin-HA (HE-HA) hydrogel, created by the crosslinking of thiolated heparin and metacrylated HA, but first they adjusted the material's properties to the cells planned for transplantation, in this case the Adipose-tissue derived Stem Cells (ASCs). The group compared the HE-HA hydrogel with control hydrogels, namely a PEG-HA hydrogel with PEG-SH in the place of the thiolated heparin, heparin-PEG hydrogel with PEG-AC instead of metacrylated HA and a stiff HE-HA hydrogel with higher concentrations of precursors (to assess whether the elasticity as an effect). They confirmed that only the HE-HA hydrogel maintained the viability of the ASCs and induced their proliferation and migration. The hyaluronidase expression was high on cells encapsulated on this hydrogel, which in turn degraded the hydrogel in a dosage dependent manner. This indicates that the degradation was through HA moieties, facilitated by the hyaluronidase that the ASCs released. Regarding the hydrogel stiffness, the softer hydrogel with lower concentrations of precursors proved to be the most suitable for cell adhesion and proliferation, with significant differences. The ASCs had the receptor for HA which facilitated their recognition and consequent anchorage, and the hyaluronidases they release allowed the degradation of the hydrogel, providing the described positive results. One requisite for tissue engineering materials is their bio/cytocompatibility to avoid any side response provoked by its implantation or by the products of its degradation. Another requisite is the materials' biomimicking of native tissue (e.g. brain), trying to develop a material with the most accurate physic and biologic properties. This is the reason researchers resort to ECM-based hydrogels since they already have ECM molecules and/or peptides that will provide anchoring sites for host and transplanted cells to recognize, improving the success rate of the treatment [40]. Natural-based hydrogels have the advantage of naturally possessing biological clues for cells' proliferation, migration, recruitment, among others [1, 54, 56]. The conformation properties of the hydrogels' polymeric networks can also be utilized in our advantage, as shown by Yan [82] and co-workers that developed silk-fibroin hydrogels via horseradish peroxidase (HRP) mediated crosslinking, which spontaneously change their conformation after 7 days in physiological conditions. In the first 7 days, the hydrogels are "organized" in random coil conformation, transparent and elastic, after which they re-organize into β-sheet conformation. During the 7 days the hydrogels allowed the survival of ATDC5 cells, a chondrogenic cell line derived from teratocarcinoma AT805, but the after the β –sheet change the cells underwent apoptosis. The hydrogel was loaded with HeLa cells, a cell line derived from cervical cancer, and implanted in a chick chorio-llantoic membrane (CAM) model and the change killed the HeLa cells, suppressed tumour forma-

tion, did not allowed endothelial invasion and limited the blood supply to the tumour.

An important prerequisite for brain recovery, after any the treatment, is tissue regeneration and regain of function to the affected area(s). Also, as previously mentioned, neural network formation and synaptic transfer of information are essential events for the regain of function. Broguiere et al. [10] produced a HA hydrogel that crosslinks with the transglutaminase (TG) activity, present in activated blood coagulation factor XIII (FXIIIa). The group was able to make the material injectable and with tuneable gelation time (from seconds to hours), depending on the quantity of the added enzyme. Moreover, FXIIIa has the ability to covalently crosslink with fibrin, which enhances the chances of recognition and/or facilitates the material's functionalization with fibrin/fibrinogen peptides. The authors proved that the hydrogel adheres to mouse spinal cord tissue, and dissociated cortical neurons were successfully encapsulated within the hydrogel without losing viability, presenting 90% viability at day 1 and 81% around day 5 (Fig. 17.8). The material do not swell, shrink nor degraded after 2 months in culture (suitable for brain applications) and has proven permissive for neurite extension, as by day 21 the hydrogel presented a dense mesh of neurites that created extensive 3D networks. These networks presented large and small dendritic growth cones (process similar to *in vivo* neurogenesis) and the neurites further developed dendritic spines suggesting the beginning of the branching process (Fig. 17.9). The morphological changes were accompanied with the appearance of specific axonal and dendritic markers such as MAP-3, βIII tubulin and neurofilaments. Most importantly, they monitored the neuronal networks' spiking activity and observed synchronous spiking and high density of pre and post-synaptic proteins in the neurons, indicating the formation of active synapses with long-term and stable spiking activity (Fig. 17.10).

Another useful applicability of hydrogels is for imaging purposes, to non-invasively monitor the changes of the material overtime, and ideally, the scaffolds' performance *in vivo* would be analysed from the implantation until degradation/recovery of the scaffold. This would allow to confirm the correct implantation of the scaffold, their degradation form and rate, the cells localization and fate post-transplantation and so on, since it known that any change in the environment can cause a different cell response. Liang et al. [45] took advantage of the natural capacity of gelatin to provide a good chemical exchange saturation transfer, allowing the detection of its contrast trough MRI and developed an hydrogel composed by HA, GelinS (thiol modified gelatin) and polyethylene glycol diacrylate (PEGDA). They assessed, first each compound individually and then all compounds combined, both *in vitro* (hydrogel phantoms) and *in vivo* (Immunodeficient, rag2−/− mice) the hydrogel's aptitude to provide label-free and non-invasive imaging. *In vitro*, the individual GelinS obtained the highest CEST signal, further confirmed to be inherent to gelatin since both modified and unmodified gelatin forms have the same signal values. The full hydrogel *in vivo* produced a signal for 1 week, after which a significant decrease occur, suggesting that the hydrogel or the gelatin was degraded and that originated this decrease. Surprisingly, after recovery the hydrogel was generally well-preserved so with additional tests they discovered the gelatin digestion did not affected the signal emission (the value were similar), indicating that the loss of signal was due to the physical clearance of the gelatin, meaning the signal depends on gelatin's content and not its structure.

All these studies further remind us of the complexity of the interactions of cell to cell and cell to environment, which have to be deeply evaluated to increase the feasibility of the therapies.

17.5.3 Micro- and Nano-particles

Biomaterials in the form of micro- and nanoparticles can also offer appealing characteristics when envisioning brain tissue engineering. These systems also have application in precision regenerative medicine and diagnosis since the combination of the small size with polymer's or molecule's characteristics can offer a more per-

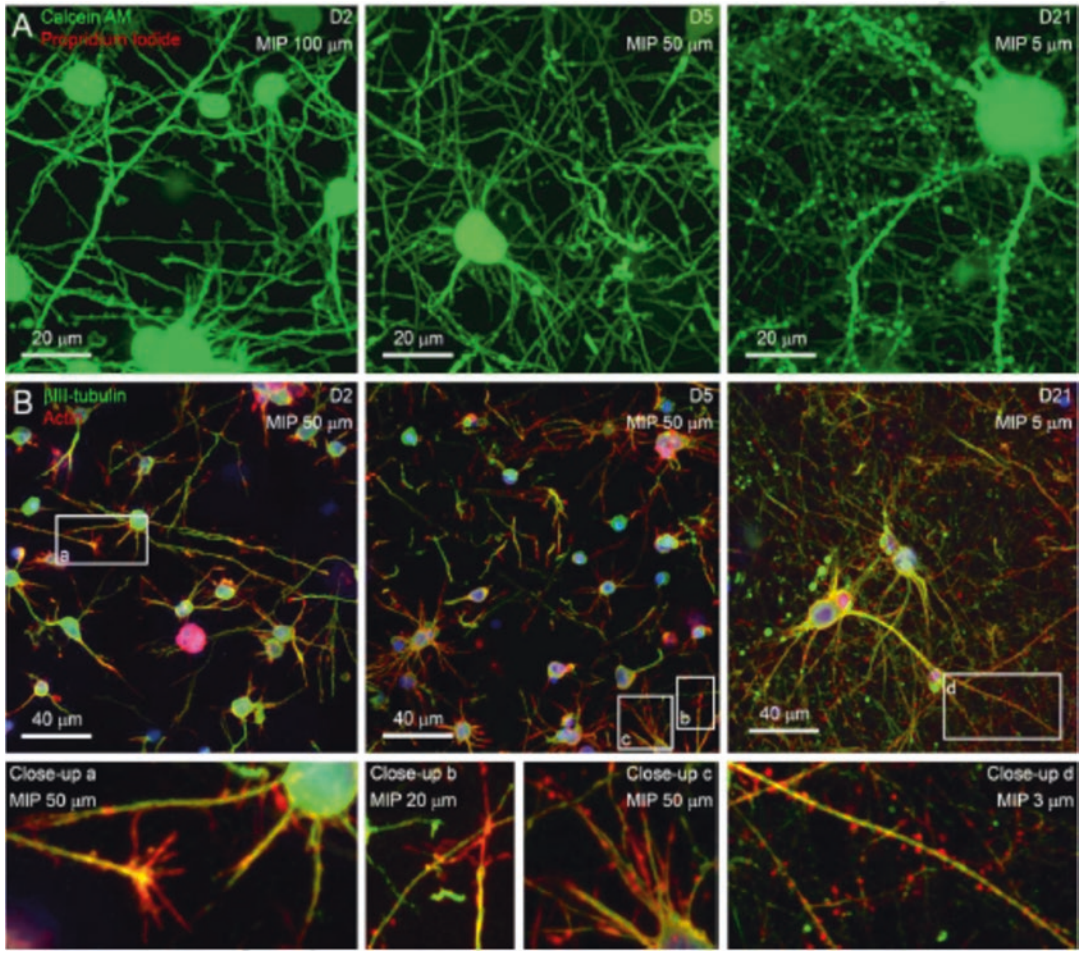

Fig. 17.8 (**a**) Neurons' viability, visible by Live/Dead assay, and (**b**) their morphology and of the new-born neurites at day 2 (D2), day 5 (D5) and day 21 (D21) of culture, immuno-stained for actin (microfilament marker) and βIII tubulin (embryonic neuron marker). Images *a–b* are close-ups of the maximum intensity projections (MIP), the co-staining indicates the growth of axons and dendrites, which become denser networks at D21. (Figure reprinted from [10])

sonalized therapy with increased goal specificity and decreased the possibility of side effects [60]. Moreover, several molecules and drugs of interest have the limitation of not being able to cross the BBB, which is a selective membrane that allows the entry of nutrients and minerals but restrings toxic and/or foreign molecules. Nano materials aid the transportation of these molecules by "disguising" their physical and/or chemical properties to allow the BBB passage, by transporting them inside the nano-systems possessing/containing biological cues that can be recognized [9].

Huang et al. [32] constructed PAMAM dendrimers conjugated with Angiopep-2 and via PEG attached a tumor necrosis factor related apoptosis-inducing ligand (TRAIL) (Fig. 17.11).

Angiopep-2 is a ligand of the LRP1, a receptor described to be present in the brain capillary endothelial cells of the BBB, and TRAIL is a signalling molecule with ability to induce cellular apoptosis (specially activated for cancer cells). This gene delivery system was tested *in vivo* (rat

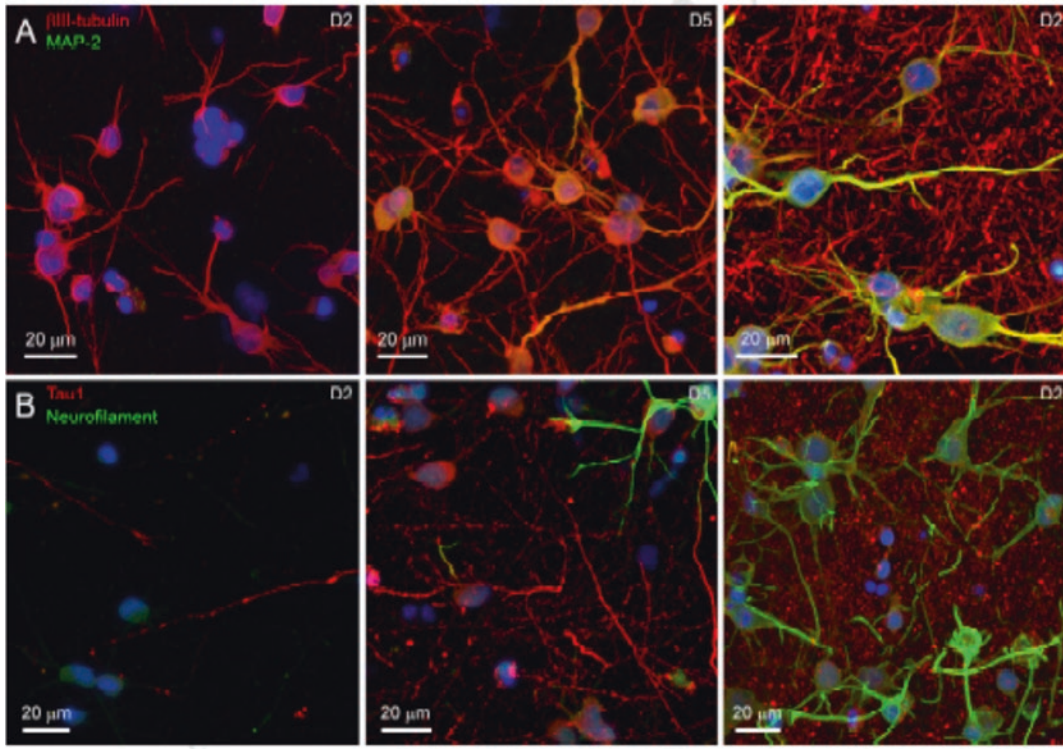

Fig. 17.9 All images are from 50 μm MIPs. (**a**) Neurons immune-stained for MAP-2 (mature dendrites marker), which is visible in some neurons at D5 and expressed along new neurites by D21. (**b**) Neurons stained for Tau1 (axonal marker) that is expressed already in D2, which increases (D5) until D21, where they are too dense to distinguish. Neurofilament staining (mature neuron marker) is visible in some neurons at D5 and by D21 all neurons express it, with most neurites also expressing the marker. (Figure reprinted from [10])

tail injection) against the controls for each used component and scored the widest particle distribution (predominantly in the tumour site) (Fig. 17.12) and induced the most pronounce apoptosis phenomenon. Moreover, when compared with the chemotherapeutical drug Temozolomide this system was able to induce higher numbers of cell death and reached the inside of the tumour site, while the chemo agent only induced apoptosis on the edges on the tumour (Fig. 17.13). The synergistic effect of this systems, namely the recognition of angiopep-2 that facilitated the BBB passage and specific targeting and apoptosis induction offered by the TRAIL, translated into a survival time of 61 days versus the 49 days of the Temozolomide-treated rats.

Nano- and micro-systems have additional advantages of being able to stabilize molecules and prolong their half-life time, decrease their bleaching/clearance from the tissues, reduce peripheral toxicity and the small size allows several routes of administration [9]. Dhar et al. [20] produced a sophorolipid-conjugated GG and gold NPs, further including the anti-cancer drug doxorubicin, and evaluated their cytotoxic effect on human glioma cell line LN-229 and human glioma stem cell line HNGC-2. The NPs were internalized by tumour cells after 3 h of incubation and surprisingly the NPs without the drug already had lower cancer cell viability on both cell lines (50%) by just including the sophorolipid, as the "blank" NPs of only GG and gold did not produced this effect. After adding the drug the viability drop to 27% at 24 h of incubation versus the 59% viability of the freely administrated doxorubicin, indicating the system stabi-

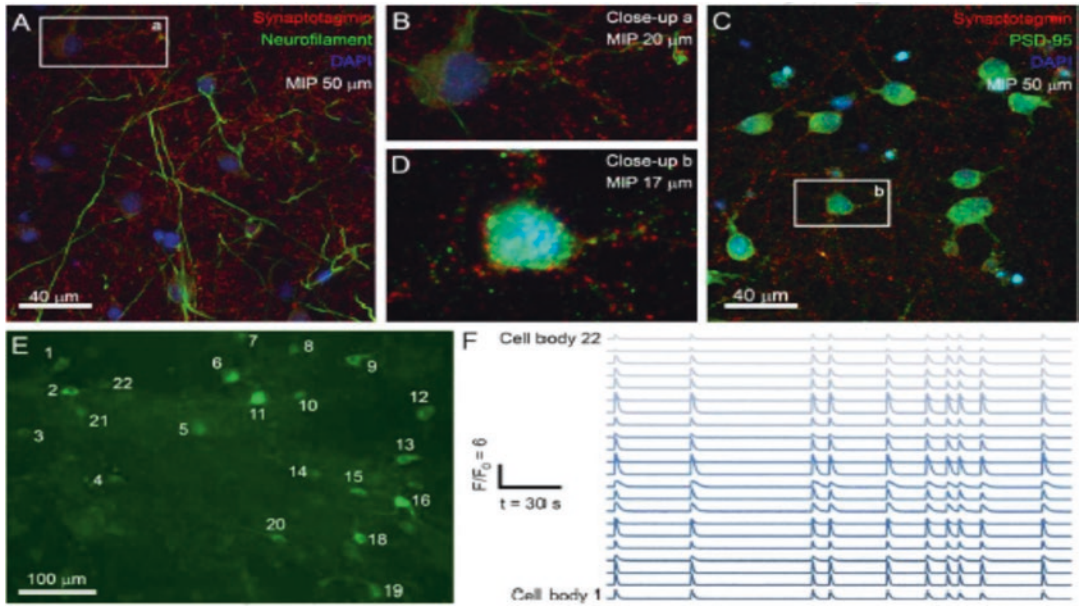

Fig. 17.10 (**a**) Neurons' cultures immuno-stained for Synaptotagmin to assess the potential of pre-synaptic densities. (**b**) close-ups showing neurons and new-born neurites heavily marked by Synaptotagmin, indicating the presence of high synaptic densities. (**c**) Neurons' immuno-stained for Postsynaptic density protein 95 (PSD-95) to show that the cultures also present high density of post-synaptic markers. (**d**) Neurons present both pre and post-synaptic densities (often in opposite sites), indicating the creation of active synapses. (**e**) The neurons were transfected with a genetically encoded intracellular calcium reporter (calcium indicator), the GCaMP, as the synaptic transfer occurs through the activation of voltage-gated calcium channels. This allowed to measure the calcium levels (**f**) in the neurons and observe they were spiking in synchrony, further demonstrating the synaptic connections between the neurons and early neural network formation. Images **a**–**d** are from 21 days of culture and **e**–**f** are from 10 days of culture. (Figure reprinted from [10])

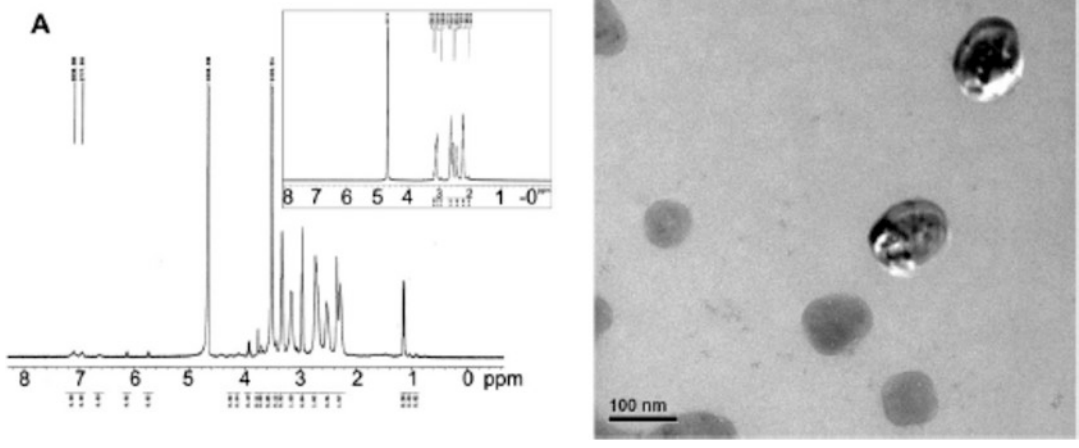

Fig. 17.11 (**a**) Spectrum from the NMR analysis performed on the PAMAM-PEG-Angiopep NPs. The small box within image (**a**) shows the NMR spectrum from only the PAMAM NPs. (**b**) Images from transmission electron microscopy (TEM) of the final PAMAM-PEG-Angiopep/DNA NPs. (Figure reprinted from [32])

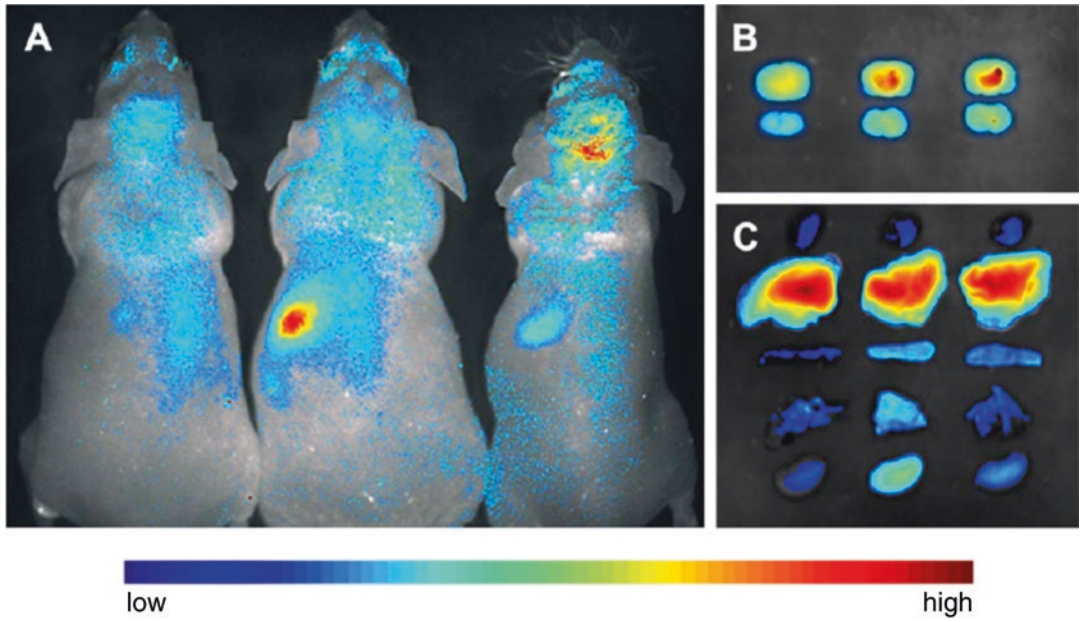

Fig. 17.12 120 min after NPs administration the following images were taken. (**a**) In vivo imaging of the distribution of PAMAM/DNA NPs (left mouse), the PAMAM-PEG/DNA NPs in the middle mouse and the PAMAM-PEG-Angiopep/DNA NPs (right mouse). The NPs distribution showed that PAMAM-PEG-Angiopep/DNA NPs provided the higher homing to the brain, especially in the tumour site. (**b**) In vitro imaging of the mice' brains shows all NPs target the brain, but the stronger signal was attained by the PAMAM-PEG-Angiopep/DNA NPs. (**c**) NPs tend to accumulate in other organs such as the spleen, lungs and kidneys. In this case, all NPs were visible in the liver but not in other organs, except from the PAMAM-PEG/DNA NPs (organs in the middle of image **c**). (Figure reprinted from [32])

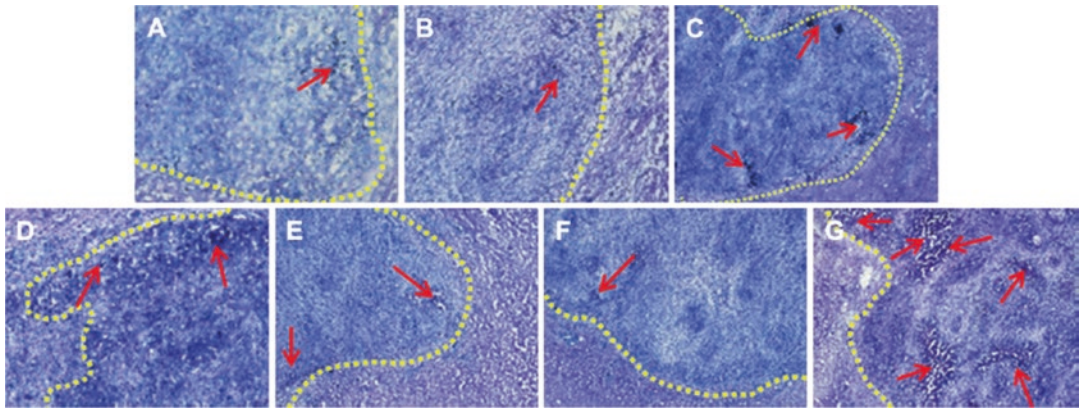

Fig. 17.13 Coronal brain tumour sections of (**a**) saline, (**b**) PAMAM-PEG-Angiopep/pGL2 NPs, (**c**) Temozolomide, (**d**) free pORF-TRAIL administration, (**e**) PAMAM/pORF-TRAIL NPs, (**f**) PAMAM-PEG/pORF-TRAIL NPs and (**g**) PAMAM-PEG-Angiopep/pORF-TRAIL NPs. The yellow lines represent the borders of the tumour and red arrows show cells undergoing apoptosis, which is almost undetectable in the saline (**a**) or PAMAM-PEG-Angiopep/pGL2 NPs (**b**). Cell death was very low on the free pORF-TRAIL (**d**), the PAMAM/pORF-TRAIL NPs (**e**) and the PAMAM-PEG/pORF-TRAIL NPs (**f**), with most of dead cells visible on the edge of the tumour. The temozolomide group (**c**) induced medium apoptosis, however it was mostly concentrated to a region, while the PAMAM-PEG-Angiopep/pORF-TRAIL NPs (**g**) induced higher apoptosis on tumour cells both on the periphery and centre of the glial tumour. Images amplification of x400. (Figure reprinted from [32])

lized the drug and promoted a sustained and localized release. These results are encouraging since the strategy killed both cancer cells and its stem cells, responsible for recurrent and more aggressive cancers.

All the aforementioned features of imaging or regenerative applications also apply to micro- and nano-particles. Rassu et al. [58] that developed chitosan chloride and methyl-β-cyclodextrin microparticles for nasal-to-brain administration of a neuroprotector agent (deferoxamine mesylate) which is unable to pass the BBB itself. The system increased the delivery rate of nasal administration, the uptake was visible in the CSF of the rats and did not produced the side effects of systemic administrations. This system is an appealing strategy for the delivery of neuroregenerative molecules into neurodegenerated brain regions.

As these systems permit the conjugation with and/or functionalization of surface with other compounds, they provide a multidisciplinary strategy to tackle the simultaneously occurring events of neuropathologies [9, 60, 70]. Microparticles have the additional advantage of allowing the incorporation or transport of larger molecules and/or cells. For example, Koh [39] and co-workers developed injectable alginate beads to encapsulate, transport and retain the cells (ASCs) in the implantation site. The particles did not produced a toxic effect, increased cell viability (for 7 days) and cell proliferation within the particle. They further confirmed this effect was related to the particles by comparing the injection of HEK-293 cells into the subcutaneous tissue of mice and through bioluminescent imaging they discovered that the encapsulated cells were visible at 7 days post-injection while the free injected cell could not be detected 7 days after the injection.

17.6 Final Remarks

The CNS is a complex tissue responsible for every involuntary and voluntary action we perform and for the psychological characteristics of each person, controlling short and long term memory, learning, social behaviour, etc. This control centre of the body is as powerful as it is sensitive, meaning that even minor alterations, be it on DNA, ECM, cells or molecules will put this structure at risk. The well-orchestrated neuronal network is interconnected and dependent on the surrounding cues and cells to perform the tasks. In pathologic events the homeostasis is disrupted and a cascade of events occur, leading to the partial or generalized loss of brain function.

The still incomplete understanding of the cellular and molecular pathophysiological processes occurring in most brain diseases and the low/null ability of the CNS to self-repair make brain regeneration an everlasting challenge, having the necessity of therapies that promote the tissue's functional recovery. Therapeutic drugs and cell delivery are appealing strategies for brain recovery as they provide trophic support and/or cell replacement, but have the drawback of being cleared from the body or cells have low engraftment rate, compromising their therapeutic effect. The use of biomaterials aids in this limitation by stabilizing the molecules/bioactive agents, promoting a sustained release and allow a targeted approach by incorporation molecules that will be recognized by the cells of interest (avoiding the side effects of systemic administrations). Biomaterials can also aid homing the therapeutic cells at the delivery site, providing the conditions for their survival. Other appealing features of natural-based biomaterials are related to their biodegradability, cytocompatibility and inherent biological effect on the engraftment, survival, proliferation and differentiation of transplanted cells. Moreover, the versatility of the materials can allow their broad application across several pathologies, while allowing the combination with other compounds for a more efficient and multidisciplinary approach. By means of combining several components to tackle multifaceted diseases, it is possible to address more than one event (e.g. inflammation, neural cell death/cancer cells imaging and targeting), taking research one-step closer to a functional recovery from brain diseases. Nevertheless, big challenges have still to be overcome before achieving full recovery, especially the difficulties in translating the obtained research to clinics and the time and

expenses needed to get a full validation of the therapies. Future research should also focus on developing new mimetic 3D *in vitro* tissue models, and validating and standardizing animal models that can allow a reliable/biomimetic model that comprises all or most of the complex process that occurs *in vivo*.

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18 Polypyrrole as Electrically Conductive Biomaterials: Synthesis, Biofunctionalization, Potential Applications and Challenges

Jifu Mao and Ze Zhang

Abstract

Electrical phenomenon is ubiquitous in any biological system. However, most synthetic biomaterials are insulators to either electrical or ionic current. To mimic the electrical and ionic conductivities of natural tissues, electrically conductive polymers have been studied and are becoming a new class of biomaterials. This chapter focuses on polypyrrole, one of the most widely investigated synthetic and intrinsically conductive polymers. Polypyrrole is a heterocyclic polymer that is both electrically conductive and ionically active. It can be easily synthesized through electrochemical polymerization or oxidative polymerization. Because of its unique properties, polypyrrole has been studied for sensing, drug delivery, and actuation. Because of its good biocompatibility, it has been used to interface electrical elements and tissues, either for recording or stimulation purpose. Polypyrrole can also be chemically modified to carry functional groups and biomolecules, allowing both specific biological recognition and electrical stimulation. This chapter also discusses a unique soft polypyrrole membrane that can be easily used as biomaterials. Hopefully, the readers of this chapter would appreciate the importance of electrical conductivity for biomaterials and the usefulness of polypyrrole.

Keywords

Polypyrrole · Electrical conductivity · Biomaterial · Biofunctionalization · Electrical stimulation · Flexible membrane

18.1 Introduction

The roles of endogenous and exogenous electricity in life are obvious. The roles of electricity in development and regenerative medicine are becoming better understood [1, 2]. Notwithstanding, it took a long time for conductive polymers to be part of the big picture of biomaterials and regenerative medicine [3]. In fact, "electrically conductive biomaterial" is still not a commonly accepted classification. Despite of that, both electrical conductivity and electroactivity are among the intrinsic characteristics of tissues and cells. Therefore there is hardly any reason not to impart electrical properties to a material that is intended to replace tissues or support cells. Among the limited numbers of conductive polymers (CPs) such as polypyrrole (PPy), poly(3,4-ethylenedioxythiophene)

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(PEDOT) and polyaniline (PANI), to name a few, PPy has been most intensively investigated in the context of biomaterials, hence will be the focus of this chapter. For a general reading of conductive polymers including their properties and routes of synthesis readers are referred to Handbook of Conducting Polymers edited by Skotheim and Reytnolds.

Although PPy has long been synthesized from early 1910s, electrically conducting PPy hadn't been reported until early 1960s [4]. And the conductive PPy has been extensively investigated only after the electrochemistry techniques became adopted in late 1970s [5]. An ideal molecule of PPy consists of repeatedly connected pyrrole (Py) rings through the α sites of Py monomers as shown in Fig. 18.1. Whereas, the linear molecules can only be formed at the initial stage of the polymerization. As a PPy molecule grows, a three-dimensional (3D) cross-linked network is formed due to the activation of the β sites. Furthermore, dopant insertions and chain distortions give birth to different inclinations such as cones or helices [6]. Consequently, PPy is very difficult to be further processed once synthesized, because the highly cross-linked structures reduce the fusibility and solubility [7]. The poor mechanical property and processability are the main drawbacks due to the PPy heterogeneity caused by the nonlinear polymerization, making PPy almost impossible to be used individually, but always composed with other materials to form composites.

Due to the positively charged backbone of the oxidized PPy, anionic counterions are always introduced to the conductive PPy molecules [8]. Conduction via polarons or bipolarons (Fig. 18.1) is considered as the dominant mechanism of charge transport in PPy [8, 9]. Bipolarons, more favorable to transfer along the PPy chains compared with polarons, are thereby considered the dominant charge carrier. There are two methods to generate a bipolaron, either to be further oxidized to remove the unpaired electron in a polaron, or to link two independent polarons between the adjacent chains through a charge transfer reaction. To note, the morphology and characteristics of PPy are inevitably associated with how it is synthesized, for example by electropolymerization (EP) or by chemical polymerization.

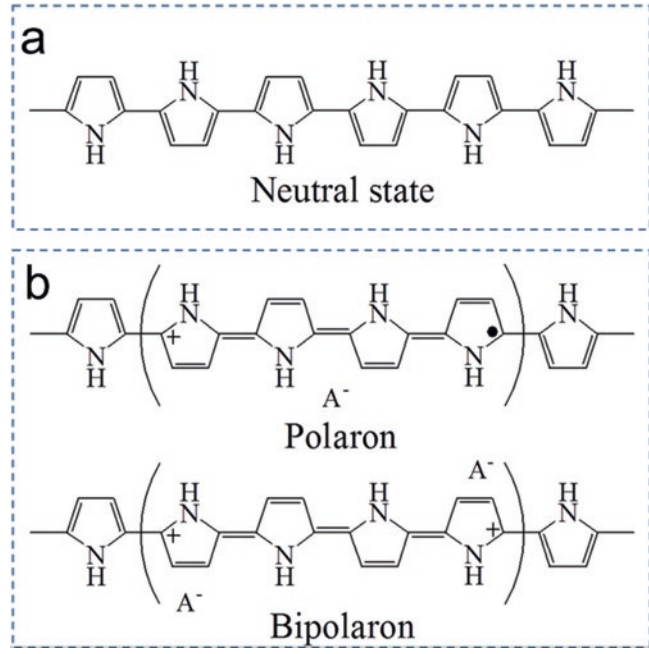

Fig. 18.1 Neutral (**a**) and oxidized PPy (**b**), showing polaron and biopolaron chemical structures. +: charge; •: unpaired electron; A^-: dopant/counterion

18.2 Preparation Methods of PPy

18.2.1 Electrochemical Polymerization

The first well recognized conductive PPy film (100 S cm^{-1}) was synthesized by Diaz via EP in 1979 [5]. This momentous event opened the prologue of researches on CPs using EP. As shown in Fig. 18.2, in a classic EP process, a uniform and highly conductive PPy film is synthesized on the working electrode in a three-electrode configuration (working, counter, and reference electrodes). The main advantages of EP are summarized as follows [8, 10]: (1). the nature of being electroactive with high conductivity; (2). the doped state after synthesis; (3). the control of film mass and thickness; (4). the in situ coating of PPy on a substrate of complex configuration.

In a traditional EP, the morphology and characteristics of the PPy film largely depend on synthesis parameters including the nature of solvent, pH, temperature, electrolyte, electrode system, purity and concentration of monomers, and deposition time and charge [9, 10]. However, stripping the PPy film off the working electrode is not always achievable due to its strong adhesion and its fragility. And, another main restriction in EP is that the area of the PPy film completely depends on the size of the substrate or electrode.

18.2.2 Chemical Polymerization

Pyrrole is vulnerable to oxidation and can be chemically polymerized in different aqueous and non-aqueous solvents thanks for its low oxidation potential (< 0.8 V) [12]. Chemical polymerization shows advantages over EP when large quantities of CPs are needed. The product of chemical polymerization, powder or surface coating, can function as fillers or a coating layer to impart conductivity to the composites. Besides, there are many other benefits when using chemical polymerization, for example, the low cost, simple process, high efficiency, and great yield. Features of the PPy prepared via chemical polymerization are inseparably correlated to the nature and concentration of the oxidant, the monomer feeding, reaction temperature and time, and the selection of solvent, surfactant and additives.

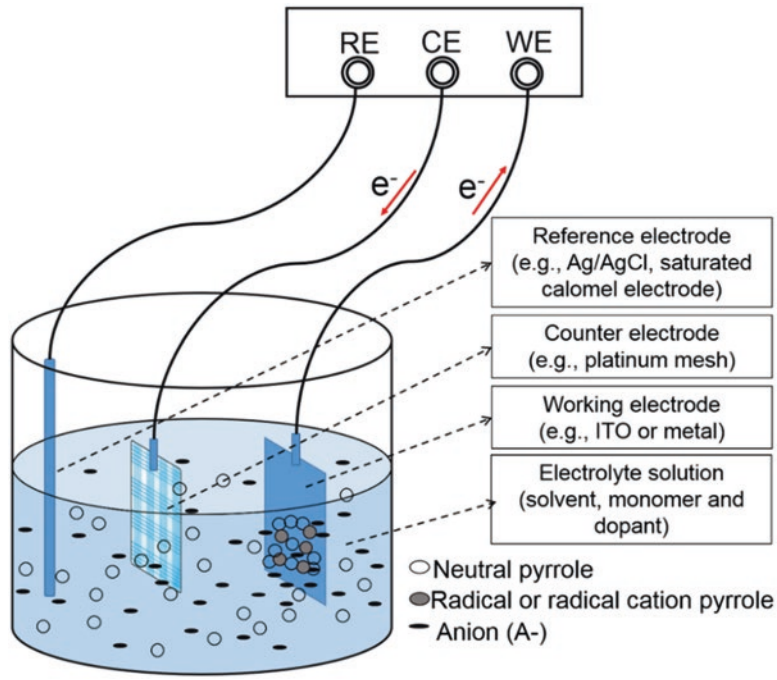

Fig. 18.2 Three-electrode system for electrochemical polymerization: reference, working, and counter electrodes submersed in a monomer and electrolyte solution. (Figure courtesy of [9, 11])

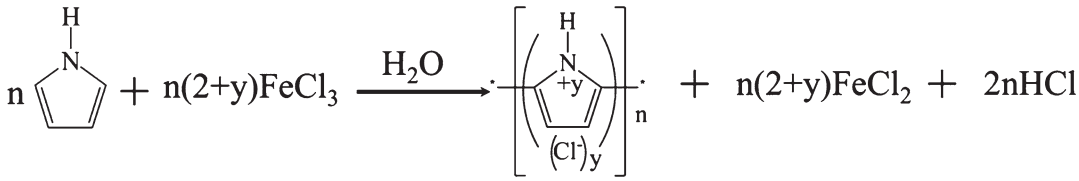

Fig. 18.3 A stoichiometric chemical polymerization of PPy with ferric chloride oxidant

Many oxidants exist to chemically synthesize PPy, such as H_2O_2, $K_2S_2O_8$, $(NH_4)_2S_2O_8$, and various salts containing transition metal ions or their mixtures, for instance, Fe^{3+}, Cu^{2+}, Cr^{6+}, Ce^{4+}, Ru^{3+}, and Mn^{7+} [13, 14]. Among them, $FeCl_3$ has been extensively studied [13]. The overall stoichiometric reaction between Py and $FeCl_3$ can be summarized in Fig. 18.3 [10, 12, 15], where y is the doping level revealing the oxidation degree of PPy. In this case, the polymer is spontaneously doped by Cl^- anions during polymerization. As described above, PPy does not always form a linear propagation during polymerization [6]. The reaction temperature has a major impact on both polymerization kinetics and side reactions [16]. Previous study indicated that with a Fe^{3+}/pyrrole ratio of 2.33 and the reaction taking place in methanol for a short reaction time at a low temperature, the highest conductivity of PPy can approach 220 S cm^{-1} [17]. Moreover, selecting suitable surfactants in an emulsion system is also an effective way to further improve the conductivity and yield [18, 19].

Furthermore, chemical polymerization is superior to EP in being able to combine with templates to prepare PPy with designed nanostructures, which demonstrates great conductivity, high specific surface area, light weight, rapid ion exchange, and superior mechanical properties [20]. In general, both soft and hard template methods were developed to synthesize nanostructured PPy. The soft template strategy is typically composed of self-assembled micelles formed by surfactants for guided PPy molecule growth. As a result, the morphology and features of PPy are controlled by nature of the surfactants [19, 20]. For the hard template method, physically strong scaffolds are selected as templates, such as metallic particles and nanoarrays, oxides, silica, and carbon [21]. Some template-free methods were also developed to synthesize PPy with less well-defined nanostructures compared to the template method [22].

The combination of chemical polymerization and template is a simple and effective strategy to prepare nanostructural PPy, along with a low cost. Moreover, as shown in Fig. 18.4, PPy can be synthesized to have designed nano-sized morphologies by altering the templates and chemical polymerization systems. Through different combinations of chemical polymerization and template, PPy can be prepared into cauliflower-like particles, nanoparticles/nanocapsules, nanowires/nanofibers/nanotubes, thin films/membranes, and hydrogels/sponges. In fact, the cauliflower-like nodular PPy is formed by PPy nanoparticle aggregations.

18.2.2.1 Nanoparticles/Nanocapsules

In previous decades, PPy nano-spheres (0D) were synthesized by chemical polymerization to fabricate the highly dispersed heterophase composite materials [32]. Dispersion or emulsion polymerization assisted by soft template is the major method to obtain homogeneous PPy nanoparticles. Various stabilizers are adopted to avoid macroscopic precipitation in dispersion polymerization [33]; and the nature and feeding of the stabilizers have an obvious impact on the morphology and size of the PPy nanoparticles. For example, PPy nanoparticles with a diameter of 20–60 nm were chemically polymerized by adjusting the molecular weight and concentration of the stabilizer (polyvinyl alcohol, PVA) [34]. However, the conductivity of PPy nanoparticles synthesized through dispersion polymerization is relatively low owing to the difficulty of removal of the steric stabilizer. Thus, emulsion

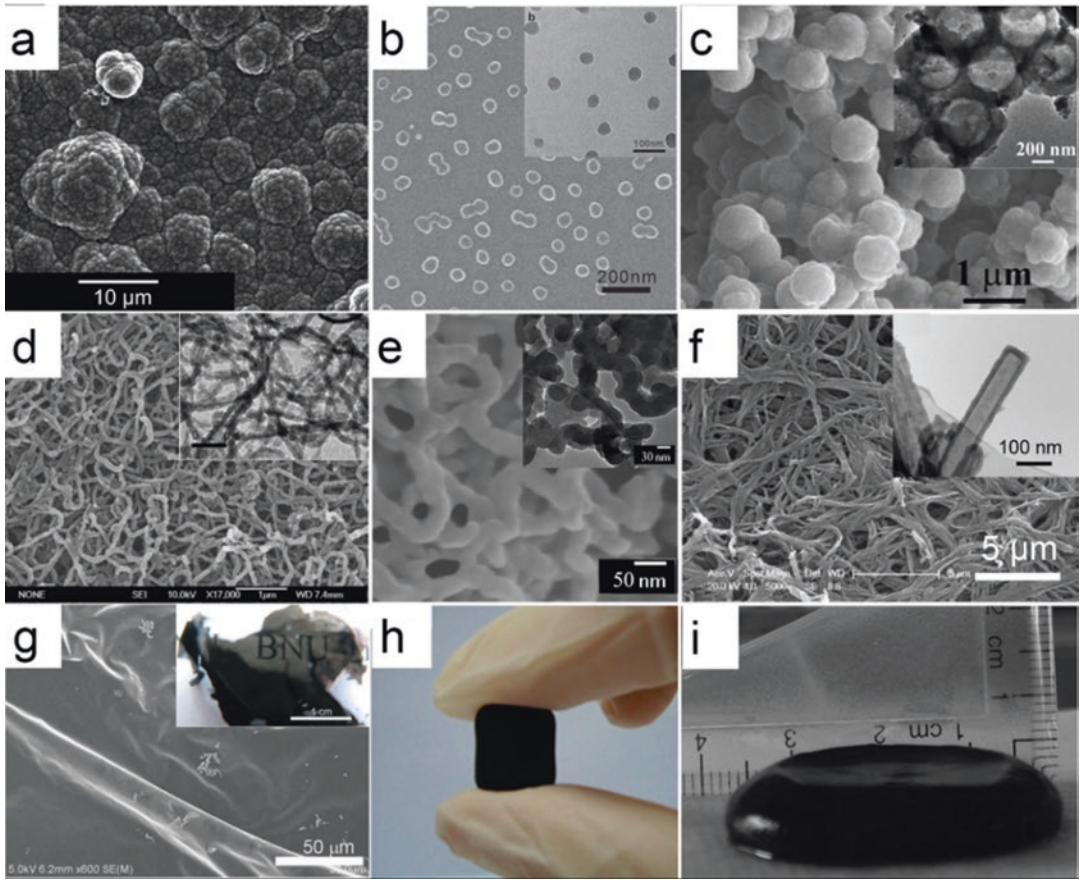

Fig. 18.4 Morphology of PPys: (**a**), cauliflower-like particles [23]; (**b**), nanoparticles [24]; (**c**), nanocapsules [25]; (**d**), nanowires [26]; (**e**), nanofibers [27]; (**f**), nanotubes [28]; (**g**), film [29]; (**h**), sponge [30]; (**i**), hydrogel [31]. Reproduced from Refs. [23–31] with permission

polymerization as an alternative method was widely employed to prepare PPy nanoparticles [35]. PPy nanoparticles with controllable diameter (30–50) and good conductivity (2.1 S cm^{-1}) were prepared by emulsion polymerization using DBS as surfactant [36]. An alternative oxidant (ammonium peroxydisulfate) and surfactant (sodium dodecyl sulfate), and an additive (n-amyl alcohol) were suggested to further improve the conductivity of the PPy nanoparticles to 61.9 S cm^{-1} [19]. Hard template was recommended to synthesize hard template@PPy core-shell nanoparticles by similar dispersion or emulsion polymerization. Many hard templates have been adopted for the preparation of the core-shell PPy nanoparticles, such as polystyrene latex spheres [37], SiO_2 spheres [38], metal [39], and metal oxide [40]. Additionally, some cores (hard template) can be removed subsequently to fabricate PPy nanocapsules [41, 42].

18.2.2.2 Nanowires/Nanofibers/Nanotubes

Recently, one dimensional (1D) PPys, i.e., nanowires, nanofibers, and nanotubes, have been widely explored because of their high specific surface area and efficient transport of charge and energy [20]. A soft template made of a lamellar inorganic/organic system (cetyltrimethylammonium bromide/ammonium persulfate) was designed to prepare PPy nanowires

[43]. Meanwhile, PPy nanowire arrays with a high surface area of 75.66–172.90 $m^2\ g^{-1}$ were synthesized using an alumina membrane with nanochannels inside as the hard template [44]. PPy nanofibers [45, 46] were also prepared by electrospinning, in which the natural nanofibres and carbon nanomaterials were employed to play the role of hard templates. After removal of the hard templates, PPy nanotubes were easily obtained from those PPy coated nanofibers [47]. In addition, PPy nanotubes have been prepared by the simple emulsion polymerization with sodium bis(2-ethylhexyl) sulfosuccinate as surfactant [48]. Recently, the fibrillary complex of $FeCl_3$ and methyl orange (MO), as a reactive hard template, has been developed to synthesize PPy nanotubes [49].

18.2.2.3 Film/Membrane

Until 2017, it was a challenge to prepare a large-sized PPy film or membrane (two dimensional, 2D) without any supporting substrate due to the insolubility and infusibility of PPy. There were only a few reports on the synthesis of PPy films by photopolymerisation and interfacial chemical polymerization. A thick PPy-Ag nanocomposite film (200 μm in thickness) and a thin film (<1 μm in thickness) were simultaneous photopolymerized respectively at the dichloromethane/water and air/water interfaces [51]. Free-standing PPy films (100 nm in thickness) with up to 2000 S cm^{-1} in conductivity were prepared by *in situ* freezing interfacial polymerization [29]. Additionally, via keeping the reaction system at room temperature, this research group prepared semitransparent free-standing PPy films with 50–500 nm in thickness and 150–560 S cm^{-1} in conductivity at the cyclohexane/water interface [52]. Similarly, PPy films were also prepared by interfacial chemical polymerization using Bmim [$FeCl_4$] as the oxidant [53]. However, all those PPy films are so thin and fragile that it is particularly difficult to collect the films from the reaction system and to further conduct handling and drying. Actually, most reported flexible and large-sized PPy films or membranes are formed by coating PPy onto a support matrix, which determines the mechanical properties and processability of the composite membrane [54, 55]. In 2017, for the first time, our group designed a template assisted interfacial polymerization (TIP) and successfully synthesized a soft and mechanically processable PPy membrane without modification of the monomers or support by other materials [50]. As shown in Fig. 18.5, although PPy has a high glass transition temperature (> 100 °C), the single-component PPy membrane presents an extraordinary softness and flexibility even in liquid nitrogen (−196 °C). The unique multilayered structures and connections among the structures were considered responsible for the excellent flexibility. Furthermore, this PPy membrane has a good mechanical processability, which can be cut, tied, folded into a cubic box, and also be laminated and rolled into elastic tubes.

18.2.2.4 Hydrogel/Sponge

PPy hydrogel has a three dimensional (3D) highly porous network resembling natural tissues. Usually, PPy hydrogels are prepared either by polymerizing the monomers onto a nonconductive hydrogel, or by mixing PPy into a nonconductive hydrogel [31, 56]. However, the performance of those PPy hydrogels is restricted by some issues, such as the loss of PPy from the hydrogel during the swelling or shrinking process, and the decline of conductivity. Compared to the conventional gels, the PPy hydrogels fabricated by using the reactive MO-Fe^{3+} complex template presented much faster swelling-shrinking behaviors and size changes [31]. The formation of this PPy hydrogel depends on π-π interaction and hydrogen bonding among PPy nanotubes and granules. Based on that, Lu used a deficient oxidant to slow down the polymerization to obtain an elastic PPy hydrogel [57]. The decreased joint density and coarsened joints of initial network was suggested to explain why the PPy hydrogel is elastic even in a dry condition. However, further research is still required to improve the mechanical properties and processability of the 3D PPy, particularly in a dry condition.

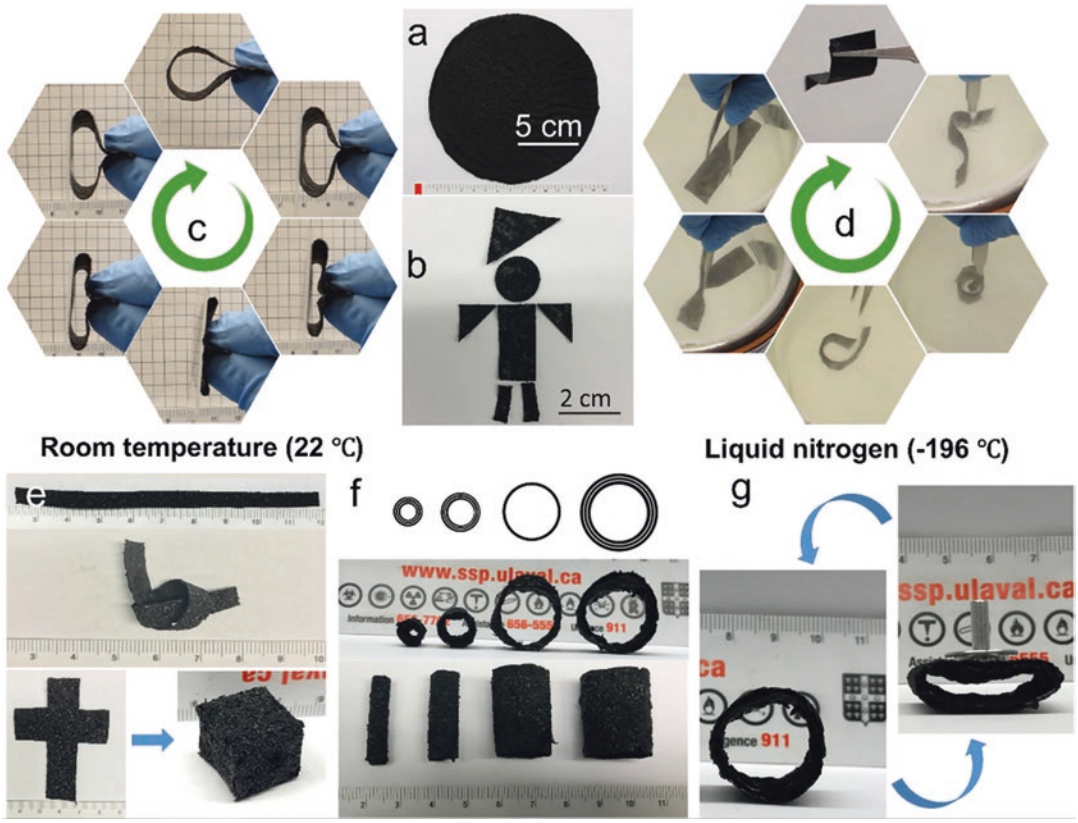

Fig. 18.5 Physical properties and processability of PPy membrane synthesized through TIP method. (Reprinted with permission from Ref [50]. Copyright (2017) American Chemical Society). (**a**) An as-prepared membrane 15 cm in diameter. (**b**) Cutout. (**c**) Cyclic bending at room temperature (7.5 cm × 2.5 cm). (**d**) Waving in liquid nitrogen (7.5 cm × 1.0 cm). (**e**) Tie and cube. (**f**) Rolling and laminating. (**g**) Radial elasticity of a triple-layer tube

18.3 Potential Applications in Biomedicine

Biocompatibility is critical for CPs to be used in biomedical applications. In the last decades, numerous studies have been conducted to evaluate the biocompatibility of PPy. Williams and Doherty proved the cytocompatibility of PPy [59]. And then, systemic toxicity of the aqueous extract of PPy powders was tested, and the results indicated that there was no acute toxicity, mutagenesis, pyrogen, haemolysis or allergic response [60]. Many *in vivo* experiments in animal models also demonstrated that PPy presented no significant long-term effect [61] or induced only a minimal tissue response [59]. Furthermore, PPy has the capacity to support the adhesion, growth and differentiation of various types of cells [7]. Consequently, as shown in Fig. 18.6, PPy has been extensively investigated for various biomedical applications, including tissue engineering, neural prostheses, biosensors, drug delivery, and artificial muscles.

18.3.1 Tissue Engineering and Regenerative Medicine

Because various cell types are responsive to electrical stimulation, PPy has been prepared into conductive scaffolds or substrate, hoping being able to regenerate functional tissues with the help of electrical stimulation [62]. To this end, numerous researches have focused on the conductive

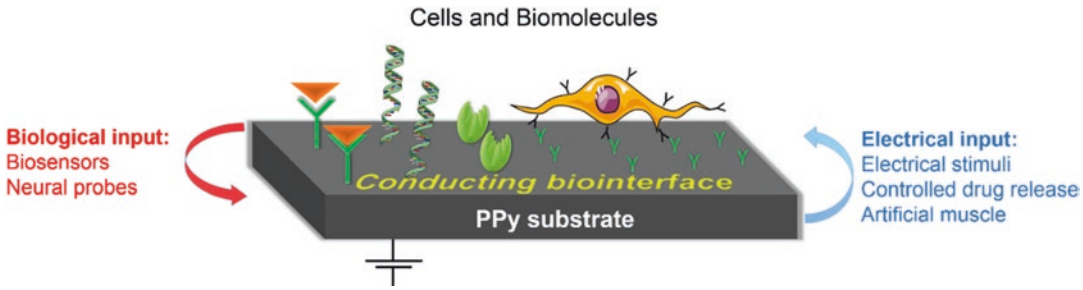

Fig. 18.6 PPy serving as a conducting biointerface for various biomedical applications. (Figure courtesy of [58])

PPy scaffolds for the purpose of neural repair and wound healing. Schmidt demonstrated that electrical stimulation contributed to the rat PC-12 cell attachment and neurite extension [63]. Thereafter, her group developed a biodegradable conductive polymeric scaffold that enhanced the adhesion and proliferation of human neuroblastoma cells *in vitro* and presented tissue compatibility *in vivo* [64]. Moreover, the PPy scaffolds loaded nerve growth factors (NGF) could further improve neurite formation and growth [65]. Our group pioneered and conducted a great deal of studies on the PPy-based conductive scaffolds and electrical stimulation for wound healing [66–71]. The results indicated that the migration, growth and differentiation of human skin fibroblasts and keratinocytes and the secretion of growth factors/cytokines can be regulated by both constant and pulsed electrical stimulations. Additionally, the electrical stimulation treated human skin fibroblasts can transdifferentiate into the myofibroblast phenotype and maintained this phenotype after 15 days of implantation in nude mice [67], showing for the first time that the electrically activated characteristics in mammalian cells can be transferred to new generations.

18.3.2 Neural Prostheses

Neural prosthesis is a medical implant containing several probes to electrically stimulate neurological activities in neural tissue and to achieve the recording functions [72]. The implantable microelectrodes for neural prostheses are normally made of metal or silicon. However, the recording and therapeutic ability of neural prostheses often decrease due to the capsulation of connective tissue during chronic implantation [73]. Modification of the electrode surface is required to solve this adverse host response and to improve the poor interface between microelectrodes and neural tissue. PPy has been coated onto the electrodes to improve the biocompatibility of neural prostheses [72, 74]. In addition, fibronectin fragments (SLPF) and nonapeptide CDPGYIGSR were entrapped into the PPy coating layer to enhance neural cell attachment and growth on the probes [75]. Cui's research group incorporated an anti-inflammatory drug (dexamethasone) into the PPy-based neural electrodes in order to decrease host response [76]. Similarly, neurotrophin-3 was also combined onto the PPy-based neural probes to promote neuron migration towards the electrodes and to prevent the capsule formation [77]. It was also demonstrated that immobilization of NGFs onto PPy through covalent bonds could significantly increase neurite extension [78]. The PPy coating layer on the neural electrodes could efficiently preserve the electrical communication between neurons and probe, and the modified surface can also improve probe sensitivity, durability and biocompatibility.

18.3.3 Biosensors

Serving as the detector of an analyte, biosensor is a device that combines a biological component with a physicochemical detector. As shown in Fig. 18.7, a biosensor contains three parts

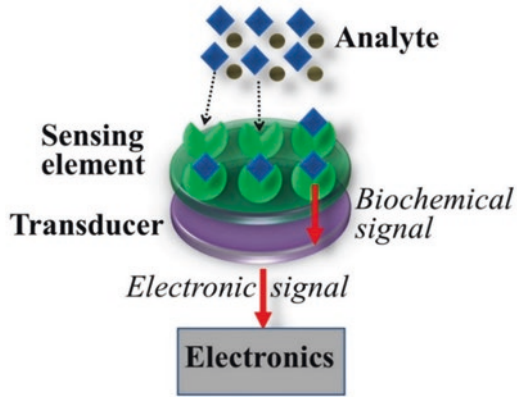

Fig. 18.7 Schematic of a biosensor. The sensing element (e.g., biomolecule) detects the analyte, and then a series of signal will be produced and monitored by an electronic device

including a sensing element, a transducer, and the associated electronics or signal processors. Once the analytes couple onto the sensing element, there will be electrochemical or physical changes due to the biochemical reactions, which can be detected quantitatively and qualitatively by means of amperometric, potentiometric, conductometric, optical, calorimetric and piezoelectric detections [79, 80]. As the core of the device, the sensing element is made of biomolecules, such as enzymes, nucleic acids, antibodies, or multi-components. In particular, PPy has been widely investigated in biosensors owing to the practicability of coupling biomolecules onto the PPy matrix. According to the type of biomolecule in the sensing element, biosensors generally include enzyme biosensors, DNA biosensors, and immunosensors.

18.3.3.1 Enzyme Biosensors

Enzyme biosensing is based upon the specific reactions between the enzymes settled on electrode and the analytes. Then the reaction, normally a redox reaction, will generate measureable signals such as electrical current or potential changes [81]. In recent years, by means of doping and covalent grafting, various enzymes were immobilized onto PPy electrodes to construct enzyme biosensors to detect diverse target analytes. By incorporating glucose oxidase (GOD) into the PPy-based composites, a glucose biosensor was assembled and showed a fast response (5 s) to glucose and a linear current-time relation over a concentration range from 0.5 mM to 147 mM [82]. Urease was also immobilized onto a PPy film to form a urea sensor with a high selectivity and efficiency at urea concentrations lower than 3.0 mmol L^{-1} as well as a sensitivity of 2.41 μA cm^{-2} $mmol^{-1}$ L [83]. However, several issues should be overcome to enhance the sensitivity, such as the loss of the quantity and activity of the enzyme because of desorption and the synthesis process. These issues could be effectively solved through covalent grafting of the enzymes onto the nanostuctural PPy-based electrode. In fact, by using covalent linkages, GOD was grafted onto the functionalized PPy composite nanowires to increase the sensitivity (33.6 μAm M^{-1} cm^{-2}), detection limit (0.63 μM) and stability of glucose biosensors [84].

18.3.3.2 DNA Biosensors

DNA biosensors have been widely studied on account of enormous applications in clinical examination of genetic diseases, rapid diagnosis of pathogenic infections, and screening of c-DNA colonies for molecular biology [79]. Normally, a DNA biosensor is formed by immobilizing a single stranded DNA (ssDNA) [85] or a double stranded DNA (dsDNA) [86] onto the PPy electrodes. Initially, DNA was incorporated to the PPy electrode surface by adsorption [87]. After that, Ko et al. synthesized carboxylic acid functionalized PPy nanotubes and grafted ssDNA to the nanotubes by covalent bond [88]. Both DNA and RNA have been verified to generate redox signals after hybridization based on their electroactive compounds [89]. However, for a long time, it was difficult to detect the signals with a dsDNA biosensor. But the signal was observed on the oscillopolarograms of the ssDNA-based DNA biosensor. In order to solve this problem, labels, such as redox-active molecules or enzymes, have been introduced to DNA sensors to offer detectable signals. Redox-active guanine was later recognized as the culprit constraining detectable signaling [90]. For this reason, label-free DNA biosensors have been

prepared by the immobilization of guanine-free probes [88, 91]. The labeling approach enhances the sensitivity and selectivity, and also means a higher cost. In contrast, the label-free biosensor provides a readable signal in real time, but it requires a complex modification process to substitute guanine by insoine.

18.3.3.3 Immunosensors

Immunosensors, usually antibodies based, are also extensively explored for pharmaceutical research, food safety, and environmental monitoring [92]. The antibody-antigen layer on the sensing electrode significantly disturbs ion diffusion and also changes the electrical capacitance and optical properties. And these changes can be converted into readable electrical signals by electrochemical impedance spectroscopy (EIS) [93] and surface plasmon resonance (SPR) [94]. In order to immobilize antibodies onto the PPy-based immunosensors, some methods have been developed, including adsorption, doping, and covalent binding. Li's group conducted a series of study on the immobilization of proteins to PPy probes [94–96]. The functionalized PPy-based probes were investigated to incorporate BSA by absorption [95], goat IgG by covalent binding [94], and anti-leptin IgG by the assistance of protein G [96]. Unquestionably, covalent binding is a method that can guarantee the superior stability and density of protein immobilization. Moreover, a PPy-based immunosensor probe with high sensitivity was prepared through oriented antibody immobilization with the help of protein G, which meaningfully improved the antibody density on probe surface and the antigen binding capacity, bringing a high sensitivity [93]. The human chorionic gonadotropin (HCG), a tumor marker in gestational trophoblastic disease and germ cell tumors, can be sensitively detected by the PPy-based HCG-immunosensor [97]. Similarly, other biomarkers such as hormone (human growth hormone) [92], growth factor (vascular endothelial growth factor) [98] and cancer marker protein (CA 125) [99] were examined via the PPy-based immunosensors. Pathogen is one of the essential detection targets in food safety testing and environmental monitoring. Anti-Listeria [100] and a plant pathogen specific antibody [101] were covalently conjugated on the surface of PPy to fabricate a recognition system to detect Listeria and cucumber mosaic virus.

18.3.4 Drug Delivery

Anions are automatically incorporated into the oxidized PPy molecules to balance the positive charges during polymerization. And a controllable release of those anions can be achieved during the reduction of PPy via electrical stimulation (Fig. 18.8). Numerous anionic drugs were entrapped into PPy matrix as dopants to form drug delivery systems to controllably release the loaded drugs such as glutamate [102], dexamethasone [76], heparin (HE) [103], and anthraquinone-2,6-disulfonic acid [104]. Some cationic and neutral drugs have also been

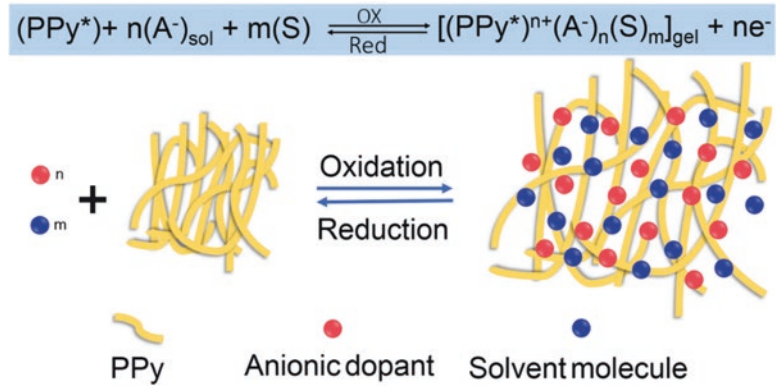

Fig. 18.8 The oxidation-reduction reaction of PPy in solution. (Figure courtesy of [58]). A^-: an anionic dopant (red dots); S: solvent molecule (blue dots)

entrapped into PPy matrix thanks for the physical entrapment, electrostatic interactions and hydrophobic forces [105, 106]. Svirskis summarized the strategies of preparing the PPy-based drug delivery systems, and demonstrated what impacted drug release such as the thickness and density of PPy matrix, the media properties, and electrochemical parameters [107]. However, the activity and stability of the drugs are still the key issues in PPy-based drug release. The drugs are always combined into the PPy matrix through polymerization, and this procedure may cause deactivation of the loaded drugs. The loaded drugs in the PPy matrix usually have a tendency to leach out through diffusion, resulting in the loss and uncontrollable release of drugs (i.e., initial burst release). Biotin has been adopted as dopants to offer the sites for covalent grafting of biomolecules, obtaining a uniform release kinetics upon electrical stimulation [108]. Thus, studies are required to increase the amount of drugs as dopant and at same time to reduce non-specific physical absorption.

18.3.5 Artificial Muscle

As illustrated in Fig. 18.8, the volume of a PPy matrix will change due to "immigration and emigration" of ions or electrolytes during the oxidation-reduction reactions (doping and dedoping processes), making it possible to use PPy in artificial muscles [58]. The simplest PPy-based bilayer artificial muscle comprises two components, i.e., a PPy layer and a non-conducting layer (ex: plastic tape). The volume of the PPy film increases relative to the tape during oxidation due to the "immigration" of ions or electrolytes to generate a bending deformation, and an opposite deformation happens during reduction. A sandwich-like three-layer (PPy/tape/PPy) device was also fabricated to significantly raise the efficiency due to the opposite volume changes in the two PPy layers with the same current at the same time [109]. It is still highly desirable to prepare a large-sized and flexible PPy film containing a large amount of anionic dopants in order to obtain large deformation.

18.4 Biofunctionalization of PPy

As a "bio-inert" material, PPy needs to be functionalized by bioactive molecules to obtain a bioactivity or specific biofunctions. As shown in Fig. 18.9, three strategies were developed to biofunctionalize PPy including physical adsorption and entrapment, doping, and covalent immobilization.

18.4.1 Physical Adsorption

Biomolecules can be physically adsorbed to a PPy matrix thanks for the physical interactions including electrostatic force, hydrophobic force, and van der Waals forces. Dicks et al. firstly fabricated a PPy-based glucose enzyme biosensor via a simple adsorption of glucose oxidase onto a PPy electrode [111]. A twelve amino acid peptide (T59) was also used to modify the PPy scaffold through electrostatic adsorption to enhance neuron adhesion and neurite extension [112, 113]. Because a nanostructure is helpful to adsorb biomolecules, PPy nanotubes were used to prepare glucose biosensors with increased enzyme loading efficiency and sensing area [114]. However, physical adsorption is infrequently employed now owing to the weak binding between biomolecules and PPy substrate, leading to the uncontrollable loss of biomolecules.

18.4.2 Doping

Doping is essential for PPy to form charge carriers (polarons and bipolarons) in its chains to gain conductivity (Fig. 18.1). Because PPy is a p-doped polymer, negatively charged molecules are always required as dopants to counterbalance the positive charges on the polymeric backbone. This provides a possibility for the negatively charged biomolecules to be incorporated into a PPy matrix as dopants. For example, proteins (enzymes and antibodies) at pH higher than their isoelectric points are negatively charged, which can be doped into PPy during an oxidation process to assign specific biological functions to

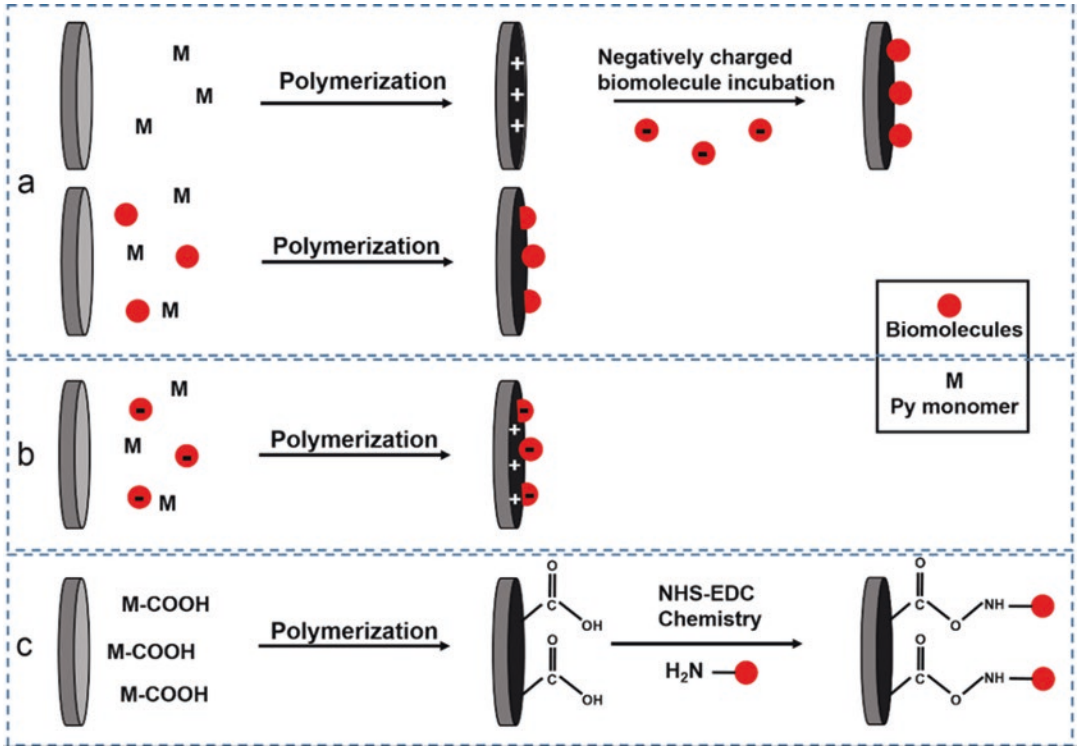

Fig. 18.9 A schematic representation of functionalization of PPy by biomolecules via (**a**) physical adsorption; (**b**) doping; (**c**) covalent immobilization. (Figure courtesy of [110])

PPy. Doping offers a straightforward and prolonged immobilization of stable biomolecules with respect to physical absorption [110].

In tissue engineering, various biomolecules (extra cellular matrix (ECM) components, proteins, and peptides) are frequently selected as dopant to improve the affinity, bioactivity, and specific biofunctions of the PPy conductive scaffolds for different cell types [115]. In our group, HE was adopted as dopants for PPy scaffolds to improve the cell adhesion and electrical stability [116]. Besides, various biomolecules such as dextran sulphate (DS), hyaluronic acid (HA), chitosan, collagen, growth factors, laminin fragments, chondroitin sulfate A (CS), oligodeoxyguanylic acids and adenosine triphosphate (ATP) have all been introduced as dopants to form bioactive PPy scaffolds [7, 115, 117]. Remarkably, these biomolecular dopants not only can render a specific bioactivity to PPy but also can change the physicochemical and other properties of the PPy scaffolds. For example, Wallace's group demonstrated the effect of different dopants on the physical properties of PPy films [118], and proved that PPy/poly(2-methoxyaniline-5-sulfonic acid (PMAS) and PPy/CS films had a lower surface roughness and Young's modulus than the PPy using other dopants such as para-toluenesulfonic acid (pTS), HA, and DS. To construct the recognition center of a biosensor, the receptors (ex: enzymes, antibodies) were also incorporated into PPy electrodes via doping [119–121]. The doping-dedoping process of PPy materials has been found particularly applicable in drug delivery (Fig. 18.8). As mentioned above, various anionic drugs such as glutamate [102], dexamethasone [76], HE [103], and anthraquinone-2,6-disulfonic acid [104] have been entrapped into PPy as anionic dopants to form drug delivery systems. The cationic drugs, such as dopamine [122] and chlorpromazine [123] were also incorporated into PPy substrates

through a post-synthesis method. However, the possibility of denaturing or deactivation of the proteins is still a key issue and needs to pay a particular attention on it. In addition, there are still some limitations for the incorporation of biomolecules by doping, including the low loading, decrease in conductivity, and uncontrollable loss of biomolecules due to diffusion. Noticeably, the biomolecules incorporated in a PPy matrix by doping or physical adsorption present throughout the PPy substrate, that is, only a small amount of the biomolecules is available on the surface for reactions, resulting in a low efficiency of the biomolecules.

18.4.3 Covalent Binding Immobilization

The method of surface covalent immobilization of biomolecules has gained more attention considering the limits of physical adsorption and doping. Due to a variety of functional monomers, copolymers made of pyrrole and functional pyrrole derivatives are favourable candidates to immobilize biomolecules covalently. As shown in Fig. 18.9c, many biomolecules are straightforwardly immobilized onto the PPy surface by using a cross-linker. The advantages of covalent binding immobilization generally include the minimal loss of biomolecules over time, tight control over grafting, high accessibility to analytes, and controllable biomolecule orientation [9]. On account of above reasons, covalent immobilization of biomolecules onto the functional PPy surface is regarded as the most efficient and stable strategy [124]. As shown in Fig. 18.10, depending on the substituted position, there are three types of functionalized Py monomers including N-substitution, α-substitution and β-substitution.

Because α-position is the reactive site for PPy molecule chain growth, only a few studies have been conducted on α-substituted PPy. Most researchers prefer the β-substituted and N-substituted Py. For example, Roy et al. copolymerized Py and pyrrole-3-carboxylic acid by EP for the covalent grafting of polyethylene glycol (PEG) to gain an antifouling property [125]. Similarly, antihuman IgG was also immobilized onto a poly(pyrrole-3-carboxylic acid) homopolymer film to prepare a biosensor [126, 127]. A poly(pyrrole-3-carboxylic acid) homopolymer was also synthesized through chemical polymerization, to which lysine was grafted for the adsorption of bilirubin [128]. However, owing to the difficulty in altering the β position in pyrrole ring, the affordable and readily available N-substituted Py has gained more research interest.

18.4.3.1 N-Functionalized PPys

Py derivatives with reactive moieties such as cyanol (-CN), amine (-NH_2), carboxyl (-COOH), hydroxyl succinimidyl ester (-NHS), hydroxyl (-OH), and epoxy (C_2H_2O) groups can be homopolymerized or copolymerized with Py to obtain various N-functionalized PPys (NPPys). Among numerous Py derivatives, N-(2-cyanoethyl)pyrrole (PyCN) is one of the most important Py

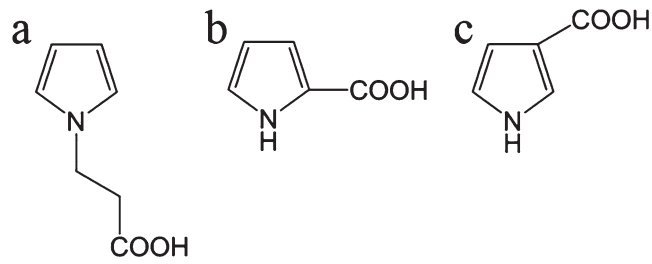

Fig. 18.10 Chemical structures of carboxylic acid-functionalized Py monomers: (**a**) N-position substituted 1-(2-carboxyethyl)pyrrole; (**b**) α-position substituted pyrrole-2-carboxylic acid; (**c**) β-position substituted pyrrole-3-carboxylic acid

derivatives [129], because many N-substituted Py monomers can be synthesized from PyCN. For example, 1-(2-carboxyethyl)pyrrole [130] and N-3-aminopropyl pyrrole [131] can be obtained by a simple nitrile hydrolyzation and a reduction of PyCN, respectively. Furthermore, the carboxyl group of PyCOOH can be changed to a hydroxyl succinimidyl ester group through the N-hydroxysuccinimide (NHS)/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry [132]. N-glycidylpyrrole was also synthesized by the alkylation between Py and epichlorohydrin to introduce the epoxy group for the covalent grafting of protein molecules [133]. Many NPPy homopolymers and copolymers have been polymerized, such as poly(pyrrole-co-1-(2-carboxyethyl) (PPyCOOH) [134], poly(N-3-aminopropyl pyrrole-co-pyrrole) (PPy-NH_2) [131], poly(pyrrole-co-succinimidyl pyrrole) (PPy-NHS) [135], and poly(pyrrole-co-N-glycidylpyrrole) (PPy-PO) [133]. However, most of these copolymers are synthesized by EP. Rajesh et al., for the first time, copolymerized Py and Py-NH_2 through EP to generate a PPy-NH_2 film for the covalent grafting of urease to form a urease enzyme biosensor [131]. Similarly, urease was also immoblized to a PPy-PO film to construct a biosensor to detect urea [133]. This urease enzyme biosensor presented a fast response time (4 s) and a linear response to urea concentrations from 0.1 to 0.7 mM with a sensitivity of 4.5 mA mM^{-1}.

As shown in Fig. 18.11, PPy-NHS can be regarded as the downstream of PPyCOOH before reacting with biomolecules. To synthesize PPy-NHS, two strategies were designed including the homo/copolymerization of Py-NHS and Py, and the modification of PPyCOOH by NHS-EDC chemistry. Py-NHS was electrochemically polymerized onto the surface of metal substrate, and then BSA was grafted to further improve biocompatibility [132]. However, due to the instability of Py-NHS, this method was gradually abandoned. Now more attention was paid to the latter strategy. In this method, the carboxyl groups of the PPyCOOH copolymer are modified to PPy-NHS by NHS-EDC chemistry, and thereafter biomolecules are immobilized. Additionally, the carboxyl groups of the PPyCOOH polymer can be converted to primary and secondary amines by simple chemical reactions [136]. Thus, PPyCOOH has been extensively explored in the biofunctionalization of PPy for biomedical applications.

18.4.3.2 Poly(Pyrrole–Co–(1–(2–Carboxyethyl)Pyrrole))

PPyCOOH homopolymers and copolymers (synthesized from Py and PyCOOH) have been extensively studied in biomedical area, as summarized in Table 18.1. Various biomolecules could be grafted onto PPyCOOH through covalent bonds by NHS-EDC coupling. It was demonstrated that the conductivity of the PPyCOOH copolymers decreased with the increase in the feeding ratio of PyCOOH [137]. Noticeably, as shown in Table 18.1, most PPy-COOH polymers in literature were synthesized by EP and so were

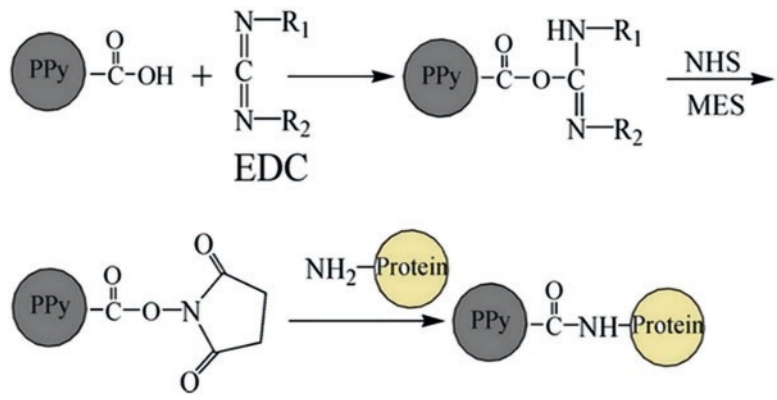

Fig. 18.11 Schematic illustration of grafting proteins onto PPyCOOH particle surface based PPy-NHS and NHS-EDC chemistry

Table 18.1 Biomedical applications of PPyCOOH

Synthesis method[a]	Ratio of PyCOOH	Grafted molecules	Conductivity	Performance	Suggested applications	References
CP	100%	RGD	2.83×10^{-4} S cm^{-1}	–	Tissue engineering	[142]
CP	50%	NGF	64 ± 50 kΩ □$^{-1}$	Increase neuron growth and neurite length	Tissue engineering	[65]
CP	100%	Copper ions	–	High adsorption capacity and good selectivity to bovine hemoglobin	Protein purification	[143]
EP	100%	RGD	4.65×10^{-4} S cm^{-1}	Improve cell adhesion and proliferation	Tissue engineering	[142]
EP	100%	GOx	–	Linear range of 1–80 mM, sensitivity of 1.7 μA cm^{-2}	Enzyme biosensor	[144]
EP	0–100%	GOx	10^{-8}–10^{-3} S cm^{-1}	Amperometric response to glucose	Enzyme biosensor	[137]
EP	100%	GOx	–	Linear range of 1–18 mM, sensitivity of 0.42 mA mM^{-1}	Enzyme biosensor	[145]
EP	100%	Alcohol dehydrogenase	10^{-4}–10^{-3} S cm^{-1}	Amperometric response to ethanol	Enzyme biosensor	[146]
EP	5%	Alcohol dehydrogenase	6.6×10^{-4} S cm^{-1}	Sensitivity of 10 μA cm^{-2} at 14 mM	Enzyme biosensor	[147]
EP	100%	Anti-mouse IgG	–	Sensitivity of ~20 pg mL^{-1}	Immunosensor	[148]
EP	0–30%	Goat IgG	–	Sensitivity of 1 μg mL^{-1}	Immunosensor	[94]
EP	20%	Protein G/ anti-leptin IgG	–	Linear range of 10^{1}–10^{5} ng mL^{-1} with sensitivity of 3.1 Ω mg^{-1} mL^{-1}	Immunosensor	[96]
EP	100%	Anti-rabbit IgG	–	Linear range of 119 pg mL^{-1} – 5.95 ng mL^{-1}	Immunosensor	[149]
EP	100%	Anti-hormone prolactin	–	Linear range of 10^{-2} –10^{4} ng mL^{-1}	Immunosensor	[150]

[a]*CP* chemical polymerization, *EP* electrochemical polymerization

unavoidably associated with drawbacks including small size, high cost, and the need for special equipment. In contrast, chemical polymerization is more suitable to synthesize PPyCOOH polymers in large-scale. For example, PPyCOOH was copolymerized onto silica microparticles [130] and polystyrene (PS) particles using $FeCl_3$ as oxidant [138, 139]. In both work, the silica and PS particles were used as the template, and surface treatments were required to improve the compatibility between the core particles and the PPy shell. Based on a new reactivity-driven mechanism, we synthesized the core-shell structured P(Py-PyCOOH) particles, containing a PPyCOOH homopolymer as the shell and a Py dominated P(Py-PyCOOH) copolymer as the core [140, 141]. By using this method, functional PPy particles can be chemically synthesized in large scale. And the conductivity, surface and bulk chemistry, and overall yield of the particles can be controlled by the feedings of monomers and oxidants or by reaction time [141]. The contradiction between the particle conductivity and surface reactivity is solved by this core-shell structure. Furthermore, as a model molecule, anti-human serum albumin antibody (anti-HSA) was covalently grafted onto the particle surface by using NHS-EDC chemistry and showed its specific reactivity to the antigen (human serum albumin, HSA) [140].

18.5 Manufacture of Polypyrrole–Based Materials

As previously mentioned, PPy is rigid, infusible and insoluble, and it is difficult to do further processing once synthesized. Thus, for now, it is still a major challenging to utilize PPy in almost any application without adding other supporting component due to the inferior mechanical properties and processability of PPy. A common method to solve this problem is to integrate PPy with other materials to form a composite that have good mechanical properties and processability. Recently, a certain level of mechanical properties of pure PPy were achieved via micro/nanostructure designs [151, 152].

18.5.1 Polypyrrole Composites

18.5.1.1 Surface Coating

Surface coating by chemical or electrochemical deposition is a primary and effective method to introduce a thin PPy layer onto the surface of supporting substrates. In this type of strategy, the thin PPy coating layer renders a favourite conductivity, and the substrate assumes the responsibility of not only a template to guide PPy growth, but also a framework to provide mechanical strength. Compared to electrochemical deposition, chemical deposition has apparent advantages in application for both conductive and insulating substrates. In our group, PET fabrics were coated with PPy through a two-step chemical deposition method [69, 71]. The PPy/PET fabric had a good surface electrical resistivity of 63.4 kΩ $\square^{-1}$ and about 80% of the conductivity was retained after an incubation in cell culture medium for 24 h.

In order to mimic the features of ECM, tissue engineering scaffolds are often fabricated into nanofibrous structures. Thus, nanostructured substrates were coated with PPy to fabricate the conductive nanofibrous composite scaffold. For example, PPy was coated onto a bacterial nanocellulose substrate, and this coating was found to improve PC12 cell adhesion and proliferation without electrical stimulation [153]. In addition, this conductive nanofibrous scaffold can be used to regulate cell activity by using electrical stimulation. Based upon the electrospinning techniques, numerous polymers can now be prepare into nanofibrous matrix for PPy coating to form the PPy-based nanofibrous scaffolds. PPy has also been coated onto nanofibrous matrix in an aqueous solution using $FeCl_3$ as the oxidant [45]. Vapor-phase polymerization is also an effective method to obtain a fine PPy coating layer on nanofibers [154]. The conductivity of those PPy-based composites seems to be controlled principally by the quantity of PPy on the surface, which is affected by the feedings of monomer and oxidant, reaction time, dopants, and doping level [45]. Due to the low affinity between PPy and most of the substrates, those PPy composites still have a crucial issue in terms of the separation between the PPy substrate [155].

18.5.1.2 Particle Filling

Blending PPy particles with other polymers is another popular method to fabricate the PPy-based composites. Compared with the commercial carbon black, PPy particles have gained much more attention as a conductive filling material in biomedical area owing to its excellent biocompatibility. The conductivity of the PPy-based composites results from the conductive paths constructed by the PPy particles in the substrate, which depends on the concentration and nature of the PPy fillers, state of dispersion, and geometry [156]. Among these factors, concentration of the PPy particles is regarded as the major factor for the conductivity of the composite. As shown in Fig. 18.12 [157], the critical feeding of the conductive fillers is recognized as the percolation threshold. Above that, conductivity of the composite dramatically grows (orders of magnitude) with the increase in the loading of the conductive fillers, following the percolation theory. The continuous conductive paths within the composite are formed when the loading of the PPy particles reaches the percolation zone. To fabricate the PPy particle filled composites, several methods were developed generally including solution mixing, *in situ* polymerization, and mechanical blending. In the solution mixing method, the PPy particles are mixed with a solution of the polymer, and then the solvent is removed by, for example, membrane casting and evaporation. In our previous reports, PPy/PLLA composite membranes (10^2–10^3 Ω □$^{-1}$) were fabricated by mixing PPy particles (5%) with a PLLA/$CHCl_3$ solution. Extensive studies have been conducted to electrically stimulate skin cell cultures for wound healing based upon this composite [66, 70, 103, 116, 158]. Through the same method, a PPy/chitosan composite membrane was also prepared presenting a conductivity of 10^{-3} S cm^{-1} with 2.5% (w/w) PPy loading [159]. The *in situ* polymerization method has also gained an enormous amount of research effort to prepare the PPy-based composites. For example, poly(D,L-lactide) (PDLLA) was introduced into the PPy emulsion polymerization system to synthesize the PPy/PDLLA composite materials [160]. After a serial dilutions with a PDLLA solution, PPy/PDLLA composite membranes with a controllable PPy loading from 1% to 17% and an adjustable surface resistivity from 2×10^7 to 15 Ω □$^{-1}$ were easily attained [161].

However, those composites may struggle with the problems such as loss of conductivity, agglomeration of the particles, and delamination owing to the inferior affinity or compatibility between PPy and supporting polymers [162, 163]. Furthermore, aging and degradation of the supporting polymers will cause the performance weakening, or even the destruction of the

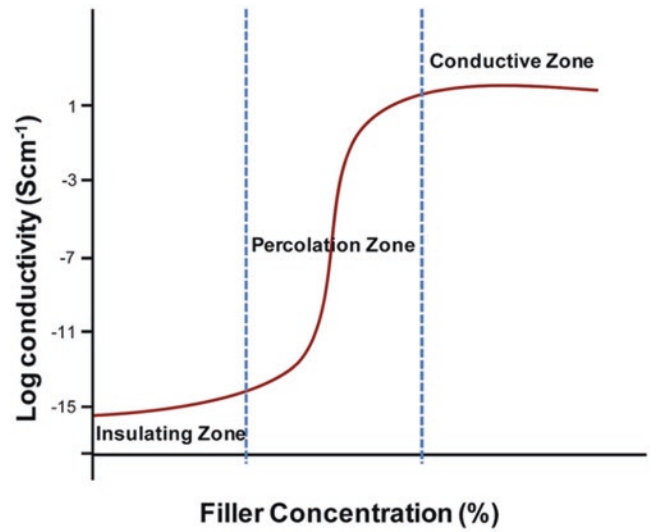

Fig. 18.12 Conductivity of composites as function of conductive filler concentration [156]. Open access article published by The Royal Society of Chemistry

structure when facing extreme temperature, pH, humidity, and environmental radiation.

Clearly, compounding PPy with other polymers to gain mechanical property and processability is on the expense of the environmental stability and electrical conductivity of PPy. Therefore, it is highly desirable to prepare the single-component PPy materials with considerable mechanical strength and processability.

18.5.2 Micro/Nanostructure Design

In order to prepare pure PPy materials with suitable mechanical properties, many attempts have been conducted through nano/microstructural designs rather than introducing another supporting component. For example, Wang et al. fabricated a free-standing 3D PPy porous foam by using a removable template [152], in which a high flexibility and a meaningful mechanical strength (~180 kPa) were achieved due to the unique microstructure and the combination with graphene oxide. Similarly, an elastic PPy framework constructed by interconnected hollow-spheres was obtained through a multiphase chemical polymerization at low temperature [151]. It was believed that the unique spherical shell geometry could help the intrinsically brittle materials to gain a variable elastic modulus. Recently, a free-standing PPy membrane with a certain degree of flexibility was constructed by PPy nanotubes [28, 49]. However, such a membrane exhibits a fragile performance rather than an incommensurable flexibility compared with the PPy composite membranes. These significant attempts mentioned above provide new thoughts about improving the mechanical strength of the single-component PPy membrane through nano/microstructure designs. Based upon these attempts, our group proposed the TIP method and succeeded in synthesizing the PPy membranes with an asymmetrical morphology on two sides, i.e., a bubble side and a nanotube side [50]. The bubble side is formed by the overlapped and thin-walled PPy bubbles, and the other side is covered by PPy nanotubes with 300 nm in diameter and 30 nm in wall thickness. It is these fundamental structures and their very small thickness that lead to the unprecedented material flexibility and mechanical processability.

18.5.3 "Soluble" PPy

The insolubility and poor processability of PPy and other intrinsically conductive polymers stem from their macromolecular structures, i.e., the strong intermolecular interactions and a high degree of crosslink. These make PPy hardly be truly dissolvable in any known solvent. Nonetheless, there are reports about the "soluble" PPy, which however is either of low molecular weight or with the help of a bulky dopant such as dodecylbenzene sulfonic acid [152] or polystyrene sulfonate [164]. Even so, such solutions can be a colloidal suspension rather than a solution at molecular level, as in the case of PEDOT:PSS. Nevertheless, no matter what level of solubility of the PPy molecules in such solutions, they are practically very useful and can be easily coated onto a substrate. Efforts have also been made to introduce polar groups at the β and N positions of pyrrole rings, hopefully to increase solubility [165]. These polymers, which actually can no longer be called PPy, become soluble at the expense of low conductivity and often low molecular weight as well. The route of synthesis of such pyrrole derivatives is much more complicate than that of pyrrole. The reaction speed and yield are also relatively lower.

While not soluble, PPy can be compounded with other polymers such as polylactide to form a partially biodegradable scaffold, to synthesize directly to the surface of a medical implant, to be polymerized in form of a hydrogel, or to form mechanically processable soft membranes, as described in previous sections. In such a way PPy has been investigated for a variety of biomedical applications. With the increasing research interest in conductive polymers as a new category of biomaterial, innovative technologies are expected to improve the processability of PPy.

18.6 Conclusion and Perspective

Electrical conductivity and electroactivity are among the inherent properties of human tissues. Most biomaterials however are yet to acquire such properties. This reality testifies the great potential in developing electrically conductive biomaterials. Among the synthetic conductive polymers, PPy is the most intensively investigated one for biomedical applications. It is cytocompatible and tissue compatible; it supports cell growth and interfaces electrical communications between cells and electrical devices. However, only a few commercial PPy products are available in the market due to its deficient mechanical property, poor processability, and insufficient functionality. Because there is no any functional group in PPy chains, the biofunctionalization of PPy using reactive pyrrole derivatives is of a great importance in the development of new electrically conductive biomaterials. Especially for biosensors, functionalization of PPy with biomolecules is imperative to construct the recognition center. Also, new technology is required to improve the mechanical property and processability of PPy, which has severely limited its application. Only with the development of new conductive materials, may one envision biomaterials more resemble natural tissues.

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19 Design of Temperature-Responsive Cell Culture Surfaces for Cell Sheet-Based Regenerative Therapy and 3D Tissue Fabrication

Jun Kobayashi, Yoshikatsu Akiyama, Masayuki Yamato, Tatsuya Shimizu, and Teruo Okano

Abstract

This chapter describes the concept of "cell sheet engineering" for the creation of transplantable cellular tissues and organs. In contrast to scaffold-based tissue engineering, cell sheet engineering facilitates the reconstruction of scaffold-free, cell-dense tissues. Cell sheets were harvested by changing the temperature of thermoresponsive cell culture surfaces modified with poly(*N*-isopropylacrylamide) (PIPAAm) with a thickness on the nanometer scale. The transplantation of 2D cell sheet tissues has been used in clinical settings. Although 3D tissues were formed simply by layering 2D cell sheets, issues related to vascularization within 3D tissues and the large-scale production of cells must be addressed to create thick and large 3D tissues and organs.

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Keywords

Temperature-responsive polymer · Poly(*N*-isopropylacrylamide) · Tissue engineering · Cell sheet · Regenerative medicine

19.1 Cell Sheet Engineering Using Temperature-Responsive Cell Culture Surfaces

Biological tissues and organs form their own shapes and exhibit specific functions by assembling multicellular cells with extracellular matrices (ECMs). The concept of "tissue engineering" [42] is emerging as a means to generate three-dimensional (3D) cellular tissues *in vitro* and to utilize them for replacement of damaged tissues. In typical tissue engineering, natural or artificial scaffolds composed of biodegradable materials are temporally used to form the shape of desired tissues. After implantation, sacrificial scaffolds in the engineered tissues should be ideally replaced with host ECMs and cells. To date, several biodegradable scaffolds, including porous synthetic polymers [43] and hydrogels [11], have been proposed for matching physical properties (e.g., the elastic modulus), exhibiting mass transport properties (e.g., the diffusion of oxygen and nutrients), cellular adhesiveness to scaffolds, and cellular signaling ligands.

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By contrast, artificially assembling cells into 3D tissues without scaffolds offers fundamental advantages in forming tight connection among cells (e.g., the formation of gap junctions in myocardium) [21] and in preventing unfavorable host responses to biomaterials (e.g., inflammatory reactions accompanied by the degradation of scaffolds) [95]. Two-dimensional cellular tissues referred to as "cell sheets" resemble native tissue structures that maintain cell-cell binding as well as adhesive proteins on the basal side. For example, epithelial tissues, including epidermal, esophageal, stomach and intestinal tissues, form sheet-like structures via cell junctions and adhere to connective tissues through a basement membrane. Therefore, cell sheets are highly effective at artificially constructing epithelial tissues.

"Cell sheet engineering" involves creating and manipulating transplantable 2D cell sheets and/or building 3D tissues by layering cell sheets [92]. Cell sheets are fabricated using a temperature-responsive cell culture dish where the surface is grafted with a temperature-responsive polymer. This chapter focuses on the design of temperature-responsive cell culture dishes for clinical applications of 2D cell sheet-based regenerative therapies. Issues related to the fabrication of 3D cell sheet-based tissues and organs are also described.

19.1.1 Temperature-Responsive Cell Culture Surfaces

The temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) has been used in several biomaterial applications because PIPAAm exhibits reversible temperature-dependent hydration and dehydration across the lower critical solution temperature (LCST) of 32 °C, which is close to body temperature, 37 °C (Fig. 19.1a) [23]. When temperatures fall below 32 °C, PIPAAm is dissolved and exhibits an expanded chain conformation in water because of hydration of PIPAAm, whereas above the LCST, PIPAAm is aggregated and insoluble in water due to the dehydration of isopropyl groups of PIPAAm. Owing to the reversible changes of hydration and dehydration, PIPAAm-grafted surfaces undergo temperature-dependent hydrophilic and hydrophobic changes across the LCST [73]. In addition, the conformation of the grafted PIPAAm chain and its chain mobility affect wettability changes occurring across the LCST on a PIPAAm-grafted surface [87, 88].

A pioneering study on temperature-responsive cell culture surfaces was conducted by Yamada and Okano et al. in 1990 [89]. A temperature-responsive cell culture surface, cross-linked PIPAAm-grafted tissue culture polystyrene (TCPS) (PIPAAm-TCPS), was prepared via electron beam (EB) irradiation. In this report, the authors showed that almost all hepatocytes adhered onto PIPAAm-TCPS at 37 °C were only detached by lowering the temperature. To our knowledge, PIPAAm-TCPS is the first temperature-responsive surface to achieve cell adhesion/deadhesion switching. On the other hand, Takezawa et al. found that human fibroblasts can be cultured on a TCPS surface coated with a mixture of PIPAAm and collagen [74]. Although the cells did not adhere onto PIPAAm-coated TCPS, cell adhesion and proliferation occurred on TCPS coated with a mixture of PIPAAm/collagen. For the PIPAAm coating, the addition of collagen proved essential for cell adhesion. When temperatures were decreased to 15 °C, the cells became detached and floated in the medium. The re-adhesion of the floating cells onto another plastic dish resulted in the formation of multicellular spheroids and in the outgrowth of fibroblasts from the spheroids.

Grafting of a nanoscale PIPAAm layer is a key factor for PIPAAm-TCPS to exhibit temperature-dependent alteration of cell attachment and detachment. To reveal the effects of grafted PIPAAm thickness, the temperature-dependent adhesiveness of cells was investigated for two types of PIPAAm-TCPS, for which the graft PIPAAm layers were thick (30 nm) and thin (20 nm) [3]. Bovine carotid artery endothelial cells did not adhere to the PIPAAm-TCPS surface with a 30 nm-thick PIPAAm layer even at 37 °C, where PIPAAm chains were dehydrated (Fig. 19.2a)). By contrast, cells adhered to the PIPAAm-TCPS surface with a thinner PIPAAm

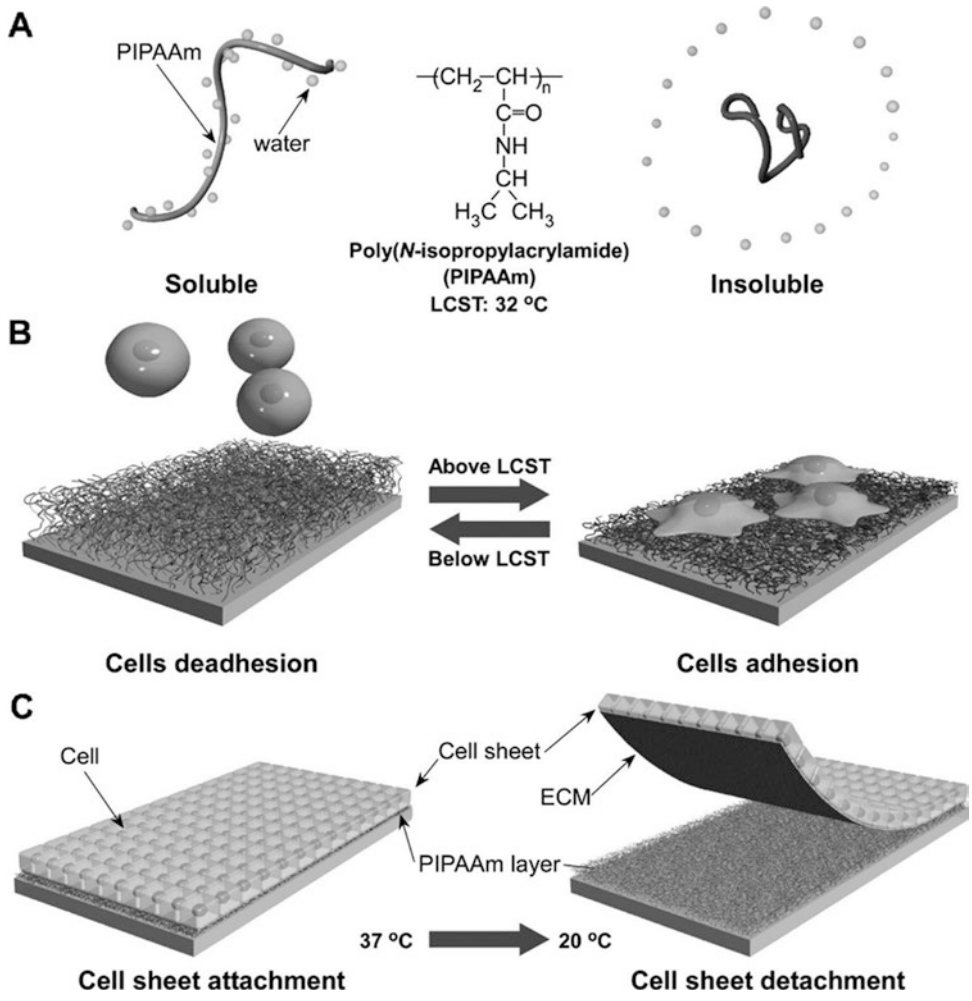

Fig. 19.1 Schematic illustration of the temperature-responsive character of (**a**) PIPAAm in aqueous solution and (**b**) PIPAAm grafted on a substrate and of (**c**) cell sheets recovered from a PIPAAm grafted substrate. (**c**) Attached cells proliferate to confluence 37 °C (right) and detach as a contiguous sheet with ECM from the PIPAAm-grafted substrate when the temperature is decreased to 20 °C (right). (Reprinted permission from [78]) with permission from Oxford University Press)

layer (20 nm) at 37 °C. By decreasing the temperature to 20 °C, the adhered cells detached from the thinner PIPAAm grafted surface. The non-cell-adherent character of layers thicker than 30 nm PIPAAm was supported by the fact that no detectable fibronectin was found on the surface.

We considered the hypothesis that PIPAAm chains at the interface of basal TCPS were aggregated and strongly dehydrated due to the hydrophobic TCPS. In contrast to flexible PIPAAm chains, such as soluble polymers and hydrogels, the mobility of covalently immobilized PIPAAm chains on TCPS was restricted. This restriction and dehydration of PIPAAm chains at the interface of the TCPS surface gradually extended toward the outermost PIPAAm-TCPS region. For the thin PIPAAm layer (15–20 nm), this gradual restriction and dehydration promoted the dehydration of PIPAAm chains in the outermost surface region, generating sufficient hydrophobicity for cell adhesion to PIPAAm-TCPS surfaces at 37 °C. On the other hand, for the thick PIPAAm layer (above 30 nm), the dehydration of PIPAAm chains at the outermost surface did not occur as

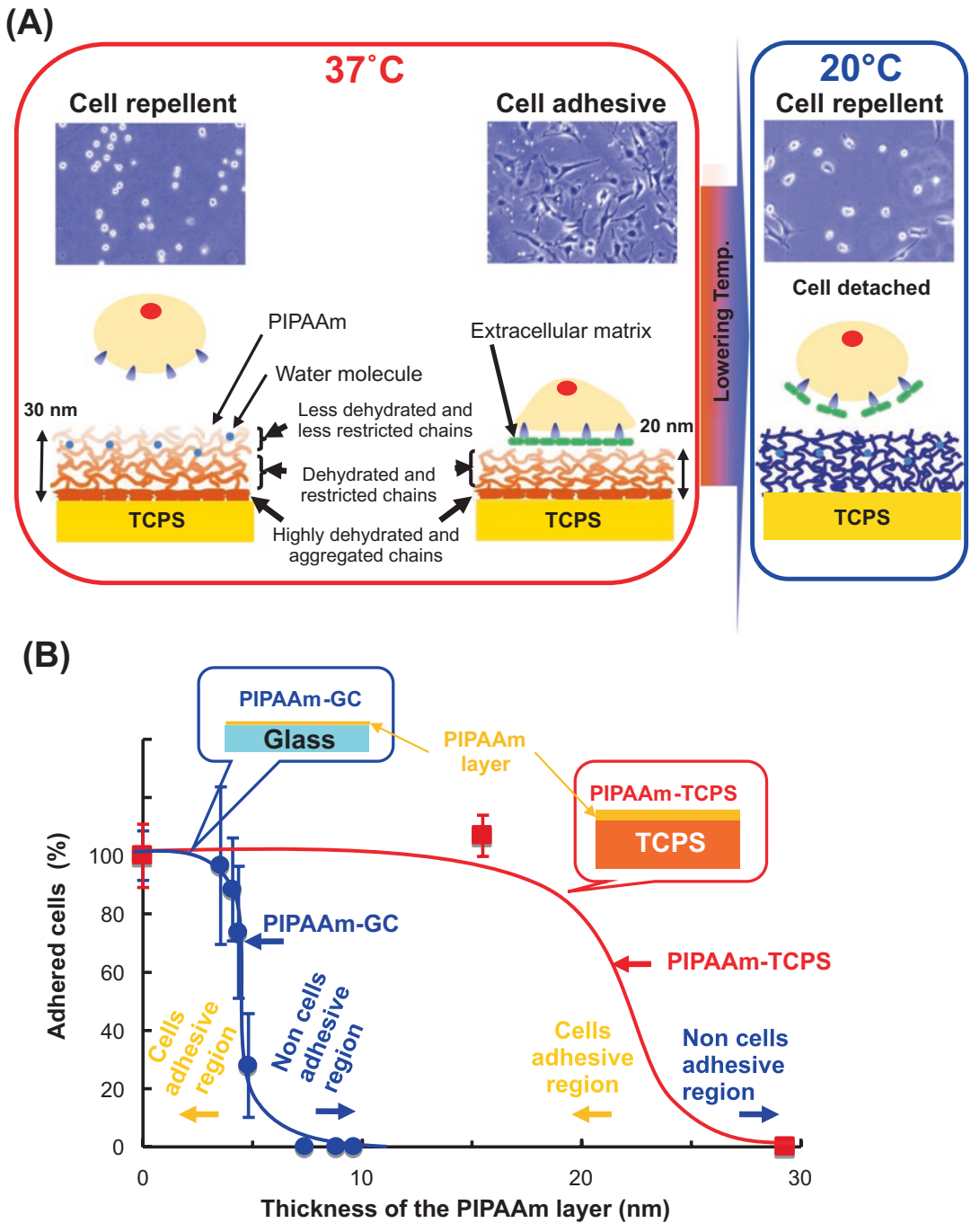

Fig. 19.2 The dependence on the graft polymer thickness of the subsequent cell adhesion behavior of temperature-responsive cell culture surfaces. (**a**) The influence of the mobility of grafted PIPAAm chains with different thickness on the hydration of polymer chains at 37 °C (left red-colored box) and 20 °C (right blue-colored box). Hydrophobicity of TCPS promoting aggregation and dehydration are shown as the black TCPS region. The molecular mobility of the grafted polymer chains increases according to the distance from TCPS interfaces. (**b**) Correlation between the spread cell density and thickness of the grafted PIPAAm. Red-colored circles and blue-colored diamonds denote PIPAAm-TCPS and PIPAAm-GC values, respectively. (Reprinted from [2, 17]) with permissions from John Wiley and Sons and The Society of Polymer Science, Japan, respectively)

efficiently. Therefore, cells were unable to adhere to the thicker PIPAAm-TCPS even at 37 °C. Based on this technology regarding the controlled thickness of grafted PIPAAm, a temperature-responsive cell culture dish, UpCell®, has been made commercially available.

Phenomena wherein the grafted polymer thickness dominates subsequent cell attachment behavior have also been found for another substrate, PIPAAm-grafted glass (PIPAAm-GC) surfaces (Fig. 19.2b) [16, 17]. By contrast to PIPAAm-TCPS, grafted PIPAAm layers 4.8 nm thick on a glass surface were found to be optimal for the temperature-dependent alteration of cell attachment and detachment. Above this optimal polymer thickness, cells did not adhere to PIPAAm-GC even at 37 °C. Such polymer thickness dependence is explainable in terms of graft PIPAAm chain mobility (Fig. 19.2a). Furthermore, the optimal graft PIPAAm layer thickness on glass for facilitating the temperature-dependent alteration of cell attachment/detachment was found to be thinner than that for TCPS, probably because the density and architecture of graft PIPAAm chains vary among these substrates [17].

Our findings regarding the dependence on the thickness of grafted PIPAAm have been recognized as part of a broader strategy to develop temperature-responsive cell culture surfaces through various methods [1, 81] (e.g., polymer coating [47], plasma irradiation [54], photoirradiation [48] and surface-initiated living radical polymerization methods, including atom transfer radical polymerization (ATRP) [45, 46] and reversible addition-fragmentation chain transfer polymerization (RAFT) [67]). Among these, surface-initiated ATRP and RAFT precisely controlled lengths of PIPAAm (molecular weight) with dense brushes. An intensive investigation of PIPAAm brush surfaces shows that the chain length and density of PIPAAm brushes and subsequent protein adsorption behavior significantly influence cell attachment behavior. When the molecular weight (M_n) and density of graft polymer chains are optimally tuned to 23,000–58,000 and 0.03–0.04 (chains/nm^2), respectively [1, 81], a PIPAAm brush surface undergoes a temperature-dependent alteration of cell attachment and detachment. More importantly, under optimal PIPAAm brush conditions, temperature-dependent changes in cell attachment and detachment were found on adsorbed fibronectin between PIPAAm brushes, and this is referred to as ternary adsorption [20, 86].

Recently, facile methods for the preparation of temperature-responsive cell culture surfaces have been developed without the use of special equipment such as EB and plasma irradiation systems. These methods are beneficial because they allow researchers to prepare temperature-responsive cell culture surfaces by themselves without using specialized equipment. Polymer coatings and visible light irradiation serve as facile alternative tools for preparation owing to their simplicity and cost effectiveness [18, 47 85].

19.2 Cell-Sheet-Based Regenerative Therapy

Alternation from a hydrophobic to a hydrophilic surface with low temperature treatment allows adhered cells to be noninvasively detached from PIPAAm-TCPS surfaces without cells being damaged while conventional cell recovery treatments using trypsin or chelate agents digest membrane proteins and ECM components of cultured cells [24, 38, 39, 93].

Cells on PIPAAm-TCPS, as well as TCPS, proliferate to confluency. After cell growth achieves confluency, temperature changes detach intact cell sheets from PIPAAm-TCPS while maintaining proteins among cell-cell bindings as well as on the cell surface. The detached cell sheet preserves the ECM component deposited on the basal side of the cell sheet. The recovered cell sheet is readily stacked onto another cell sheet or is transplantable to tissues and organs, as the ECM functions as biological glue and promotes adhesion between the cell sheet and another cell sheet or tissue [63, 94]. The deposited ECM allows the cell sheet to be transplanted and adhere to target tissues and organs without suture.

Therefore, cell sheet-based tissue engineering and regenerative therapy have attracted attention

as novel medical treatment approaches. In this section, cell sheet-based regenerative therapies for treating human diseases are described.

19.2.1 Autologous Cell-Sheet-Based Regenerative Therapy

The first trial for clinically transplanting cultured cell sheets was executed in 1981 for burn treatments by Green et al. [51]. Cultured autologous epidermal sheets were recovered by dispase treatment and were provided to patients with burn injuries. On the other hand, cell sheets that had detached from the temperature-responsive culture dish held ECMs on the bottom of the sheets due to the lack of enzymatic treatments (e.g., trypsin and dispase). This characteristic is expected to facilitate the efficient transplantation of cell sheet tissues to targets.

Table 19.1 shows clinical applications of cell sheet regenerative therapy. As epithelial cell sheet tissues, including epithelial keratinocyte sheets, corneal epithelial cell sheets, and oral mucosal epithelial cell sheets, resemble native sheet-like structures in the body, these cell sheet tissues replace damaged tissues after transplantation.

Since 2002, we have conducted collaborative research with K. Nishida on corneal epithelial regeneration treatments using cell sheets [49, 50]. From patients who had lost corneal epithelial cells due to alkali burns or drug side effects, ~2 mm^2 tissues containing epithelial stem cells at the boundary between the cornea and conjunctiva were collected. The cells collected were expanded and cultured to form corneal epithelial cell sheets [49]. In the case of binocular diseases, alternative epithelial cell sheets were prepared from oral mucosa collected from patients [50]. Both epithelial cell sheets regenerated human corneal epithelium after they were transplanted onto injured eyes, improving vision capabilities. Based on this achievement, CellSeed Inc., Japan, a venture company based out of Tokyo Women's Medical University (TWMU), initiated clinical trials in collaboration with Hospices Civils de Lyon in France in 2007 that were completed in 2011, indicating its effectiveness [9].

Collaborative research with Y. Sawa revealed that the regeneration of ischemic myocardium is induced by the attachment of skeletal myoblast sheets. Autologous skeletal myoblast sheets were generated from patient leg muscles and were stacked into three layers for transplantation. As a first clinical trial, autologous skeletal myoblast sheets were transplanted onto the heart surfaces of a patient who had experienced serious heart failure due to dilated cardiomyopathy. The patient's cardiac functioning was improved, eliminating the need for a left ventricular assist device (LVAD) and for cardiac transplantation. This recovery is attributed to angiogenetic effects of cytokines secreted from transplanted sheets in the proximity of the transplanted site and to paracrine effects inducing the recruitment of stem cells. Based on this method [59], a new medical "Heart Sheet" product for patients with ischemic heart disease was approved on September 18, 2015 and has been launched by Terumo Corporation, Japan.

In the field of esophageal research, clinical studies on the treatment of artificial ulcers accompanied by early esophageal cancer resection with endoscopic submucosal dissection (ESD) were first initiated by T. Ohki in April 2008. ESD even of large lesions is less invasive than surgical approaches. However, frequent extension using balloons is required to prevent stenosis due to artificial ulcer scarring after extensive ESD treatment. To prevent stenosis without invasive balloon extension, autologous oral mucosal epithelial cell sheets were endoscopically transplanted onto the ulcer surfaces of 10 patients. This procedure was found to promote the re-epithelialization of the esophagus and to prevent esophageal narrowing after ESD, leading to ESD therapy without lowering each patient's quality of life [53]. Additionally, collaborative clinical studies have been conducted for the treatment of 10 patients at Nagasaki University Hospital [90] and for the treatment of 10 patients suffering from Barrett's esophagus at the Karolinska Institute, Sweden. Based on these treatment outcomes, CellSeed is conducting clinical trials in collaboration with National Cancer Center Japan for the treatment of artificial ulcers.

Table 19.1 Cell-sheet based regenerative therapies using autologous cell sources

Tissue/organ	Target illness	Cell sheets	Implementation site (country)	Comments
Corneal epithelium	Limbal stem-cell deficiency	Corneal limbal-derived cell sheet	Osaka University (Japan)	Clinical research from 2002
		Oral mucosal epithelial cell sheet		
		Oral mucosal epithelial cell sheet	Les Hospices Civils de Lyon (France)	Clinical trials during from 2007 to 2011
			Collaborator: Cellseed, Inc. (Japan)	
Myocardium	Severe cardiac disease (e.g. ischemic heart disease, dilated cardiomypathy)	Myoblast sheet	Osaka University (Japan)	Clinical research from 2006
			Terumo Corporation (Japan)	Approved on September 18, 2015, and have launched Japan
			Collaborator: Cellseed, Inc. (Japan)	
Esophagus	Prevention of stenosis after endoscopic submucosal dissection of esophageal cancer	Oral mucosal epithelial cell sheet	Tokyo Women's Medical University (Japan)	Clinical research from April 2008
			Tokyo Women's Medical University (Japan)	Tissues and cell sheets were transferred between Tokyo and Nagasaki by airplane, and transplanted in Nagasaki
			Nagasaki University (Japan)	
			Cellseed, Inc. (Japan)	Clinical trial has started from 2017
			National Cancer Center (Japan)	
			Tokyo Women's Medical University (Japan)	
	Barrett's esophagus	Oral mucosal epithelial cell sheet	Karolinska Institute (Sweden)	10 cases of clinical research from 2011
Periodontal ligament	Periodontal disease	Periodontal ligament-derived cell sheet	Tokyo Women's Medical University (Japan)	10 cases of clinical research from 2011 to 2014
Cartilage	Knee cartilage injury	Cartilage cell sheet	Tokai University (Japan)	Clinical research from 2011 to 2014
Middle ear mucosa	Removed mucosa in middle ear cavity after tympanoplasty	Nasal mucosal cell sheets	Jikei University School of Medicine (Japan)	Clinical research from 2014
Lung	Closure of air leakage after pulmonary resections	Dermal fibroblast sheet	Tokyo Women's Medical University (Japan)	Clinical research from 2018

In the dental field, the transplantation of periodontal ligament-derived cell sheets has been carried out for the regenerative treatment of periodontal tissue, specifically of periodontal ligaments [28]. Periodontal disease induces the loss of periodontal tissue, resulting in tooth loss or instability. Therefore, the regeneration of periodontal tissues is essential to preventing tooth loss. Ten clinical studies were conducted from 2011 to 2014 using autologous cell sheets derived from periodontal ligaments of extracted teeth (e.g., wisdom teeth). Three-layered periodontal ligament cell sheets were attached to tooth surfaces in combination with bone prosthetic materials. Eventually, absorbed alveolar bone was regenerated, preserving teeth that were originally to be extracted [29].

In the orthopedic field, cartilage damage is typically treated via chondrocyte implantation, mosaic formation or microfracture procedures. Recently, tissue-engineered cartilage has been applied to repair articular cartilage damage using various transplanted cells [13]. Clinical research on joint repair using autologous chondrocyte sheets derived from cartilage tissue has been conducted by M. Sato since 2011. Safe outcomes, improved clinical symptoms, and the regeneration of hyaline cartilage were confirmed in all cases, showing that positive therapeutic effects were obtained. This therapeutic method will be made available with approval from the Ministry of Health, Labour and Welfare, Japan.

In the field of otolaryngology, cell sheet-based regenerative therapy has been applied to tympanoplasty in middle ear surgery. Poor mucosal regeneration in the resected area of the mastoid cavity occurs after conventional tympanoplasty, resulting in the frequent re-adhesion of tympanic membranes and the recurrence of adhesive otitis media. Our collaborative clinical research with H. Kojima and K. Yamamoto was conducted for the transplantation of autologous mucosal cell sheets onto the surfaces of exposed bone from which mucosa had been removed during tympanoplasty. As a surrogate of middle ear mucosa, autologous mucosal cell sheets were derived from an approximately 10 × 10 mm^2 piece of nasal mucosal tissue. Our clinical research shows that all of the patients tested exhibited good postoperative course without adverse events or complications and that the patients' hearing abilities remained strong after transplantation [91].

In the field of thoracic surgery, the clinical application of autologous skin fibroblast sheets for the closure of lung air leakages has been executed in collaboration with M. Kanzaki. Air leakages are observed after lung resection, causing severe complications when proper treatments are not applied. Therefore, the prevention of air leakages is essential for managing patients after lung resection. Autologous skin fibroblast sheets fabricated from 5 × 15 mm pieces of patient skin were applied to seal air leakages for a 44-year-old male patient with multiple bullae following pulmonary resections by video-assisted thoracoscopic surgery. Air leakages were fully sealed with the attachment of cell sheets. No adverse events were observed after 7 months, and the patient recovered fully [31].

In addition to the abovementioned therapeutic studies, several preclinical studies using haptic cell sheets [52], pancreatic cell sheets [55], and iPS-derived cell sheets including cardiomyocytes [44] have been ongoing.

19.2.2 Allogenic Cell Sheet-Based Regenerative Therapy

Human mesenchymal stem cells (MSCs) are a promising cell source for clinical applications [10], as the isolation from various tissues and expansion of these cells are relatively easy. In addition, it has been reported that MSC can supply growth factors and cytokines while also exhibiting immunomodulatory properties [10]. Typical regenerative treatments using MSCs have been conducted through the injection of suspensions. However, the injection method using an enzymatically treated single cell suspension presents disadvantages in terms of efficient transplantation due to the limited stability of cells at the target site. In contrast, MSC sheets facilitate attachment to tissue surfaces while maintaining cell-cell binding, leading to highly efficient transplantation.

Diabetic foot ulcers can form as a complication, and their treatment in many cases is difficult due to the occurrence of reduced blood flow and neuropathy. The progression of diabetic ulcers results in the development of gangrene, which can mostly require the amputation of the patient's foot. As a means of healing diabetic ulcers, a new therapy using allogenic MSC sheets has been developed [32]. In a preclinical study, allogenic adipose-derived MSC sheets were transplanted into a type 2 diabetic model with obesity (Zucker rats). For the wound healing model, full-thickness skin deficiencies were created on the cranial 10 × 15 mm area where cell sheets were transplanted in combination with artificial dermis. Seven days after transplantation, the degree of wound closure was significantly higher in the cell sheet transplantation group (transplant group) compared to the rats with artificial dermis (control group). The mean time to wound closure was 34.2 days for the control group, whereas it was shortened to 25.6 days for the transplant group. Through histological analyses, angiogenesis was found to be significantly present in the transplant group. Vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and other growth factors were also found to be secreted during *in vitro* cultivation. The transplantation of GFP-expressing MSC sheets revealed that GFP positive cells were positioned around newly generated blood vessels, suggesting that transplanted MSCs may support the formation of blood vessels. Consequently, the transplanted MSCs not only promoted wound healing by secreting angiogenic factors but also were involved in the construction of blood vessels.

MSC transplantation was also applied for the treatment of osteonecrosis due to drug side effects. Bisphosphonate (BP) is a primary choice for the treatment of osteoporosis and is used to treat cancer and diseases involving bone decline. Recently, BP-related osteonecrosis of the jaw (BRONJ) was found to occur in patients receiving BP. To limit the occurrence of BRONJ, MSC sheets were transplanted onto bone exposed after tooth extraction in BRONJ-like rats [30]. After a two-week transplantation period, osteonecrosis healing was significant in the MSC sheet-transplant group relative to those for the control group of BRONJ rats receiving sham surgery. Neovascularization was found in the transplant group due to the secretion of angiogenic factors from transplanted MSCs. Furthermore, the gene expression of receptor activators of nuclear factor κ-B ligand (RANKL), which induced the differentiation of osteoclast precursor cells into osteoclasts, was detected from MSCs. In addition to angiogenetic effects, the transplantation of MSC sheets contributed to the promotion of bone turnover.

Recently, treatments using allogenic chondrocyte sheets have been applied by M. Saito et al. at Tokai University. Typically, young patients of approximately 1 year old suffering from polydactyly were treated by removing unnecessary fingers. These fingers are ordinarily considered medical waste, but they contain chondrocytes with the potential to proliferate. Using fingers amputated from patients suffering from polydactyly, allogenic chondrocyte sheets have been prepared. Clinical research on joint repair using allogenic chondrocyte sheets has been conducted at Tokai University.

19.3 Design of Temperature-Responsive Cell Culture Surfaces for 3D Tissue Fabrication

As noted above, a few layered cell sheets have been clinically transplanted onto the superficial layers of tissues and organs due to ease of transplantation. In regard to challenging issues of the next generation of tissue engineering, much attention has been paid to creating 3D tissues and organs. One approach involves mimicking emergence processes (e.g., cell proliferation and differentiation in the developmental stage of biology). Self-organized multicellular bodies have been formed through the proliferation and differentiation of pluripotent stem cells such as ES and iPS cells under appropriate ECMs (e.g., Matrigel®) and growth factors, resulting in the formation of small tissues and organs referred to

as organoids [15]. Several researchers have generated organoids including teeth [25], liver tissue [72], optic-cups [14] and intestinal tissue [19]. In general, organoid sizes are limited to miniaturized units of tissues and organs mainly for investigating biological events *in vitro* and surrogates to test drug toxicity levels and effects.

Another approach to 3D tissue and organ generation involves cell sheet engineering, which enables one to create cell-dense assemblies and hierarchical architectures through the sequential lamination of cell sheets. Key technologies for constructing 3D structures from cell sheets and for transplanting them are described in the following sections.

19.3.1 Materials and Devices for Manipulating Recovered Cell Sheets

As single cell sheets are flexible, the development of cell sheet manipulation techniques is essential for the fabrication of cell-dense 3D engineered tissue and for the transplantation of cell sheets onto damaged tissues and/or organs. Detached cell sheets float in a medium and are manipulated with a pipette. Support membranes, such as chitin [33], porous polyethylene terephthalate (PET) [33], poly(vinylidene difluoride) (PVDF) [24], and parchment paper, are used to facilitate the manipulation of cell sheets. Prior to manipulation, a support membrane is overlain on the apical side of confluent cells, which are cultured on temperature-responsive cell culture surfaces at 37 °C. During low temperature treatments, confluently cultured cell sheets detach with the support membrane while all sheets are physically attached to the support membrane. When the deposited support membrane is peeled off, cultured cells are harvested as a cell sheet together with the support membrane. The harvested cell sheet is adhered to another TCPS surface to maintain the ECM composition of the basal side of the cell sheet. The cell sheet is in turn readily detachable from the support membrane by using an excess volume of aqueous media. However, special skill is required to manipulate cell sheets with support membranes and a pipette.

To promote the adhesion of cell sheets, cell adhesive materials, such as the polyion complex hydrogel composed of (poly(*N,N*-dimethylacrylamide-*co*-2-acrylamido-2-methylpropane sulfonic acid) (P(DMAAm-*co*-AMPS) and poly(*N,N*-dimethylacrylamide-*co*-2-acryloxyethyltrimethylammonium chloride) (P(DMAAm-*co*-AETA-Cl), fibrin, and gelatin, have been coated on the surfaces of support membranes and plunger devices [58, 79]. Tang et al. modified polyion complex hydrogels on porous PVDF membranes through cross-linking and grafting by EB irradiation [79]. They successfully transferred cultured fibroblast sheets from temperature-responsive cell culture surfaces to a collagen-coated surface using the polyion complex-coated membrane.

Sasagawa et al. developed a plunger device for cell sheet manipulation (Fig. 19.3a). Cell adhesive natural hydrogels, such as fibrin and gelatin, were coated onto the surface of the plunger [58]. The hydrogel-coated plunger was placed onto confluent cells cultured on the temperature-responsive cell culture surface. By incubating the entire plunger on the cell culture surface at a low temperature (20 °C), the cells detached from the surface while remaining attached to the hydrogel surface. By lifting the plunger, the cell sheet can be readily harvested and transferred to another cell sheet cultured on a temperature-responsive cell culture surface without damaging cells. These two cell sheets bind together to form double-layered cell sheets at 37 °C. Repetitively layering cell sheets with this device allows for the fabrication of thick cardiac tissues and hierarchically aligned structures [22, 56, 69, 84]. Using the plunger cell sheet manipulator technique, automatic apparatuses for cell sheet stacking have been developed. Five-layer cell sheets 70–80 μm thick have been automatically fabricated with this apparatus [34].

Recently, Tadakuma et al. developed a new device that enables one to rapidly transfer and

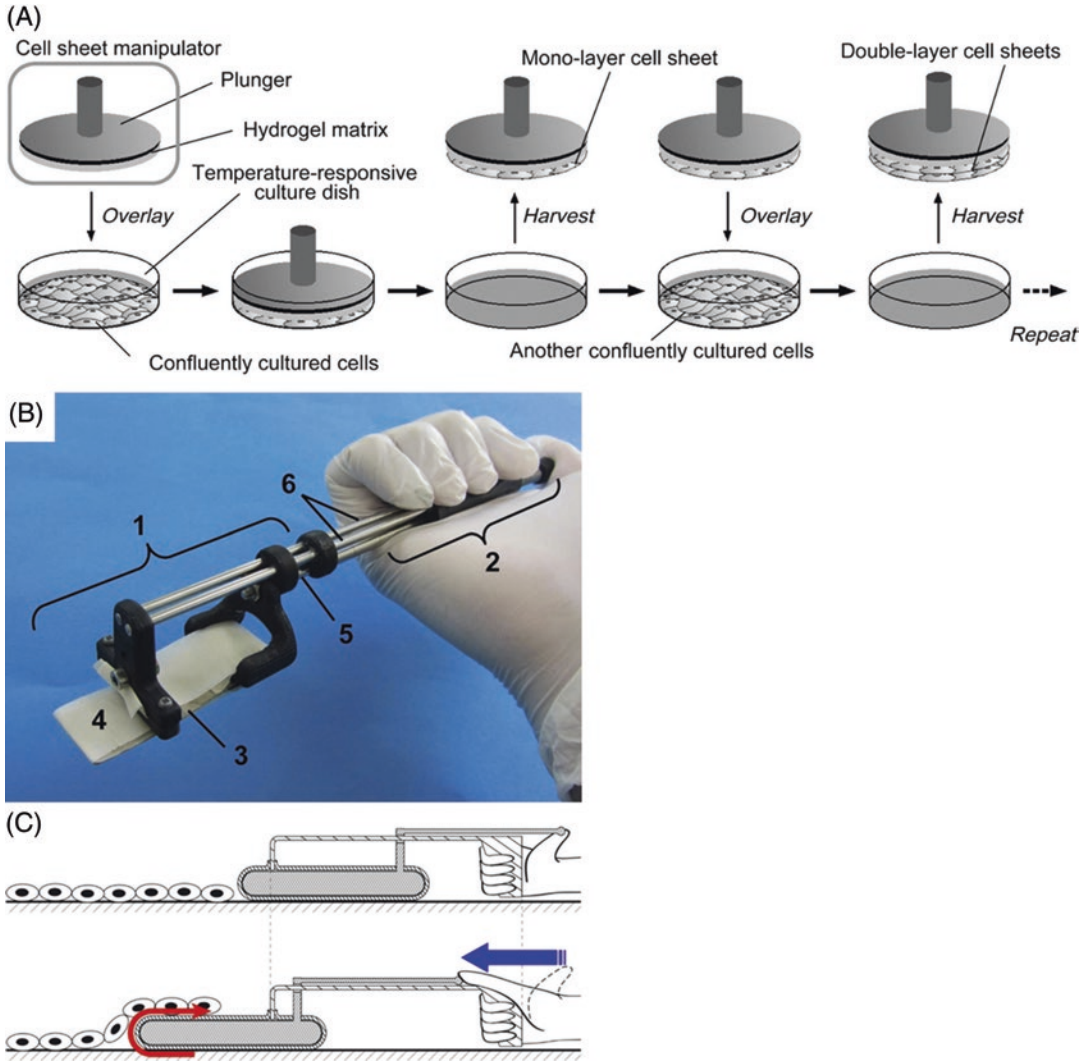

Fig. 19.3 Cell sheet layering manipulation procedures and of new cell sheet transfer/transplantation devices. (**a**) Schematics of layering cell sheets utilizing temperature-responsive culture dishes and a cell sheet manipulator. Upon repeated application of this procedure, cell sheets can be stacked. (**b**) Photograph of a new cell-sheet transfer/transplantation device consisting of several parts (a scoop (1), a handle (2), an inner plate (3), an outer movable belt (4), a pushing rod (5), and stainless rods (6)). (**c**) Schematic illustration of cell sheet scooping mechanism of the device shown in (**b**). The outer removal belt comes into direct contact with cell sheets and dish surfaces. The outer removal belt surface is polytetrafluoroethylene, which is non-adherent, low friction, and low wearing. The manipulation of the device does not affect the cell sheet when scooping and releasing the cell sheet. (Reprinted from [58, 65]) with permissions from Elsevier)

transplant recovered cell sheets onto different surfaces or tissues (Fig. 19.3b, c) [65]. The device is mainly composed of two parts: scooping and handling parts (Fig. 19.3b). Compared to conventional cell sheet transfer methods involving the use of a pipette, this device allows one to retrieve recovered cell sheets and to then transfer them in several seconds without requiring any special skills. The device also allows for the rapid transplantation of cell sheets onto soft, rugged and uneven surfaces and rat subcutaneous tissues within 30 seconds.

19.3.2 Temperature-Responsive Cell Culture Surfaces for the Acceleration of Cell-Sheet Detachment

Novel rapid cell-sheet detachment has been investigated as a means to maintain biological activity and physiological cell-sheet functioning when applied for the subsequent fabrication of cell-sheet-based tissue or transplantation. In addition, rapid cell sheet recovery techniques are anticipated to support rapid cell sheet transplantation, reducing the patient burden in clinical application. In the case of conventional temperature-responsive cell culture surfaces, grafted PIPAAm chains are gradually hydrated from the periphery of the temperature-responsive cell culture surface to the central region as the temperature decrease across the LCST [40]. Cell sheet can be quickly detached by accelerating the hydration of grafted PIPAAm chains. Several approaches developed involve grafting cross-linked PIPAAm onto porous membranes [40], incorporating hydrophilic units into PIPAAm [12, 41] and hierarchically grafting PIPAAm onto hydrophilic polymers (Fig. 19.4) [4, 35].

Porous membrane surfaces have been used as base materials to enhance water permeability [40]. Cell sheets detach more quickly from PIPAAm-grafted porous membranes than from PIPAAm-TCPS. The incorporation of hydrophilic components, such as poly(ethylene glycol) (PEG) and 2-carboxyisopropylacrylamide (CIPAAm), into grafted PIPAAm also accelerates the detachment of cells (Fig. 19.4a–c) [12, 41]. As hydrophilic components are readily hydrated, changes in the hydrophilicity of polymer-grafted surfaces are accelerated when temperatures are decreased to below the LCST. Furthermore, the introduction of a hydrophilic interface layer between PIPAAm and TCPS affects the kinetics of temperature-responsive change in cell culture surfaces (Fig. 19.4d). By successively grafting PAAm and PIPAAm, double polymeric layers and polyacrylamide (PAAm) and PIPAAm layers form on a TCPS surface (PIPAAm-PAAm-TCPS). An interpenetrating polymer network (IPN) of PIPAAm and PAAm in turn forms on the basal TCPS surface. At 37 °C, cells fail to adhere to PIPAAm-PAAm-TCPS surfaces with high PAAm content, while cells adhere to and proliferate on PIPAAm-PAAm-TCPS with low PAAm content. Under lowered temperatures, cell sheets rapidly detach from PIPAAm-PAAm-TCPS with low PAAm content. Hydrated water molecules within PAAm components are considered to be supplied to the grafted upper PIPAAm layer as temperatures decrease to below the LCST.

In addition, Tang et al. found that the grafted architecture of thin PIPAAm influences cell sheet detachment (Fig. 19.4e) [80]. The comb-type PIPAAm hydrogel contains grafted PIPAAm chains with free mobile terminals, which enhance the deswelling/swelling rates of PIPAAm hydrogels [97]. By increasing temperatures across the LCST, only free mobile terminals of grafted PIPAAm chains rapidly dehydrate and aggregate. Such dehydration and aggregation induce a more rapid deswelling of comb-type PIPAAm hydrogels compared to conventional cross-linked PIPAAm hydrogels. By contrast, with decreasing temperature across the LCST, the rapid hydration of PIPAAm chains with free mobile terminals accelerates the swelling of comb-type PIPAAm hydrogels. To accelerate swelling, comb-type thin PIPAAm hydrogels have been grafted onto TCPS [80]. This surface undergoes more rapid cell sheet recovery than PIPAAm-TCPS, which does not contain free mobile PIPAAm chains.

19.3.3 Heparin-Immobilized Thermoresponsive Surfaces for the Sustained Stimulation of Cellular Receptors and the Recovery of Cultured Cells

Stimulation with growth factors in the culture system is required to express the phenotype of a specific cell type (e.g., hepatocytes) in a differentiated state or to maintain undifferentiated embryonic stem cells. Typically, growth factors in soluble form are supplemented in the culture medium. This supplementation must occur

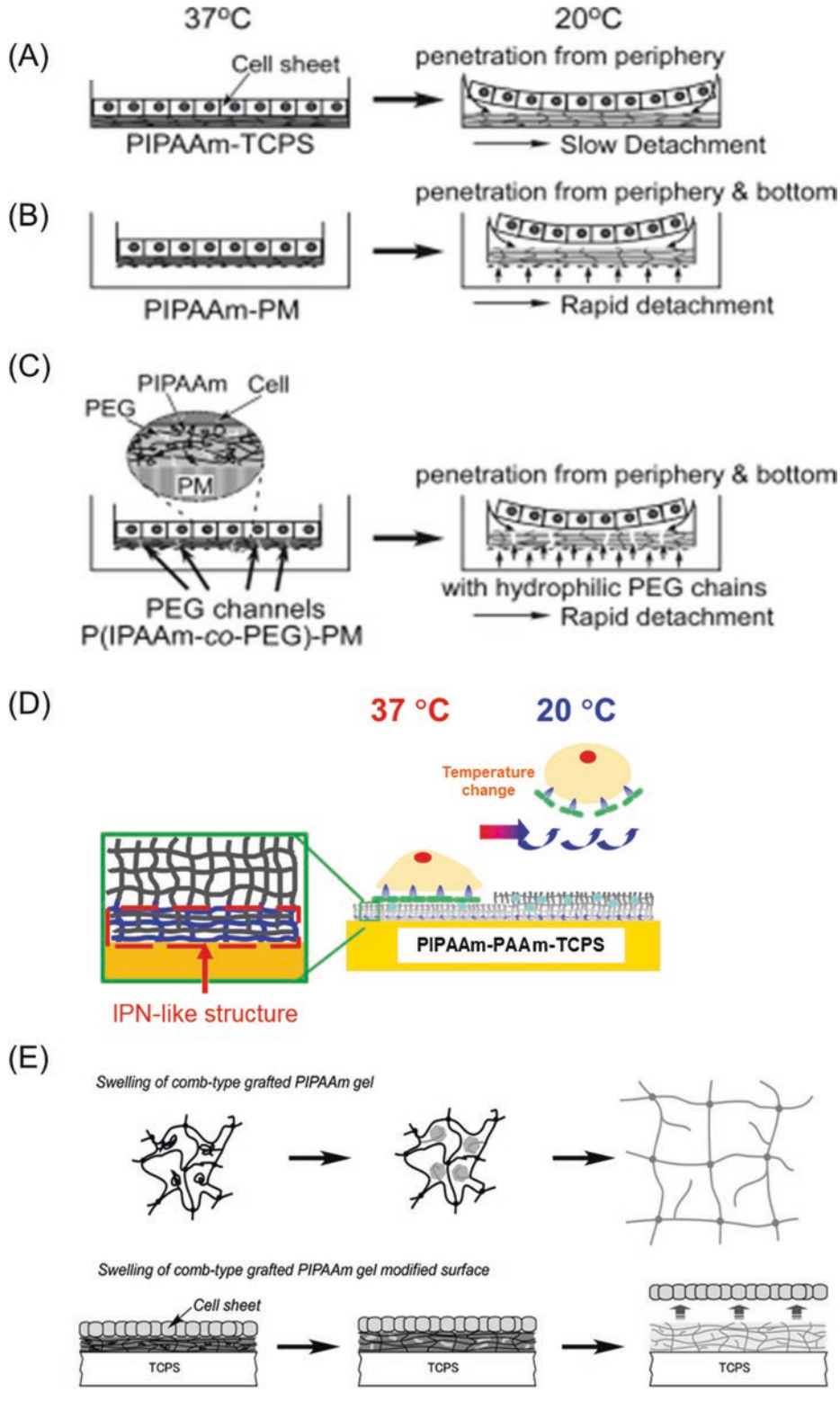

Fig. 19.4 Schematic illustration of the rapid hydration of grafted PIPAAm chains. (**a**) PIPAAm-TCPS, (**b**) PIPAAm-grafted PM, (**c**) P(IPAAm-*co*-PEG)-grafted porous membrane (PM), (**d**) PIPAAm-PAAm-TCPS, (**e**) comb-type PIPAAm (upper illustration) and comb-type PIPAAm gel-grafted TCPS (bottom illustration). (Reprinted from [4, 81]), and [80]) with permissions from John Wiley and Sons and Elsevier)

frequently, and it is difficult to maintain its effectiveness over the long term due to the down-regulation of cellular receptors. By contrast, covalently immobilized growth factors on a cell culture substrate escape receptor down-regulation, resulting in prolonged cell stimulation by immobilized growth factors [27, 37]. However, during passage with trypsin treatment, proteolytic activity induces the degradation of receptor proteins and eventually the deterioration of cellular functions.

To achieve the sustained stimulation and non-invasive recovery of cultured cells, temperature-responsive surfaces covalently conjugated with ECM molecules have been designed [36]. In particular, proteoglycan-mimicked temperature-responsive surfaces resemble a basement membrane that supplies cellular microenvironments. Negatively charged heparan sulfate chains on proteoglycans stabilize growth factors through the formation of complexes, leading to the maintenance of activity and to the suppression of growth factor diffusion. In turn, the introduction of heparin (with the same structures and functions as heparan sulfate) onto poly (IPAAm-*co*-CIPAAm)-grafted surfaces mimics ECM microenvironments [5–7]. Heparin typically exhibits an affinity with various heparin-binding proteins such as basic fibroblast growth factor (bFGF, or FGF-2), heparin-binding epithelial cell growth factor-like growth factor (HB-EGF), and VEGF.

Heparin-immobilized temperature-responsive surfaces have been used to form hepatocyte sheets with hepatic functions maintained (Fig. 19.5) [7]. Liver tissue engineering using hepatocytes serves as an attractive approach for the treatment of liver diseases. In a preclinical study, hepatocyte sheets transplanted into a subcutaneous site stably persisted over more than 200 days and secreted specific proteins [52]. In contrast, cultured hepatocytes rapidly lose their viability and phenotypic functions when isolated from the natural *in vivo* microenvironment of the liver. Soluble EGF or HB-EGF in the cell culture system is essential for the survival of hepatocytes. Thus, heparin-immobilized temperature-responsive surfaces bound with

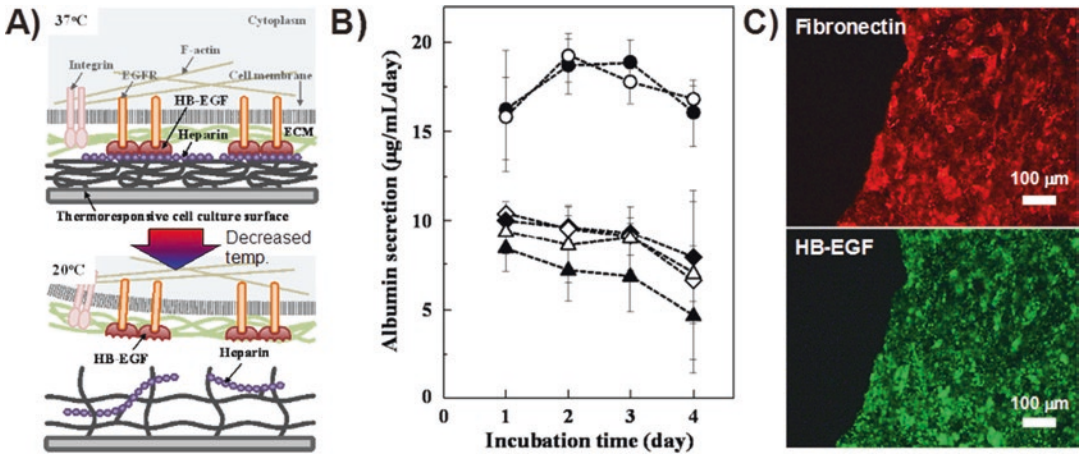

Fig. 19.5 Design of heparin-immobilized temperature-responsive surfaces for hepatocyte sheets with maintenance of their functions. (**a**) Adhered hepatocytes survive on HB-EGF-bound heparin-immobilized temperature-responsive surfaces at 37 °C (upper). Cultured hepatocytes detach at 20 °C, presumably due to a reduction of affinity between EGF receptors, HB-EGF and surface-immobilized heparin (lower). (**b**) Timing of albumin secretion from hepatocytes on heparin-immobilized temperature-responsive surfaces bound with 76.2 ± 3.8 ng/cm^2 HB-EGF (circles), PIPAAm surfaces with 100 ng/cm^2 soluble HB-EGF and 1.0 mg/cm^2 soluble heparin (triangles), and collagen-coated tissue culture polystyrene surfaces (diamonds) subjected to culturing with epidermal growth factor (EGF, 10 ng/mL, open symbols) or without EGF (closed symbols). (**c**) The cultured hepatocyte sheet was detached with fibronectin (red) and HB-EGF (green) from heparin-immobilized surfaces while lowering the temperature to 20 °C. (Reprinted from [7]) with permission from Elsevier)

HB-EGF have been used to prepare hepatocyte sheets with sustained stimulation of EGF receptors on hepatocytes. Hepatocytes survive on heparin-immobilized temperature-responsive surfaces bound with HB-EGF of 10 ng/cm^2, leading to the formation of their sheets. During cultivation, hepatocyte-specific functions (e.g., albumin secretion) were highly maintained compared with soluble HB-EGF, presumably due to the continuous activation of hepatocytes. This shows that bound HB-EGF continuously stimulates hepatocytes through EGF receptors, resulting in the maintenance of hepatic functions. It was previously reported that the viability and functioning of primary rat hepatocytes, such as albumin secretion and urea synthesis, are preserved on substrates with covalently immobilized EGF [37]. By contrast, cultured hepatocytes on heparin-immobilized temperature-responsive surfaces are detached as a contiguous sheet with fibronectin and HB-EGF when temperatures are lowered to 20 °C. Therefore, heparin-immobilized temperature-responsive cell culture surfaces facilitate the manipulation of hepatocyte sheets while maintaining hepatic functions by simply changing the culture temperature.

Furthermore, heparin-immobilized temperature-responsive surfaces promote cell proliferation and cell sheet formation [6]. Heparin-immobilized temperature-responsive surfaces bound with bFGF accelerate the proliferation of fibroblasts. After 3 days of culture, bFGF-bound heparin-immobilized temperature-responsive surfaces are able to hold 2–3 times more fibroblasts than surfaces with the same amount of soluble or physisorbed bFGF. The incubation period required for fibroblasts to reach confluence on bFGF-bound heparin-immobilized temperature-responsive surfaces is 3 days. However, cultured fibroblasts on PIPAAm surfaces with physisorbed bFGF reach confluence after 5 days. This prolonged period is considered to be due to the denaturation and/or random orientation of bFGF through physisorption, leading to decreasing bFGF activity. In contrast, binding bFGF to immobilized heparin preserves the activity and stabilizes the formation of bFGF/FGF receptor/heparin complexes. Therefore, bFGF bound to heparin-immobilized temperature-responsive surfaces promotes cell proliferation.

Finally, heparin-immobilized temperature-responsive surfaces offer two advantages with respect to cost effectiveness. First, heparin-immobilized temperature-responsive culture dishes decrease the amount of HB-EGF needed for culturing a hepatocyte sheet. No additional HB-EGF in the medium is required during the 4-day incubation period. On PIPAAm-grafted surfaces without heparin, by contrast, the medium containing HB-EGF as a soluble form is exchanged three times over 4 days. Therefore, cell culture systems using heparin-immobilized temperature-responsive surfaces have been shown to be beneficial for reducing the total cost of cell cultures associated with the amount of HB-EGF required. Second, heparin-immobilized temperature-responsive surfaces accelerate cell sheet fabrication. Shortening the time required for cell sheet preparation fabrication reduces costs. bFGF bound to heparin-immobilized temperature-responsive surfaces shortens the culturing period required to achieve the confluence of fibroblasts by 2 days. In addition, it has been found that the detachment of fibroblast sheets from heparin-immobilized temperature-responsive surfaces was faster than that from PIPAAm surfaces, presumably because surfaces are hydrated with immobilized heparin. Consequently, a cell culture system using heparin-immobilized temperature-responsive surfaces has been shown to be beneficial in terms of reducing total costs related to the growth factors and media required.

19.3.4 Design of Micropatterned Temperature-Responsive Cell Culture Surfaces for Generating Microscopically Aligned Tissues

Tissues and organs comprise heterogeneous cell species that express and maintain their physiological functions through complex interaction

between cells and ECMs. Micropatterning technologies have been applied to spatially position cells to construct biomimetic tissue *in vitro* ([8]). Micropatterned temperature-responsive cell culture surfaces are designed for fabricating microscopically aligned tissue contracts [26, 68, 69, 82–84, 96]. In particular, the use of micropatterned temperature-responsive surfaces facilitates the transfer of patterned cells without the structural destruction of cell-cell binding.

A temperature-responsive cell culture surface patterned with two different temperature-responsive polymers (PIPAAm and poly(*n*-butyl methacrylate) (PBMA)-*co*-grafted PIPAAm) has been developed by EB irradiation with a stainless mask [82]. The LCST of the co-graft polymer area is lower than that of PIPAAm. At 27 °C, primary rat hepatocytes were selectively adhered to the co-grafted domain (1.0 mm in diameter), as the PIPAAm grafted area was sufficiently hydrophilic to be non-cell adhesive [82, 83]. Sequentially, by increasing the temperature to 37 °C, bovine aortic endothelial cells were favorably attached to the grafted PIPAAm area. A co-culture of hepatocytes with bovine aortic endothelial cells enhanced the hepatic physiological functions of albumin secretion and urea synthesis relative to monocultures of hepatocytes [83]. The co-culture system was useful for the fabrication of liver tissue structures and for liver regeneration. By decreasing the temperature to 20 °C, co-cultured cells were recovered as a contiguous co-cultured cell sheet maintaining hepatocytes and endothelial cell interactions.

Micropatterned temperature-responsive surfaces wherein striped PIPAAm and brush PIPAAm-*b*-poly(*N*-acryloylmorpholine) (PIPAAm-*b*-PAcMo) domains are fabricated in combination with surface-initiated RAFT polymerization via the photolithographic method have been found to direct not only the orientation of cultured cells but also the alignment of recovered cell sheets [68]. In the case of polymer brush surfaces prepared through surface-initiated RAFT polymerization, reactive groups remain at polymer brush terminals. Additional polymer chains are grafted to the terminals of grafted polymer brush surfaces. As shown in Fig. 19.6, reactive dithiobenzoate (DTB) groups at the end of grafted PIPAAm brushes have been converted into inert maleimide groups by spatial masking with photoresist. After the removal of photoresist, another PAcMo was grafted to the terminal of PIPAAm brush surfaces, where reactive DTB

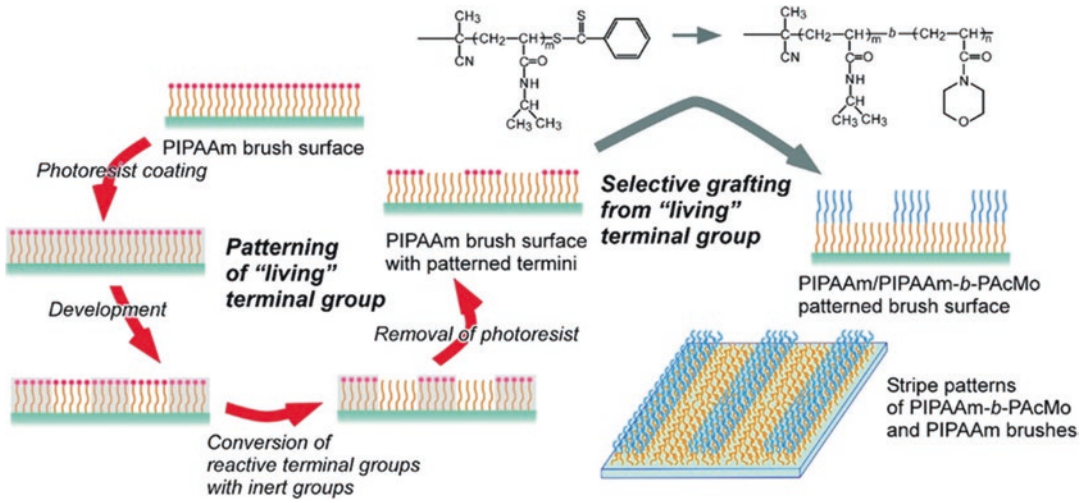

Fig. 19.6 Schematic illustration of the fabrication of micropatterned temperature-responsive PIPAAm and PIPAAm-*b*-PAcMo brush surfaces using a two-step RAFT polymerization procedure. PAcMo chains are further grafted from the terminal of PIPAAm chains *via* reactive DTB groups. (Reprinted from [68]) with permission form American Chemical Society)

remained. Normal human dermal fibroblasts (NHDFs) preferentially adhered to the microstripe patterned PIPAAm brush domain at 37 °C and were oriented in a direction parallel to the stripe patterns after 24 hours only when the PIPAAm brush domain was 50 μm in width. By contrast, NHDFs randomly adhered to PIPAAm brush surfaces without micropatterning. Further incubation caused the patterned NHDFs to migrate and proliferate on the patterned PIPAAm-*b*-PAcMo brush domain. Eventually, all of the cultured NHDFs preserved their orientations. Finally, after 5 days of cell culture, adhered cells became confluent and oriented on the line-striped micropatterned PIPAAm and PIPAAm-*b*-PAcMo brush domains. Fluorescence photographs showed that actin filaments in cultured cells on patterned domains also became oriented. By decreasing the temperature to 20 °C, NHDFs with well-oriented structures can be harvested as an anisotropic cell sheet, with different shrinking rates observed for the vertical and parallel directions of the cell sheet.

Once the anisotropic cell sheet detaches from the surface and is spontaneously shrunk, the unique oriented cell structure is lost due to cytoskeleton rearrangement. To prevent shrinkage, a cell sheet manipulator device is used to harvest anisotropic cell sheets while maintaining their unique cell orientations [69]. When this anisotropic cell sheet is translocated to TCPS using a manipulator device, its anisotropic character is structurally maintained for 7 days. Biological assays for anisotropic NHDF sheets show that VEGF secretion is enhanced relative to isotropic NHDF sheets, which comprise randomly oriented cells, while type-I collagen and transforming growth factor-β1 (TGF-β1) are independent of the orientation of culture cells. Anisotropic myoblast sheets are also applied for neuron-muscle tissue fabrication, sandwiching neurons and endothelial cells [70].

This micropatterned temperature-responsive cell culture surface is further used for the fabrication of tubular neural tissues using human-iPS cells derived from neurons and human astrocytes [71]. Astrocyte-sandwiching neurons are cultured on a micropatterned temperature-responsive cell culture surface. For striped patterns of appropriate widths, seeded astrocytes selectively align onto the cell adhesive striped pattern. Seeded neurons then spread and are oriented in the same direction as the aligned astrocyte. Finally, astrocytes are seeded again on the co-cultured and striped cells. The co-cultured cells form tubular structures wherein neurons are wrapped by astrocytes. One month of cell culture allows co-cultured cells to elongate and form tightly bundled structures that mimic neural tissue-like constructs. Patterned temperature-responsive cell culture surfaces may be useful for the fabrication of well-organized oriented cells and/or ECM as well as native tissues in the body.

19.4 Challenging Issues Related to Thick and Large 3D Tissue Fabrication

19.4.1 Vascularization

Layering multiple cell sheets allows for the fabrication of thick and functional tissue structure as described above. However, four-layered cardiomyocyte sheets undergo necrosis within the structure due to the limited diffusion of O_2 and nutrients [64]. Therefore, the formation of vascular networks is required to supply O_2 and nutrients to transplanted cell sheets. Endothelial cells (ECs) derived from the hearts of neonatal rats contribute to the formation of capillary-like networks within cardiac cell sheet tissues with stimulation by VEGF [57, 62, 66]. In the absence of endothelial cells, such cell network formation does not appear. The transplantation of triple-layered cardiac cell sheets including EC networks into rat dorsal subcutaneous tissues or rat infarcted myocardial tissues induces the migration of ECs to host tissues over a few days [60, 64]. Tubular microvessel networks form from these ECs in transplanted cell sheets and connect to the host vessel over several days. Transplanted cell sheets can survive for longer periods without necrosis. Furthermore, the transplantation of cardiac cell sheets containing EC networks improves the functioning of infracted myocardial tissues [60].

Additional transplantation of cardiac cell sheets containing EC networks facilitates the connection between the EC network and newly formed microvessels in implanted cardiac cell sheets. More specifically, the stepwise transplantation of triple-layered cardiac cell sheets allows for the fabrication of ca. 1 mm-thick cardiac tissues composed 30 layers of cardiac cell sheets with well-organized vascular networks *in vivo* [64].

Based on the insight gained from these experiments, novel bioreactor systems are developed for the fabrication of engineered cell sheet-based tissues with vascular structures formed *in vitro* [56, 61]. Two different types of vascular beds (a resected section of femoral tissues with arteries and veins preserved or collagen gel) are used in the bioreactor system. A cell culture medium with growth factors, such as VEGF and bFGF, is perfused to the vascular bed, on which cardiac cell sheets containing ECs are cultured. In both vascular bed systems, ECs in the cell sheets migrate toward the vascular bed, forming a vascular tubular structure. This tubular structure connects to vascular beds, capillaries of resected femoral tissues and microchannels in the collagen gel. Finally, the vascular structure matures to supply nutrients and O_2 to the deposited cell sheets. In the same way, thick and cell-dense tissues and multi-layered cardiac cell sheets are constructed on the vascular bed through step-by-step cell sheet transplantation as described above.

On the other hand, ECs seeded on a single myoblast sheet do not form such a vascular network. When sandwiching ECs between two myoblast sheets, ECs spontaneously form vascular networks with random orientations [70]. This shows that the 3D environments around ECs may dominate vascular network formation. More interestingly, when ECs are sandwiched between two anisotropic cell sheets, they form vascular formations oriented in the same directions as anisotropic myoblast cell sheets. ECs likely recognize the anisotropy of myoblast sheets. This result may suggest that EC vascular networks are controlled by anisotropic myoblast sheets.

Liver tissues are also composed of thick cell-dense tissues and require vascular formation in transplanted hepatic cell sheet tissues to survive and maintain liver-specific functions for long periods. In actuality, hepatic cell sheets transplanted into the vascularized mouse subcutaneous space generated by a bFGF releasing device prior to engraftment survive for more than 230 days with sustained secretion of albumin and α1-antitrypsin [52]. By contrast, hepatic cells in a biodegradable scaffold do not survive for long periods in a normal subcutaneous space. In addition, the transplanted hepatic cell sheet proliferates and grows in response to a regenerative stimulus (two-thirds liver resection for a recipient mouse). These results denote the importance of vascular network platforms for subsequent mini-liver construction.

Cell-based therapies for type 1 diabetes mellitus are developed using pancreatic islets. In clinical trials, islets are injected and transplanted into the liver through the portal vascular system. However, injected islets are not efficiently transplanted into the liver, probably due to blood-mediated and leukocyte inflammatory reactions, etc. To improve the transplantation efficiency of islets and their survival for the treatment of diabetes, islet-derived cell sheet-based therapy has been investigated based on vascularized subcutaneous spaces of diabetic-severe combined immunodeficiency (SCID) mice [55]. The nonfasting blood glucose levels in recipient SCID mice transplanted with islet-derived cell sheets decrease to a state of normoglycemia within a week, while mice without transplantation maintain a hyperglycemic state. The normoglycemia state is stably maintained for 110 days. By removing the transplanted islet-derived cell sheet, the nonfasting blood glucose levels in SCID mice immediately return to hyperglycemic levels. Histological and immunohistochemical analyses demonstrate that the islet-sheet not only newly forms a vascular network therein but also secrets insulin 4 days after transplantation into the vascularized subcutaneous space. The vascular formation releases insulin into the systemic circulation.

19.4.2 Temperature-Responsive Microcarriers for the Large-Scale Production of Cultured Cells

Much attention has been dedicated to the large-scale culture of undifferentiated cells, such as human MSCs, for therapeutic applications. Large-scale cell culture systems using microcarriers (MCs) are promising because of the large surface area to volume ratios of small particles. MC systems enables one to expand adherent cells such as stem cells to generate sufficient quantities for practical tissue engineering and cell therapy applications.

On the surfaces of MCs, cultured cells grow as monolayers suspended in a medium with gentle agitation. Subcultures of cells on MCs require ongoing trypsin treatment, causing damage to the cell membrane. Thus, non-invasive subculture systems of MCs are considered to preserve the viable and subsequent proliferative properties of recovered cells. Surface modification technologies with temperature responsiveness have been applied to MCs to collect cultured cells by lowering the temperature [75–77]. On the surfaces of chloromethylated polystyrene beads, surface-initiated ATRP has been applied to immobilize PIPAAm brushes on surfaces [75, 76]. Chinese hamster ovary (CHO-K1) cells on temperature-responsive MCs were found to expand 49.8-fold in a stirred suspension culture over 7 days. When temperatures were lowered to 20 °C, the kinetics of CHO-K1 cell detachment from temperature-responsive MCs was greatly influenced by both the grafted amount of PIPAAm and bead diameter. Typically, cell harvest efficiency increased with the bead diameter, presumably because the cell-cell junction on the surface is disrupted. The cell harvesting efficiency achieved using the optimized PIPAAm-immobilized MCs was 76.1 ± 16.3% with low temperature treatment.

Furthermore, the introduction of positively charged moieties into PIPAAm brushes improves the efficiency of cell detachment from MC surfaces while maintaining cell adhesion and proliferation [77]. Copolymerized PIPAAm brushes with quaternary amino groups show larger amounts of adsorbed anionic BSA than nonionic PIPAAm MCs. Hydration and/or repulsive electrostatic interactions on positively charged PIPAAm brushes facilitate the dispersion of MCs in cell culture medium containing CHO-K1 cells, resulting in enhanced cell proliferation. In addition, after temperatures are lowered to 20 °C, the efficiency of cell detachment for the positively charged MCs was larger than that for nonionic MCs. This higher efficiency is likely attributable to the enhancement of hydration with the introduction of positively charged moieties.

Consequently, temperature-responsive MCs exhibiting temperature-dependent cell adhesion/detachment properties can be utilized to develop therapeutic cells using large-scale cell culture.

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Harnessing Nanotopography of Electrospun Nanofibrous Nerve Guide Conduits (NGCs) for Neural Tissue Engineering

Jeong In Kim, Cheol Sang Kim, and Chan Hee Park

Abstract

The anatomical recovery of nerve defects with their neurological functions after an injury caused by diseases or accidents is an important clinical issue. The most efficient surgical technique so far to the nerve defects, which are unrepairable by direct end-to-end suture, can be autograft transplantation. The autograft transplantation, however, has disadvantages including multiple rounds of surgery, a shortage of nerve donor, and function loss at the donor site. Tissue-engineered nerve guide conduits (TENGCs) have emerged as a potential alternative to autologous nerve grafts for nerve regeneration and functional recovery. Various TENGCs researches are being carried out to improve characteristics and enhance functionality such as material selection, biomimetic, topography, and enhancement by the biomolecules additions. Among them, the customizable surface nanotopography of aligned fibrous TENGCs foster neural repair by providing a cell-friendly environment, permissiveness, guidance cues, and directional growth of the cells. Fibrous nerve guide conduits (NGCs) made of longitudinally ordered fibers is a promising candidate for nerve tissue engineering.

Keywords

Nerve guide conduits · Nanotopography · Neural repair · Aligned fibers · Electrospinning

Abbreviations

BMSCs	Bone marrow stem cells
CNS	Central nervous system
ES	Electrospinning
ECM	Extracellular matrix
FGF	Fibroblast growth factor
GGF	Glial growth factor
IGF-I	Insulin-like growth factor –I
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NGF	Nerve growth factor
NGCs	Nerve guide conduits
N-CAM	Neural cell adhesion molecules

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NSCs	Neuronal stem cells
OECs	Olfactory ensheathing cells
OEG	Olfactory ensheathing glia
OEGCs	Olfactory ensheathing glia cells
OSCs	Olfactory Schwann cells
PE	Polyethylene
PNS	Peripheral nervous system
PLLA	Poly (L-lactic acid)
PLA	Poly (L-lactide)
PLGA	Poly lactic-co-glycolic acid
PCL	Polycaprolactone
PCLA	PCL acrylate
PU	Polyurethane
SEM	Scanning electron microscope
SWCNT	Single-walled carbon nanotube
TENGCs	Tissue-engineered nerve guide conduits
VEGF	Vascular endothelial growth factor

20.1 Introduction

20.1.1 Nervous System

The human nervous system consists of two sections: the central nervous system (CNS) and the peripheral nervous system (PNS), which are based on functions and location [1]. The CNS is the main part of the nervous system consisting of the spinal cord and the brain, and they integrate information and influence the activity of our body via the PNS as shown in Fig. 20.1 [2]. The PNS that is one of the two sections of the nervous system connects the CNS to the organs and limbs throughout the trunk and extremities.

At the cellular level, the nervous system can be defined by the presence of neurons, which have special constructs to send various signals precisely to other target cells [3, 4]. The neurons generate signals of electrochemical waves delivering along axons that cause

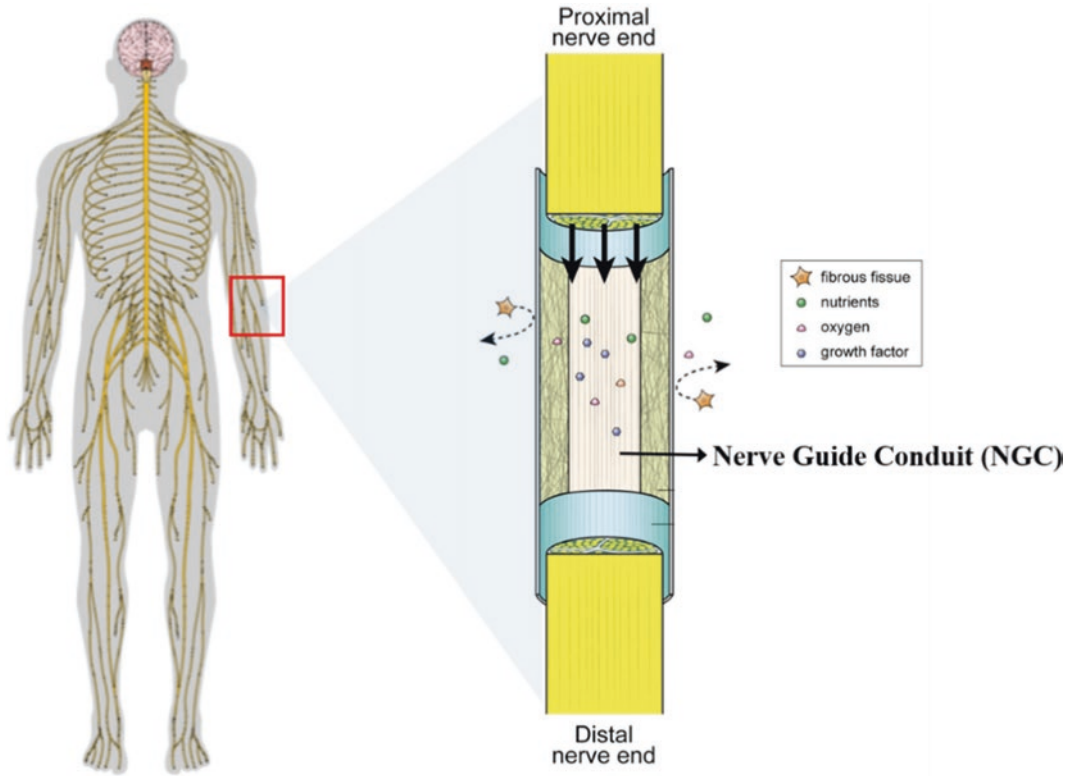

Fig. 20.1 Illustration of nervous system distribution and nerve conduit in the human body

neurotransmitters to be released at synapses [5]. The nervous system consists of neurons as well as other specialized cells named glias that provide metabolic and morphological supports.

20.1.2 Nerve Injury

Injury of nervous tissue caused by thermal, mechanical (too much pressure, stretching, or cut), chemical, or ischemic factors can impair cognition, movement, and language that are so important for peoples' lives [3, 6]. Nerve injury can be classified in five stages, depending on the degree of damage to the nerves and surrounding connective tissue, as the glial cells might affect the surrounding nervous tissue [7].

The events which occur in peripheral nerve regeneration could be divided into the following main processes: Wallerian degeneration (a clearing process of the axon distal to a transection site), axonal regrowth, and end-organ reinnervation as shown in Fig. 20.2 [8]. During the process of Wallerian degeneration, macrophages and Schwann cells interact to make the removal of debris such as damaged axons [9, 10]. After that, un-innervated Schwann cells grow and the Büngner bands which guide the regenerating axon are formed in the nerve injury site [8]. Axonal regeneration can be characterized by the growth cone formation and digestion of debris which remains in the path of nerve regeneration. The nerve fibers can regenerate about one inch a month under ideal conditions after proper surgery [11]. Depending on a variety of factors, including the length of the nerve fibers to finish regenerating, it can take several months to complete nerve regeneration after injury.

20.1.3 Nerve Guide Conduits (NGCs)

Peripheral nerve defects result from traumatic injuries caused by surgical intervention, accidents, diseases, and physical conflict [12, 13]. Restoration of neurological functional recovery after injury of peripheral nerve continues to be a challenge [14]. The autograft transplantation that is unrepairable by direct end-to-end suture has been used for injured peripheral nerve repair as a first-line therapy [11, 15]. But, the transplantation of autologous nerve graft has the various limitation of a mismatch between the graft and nerve, multiple rounds of surgery, limited supply of available grafts, and permanent loss of functions of the donor nerve [16]. TENGCs have been accepted as a potential alternative that creates a nerve cell-friendly environment for improved nerve regeneration

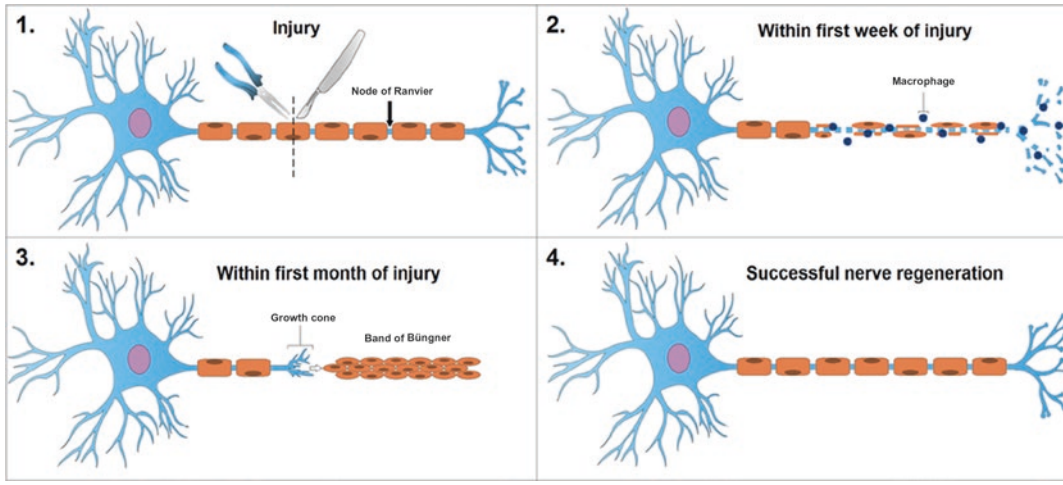

Fig. 20.2 Schematic representation of Wallerian degeneration and nerve regeneration. © Wiley. (Reproduced with permission)

[1]. Material selection, topographical cues, and biomimicry can be important requirements for desirable TENGCs. The artificial NGC is a supporting tubular type scaffold proposed complementary therapeutic technology for the autologous nerve graft. A lot of researchers are being carried out to improve properties and promote neurological functionality of NGCs such as biocompatibility, a permissive ability for cell activity, proper material selection, biomolecules addition, and topography [13, 14, 17]. In particular, topographical cues could be significant to sustain a micro-environment capable of controlling the fate of the neural cells [18, 19]. The influences of the surface topography of NGCs on accelerated and enhanced directional movement, migration, and proliferation of neural cells were investigated on many types of research.

20.1.3.1 Requirements of NGCs

There are a lot of requirements for an ideal NGCs, including selective permeability that can permeate oxygen and nutrients but prevent from an invasion of scar tissues (nerve regeneration inhibitors), proper mechanical properties for the maintenance of a structure and inner space, and revascularization for nerve regeneration [20]. Also, TENGCs should be made of biodegradable or biocompatible biomaterials to prevent undesirable immune and inflammatory response [13, 21, 22]. Natural and synthetic polymers have been investigated as NGCs to fulfill their requirements. The natural polymers used for manufacturing NGCs include collagen, chitosan, gelatin, and silk. These natural polymers support cell adhesion and functions, provide superior biocompatibility, avoid serious immune reactions, and could degrade by naturally occurring enzymes. But, natural polymers degrade relatively fast in-vivo condition having lack adequate mechanical strength. Often, natural polymers should be chemically cross-linked, modified, or mixed with synthetic polymers for enhancing the mechanical properties. Synthetic polymers have profits such as tailorable properties through chemical structure modification and unlimited supply compared to naturally derived materials. The ease of copolymerization facilitates the control and optimization of material properties such as thermal properties, wettability, mechanical behavior, and decomposition performance. Synthetic polymers used in NGC manufacturing include polyethylene (PE), polycaprolactone (PCL), PCL acrylate (PCLA), or poly(L-lactic acid) (PLLA). In the initial approaches using polymers, the most tubular NGCs not only had no surface topography but also had a dense wall having impermeability [23]. However, this structural limitation leads to suppressed neural repair because of hindered oxygen and nutrient supply and non-directional growth of the cells [20]. Many researchers have been developed to nerve scaffolds with topographical cues and proper porosity (Fig. 20.3).

Tubular type TENGCs have been applied for neural defects as early as 1879, with the application of bone scaffold used as a NGC, however, the experiment failed due to the scar tissue formation in the implanted NGC [24, 25]. An ideal NGC to reduce nerve regeneration failure rate needs to get characteristics such as high porosity, compliance, flexibility, biocompatibility, and appropriate surface modification [24, 26]. Furthermore, when biological polymers are used as an alternative treatment for effective neural repair, polymer-based NGCs can be tubular constructs with porous walls with an incorporation of cells, growth factors, or bioactive agents (Fig. 20.3) [22]. Biomaterials with the proper properties have been evaluated as NGCs for neural repair.

20.2 Electrospun Nanofibrous NGCs

A lot of techniques are available to process natural and synthetic biomaterials into NGCs. These include conventional methods, such as gas foaming, phase separation, freeze-drying, salt leaching, and electrospinning, among others [27, 28]. Among those fabrication techniques, the electrospinning (ES) is widely used because of its superior adjustability in surface topography, fiber alignment, signal encapsulation of fibers, diame-

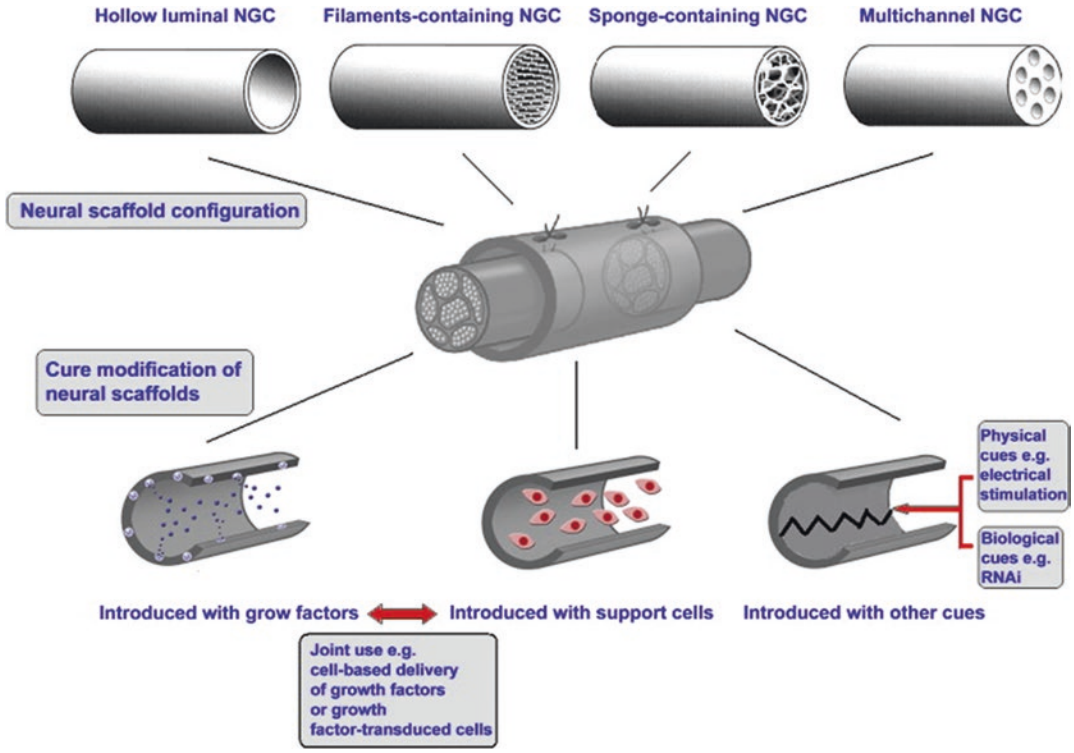

Fig. 20.3 A schematic diagram showing how an ideal tissue engineered nerve graft is constructed by incorporating a diverse array of physical and biological cues to a neural scaffold with different configurations. ©Elsevier. (Reproduced with permission)

ter, and porosity [29, 30]. ES is a fabrication method utilizing electricity to draw nano-scale fibers [31]. Many significant research in ES have allowed creating various scaffolds with biomaterials and, hence, the ES method has gained a popularity in tissue engineering application [32]. During ES process for the scaffold fabrication, the following parameter of ES can be very significant with respect to morphology of nanofibers: voltage, flow rate(ml/h), distance between needle tip to collector, polymer conductivity, viscosity, and surface tension [33]. Electrospun nanofibrous scaffold with high surface area to volume ration is suitable for biomedical application.

Electrospun nanofibrous constructs represent a potential class of biomaterials for the fabrication of NGCs because of their mimicking topography and architecture [15, 17, 34]. Although the benefits of fibrous structures in promoting neural repair have been demonstrated, the effects of in-vivo nerve regeneration according to the diameter or size of electrospun fibers were not evaluated. For this reasons, Jiang et al. Analyzed the influence of fiber size of electrospun NGCs on neural repair across a critical sciatic nerve defect (15 mm) in a rat [35]. Electrospun fibrous nerve tubes comprised of ordered polycaprolactone (PCL) nanofibers (251 ± 32 nm) or microfibers (981 ± 83 nm) were fabricated. Axonal regeneration was completed in the sciatic nerve gap of all rats which received the aligned nanofibrous (251 ± 32 nm) NGCs at 3 months post-implantation. Nanofibrous NGCs resulted in significantly thicker myelin sheaths and higher amount of myelinated axons compared to microfibrous samples.

20.2.1 Nanotopography of NGCs

The architectures and surface topography of nanofibers are known to modulate effects on a variety of cell activity and response [19, 36]. In particular, aligned nanofibrous scaffolds exhibit significant advantages in terms of their topographical cues, mechanical properties, and cell viability processes, such as directional migration, proliferation, and differentiation of the cells. The directional growth and polarization of the cells have been investigated with surface patterns of electrospun scaffolds which have modifications such as isotropic or anisotropic nanotopography [9]. Various cell types cultured on the electrospun scaffolds with nanotopography were reported to prefer directional elongation and polarization with alignment to the nano-patterns [19, 37–40]. For example, the neuronal cells are polarized along the nano-patterns and their viability rate can be sensitive to the morphology and size of the nano-architectures [41]. Also, bone marrow stem cells (BMSCs) on electrospun aligned fibrous membrane showed a tendency to differentiate into neural cells [36].

20.2.2 Aligned Nanofibers

Anisotropy of NGCs has been an important strategy to control cellular directional movement and neuritogenesis [42]. Indeed, alignment of cells could be a superior precursor to in-vivo regeneration and repair [43–45]. A lot of research teams have achieved optimal in-vitro cell alignment with ES methods based on nano to micro-scale patterns [10, 34]. Aligned electrospun nanofibers can play a significant role in better providing topographical cues for polarization and neural differentiation of the neuronal cells compared to randomly-oriented nanofibers. The reason is that aligned nanofibers induce directional regeneration fo the nerve with minimal contact points in one direction compared to randomly-oriented nanofibers as shown in Fig. 20.4. The difference in the degree of alignment can also be clearly distinguished from the Scanning electron microscope (SEM) images (Fig. 20.4). Randomly-oriented nanofibers are generally fabricated without preferential direction through a conventional ES method because of the electrified and disordered polymer jet which travels from a needle tip to a conductive collector [46, 47].

Although random nanofibers have been employed in various tissue engineering applications, the randomly-oriented nanofibrous scaffold might be undesirable in specific application requiring highly aligned architecture or surface topography, such as TENGCs for the directional growth of nerves [13]. Huang et al. developed a novel ES technique of the nanofibrous NGC consisting of highly-ordered fibers with longitudinal grooves on the surface of fibrous conduit for a sciatic nerve-injured rat model [35]. The grooved and aligned fibrous samples have been shown to effectively enhance sciatic nerve repair in transplanted rat electrophysiological, gastrocnemius muscle, walking track, triple-immunofluorescence, and immunohistological analysis results. Kim et al. developed a modified ES technique in order to fabricate aligned fibers

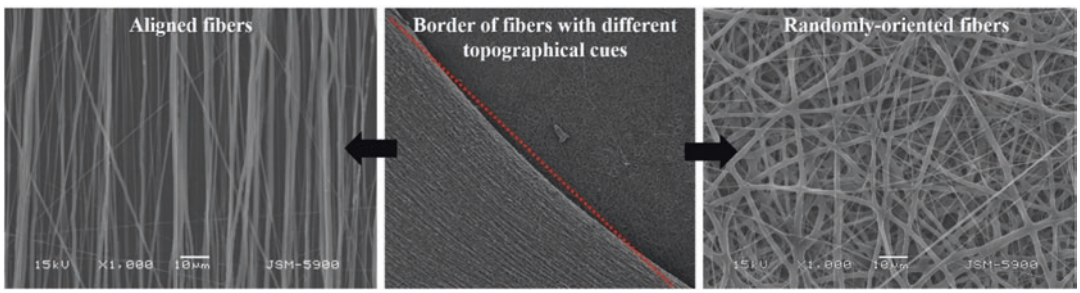

Fig. 20.4 SEM images of aligned nanofibers, randomly-oriented nanofibers, and intersection of the two fibers

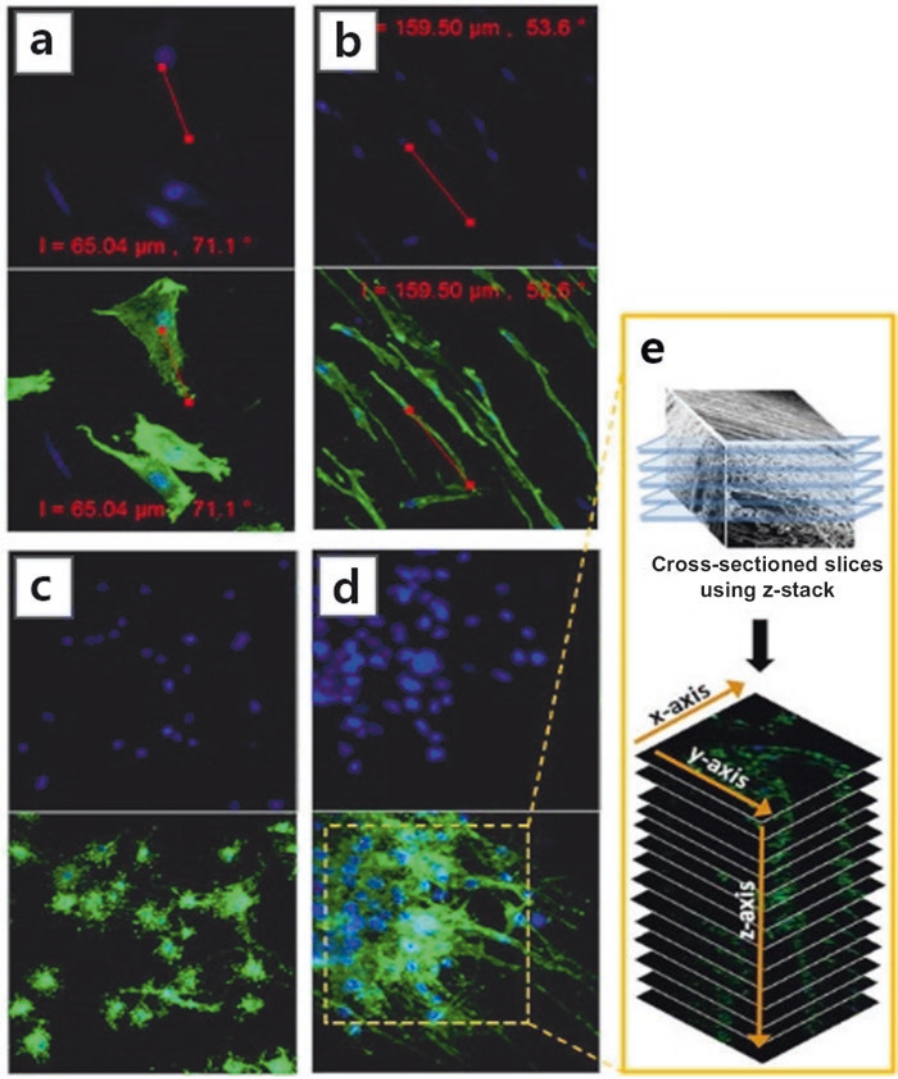

Fig. 20.5 Confocal microscopy images of PC12 cells attached after 5 day of culture on a (**a**) randomly oriented and (**b**) aligned nanofibrous PLGA scaffolds, Confocal microscopy images of S42 cells attached after 5 day of culture on a (**c**) randomly oriented and (**d**) aligned nanofibrous PLGA scaffolds (**e**) Z-stack from a PLGA scaffold with aligned nanofibers in which S42 cells. Images were collected at 0.37 µm intervals. (From Ref. [11] by Kim et al., licensed under CC BY 4.0)

and random fibers on a single membrane via a single step processing [11]. In this study, PC12 and S42 cells were cultured on the aligned and randomly oriented PLGA fibrous membrane fabricated using a modified ES method. The cells appear to be well-attached and proliferate to the surface of the aligned fibrous mat compared to the random fibrous mat as shown in Fig. 20.5. The central portion of the NGC is double-coated with random fibers over ordered electrospun fibers, strengthening the tensile strength of the ordered fibrous membrane. The inner part of the proposed NGC is made of highly-ordered fibers to promote nerve regeneration. Compared to the NGCs fabricated in a single layer of ordered electrospun fibers, multi-layer TENGCs with randomly-oriented and aligned fibers in the inner and outer layers can be more robust and

tear-resistant during surgery. For this reason, Xie et al. evaluated that the randomly-oriented nanofibers would interfere with ordered fibers to change the extension morphology of the neurites and impede regeneration [48]. The randomly-oriented and aligned fibrous double-layer exerted negative effects on the neurite extension with less aligned constructs compared to those grafted on a single layer fabricated with aligned fibrous membrane. Xie et al. reported that the negative effects of the randomly-oriented fibers can be mitigated by preculturing with Schwann cells [48]. Li et al. reported that the uniform poly(l-lactic acid) (PLLA) nanofibrous yarn type NGCs constructed by directionally aligned fibers were manufactured using a dual spinneret system, subsequently incorporated into a hollow tube [49]. The biocompatibility and cell morphology on the PLLA yarn type scaffolds were assessed by in-vitro experiments. Schwann cells showed a better proliferation rate of the PLLA yarn type scaffold than that of PLLA membrane.

20.2.2.1 Fabrication Methods for Aligned Nanofibers

NGCs made of ordered nanofibers can be favorable for neural repair due to their superior proliferation and directional migration of neuronal cell and neurite. But, this is challenging to fabricate a neat membrane with ordered fibers for tissue engineering application as NGCs because of the insufficient mechanical strength. The polymer jet spreads over an entire portion of the rotating collector, resulting in randomly-oriented fibers when a conventional electrospinning method is used.

A lot of researchers have studied on the fabrication method of ordered fibrous membrane with proper mechanical properties to meet requirements for the development of TENGCs as shown in Fig. 20.6. Edwards et al. reported the influence of the rotating collector with high speed on the surface architecture of electrospun PCL nanofibers. When rotating collector speed was faster than the spinning rate of nanofibers, highly aligned nanofibers were observed. Li et al. investigated the effects on surface nanotopography of electrospun fibrous mat fabricated by ES with parallel conductive bars with gap (Fig. 20.6) [50]. Also, Afifi et al. designed a new collector consists of a rotating collector around which conductive pins were attached for taking up ordered poly(L-lactide) (PLA) fibers. 60% of the electrospun fibers were aligned relative to the rotating direction of the rotor [35]. But, PLA nanofibers were formed in a bundle morphology and deposited in less quantity because of the repulsive wind force created from the conductive pins. Kim et al. demonstrated a collector modification with semi-conductive membrane and cooper wires for the fabrication of a single mat deposited with aligned and randomly-oriented fibers [11]. The developed polyurethane (PU) and poly lactic-co-glycolic acid (PLGA) NGCs fabricated by the modified ES technique has a high transparency that plays important role in follow-up close observation during a surgery. Sun et al. proposed a grooved collector for the fabrication of highly-aligned nanofibrous membrane [51]. The electrified polymer jet is spun and stretched across a gap between grooves of the collector. Theron et al. used a thinner disk-shaped rotor for fabricating continuous ordered fibers around its circumference (Fig. 20.6) [52]. However, the fibers on a disk collector were not perfectly aligned and uniform due to the residual electricity of the electrospun fibers which reached the collector repelled the following ones.

20.2.3 Other Improvement of Electrospun NGCs

After a series of studies in efforts for the improvement of the electrospun NGCs, several requirements have been explored and optimized. The electrospun NGCs requires additional improvements besides topographical cues to avoid rejection and additional inflammatory responses.

20.2.3.1 Neurotrophic Factors

The process of nerve regeneration can be dependent on the effects of neurotrophic factors secreted by the nerve cells. A variety of neurotrophic factors are released from different sensory neurons to exert their biological functions. Neurotrophic factors control and regulate

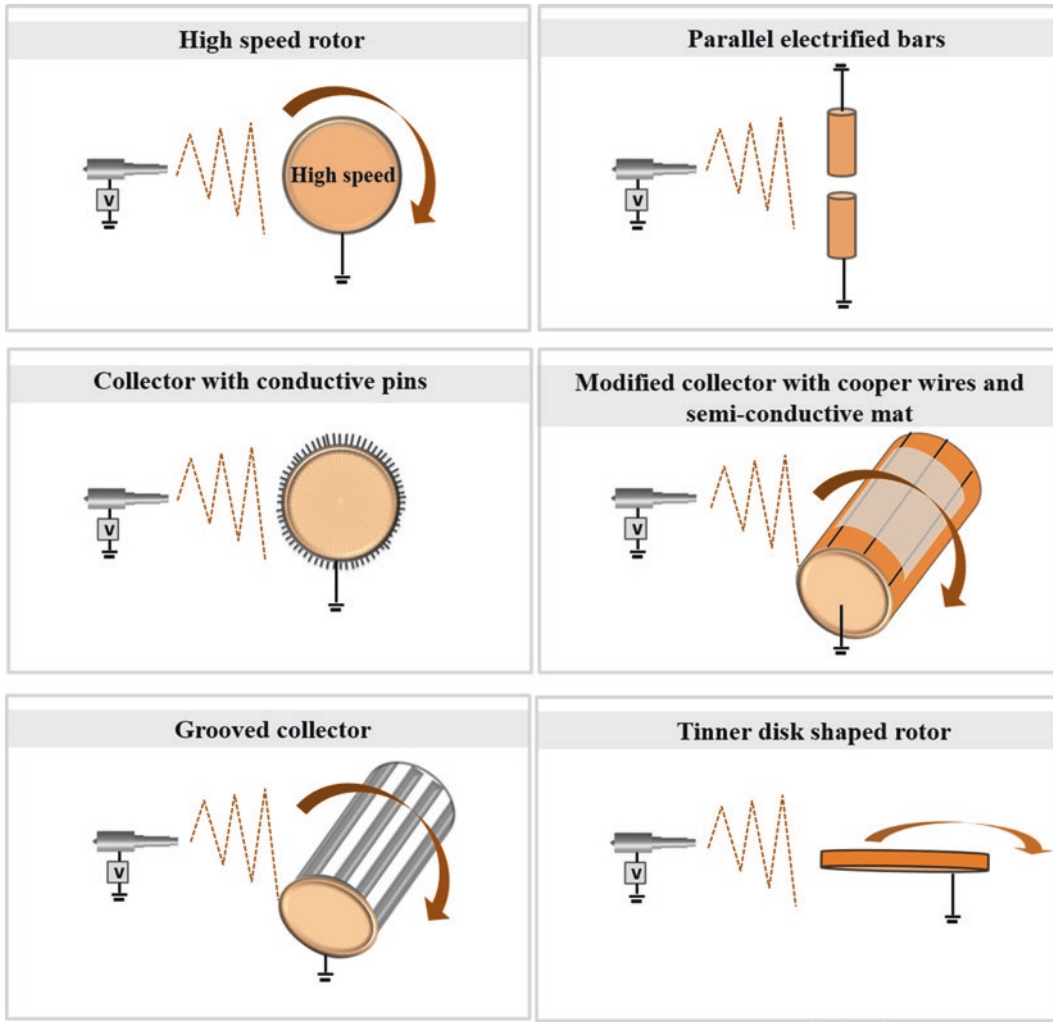

Fig. 20.6 Illustration of various electrospinning techniques for the fabrication of aligned fibrous membrane

the cellular activity, viability, migration, and differentiation in PNS and CNS. Therefore, NGCs having neurotrophic factors, along with other components, could be significant tools for neural repair.

The use of TENGCs is limited to short nerve gaps, up to about 30 mm. For large nerve defects, an inadequate fibrin formation between the proximal nerve end and distal nerve end limits the movement of Schwann cells and the Büngner band formation, which are the neurotrophic guidance for the regeneration of axons. For this reasons, an insufficient trophic supports in TENGCs could be a significant issue. The treatment of NGCs accompanied with cellular elements might require the various neurotrophic factors (vascular endothelial growth factor (VEGF), nerve growth factor (NGF), insulin-like growth factor -I (IGF -I), fibroblast growth factor (FGF), and glial growth factor (GGF)) to facilitate the nerve regeneration processing.

20.2.3.2 Cell Transplantation

Glias and neurons are two major cell types in the PNS and CNS. Glias are supportive cells which refer to oligodendrocytes and Schwann cells [1]. Neurons consist of axons, dendrites, and ganglia which are clusters of soma (sensory nerve).

Axons perform electric impulses away from the cell body of neurons, but dendrites transmit electrical signals received from other neurons to sensory nerve which called soma. Besides glias and neurons, other cell lines could play a significant role in the PNS and CNS. In particular, various stem cells which can differentiate into the neuronal lineages such as astrocyte, oligodendrocytes, and neurons can promote the maturation of a nervous system.

Mesenchymal Stem Cells (MSCs)

MSCs are multipotent stromal cells which could differentiate into a variety of connective tissues like cartilage, bone, or tendons. Ankeny et al. evaluated the influences on nerve defects with transplanting previously cultured MSCs to a spinal cord injury site of a rat [53]. The transplantation of MSCs could not change the extent of overground locomotor recovery, but, they induced hindlimb air stepping. Also, the neurites and axon in MSCs graft samples were highly aligned with their long axis parallel to that of the spinal cord [53]. Mimura et al. proposed BMSCs could be induced to form Schwann cells after treatment with β-mercaptoethanol and retinoic acid in transected sciatic nerve injury of a rat [7]. Cooney et al. investigated the efficacy of intravenous MSCs administration to augment neural regeneration in a rat model. The rats treated with injection of MSCs showed significant improvement in neural recovery of axon counts and action potential amplitudes of compound muscle compared to control groups [54].

Neuronal Stem Cells (NSCs)

NSCs are self-renewing, multipotent cells which could differentiate into astrocytes, oligodendrocytes, and neurons. Many researchers have reported that transplanted NSCs can enhance regeneration of axons through enervated nerve because NSCs secreted metalloprotease-2 and various neurotrophic factors. Lee et al. reported that implantation of a TENGC cultured with NSCs can promote the nerve regeneration, likely because of the implanted NGCs support and guide axonal regeneration from a proximal nerve end to distal nerve end, while retaining trophic factors and preventing ingrowth of fibrous tissues [55]. Also, the cultured NSCs can differentiate into Schwann cells, maintaining a neuronal growth factors in microenvironment of NGCs. Cheng et al. investigated that NSCs can promote neural repair after transplantation into acute traction injury, and this result can be seen on magnetic resonance imaging (MRI) by a pronounced increase in the T1 and T2 values of the nerve defects during the regeneration phase [56]. In addition, with well-controlled electrospun fibrous patterned scaffolds, stem cells were cultured and evaluated in-vitro and in-vivo. Beigi et al. reported that the stem cell-cultured matrixes showed better performance than that without stem cell culture in terms of both physical and functional repair.

Olfactory Ensheathing Cells (OECs)

OECs are known as olfactory ensheathing glia cells (OEGCs) or olfactory ensheathing glia (OEG). They behave similarly as astrocytes and Schwann cells ensheath non-myelinated neurons as shown in Fig. 20.7, so they are also known as olfactory Schwann cells (OSCs). When OECs were transplanted to repair the injury site of spinal cord, the implanted OECs express neurotrophic factors and NGF to the site [3, 57–59].

Fairless et al. proposed that OECs are a distinct glial cell type and possess characteristics that could make them suitable for the regeneration of CNS defect sites [60]. Kabiri et al. evaluated the efficacy of a multi-layered conductive nanofibrous hollow NGCs cultured OECs to enhance the neural repair. Harnessing topography and electrical cues of the proposed single-walled carbon nanotube/poly (L-lactic acid) (SWCNT/PLLA) NGCs along with the trophic features of OECs in a neural tissue engineering application [58]. An OEC-cultured NGC implanted to the transected sciatic nerve promoted functional and morphological recovery compared to a NGC without any seeded cells.

Schwann Cells

Schwann cells play a role in myelinating and ensheathing nerve fibers in a nervous system.

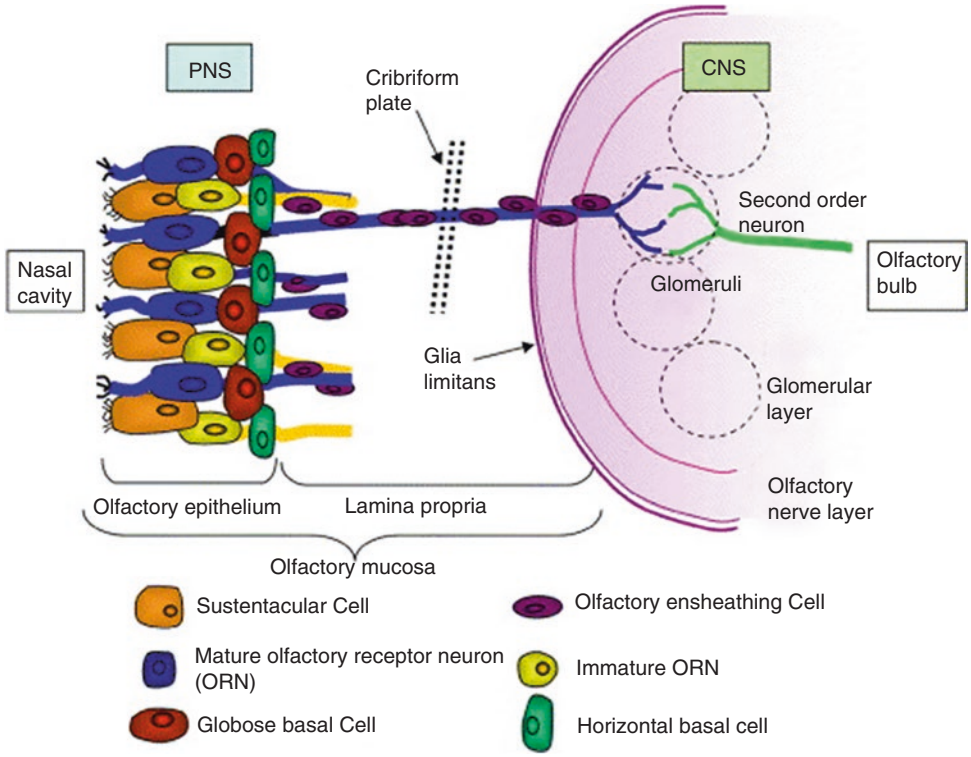

Fig. 20.7 The peripheral olfactory system. The olfactory system is composed of PNS and CNS tissue. The PNS component contains the olfactory mucosa, which is subdivided into the olfactory epithelium and lamina propria. The olfactory epithelium contains olfactory receptor neurons (ORNs, which project cilia into the nasal cavity), sustentacular cells (non-neuronal supporting cells), globose basal cells (putative stem cells for the epithelium), horizontal basal cells (putative stem cells) and Bowman's gland and ducts. The lamina propria consists of loose connective tissue and olfactory ensheathing cells (OECs), which wrap around bundles of olfactory receptor axons extending from the olfactory epithelium. The ORN axons extend through the cribriform plate and enter the outer, olfactory nerve layer of the olfactory bulb, which is a CNS tissue. It is in the glomerular layer where the axons synapse to form glomeruli with the second order neurons (mitral/tufted cells). © Elsevier. (Reproduced with permission)

Furthermore, Schwann cells produce biomolecules of extracellular matrix (ECM) and secrete neurotrophic factors that promote nerve regeneration. Regenerating axons are aligned and migrated with Schwann cells. In contrast, the deformation of Schwann cells can interfere with axonal regeneration and lead to failure of neural repair. Guenard et al. evaluated the potential use of Schwann cells which enhances nerve regeneration [61]. Schwann cells enhance remyelination and axonal regeneration by secretion of neural cell adhesion molecules (N-CAM), collagen, laminin, and adhesion molecules L1. Wang et al. proposed the effects of Schwann cells alignment along the ordered electrospun fibrous membrane on axonal regeneration [62]. Electrophysiological and functional recovery occurred in the aligned fibrous groups with the cells, and histological analysis results also revealed that the myelinated axons sprouting occurred vigorously in the ordered fibrous group.

20.3 Conclusion

A lot of developments of therapies for the injury of CNS and PNS have led to various biological treatments including cell graft transplantation. Developed strategies which bridge the nerve injury site and allow improved regeneration rate promise to have a significant impact on restoring high quality of patient's life. In particular, ordered fibrous architectures have been shown to support axonal regeneration and guide directional neurite growth, mimicking ECM. ES is the most common technique of producing nanofibrous NGCs with aligned topography which can achieve directed migration of neuronal cells. Various neural cell types grafted on the electrospun NGCs with alignment migrate directionally due to the directional elongation and polarization according to aligned nano-patterns. Also, the electrospun fibrous NGCs can be grafted with cells such as OECs, Schwann cells, and stem cells to remyelinate axons and support them integrate with the target spinal cord.

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Biomechanics in Annulus Fibrosus Degeneration and Regeneration

21

Genglei Chu, Chen Shi, Jun Lin, Shenghao Wang, Huan Wang, Tao Liu, Huilin Yang, and Bin Li

Abstract

Degenerative disc degeneration (DDD) is the major cause of low back pain, which seriously affects the life of patients. Current surgical and conservative treatments only relieve the pain temporarily, yet fail to restore the normal biomechanics and functions of healthy spine. Indeed, high recurrence of disc herniation commonly happens after discectomy. Degenerative changes in biomechanical and structural properties of the intervertebral disc (IVD), including fissures in annulus fibrosus (AF) and volume loss of nucleus pulposus (NP), mainly contribute to DDD development. AF plays a critical role in the biomechanical properties of IVD as it structural integrity is essential to confine NP and maintain physiological intradiscal pressure under loading. Maintaining the homeostasis of AF and NP, and thereby IVD, requires regulation of their biomechanics, which is also involved in the onset and subsequent development of AF degeneration. Therefore, it is essential to understand the biomechanical changes of AF during degeneration, which can also provide valuable insights into the repair and regeneration of AF. In this review, we focus on the biomechanical properties of AF tissue associated with its homeostasis and degeneration, and discuss the biomechanical stimulus required for regeneration of AF. We also provide an overview of recent strategies to target and modulate cell mechanics toward AF regeneration.

Keywords

Annulus fibrosus · Biomechanics · Biomaterial scaffold · Degeneration · Regeneration

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21.1 Introduction

Lower back pain (LBP) is one of the top 3 causes of disability in developed countries which seriously impacts the quality of patients' life and incurs significant amount of medical cost [56]. Degenerative disc disease (DDD), associated with degeneration and breakdown of intervertebral discs (IVD) of spine, is the leading cause of LBP. Surgical interventions, such as discectomy, disc replacement and spinal fusion, are current positive treatments if the conservative treatment fails [25, 42]. However, these treatments can only alleviate the pain temporarily without restoring the normal function of healthy IVD, and the post-surgery recurrence is high [6]. Therefore, new approaches to treat DDD are needed.

Anatomically, a healthy IVD is a three-component construct consisting of a gelatinous proteoglycan-rich nucleus pulposus (NP) in the center and a multi-lamellar collagen-rich fibrocartilage annulus fibrosus (AF) in the surrounding, which is connected by the cartilaginous endplates (CE) of the vertebral bodies. NP is highly pressurized and AF prevents radial disc bulge of NP by generating large hoop stresses (Fig. 21.1a). AF can also resist large tensile and compressive strains as the disc undergoes 6 degree of freedom motion including twisting and bending. Under normal conditions, NP and AF work in concert to provide appropriate gross mechanical properties of IVD to sustain the high stress load on the vertebral body. However, degenerative changes in biomechanical and structural properties of NP and AF usually occur as human ages, resulting in loss of normal function of IVD and serious degenerative diseases like DDD and LBP. In the past few decades, novel strategies such as gene therapy, growth factor treatment, cell-based therapies and tissue engineering approaches have been developed aiming to ameliorate IVD degeneration or promote its regeneration [15]. While considerable progress has been achieved in the treatment and regeneration of NP, much less is achieved in that of AF. As a crucial supporting component in the biomechanical properties of IVD, the structural and mechanical integrity of AF is essential in confining NP, and tears or fissures in defects of AF are closely associated with the onset and development of DDD [2, 32, 58]. Therefore, understanding the changes of mechanics and

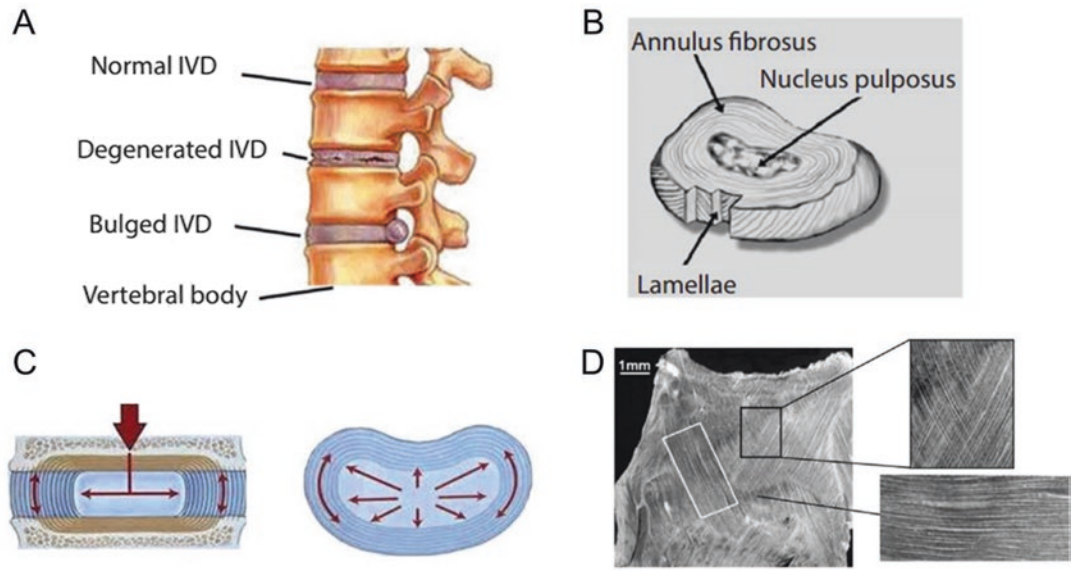

Fig. 21.1 (**a**) Schematic illustration of normal and degenerative IVDs. (**b**) Schematic illustration of the anatomic structure of an IVD. (**c**) Illustration of the mechanical force distribution in an IVD. (**d**) The hierarchical structure of AF. (Adopted with permission from [48])

structures of AF during regeneration is of critical importance to achieve repair/regeneration of IVD and ultimate treatment of degenerative diseases.

Complete AF regeneration requires the recovery of biomechanical and structural properties of healthy AF and restoration of biological behavior of resident cells in AF [21]. Balanced mechanical regulation is considered an essential factor for AF regeneration. Traditionally, mechanics were mostly considered as detrimental and correlated with AF lesions. Nevertheless, it remains unclear whether the mechanic force is a cause of DDD and herniation [14, 18]. One of the issues to be resolved is the relationship of the biological and biomechanical alterations in the development of degeneration of AF. In fact, the conflict of the difference between biology and mechanics has been resolved, as it has been well known that the cellular physiology is largely influenced by mechanical environment [16, 66]. Today, the effect of biomechanical forces on cellular function is known as mechanobiology, and it is deemed as a helpful tool in the study of development of degeneration of AF. In this review, we will address on the changes in biomechanics and structures of AF during regeneration, followed by discussion on cellular response to mechanical stimulus and required biomechanics for regeneration of AF. Finally, recent attempts to AF regeneration using biomaterial-based mechano-regulation are also discussed.

21.2 Anatomy and Mechanical Properties of AF

In contrast to the relatively simple structure of NP, AF is a highly heterogeneous tissue in terms of cellular phenotype, biomechanics, biochemistry, and microstructure, with typical gradient characteristics along the radial direction [47]. Microscopically, AF consist of 15–25 concentric layers, which are composed of alternatingly aligned oblique Collagen fibers interspersed with proteoglycans (Fig. 21.1b, d) [10]. The lamellas in AF intercross with each other and possess prominent mechanical nonlinearity and anisotropy, contributing to the mechanical properties of AF [52]. The outer layers of AF are more oriented between layers than the inner ones. Moreover, the outer zone of AF is more fibrous mainly consisting of collagen-I, while the inner zone is more cartilaginous, containing mostly collagen-II and aggrecan. Accordingly, the content of collagen-I increases from the inner zone to the outer zone of AF, and the opposite trend is found in the content of collagen-II and aggrecan [53, 75]. The collagen fibers of AF are tensioned by intradiscal pressure through two mechanisms: direct radial pressure from NP, and cranial-caudal stretch from the separation of the two endplates (Fig. 21.1c). Besides the microstructural heterogeneity, the mechanical properties of AF also gradually change from inner to outer layers along radial direction, leading to regional specific mechanical and biological functions. In general, the elastic modulus of AF lamellae changes from 59 MPa to 136 MPa from the inner region to outer region of AF [13, 75].

The unique composition and architecture of AF is crucial for IVD to sustain anisotropic, nonlinear and viscoelastic mechanical loading and maintain homeostasis [60]. Therefore, the repair and regeneration of AF must take into consideration the structural and mechanical characteristics of this tissue over a wide range of length scales.

21.3 Biomechanics During AF Degeneration

Understanding the pathological factors of degenerative disc diseases requires knowledge on the relation between biological and biomechanical alternations of AF. It is generally believed that DDD originates from biomechanical wear and tear [4, 12]. Following that, disorder of cellular components occurs as a result of the altered biomechanics [69]. Changes in the biochemical and cellular phenotype factors can also cause imbalanced catabolism and anabolism, resulting in accelerated degradation of AF matrix [5]. Once the steady state in metabolism is lost, the abnormal matrix components often go along with an inflammatory reaction. All of these contribute to degeneration of AF and consequently, the IVD.

21.3.1 Structure Changes of AF Induced by Biomechanical Alterations

Degeneration of IVD is often associated with changes in biomechanical environment from hydrostatic pressure to shear stress, which will induce significant changes in structures of AF and NP. According to previous studies, the biomechanical "wear and tear" is a vital cause of AF degeneration and DDD [8], as indicated by the high incidence of LBP and DDD in manual labor workers, machine drivers, soldiers carrying loads [62], and elite athletes [67], who generally experience high physical loading. In addition, most astronauts experienced LBP upon the exposure to microgravity and on their re-entry [63], which might be caused by overpressurization of their nucleus. Therefore, it can be inferred that the biomechanical factors play an important role in DDD. Previous studies have also demonstrated that NP extensively changes as well during DDD, losing its capacity to bind water under compression and developing into unorganized fibrous tissue, which further altered the biomechanical environment of AF [35, 71].

A healthy AF has a highly organized fibrous structure. The collagen fibers in AF are tensioned by cranial-caudal stretch from the separation of two endplates and radial pressure from NP. Therefore, appropriate pressure between IVD is highly essential for the maintenance of biomechanical properties and functions of AF [35]. However, in degenerated discs, the pressure was found to be much reduced between IVD, which dramatically disturbed the stress distribution in AFs [3]. This disturbance in stress distribution can generate stress gradients, which drastically increases the risk of endplate fractures or Schmorl's nodes, which are usually observed in DDD [54]. The reduction in intra-disc pressure will also lead to increased shear stresses in both nucleus pulposus and AF upon axial compression of spine [7].

To fully elucidate the mechanisms of AF and DDD, a number of biomechanical devices have been designed to induce degeneration in animal models, including tail suspension [28], hind leg unloading [29], tail or spinal compression [40, 77], tail bending [19], spinal shear stress [38], and microgravity [7]. *In vivo* studies also show that the failure limit of the production of fiber strain was over 20% with asymmetric bending and rotation. The rupture of collagen fiber initiated from the inner posterolateral AF and propagated as a radial tear. This study presented a consistent conclusion that complex loading (i.e., combined flexion plus compression, or torsion plus bending) led to high fiber strains, especially in the posterolateral AF (Table 21.1) [38].

Table 21.1 Mechanical properties of human lumbar discs

Tissue scale	Testing modality	Property	References
AF, single lamella	Uniaxial tension	80–120 MPa (E, tensile modulus)	[30]
AF, tissue level	Axial compression	0.56 ± 0.21 MPa (H_A, aggregate modulus)	[33]
	Tensile hoop stress	12.7 MPa (E, tensile modulus)	[1]
	Shear stress	25–110 kPa	[34]
Motion segment	Disc height	11.1 ± 2.5 mm	[17]
	Mid-disc area	310 ± 17 mm^2	[71]
	Axial compression	3–10 MPa	[9]
	Compressive stiffness	40–60 N (low load cycles)	[71]
		110–150 N (high load cycles)	
	Intradiscal pressure	0.5 MPa (leakage pressure)	[74]
		1.9 MPa (300 N load pressure)	
	Torsional mechanics	0.7–0.8° (with 8° pelvic rotation)	[11]
		2–9 MPa (shear modulus)	[22]

21.3.2 Cellular Physiology Changes During AF Degeneration

The changes in biomechanics can also lead to significant alteration of cellular physiology during AF degeneration. Several groups have shown that distinct compression on a spinal motion segment, either *in vivo* or *ex vivo*, could cause catabolic, anabolic and inflammatory cell responses in IVD [39]. As a result, the relation between mechanical environment and cell behaviors is considered a pivotal factor that determines the normal function and dysfunction of AF and IVD [27, 73].

Cells within AF respond to changes of hydrostatic pressure. AF cells were reported to produce roughly 20% more proteoglycan under higher hydrostatic pressure [70]. On the other hand, the production of collagen I and aggrecan was found to reduce, while that of Tissue Inhibitor of Metalloproteinase-1 (TIMP-1)increased, which could affect the remodeling of extracellular matrix (ECM) [31, 64]. This pressure sensing behavior appeared to be impaired in cells from degenerated AF as they responded less anabolically to physiologic intradiscal pressure [44, 57].

The shift of hydrostatic pressure to shear stress in the degenerated IVD was also reported to have distinct mechanobiological effects on AF cells [38, 41, 65]. Similar to other load-bearing tissues like cartilage and bone, the increase in shear stress will stimulate the formation of fibrous tissues which are rich in collagen-I. Furthermore, increased shear stress can also increase the production of nitric oxide by chondrocytes. Nitric oxide is a reactive oxygen metabolite which can reduce the production of proteoglycan and increase apoptosis in the cells within IVD [49]. Thus, the increased shear stresses in AF may accelerate IVD degeneration.

Biomechanical changes also induce inflammatory responses of IVD. Indeed, in the altered biomechanical load model of degenerated IVD, there is significant increase in the expression of inflammatory cytokines such as IL-8, IL-1β, IL-6, and TNF-α [61, 68]. These cytokines expressed by NP and AF cells remodeled the ECM from anabolism to catabolism, leading to DDD. The biomechanically induced inflammatory cytokines can also irritate the sensory nerves and cause pain response.

Overall, with the development of degeneration induced by altered biomechanical loading, there is a significant reduction in the expression of collagen-II and proteoglycans. Simultaneously, the expression of collagen-I also increases, indicating that matrix stress is changed and in turn accelerating the progress of disc degeneration. This degenerative circle not only illustrates the progressive nature of DDD, but also explains the different etiologies of DDD [43].

21.4 Biomechanics in AF Regeneration

21.4.1 Biomechanical Requirements for AF Regeneration

Repairing a damaged disc and AF involves many complicated issues, including risk of reoccurrence and disturbance of stress shielding in the tissue, both of which may exacerbate the clinical problems the repairing strategy tries to mitigate. Understanding the mechanics of normal AF and the mechanical requirement for AF regeneration will provide valuable guidance to future approach to optimizing biomaterials with biomechanical behaviors matching with those of native AF tissues. Moreover, mechanical factors are also known to significantly influence the biological response of IVD and AF. As a major component of IVD, AF is always under tension in vivo, which must be taken into consideration when designing scaffolds with proper mechanical properties for AF regeneration. Furthermore, a moderate mechanical regulation is also required for the function maintenance of cells within scaffolds.

21.4.2 Mechano-Regulation of Stem Cells for AF Regeneration

Cells are one of the major components in tissue engineering, which largely determines the

properties and behaviors of engineered tissues. Bone marrow-derived mesenchymal stem cells (BMSCs) are popular seed cells for bone tissue engineering. Advance in tissue engineering relies on the capacity to direct stem cells to differentiate into specific cell phenotypes. Recently, our group has also isolated a population of AF-derived colony-forming cells from rabbit IVD. These cells were self-renewable and could be readily induced to differentiate into different cell types including osteocytes, chondrocytes, and adipocytes. Such AF-derived stem cells (AFSCs) could potentially be a valuable source for repair or regeneration of AF tissue [50]. In addition, we have shown that transforming growth factor-β3-mediated BMSC (tBMSC) had strong tendency to differentiate into various types of AF cells and presented gene expression profiles similar to AFSCs [26].

Numerous studies have elucidated the critical role of mechanical stimulus in regulating the differentiation of stem cells [24, 46]. Jiang et al. demonstrated that applied forces, including tension, compression, stress, and hydrostatic pressure, could remarkably affect the maintenance and lineage specification of mesenchymal stem cells (MSCs) (Fig. 21.2a, b). It is believed that a diversity of mechanical signals within *in vivo* niches work together to regulate the fate of stem cells. For example, tensile forces induced osteogenic differentiation while compression forces led to chondrogenic differentiation [37]. External mechanical stimuli also influence the growth of cells and formation of ECM. In our work, we applied cyclic tensile strain (CTS) with different magnitudes (2%, 5% and 12%) when culturing AFSCs. The expression of anabolic

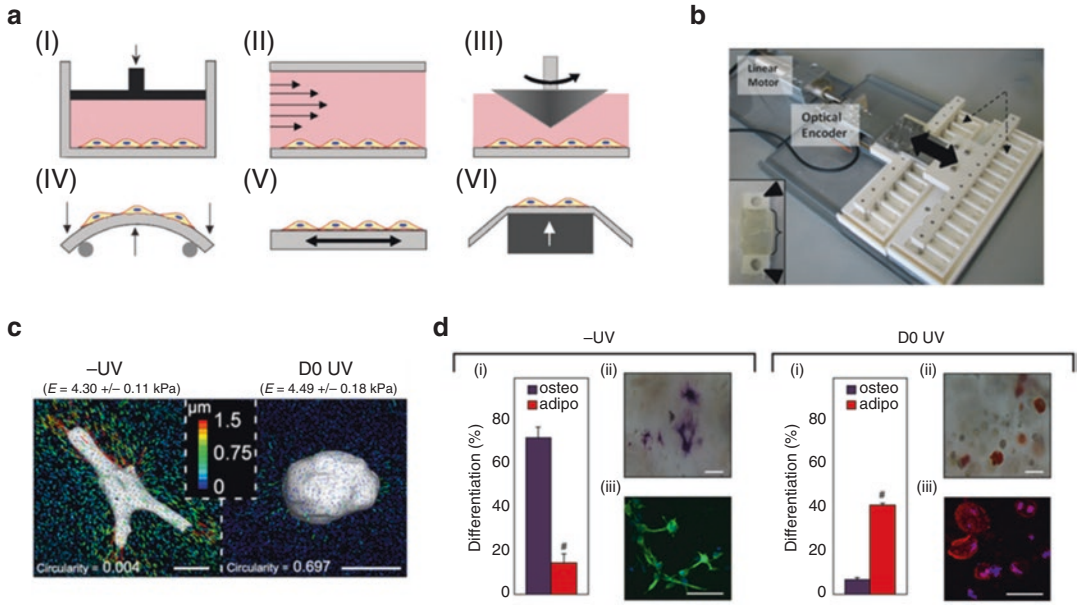

Fig. 21.2 (**a**) Representative approaches for cellular mechanobiology studies. (*i*) hydrostatic pressure; (*ii*, *iii*) fluid shear stress; (*iv*) bending; (*v*) tension; (*vi*) compression. (Reproduced with permission from [55]) (**b**) Tensile culture system. Up to 24 biomaterial constructs can be cultured in tensile wells (dotted-line arrows) and strained by the tensile rakes (double-head arrow shows direction of tensile strain) at the same time. The rake is moved by a linear motor with positional accuracy monitored by an optical encoder. Inset: individual construct with hydrogel (bracket) flanked by end blocks (arrowheads). (Reproduced with permission from [20]) (**c**) 3D traction force microscopy (TFM) images of hMSCs following 7 days of growth-medium incubation in hydrogels that were either proteolytically degradable (–UV) or photo polymerized to resist degradation (D0 UV). Bottom, hMSC differentiation following an additional 14 days of mixed-medium incubation. (Reproduced with permission from [37]) (**d**) Percentage differentiation of hMSCs toward osteogenic or adipogenic lineages in –UV (left) or D0 UV ($p < 0.005$). (Reproduced with permission from [37])

genes (aggrecan, collagen-I, and collagen-II) in AFSCs was found to increase with increasing applied CTS from low (2%) to moderate (5%) at a frequency of 0.5 Hz for 4 h. However, the anabolic genes expression decreased at high magnitude of CTS (12%) (unpublished data). Mostafa *et al.* applied varying magnitudes of equiaxial strain at different frequencies to investigate the differentiation of adipose-derived stem cells (ASCs). They found that 6% strain and 1 Hz was the optimal loading modality to induce differentiation into AF-like cells and formation of AF-like matrix. Furthermore, the equiaxial load also induced region-specific differentiation of ASCs within the inner and outer regions on their scaffolds [23].

In addition, recent advances have highlighted the critical role of internal forces due to cell–matrix interaction in MSC function. In addition, MSCs were found to be maintained and regulated by internal microscopic forces that occurred when contracting cells pulled on surrounding extracellular matrix (ECM) or on each other (Fig. 21.2c). These internal forces can be regulated by adjusting the mechanical properties of materials where cells reside, such as rigidity, topography, degradability, and substrate patterning. The seeded MSCs can generate contractile forces to sense these mechanical properties and thereby perceive mechanical information that directs broad aspects of MSC functions, including lineage commitment (Fig. 21.2d) [37, 45].

In all, the mechanical stimulation and cell–matrix interaction are believed to be essential for the maintenance and lineage specification of stem cells. More importantly, a moderate mechanical regulation is required to stimulate the anabolic expression, which can potentially be utilized for repair and regeneration of AF.

21.5 Biomaterial-Mediated Mechanotransduction

An ideal scaffold for AF regeneration should possess instant and prolonged mechanical stability and allows new competent tissue to form. Current biomaterial-based strategies for regeneration of AF tissue focus on developing scaffolds mimicking the microstructures, mechanical properties and biochemical characteristics of native AF tissue. More importantly, the scaffolds should also be strong enough to withstand the complex and multidirectional mechanical loads similar to native AF tissues so as to retain the functions upon implantation.

21.5.1 Microstructure of Scaffolds

The cellular alignment and architectural organization of the fibrous ECM in AF tissue play crucial roles in its biomechanical properties. The microstructures of scaffold significantly affect the morphology and function of cells cultured on it. By replicating the key length scales and structural features of native AF tissues, aligned fibrous scaffolds are preferred for AF regeneration, which are expected to control cell morphology and differentiation, as well as direct the ordered deposition of new ECM (Fig. 21.3). Many studies have shown that fibers orientation can affect the formation of ECM and the final mechanical properties of engineered AF constructs [76]. Our group found that aligned fibrous scaffolds could provide a favorable microenvironment for differentiation of AFSCs into cells similar to AF cells in different regions (Fig. 21.2a, b) [51]. Nerurkar et al. fabricated aligned nanofibrous scaffolds which demonstrated nonlinear dependence of modulus mimicking native AF. They found that AF cells grew along the fibers within scaffolds and formed ECM with considerable alignment (Fig. 21.3c) [59]. In a study using silk fibroin (SF) based scaffolds with fibers alignment resembling the fibrous orientation of AF tissue, the scaffolds guided the alignment of human chondrocytes and, in turn, the alignment of the deposited ECM. By offering the combined effects of cell/matrix alignment and chondrogenic redifferentiation support, these aligned SF scaffolds can potentially serve as a suitable substrate for AF regeneration [10]. In all, the topographical and structural features of scaffolds can regulate cell behaviors such as proliferation, matrix synthesis, and apoptosis, and can even direct stem

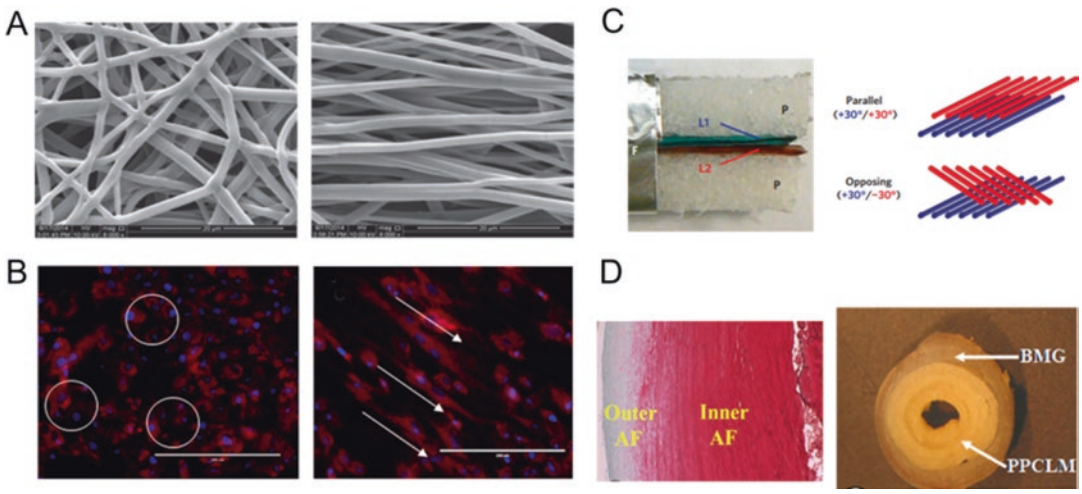

Fig. 21.3 (**a**) Scanning electron microscopy images of non-oriented and oriented electrospun biodegradable poly (ether carbonate urethane)urea scaffolds. (Reproduced with permission from [51]) (**b**) Immunofluorescence staining of collagen I protein produced in AFSCs on aligned and random scaffolds. (Reproduced with permission from [51]) (**c**) Fabrication of bi-lamellar tissue constructs. MSC-seeded scaffolds were formed into bilayers between pieces of porous polypropylene and wrapped with a foil sleeve. *P* porous polypropylene, *F* foil; L1/2: lamella 1/2. The bilayers were oriented with either parallel (+30°/+30°) or opposing (+30°/−30°) fiber alignment relative to the long axis of the scaffold. (Reproduced with permission from [58]) (**d**) Left, vertical section of normal rabbit IVD stained with Safranin-O. The outer layer (light red staining) and inner layer (dark red staining) of AF can clearly be seen. Right, a composite construct which consists of concentric PPCLM sheets surrounded by a BMG ring, mimicking the structure of the inner and outer AF, respectively. (Reproduced with permission from [72])

cells to differentiate toward specific lineages. Therefore, in order to engineer AF with similar properties as native tissues, the scaffolds should recapitulate the mechanical features of the complex structural anisotropy of AF to induce orientation of newly produced ECM.

21.5.2 Mechanical Characteristics of Scaffolds

In addition to composition and structure, the mechanical property of scaffold is also an important designing factor for AF tissue engineering. An ideal AF substitute should recapitulate the mechanical properties and distribution of native AF tissue. The mechanical properties such as stiffness can significantly affect the cell behaviors such as adhesion, proliferation, differentiation, and migration, and thereby play important roles in AF repair and regeneration. In particular, the stiffness of substrate was found to affect the differentiation of stem cells and direct their lineage specification [24, 36]. Similarly, rat AF cell were reported to be sensitive to substrate stiffness which could regulate their morphology, growth, apoptosis, and ECM metabolism [78]. In a recent study, we fabricated a series of nanofibrous polyurethane scaffolds with elastic modulus close to that of native AF tissue and studied the behaviors of AFSCs and BMSCs on the scaffolds. Depending on the elasticity of scaffold materials, AFSCs showed strong tendency to differentiate into AF-like cells. On scaffolds with low modulus, the gene expression of collagen-I in both AFSCs and tBMSCs was relatively low, whereas collagen II and Aggrecan gene expression was relatively high. The opposite trend was observed on scaffolds with high modulus. Since the inner region of AF mainly consists of collagen-II and PGs and outer regions mainly contains collagen I, it is likely that both AFSCs and tBMSCs tend to differentiate into cells in inner region of AF on the soft scaffolds, whereas they preferred to

differentiate into cells in outer region of AF on the stiff scaffolds [26, 79]. Wan et al. fabricated a biphasic elastic scaffold to elastically and structurally simulate the AF, the inner phase of the scaffold is an elastic material based on poly (polycarolactone triol malate) (PPCLM) while the outer part of the scaffold is demineralized bone matrix gelation (BMG) (Fig. 21.3d). The biphasic scaffold possessed enhanced compressive strength and tensile stress than uniphasic scaffold, making it a promising candidate for AF repair [72]. Therefore, the elastic modulus of scaffold remarkably affects the biochemical and biomechanical properties of cultured AFSCs and the matrix they produce. These findings provide new insights toward developing engineered AF with biological characteristics and mechanical functions approximating the native AF tissues.

21.6 Concluding Remarks

Natural AF possesses complex hierarchical structures and mechanical properties, and degeneration of AF involves altered biomechanics, catabolic cell response and changed ECM. Therefore, repair and regeneration AF require comprehensive understanding of the structure and mechanical property of native AF, followed by designing and fabricating scaffolds which replicate the key features of normal cellular microenvironment in vivo.

In recent years, interdisciplinary strategies addressing biologic, biomechanical and biomaterial needs for AF regeneration have been initiated. Contributions from all these areas are important to construct a biological implantation, which is expected to replicate the properties of AF and help maintain the mechanical properties of the IVD after implantation. Despite the recent progress in AF regeneration, much more challenging issues should be addressed to in the future research on AF repair and regeneration. The impacts of mechanical loading on cell behaviors should be further studied, and appropriate mechanical loading mimicking the physiological environment of native disc should be applied during cell culturing for AF tissue engineering. Evaluation of the performance of scaffolds and cell therapy under physiologically relevant loading conditions is essential to reveal the advantages and limitations for clinical application. Thus, an organ culture model with dynamic force loading would be very useful for evaluating the feasibility and effectiveness of current strategies for AF regeneration.

In summary, biomechanics offers promising perspectives for degeneration and regeneration of AF. Achieving AF regeneration relies on a comprehensive consideration of biological, biomechanical and biomaterial cues. Future development should pay more attention to making full use of mechanical stimulation or biomaterials-mediated delivery of biomechanics to effectively promote AF regeneration. In-depth understanding of the biomechanics during AF degeneration and regeneration, therefore, will help find novel approach to restoring the homeostatic mechanical properties of AF and ultimately to achieving effective therapies for DDD.

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22 Nanopatterned Scaffolds for Neural Tissue Engineering and Regenerative Medicine

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Abstract

Biologically inspired approaches employing nanoengineering techniques have been influential in the progress of neural tissue repair and regeneration. Neural tissues are exposed to complex nanoscale environments such as nanofibrils. In this chapter, we summarize representative nanotechniques, such as electrospinning, lithography, and 3D bioprinting, and their use in the design and fabrication of nanopatterned scaffolds for neural tissue engineering and regenerative medicine. Nanotopographical cues in combination with other cues (e.g., chemical cues) are crucial to neural tissue repair and regeneration using cells, including various types of stem cells. Production of biologically inspired nanopatterned scaffolds may encourage the next revolution for studies aiming to advance neural tissue engineering and regenerative medicine.

Keywords

Nanopattern · Scaffold · Stem Cell · Neural tissue engineering · Neural regenerative medicine

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22.1 Introduction

Neural tissues, which are composed of neurons and neuroglia, receive stimuli and conduct impulses in the central nervous system (CNS) and the peripheral nervous system (PNS) [1]. Neurons are important cells that transmit chemical signaling molecules and electrical signals to other neurons (Fig. 22.1a). Axons and dendrites extend from the neuron cell body, and electrical signals are transmitted from the dendrites to the axon. Neuroglia, including Schwann cells, astrocytes, and oligodendrocytes, support neuron function during signaling and metabolite transmission (Fig. 22.1b). Schwann cells are typically aligned along the axon, and they protect the neuron and provide the nutrient to neuron in PNS [3]. In contrast, oligodendrocytes cover the axons in the CNS and provide metabolites and neurotrophic factors to maintain axonal integrity [4]. Astrocytes form the blood-brain barrier and transport nutrients and metabolic precursors to neurons in the CNS [5]. These complex neural tissue structures enable the body to receive information from external and internal environments; the CNS communicates commands following processing of this information.

The nervous system is often exposed to physical injuries, trauma, tumor, and disease. However, the ability of injured and diseased the nervous system to regenerate itself in adults is often limited, and damage to the nervous system results in

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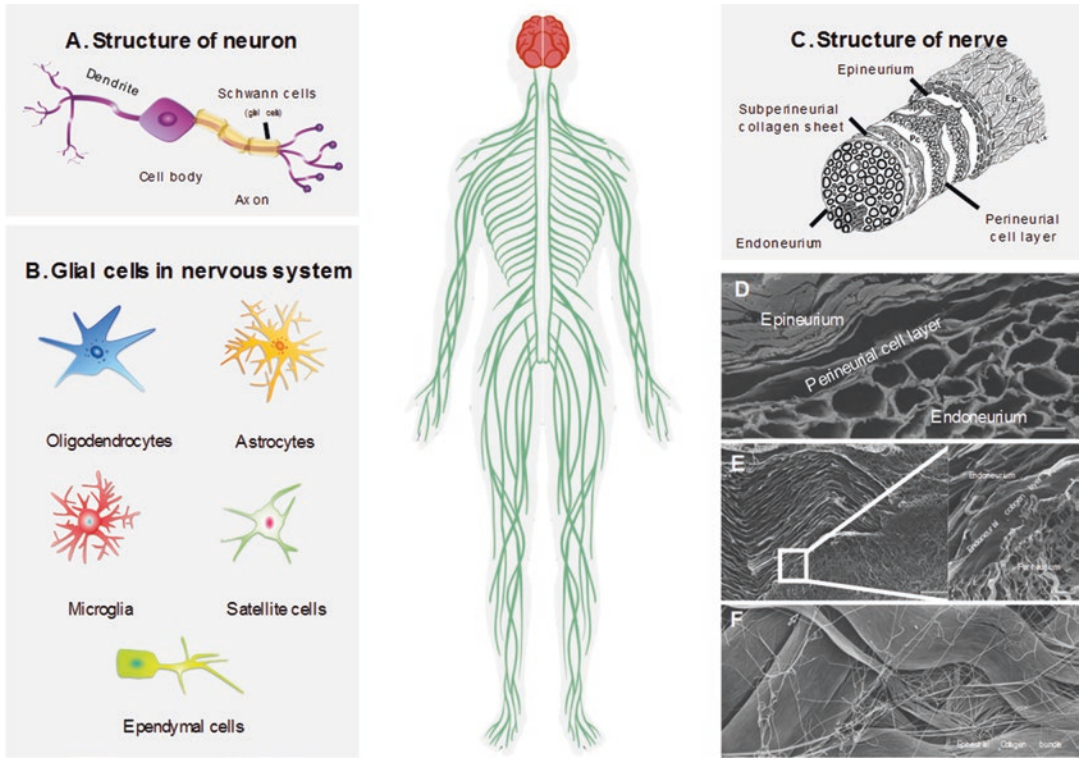

Fig. 22.1 Human nervous system consists of the central and peripheral nervous systems. The nervous system is made up of neurons and neuroglia, and signals are transmitted from dendrites to axon in neurons (**a**). Neuroglia support the neurons and include Schwann cells, oligodendrocytes, astrocytes, microglia, satellite cells, and ependymal cells (**b**). Living neuron and neuroglia are exposed to hierarchical nanotopography surface of collagen fibrils (**c**). The complexity of this structure increased as magnification increased. The nanoscale collagen fibril bundles were aligned (**d**) [2]

critical neurological disorders that may not be repairable. The incidence of nerve injuries is relatively high worldwide, and with more than 200,000 in in the US estimated to suffer annually from peripheral nerve injury [6].

From a physiological perspective, electrical and chemical signals are transmitted by neurons and other support cells. Each side of the cell membrane has an unequal distribution of ions, establishing a potential in the cell membrane. When the stimuli are generated, the potential difference is altered by opening of the sodium channel. Sodium ions pass through the channels, reducing the voltage in the membrane. As the potential reaches a peak, the sodium gates closes and the potassium gates open to restore the normal potential [7]. Impulses are transmitted through the synapse to another neuron, neuromuscular junctions, or neuroglandular synapses. Signal transmission, which conducted to integrate and propagate information within the nervous system, is affected by the axon formation [8]. Thus, polarization of the neurons has an important relationship with formation of the cytoskeleton. The cytoskeleton of neurons is naturally exposed to the extracellular matrix (ECM) structure and collagen fibrils in the nerve (Fig. 22.1c). The epineurium (EP), perineurium (P), and endoneurium (En) form collagen fibrils (collagen fibril size: 10–20 μm (in the epineurium), ≤3 μm (in the perineurium), and 1–3 μm (in the endoneurium)) [2]. Scanning electron microscopy (SEM) images revealed a distinct pattern of collagen fiber arrangement in the Ep, P, and En. As shown in Fig. 22.1e, the epineuria collagen bundle is divided into single

fibrils, which have an aligned structure. Images of the P and En revealed that the cylindrical collagen tubes also formed an aligned structure, while the nanostructure formed a microscale zigzag structure. Neurons and neuroglia were in the nanostructured environment, and thus, nanostructured scaffolds may be suitable for neural tissue engineering. Therefore, biomimetic scaffolds mimicking the unique nanostructures of neural tissue environments are important for neural tissue engineering applications to form brides between neurons-neurons, neurons-neuroglia, and neurons-neural tissue for complete tissue regeneration. In this chapter, we describe current efforts in the design, fabrication, and application of nanopatterned scaffolds for neural tissue engineering and regenerative medicine.

22.2 Design of Nanopatterned Scaffolds for Neural Tissue Engineering

Neural cells are exposed to complex 3-dimensional (3D) environments that can affect functions such as electrical signal transmission. Particularly, the nerve environment consists of collagen nanofibrils with the collagen bundle aligned in the nerve environment. The local nanotopography (e.g., nanotopographical feature sizes) greatly influences the behaviors of neural cells, and thus is an important factor in neural tissue repair and regeneration. To fabricate nanotopographically defined cellular environments, the various nanotopographical approaches including electrospinning [9], lithography [10], 3D bioprinting [11], etching [12], self-assembly [13], and hybrid composite nanomaterials [14] were proposed. Here, we describe representative techniques for designing and manipulating nanostructured scaffolds for neural tissue engineering and regenerative medicine (Table 22.1).

22.2.1 Electrospinning

The electrospinning technique is the general tool for generating nanofibers in tissue engineering and can be used to construct synthetic and natural polymers in a cost-efficient manner. Electrospun nanofibers are similar to the nanoscale ECM structure (collagen fiber diameter: 50–500 nm) [29], and can provide suitable nanotopographical environments for cells to enhance their adhesion, proliferation, differentiation, and function.

Electrospinning equipment consists of a high-voltage power supply, syringe pump, syringe, needle, and grounded collector (Fig. 22.2a). The solution on the end of the needle forms a Taylor cone according to the force of the electric field, with shape controlled by several factors, such as solution concentration, surface tension, flow rate, and applied voltages, among others [32]. Nanofibers with diameters between 3 nm and 10 μm and can be formed by electrospinning, with the direction controlled by the substrate such as a rotating mandrel and plate [33]. As described above, various materials have been used to form synthetic polymer-based nanofibers (e.g., poly(lactic-co-glycolic acid) (PLGA) [34], polycaprolactone (PCL) [35], polyacrylonitrile (PAN) [20]), and the natural polymer (e.g., collagen [36], silk [37], gelatin [38]). A proper viscosity needed to fabricate the nanofibrous scaffold for neural tissue engineering, and synthetic polymers blended with natural polymers are commonly used because of their synergistic effects, such as the viscosity of synthetic polymers and biocompatibility of natural polymers. Tian et al. developed a co-electrospun PLA/silk fibroin nanofiber scaffold for the differentiation of stem cells [39]. To optimize the conditions of this process, various parameters were adjusted, including the voltage, core flow rate, and core solution concentration. The diameter decreased as voltage was increased from 268 ± 53 to 197 ± 54 nm, and nanofiber uniformity was decreased at high concentrations, while flow rate had significant effects on nanofiber formation. Use of a PLA/silk nanofibers scaffold in combination with nerve growth factors greatly promoted the differentiation of stem cells.

It is widely accepted that neural cells are sensitive to topographical cues, and many studies have revealed the influence of nanotopographical cues on neural cell behavior. In 2004, electrospun

Table 22.1 Summary of representative nanofabrication technologies for neural tissue engineering scaffolds

Type	Material	Substrate	Characteristic		References
Electrospinning	PCL/DMF/ tetrahydrofuran (THF)	Random (rotating drum)	Distance: 20 cm, voltage: ~13 kV, flow rate: 2 ml/h	Fiber diameter: 450–1150 nm	[15]
	PCL/DCM/DMF	Random/align (custom-made drum collector)	Distance: 19 cm, voltage: 14–16 kV, flow rate: 1 mL/h	Fiber diameter: 750–1000 nm	[16]
	PLLA/chloroform	Align (rotating collection disc)	Distance: 5 cm, voltage: 10 kV, flow rate: 2 mL/h	Fiber diameter: 170–200 μm	[17]
	Collagen/silk/ HFIP	Align (parallel metal plates)	Distance: 10–20 cm, voltage: 15–25 kV, flow rate: 5 mL/h	Fiber diameter: 1–2 μm	[18]
	PLA/chloroform/ DMF	Align (rotating collection disc)	Distance: 10 cm, voltage: 10 kV, flow rate: 1.1 mL/h	Fiber diameter: 1.36–1.56 μm	[19]
	PAN/DMF	Random (rotating drum)	Distance: 12 cm, voltage: 25 kV, flow rate: 0.25 mL/h	Fiber diameter: 80–162 nm	[20]
	Collagen/PCL/ chloroform/CNTs	Random (flat plate)	Distance: 12 cm, voltage: 16 kV, flow rate: 2 mL/h	Fiber diameter: 564 nm	[21]
UV-assisted lithography	UV-curable PUA/ PUA mold	Glass coverslips (25 mm)	Ridge/groove 350 nm, height 500 nm		[22]
	PU-curable PUA	Glass coverslips (1-inch)	Spacing ratio 1:1, 1:3, 1:5 (width: spacing, width = 550 nm)		[23]
	UV-curable PUA/ PUA mold	Cover glass	Pattern spacing (250–800 nm)/PLL and laminin coating		[24]
	UV-curable PUA/ PUA mold	Glass coverslips	Ridges/high: 350 nm, grooves: 350, 700, 1050 1650 nm (grooves: 1:1, 1:2, 1:3, 1:5)		[25]
Thermo-assisted lithography	PLGA/chloroform (15% w/v)/PUA mold	Cover glass	Groove/ridge, height: 5000 nm, groove/ ridge: 800 nm, height: 600 nm, groove/ ridge: 400 nm, height: 600 nm, FN-PLL-DOPA coating		[26]
	Si mold	TCPS	Width: 5, 2, 1 μm, 750, 500 nm, pitch: 10, 4, 2, 1.5, 1 μm respectively		[27]
	Cyclic olefin copolymer	Silicon wafer	Ridge: 500 nm, depth: 350 nm, groove: 750, 1000, 1250, 1500, 2000 nm		[28]

nanofibrous platforms were applied for nerve tissue repair and regeneration. Yang et al. studied the attachment of neural stem cells to electropun poly(L-lactic acid) (PLLA) nanofibrous scaffold fabricated by dissolving PLLA into dichloromethane (DCM) and dimethylformamide (DMF). Reactions were performed under the following conditions: flow rate, 1.0 mL/h; distance between needle and collector, 10 cm; inner diameter of the needle, 0.4 mm; voltage, 12 kV. Nanofibers of 150–300 nm were prepared by electrospinning, and C17-2 cells were cultured on the prepared substrates for 10 h and 1 day. The ratio of attached cell on the electrospun nanofibrous substrates was lower than that on tissue culture polystyrene (TCPs), but increased neurite length was confirmed by SEM analysis. The following year, the same group demonstrated the differences between aligned nanofibers, aligned microfibers, random nanofi-

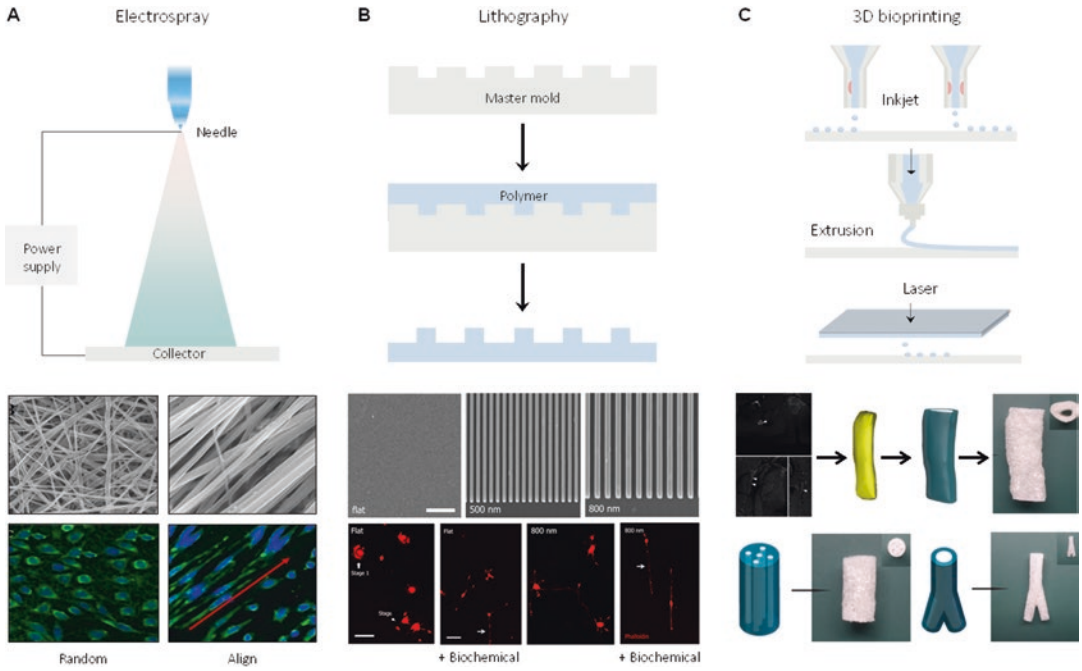

Fig. 22.2 Representative techniques for developing nanopatterned scaffolds. (**a**) Electrospinning for fabricating random or aligned nanoscale fibers [30]. (**b**) Lithography using various polymers was used to form the nanostructure-like aligned pattern, nanodot, and nanopillar [24]. (**c**) 3D bioprinting shows potential for neural tissue engineering, and is most suitable for generating 3D scaffolds [31]. Neurons and neuroglia were reacted with nanotopography of electrospun, patterned, and printed surfaces

bers, and random microfibers [40]. The order of neurite length was aligned nanofiber > random nanofiber > aligned microfiber > random microfiber, and cell orientation was not affected by diameter [9]. These findings demonstrate the importance of nanoscale fiber alignment and diameter. Prabhakaran et al. also reported the importance of fiber diameter [41]. Mesenchymal stem cells (MSCs) were cultured on the PLCL and collagen/PLCL nanofibrous scaffolds. The proliferation of MSCs was higher on the collagen-blended PLCL nanofibrous scaffolds than on the TCPs and PLCL nanofibrous scaffolds. In addition, the expression of neurofilament 200 (NF200) and nestin was increased on the collagen/PLCL, suggesting that the nanoscale topography is also important for stem cell differentiation into neurons.

Biodegradable polymer-based electrospun nanofibrous scaffolds were developed for neural tissue engineering. Potas et al. fabricated interleukin-10 (IL-10) conjugated electrospun PCL nanofibrous scaffolds with diameters of 400 ± 110 nm [42]. IL-10 is a cytokine that affects wound healing. The attachment of IL-10 was confirmed by enzyme-linked immunosorbent assay, and the prepared platforms offered a novel recovery approach for assisting in neural tissue regeneration by inducing macrophage polarization towards the M2 activated state surrounding the nerves. Kriebel et al. also demonstrated the utility of biodegradable polymer-based nanofibrous scaffolds as nerve graft platforms [43]. The artificial conduit was fabricated using PCL and collagen, and the diameter and alignment were controlled by adjusting the solution concentration. After implantation into the rat sciatic nerve, the authors conducted electromyography to confirm nerve regeneration. The results showed that clusters were formed in the autologous nerve grafts and artificial implant, while the PCL and gelatin-based nerve graft formed a single cluster, indicating inferior recovery.

To increase the function of biodegradable polymer-based nanofibrous scaffolds, various nanomaterials (e.g., carbon nanotubes [21] and graphene oxide [44]) have been introduced. Shah et al. demonstrated the use of graphene-based nanofibrous scaffolds to differentiate neural stem cells into oligodendrocytes [45]. Physical cues were used to prepare the scaffolds for neural stem cells (NSCs) and the morphology was controlled by nanotopography. The expression of the integrin-related signaling proteins involved in oligodendrocyte differentiation and development including focal adhesion kinase, Akt, integrin-linked kinase, and Fyn kinase was increased on the graphene-based PCL nanofibrous scaffolds. These findings demonstrate the synergistic effects of nanomaterials and nanotopography.

Electrospinning-based nanopatterned scaffolds may provide unique topographical cues to neural cells including stem cells to control factors such as neurite length, proliferation, and differentiation of the cells, which may play important roles in neural tissue regeneration. Recently, biodegradable polymer-based electrospun nanoscaffolds were proposed for use in neural tissue repair and regeneration (Table 22.2).

22.2.2 Lithography

Lithography is an effective method for fabricating precisely controlled nanotopographical structures (e.g., nanoscale geometry features of ECM) using various materials [58]. Representative lithography approaches include the following: electron beam lithography (EBL), photo lithography, scanning beam lithography, nanoimprint lithography, dip-pen lithography, capillary lithography, and soft lithography.

EBL is the technique used to change a structure via exposure to an electron resist (ER) and create nanoscale topography. After coating of the ER, the ER coated surface was exposed by electron beam. The ER can be classified as positive and negative and consists of high molecular-weight polymers. Positive resists show weak bonding between molecules and molecular chains (chain scission) after ER scanning which are easily removed during the developing process. Negative resists show strong bonding between molecules and molecular chains (cross-link) after ER scanning, which are not removed during the developing process [59]. Krsko et al. reported a patterned poly(ethylene glycol) (PEG) hydrogel produced through a cross-linking process under the following conditions: electron energy, 5 keV; current, 23 pA. The spacing between the individual points was 1, 2, 3, 4, or 10 μm on the 300-μm diameter circular array, with laminin coating the patterned PEG hydrogels. PEG-based substrates prevent adhesion of astrocytes but promote adhesion and oriented growth of neurons. This platform may be useful for promoting neurite outgrowth of neurons, while controlling the adhesion and ingrowth of astrocytes [60]. EBL can be used to create nanometer feature sizes by using very short wavelengths and suitable energy density characteristics. EBL-based platforms can also be used as a master mold because they are not needed for mask preparation, unlike photolithography [61].

The capillary phenomenon is an important characteristic in the manipulation of lithography-based nanostructures (Fig. 22.2b). A liquid has low free energy when it is wetting the capillary, and the wetting phenomenon causes the capillary rise of the liquid. The capillary force lithography process was conducted using the following steps: (i) an elastomeric mold was fabricated using a prepared pattern by photolithography or the electron-beam method. (ii) The mold was carefully peeled off the substrate. (iii) The prepared mold was used to cover the dropped polymer solution surface. (iv) The sample was cured by UV light or heating, and the elastomeric mold was removed [62].

The representative thermo-assisted materials include PCL, polylactide (PLA), and PLGA, which have advantageous properties such as biodegradability, biocompatibility, and high stiffness. Biodegradable materials have received attention since the Food and Drug Administration approved their use as biomaterials for clinical applications. The degradation time of PCL is ≥24 months, transition temperature (T_g) is −65 to

Table 22.2 Effect of the nanopatterned scaffolds on neurons and neuroglia for neural tissue engineering

Type	Materials	Cells	Supplement	Feature dimensions	Cellular response	References
Nanopattern	PDMS	Hippocampal neurons	NGF	Depth: 400,800 nm	Topography has the impact regardless of the presence or absence of NGF	[46]
			NGF, laminin	Width: 1,2 μm	Longer exon length on the flat pattern	[47]
		Schwann cell	PLL	–	Schwann cell was promoted adhesion at Nano and flat substrates	[48]
	PMMA	PC 12 cell	NGF	500 nm	PC 12 is stimulated by the nanopattern and attached well.	[49]
Nanofiber	PLGA	PC 12 cell	Graphene	314, 185, and 156 nm,	Improved neuronal cell proliferation and viability	[50]
	PLL	Astrocyte	Silk fibroin	1325 + 383 nm (large diameter fibers)	Increased of astrocyte spreading area on 400 nm silk fibroin scaffold	[37]
				759 + 179 nm, (intermediate diameter fibers)	PLL-silk fibroin scaffolds with smaller diameters promote the spreading of astrocytes.	
				293 + 65 nm (small diameter fibers)		
	PCLEEP	Astrocyte	–	665 ± 11 nm	Enhanced astrocyte elongation	[51]
	PCL	Schwann cell	Gelatin	232 ± 194 to 160 ± 86 nm	Cells on PCL/gelatin random nanofibrous scaffolds similar to the cell morphology on tissue culture polystyrene	[52]
			None	1.03 ± 0.03 μm (aligned)	Increased cell proliferation on PCL random nanofibrous scaffolds	[53]
				2.26 ± 0.08 μm (oriented)	Decreasing cell proliferation on flat scaffold	
	PCL & PCL/collagen	Schwann cell		~0.5 μm	Guided of direction of cell migration and axonal growth	[35]
	PLGA	Astrocyte	Carbon nanotubes	–	To guide axon regeneration and improve neural activity as biomimetic scaffolds at neural tissue injury sites.	[54]
–	PLLA	Oligodendrocyte		0.2–4.0 μm	Myelin of oligodendrocytes increased five-fold on 0.5 μm fibers	[55]
Nano-dot	PEG	PC 12 cell	–	–	Regeneration of neuron axons and neurite	[56]
–	Tantalum oxide/anodic aluminum oxide	Astrocyte	None	10–200 nm	Growth of cell viability, cytoskeleton, and adhesion, change of morphology on 50-nm nanodots	[57]

−60 °C, elastic modulus is 0.4 GPa, and contact angle is 78 ± 2°. Degradation time of PLA is ≥24 months, T_g is 60–65 °C, elastic modulus is 2.7 GPa, and contact angle is 76 ± 1. The degradation time of PLGA, which can be regulated by using the copolymers polyglycolic acid and PLA, is 1–6 months according to the molar ratio between lactic acid and glycolic acid (50:50, 65:35, 75:25, 85:15). The T_g is 40–60 °C, elastic modulus is 2 GPa, and contact angle is 72 ± 1 [63–65]. Poly(dimethylsiloxane) (PDMS) and polyurethane acrylate (PUA) are widely used to prepare micro and nanoscale structures in rapid and simple fabrication processes. However, these materials are not appropriate for implantation *in vivo* because they are non-degradable. Therefore, the development of biodegradable substrates with high biocompatibility and nanotopographical structure are required to extend the beneficial effects to *in vivo* situations [66]. Yang et al. studied the enhanced contact guidance and neuronal differentiation of human NSCs (hNSCs) on a PLGA nanopattern. The fabrication process involved the following steps: PLGA dissolved in chloroform (15% (w/v)) was coated onto the cover glass in a uniform manner. The flat PDMS mold and patterned PUA mold were placed onto the PLGA solution under heat and pressure. The pattern sizes were groove:ridge ratios of 5000:5000, 800:800, and 400:400 nm. In addition, poly-L-lysine (PLL) and fibronectin (FN) were coated on the nanopatterned substrates after pre-coating with 3,4-dihydroxy-L-phenylalanine (DOPA). Contact guidance and focal adhesion were higher for the FN-PLL-DOPA coated pattern than for the FN-PLL coated pattern, and neural differentiation was higher for the nanopattern than for the micropattern as confirmed by immunocytochemistry analysis [26]. To confirm the differentiation into neurons, hNSCs were cultured on the prepared substrates with neuron media for 4 days. Most neurons formed on the 400-nm nanogrooved substrates. The nanopatterned substrates using lithography were more suitable for providing contact guidance to cells.

Representative UV-assisted materials include PUA and norland optical adhesive (NOA), which can be used to adjust the modulus according to the different type NOA is typically used as an optical adhesive because of its high transparency and simple curing process [67]. NOA is not affected by the presence of oxygen compared to PUA or other UV-curable polymers, and has a wide-ranging elastic modulus of 2.5–6 GPa [65]. PUA was crosslinked by exposure to UV light on a flexible and transparent polyethylene terephthalate film. PUA is transparent and chemically stable and the modulus scale can be varied by 20–320 MPa by changing the amount of soft and hard modulators [68, 69].

Biochemical and biological agents are widely used induce cell functions, but these agents are costly and cause side effect in the cells. Importantly, it is difficult to determine optimal concentrations for efficient cell culture conditions. Lee et al. studied the neural differentiation of human embryonic stem cells (hESCs) on PUA for nanopatterning (ridge/groove: 350 nm). hESCs were cultured on a flat surface and nanopatterned surface for 5 and 10 days. The ratio of differentiation on the nanopattern surface was higher than that on flat surface according to immunofluorescence staining [22]. Kim et al. reported the differentiation of human MSCs (hMSCs) into neurons at spacing ratios of 1:1, 1:3, and 1:5 (width:spacing, width = 550 nm) [23]. Neurite length was higher for the 1:3 nanopatterned substrates and neuron shape was changed by the nanogroove. According to immunofluorescence staining images, the degree of neurogenesis was confirmed on the various topographies. Tuj1-positive (early neural marker) and NeuN-positive (later neural marker) cells were formed on the 1:3 nanosubstrate, and the degree of marker expression was higher on the nanostructured substrates than on the flat substrate.

Many studies have demonstrated the importance of chemical and physical cues in regulating the behaviors of living cells. Particularly, cell functions were enhanced by chemical and physical cues and neuron formation was synergistically affected by chemical and physical cues.

Park et al. reported that using laminin, a major component of the ECM, to coat the matrix nanotopography platform can control the morphology, neurite length, and type of neurons (pattern size of the platforms is flat, 250, 300, 400, 500, 600, 700, and 800 nm, followed by dip-coating in PLL and laminin). They demonstrated the neurons on the laminin-coated nanotopography were more developed than on the flat substrate. These findings indicated that ECM chemical components with nanotopography can generate both extracellular physical and chemical cues for neuronal development.

22.2.3 3D Bioprinting

3D bioprinting is a powerful technique for building 3D complex tissue for transplantation. Murphy and Atala reported three approaches for 3D bioprinting: (i) biomimicry of the human tissue microenvironment, (ii) autonomous self-assembly for appropriate cell signaling and autonomous organization, and (iii) mini tissues, which is the smallest structural and functional components of tissue [70]. Based on these approaches, the process of the 3D bioprinting is as follows: imaging of the damaged tissue in the human body → design of a scaffold → selection of suitable materials → target cell selection → bioprinting → application such as implantation, *in vitro* testing platforms, and maturation. The types of 3D bioprinting have been classified by Mandrycky et al. as inkjet printers, laser printers, and stereolithographic printers (Fig. 22.2c) [71]. 3D bioprinting can accommodate the complexities including materials, cells, and growth factor selection. However, current 3D bioprinting techniques remain limited, as improvements are needed to ensure high-resolution cell deposition, controlled cell distributions, vascularization, and innervation within complex 3D tissue [71, 72]. It is also difficult to generate nanoscale scaffolds using current bioprinting techniques. Hsieh et al. reported the use of a biodegradable polyurethane hydrogel platform to mimic the microenvironment of the brain for proliferation and differentiation into neurons [73]. By using a prepared hydrogel, NSCs secreted more neurotrophic factors than NSCs on the monolayer. The functions of adult zebrafish with brain injury improved after implantation of the hydrogel according to distribution analysis of fluorescence-labeled NSCs. Hu et al. fabricated a cellularized bioconduit consisting of a cryopolymerized gelatin methacryloyl gel cellularized with adipose-derived stem cells (ASCs) (Fig. 22.2c) [31]. The prepared conduits were implanted into rats and degraded over 1, 2, 4, and 8 weeks. The proliferation of cells on the TCPs was higher than on the prepared conduit, but the relative ratio of gene expression on the prepared conduit was higher than on the TCPs. The ASC cellularized nerve conduit showed potential for clinical application.

To fabricate nanostructured platforms using 3D bioprinting, the nanomaterials were incorporated in the bioprinting inks. For example, Zhu et al., fabricated the bioprinted scaffolds using graphene and gelatin methacrylamide (GelMA) hydrogels [74, 135]. The graphene incorporated GelMA-based hydrogels had the well-defined nanotography. The neural stem cells were cultured on prepared scaffolds, showing the excellent cell biocompatibility. To confirm the differentiation of the neural stem cells in the bioprinted hydrogel scaffolds, the staining with neural markers including β-tubulin III and Glial fibrillary acidic protein (GFAP) was conducted. After 14 days culture, the neurite elongation of neural stem cells was processed, and the expression of neural markers was verified. In addition, Huang et al., reported that the graphene-polyurethane composite hydrogel using 3D bioprinting could be used a platform for the differentiation of neural stem cells [71]. The neural stem cells were cultured on the graphene composite hydrogels, and the cell viability was increased significantly on the graphene composite hydrogel compared to that on the non-treated hydrogels [75, 136]. Furthermore, the oxygen metabolism was enhanced on the graphene composite hydrogels, and the differentiation of neural stem cells was enhanced remarkably through the enhanced protein expressions of neural biomarkers including β-tubulin and

GFAP. Although nanoscale approaches are still limited in the 3D bioprinting field, these techniques are an important approach for generating suitable 3D structures for neural tissue repair and regeneration.

22.3 Nanopatterned Scaffolds for Neural Cells and Stem Cells

22.3.1 Nanopatterned Scaffolds for Neural Cells

Neural tissues contain neurons and neuroglia [76], and it is important to form appropriate interactions between these cells to create a complete system. However, studies using primary neural cell culture have some limitations for neural tissue regeneration: (i) the number of primary cells is limited for forming a mature neural cell for clinical repair [77] and (ii) it is difficult to generate an axon from a neuron body spontaneously [78]. Therefore, various platforms for neural cell culture and neural tissue regeneration have been developed to provide more suitable environments for neurons and neural cells. The formation involves the following steps. First, embryonic hippocampal neurons form several thin filopodia from the neuron body (filopodia contain 15–20 parallel filaments and constitute a bundle with their barbed ends facing the membrane). Next, neurons form immature neurites from the filopodia. After several hours, one of these immature neurites begins to extend rapidly, becoming much longer than the other neurites. Finally, the long neurite becomes the axon, while short neurites become mature dendrites [79] (Table 22.3).

The major roles of neuroglia including Schwann cells, astrocytes, and oligodendrocytes are to support neurons for neurite outgrowth during the development and regeneration of neurons. Schwann cells form parallel collagens fibril bundles to surround neurons and support the axon of the neuron [92]. Astrocytes connect neurons to each other and neurons to blood vessels [93], and can communicate with each other (neurons, blood vessels, and astrocytes) through calcium signaling [94]. Oligodendrocytes surround the axon of neurons to form the myelin sheath [95].

In studies of the importance of the nanotopographical cues to neuroglia, various researchers demonstrated that Schwann cells were directly affected by aligned nanofibers, enhancing axonal outgrowth of neurons on nanoaligned substrates. Xie et al. showed that the neurite of neurons was altered according to the absence and presence of Schwann cells, with neurite lengths of 378 ± 13 μm (no Schwann cells with nanopattern) and 1232 ± 325 μm (Schwann cells with nanopattern), respectively (Fig. 22.3) [97]. Astrocytes, which are a type of neuroglia, showed lower proliferation on carbon nanotube (CNT)-blended nanofibers compared to CNT-blended microfibers [98]. In contrast, nanotopographical substrates guide the growth direction of astrocytes and increase adhesion. The transport efficiency of astrocytes was also greatly enhanced by incubation on 50-nm nanodots, and the astrocytes were stretched along the 50- and 100-nm nanodot. Nanotopography may also affect the constructs and functions of astrocytes, leading to regulation of hyperexcitability and epileptic activity in neurons [45].

Neurons are the functional units of the nervous tissue, and their major role is to transmit information through electrical and chemical signals. PC12 cells, a type of neuron, have been widely used to investigate cell behavior in neural tissue engineering because of their reversible adoption of several neuralcharacteristics [99]. Klymow et al. reported axonal outgrowth of PC12 cells on silicon wafers with different grooved nanotopographies (depth of 30–150 nm, pitches of 150–1000 nm). Different nanotopographies affected cell morphology including cell-orientation, axon-orientation, and axon length. The order of axon length was 1000 nm on patterned substrates >300 nm on patterned substrates >150 nm on patterned substrates > on smooth patterned substrates, while even smaller scale topographies did not affect cell morphology [100]. Genchi et al. poly(hydroxybutyrate) (PHB) microfibers (1 μm) were successfully fabricated by electrospinning

Table 22.3 Effect of nanopatterning on stem cells for neural tissue engineering

Type	Materials	Cells	Treatment/protein coating	Feature dimensions	Cellular response	References
Nanofiber	PCL	hESCs	Poly-L-ornithine/ laminin	Diameter: ~250 nm	Grown axonal outgrowth along with the direction of aligned fiber, increased the neural differentiation.	[80]
		Mouse ESCs	None		Increased the neuronal differentiation.	[81]
					Suppressed the differentiation into astrocytes.	
Nanopore	PMMA	hNSCs	None	Diameter: 10 nm, groove: 1500 nm	Promoted the neuronal differentiation.	[82]
Nanopattern	PUA	hMSCs	Gelatin	Ridge: 550 nm, groove: 550, 1650, 2750 nm, height: 600 nm	Increased the neurogenesis.	[83]
		hESCs		Ridge: 350 nm, height: 500 nm	Promoted the neuronal differentiation	[84]
	PCL	hNSCs	Fibronectin	Ridge: 300 nm, spacing: 300–1500 nm, height: 300 nm	Promoted the differentiation into neurons and astrocytes.	[85]
	PDMS	hESCs	Poly-L-ornithine/ laminin	Ridge: 250 nm, height: 250 nm	Increased the differentiation into neurons with differentiation into astrocytes.	[86]
			Fibronectin	Ridge: 600 nm, groove: 600 nm, height: 600 nm	Increased the differentiation, alignment with reduced proliferation.	[87]
		hMSCs	Air plasma treat/ bovine collagen I	Ridge: 250 nm, groove: 500 nm, height: 250 nm	Promoted the neuronal differentiation	[88]
			Bovine collagen I	Ridge: 350, 1000, 10,000 nm, groove: 700 nm, height: 350 nm	Up-regulated the neuronal marker expression.	[89]
		hiPSCs	Oxygen plasma treat	Ridge: 350, 2000, 5000 nm, groove: 350, 2000, 5000 nm, height:300 nm	Up-regulated the neuronal marker expression.	[90]
			None	Ridge: 500 nm, groove: 1000 nm, height: 150, 560 nm	Promoted the generation of neuronal cells.	[91]

with random and parallel fibers, and experiments using prepared nanofibers were conducted to confirm the cell adhesion and proliferation of PC12 cells on random PHB, parallel PHB, and collagen-coated cell culture polystyrene as a control substrate. The results suggested that parallel fibers with diameters of 200–400 nm could provide neurite guidance and induce more rapid growth of neurites. The proliferation of PC 12 cells on the random and parallel PHB substrates was higher than that on collagen-coated polystyrene as observed in immunofluorescent images. All nanoscale grooves of parallel PHB fibers could be applied as nerve conduits for peripheral nervous system regeneration [101]. Cesca et al. studied the topographical effects on neuronal growth and cell-substrate adhesion. Primary hippocampal neurons were cultured on flat and nanopatterned PCL substrates using lithography. In immunofluorescence images, only a few cells grew on the flat films, forming a sparse network. On nanopatterned substrates, neurons were healthy, as indicated by the smooth surface of cell bodies and dense network of neurites, which grew in tight adhesion with the substrate [102].

The neural tissue inspired platforms enables neurite outgrowth of neuron for their adhesion. The morphology of the neurons and neuroglia cells is critical to form the intricate neural networks of the functional neural tissue. In the case of the nanopatterns with the groove depth and width are shown to be the critical parameters for studying axonal guidance oriented neurite outgrowth, neuronal polarization and branching, respectively [28].

Furthermore, nanopatterned scaffolds with biochemical and nanomaterials have been developed with improved functions for nerve tissue regeneration. Laminin is an ECM component that is continuously synthesized after nerve injury and functions in cell migration and axonal growth [103]. Park et al. proposed the use of laminin-coated matrix nanotopography platforms for generating extracellular physical and chemical cues for neuronal development. Quantitative analysis revealed that the rate of mature neuron development was higher on laminin-coated 300- and 400-nm nano-substrates than other substrates. The rate of multipolar neurons on the PLL-coated nano platforms was higher than that on other platforms, while more bipolar neurons were formed on the laminin coated nanotopography. Neurite length was greater on laminin-coated substrates than on PLL-coated substrates [104]. Kijenska et al. fabricated laminin and PLCL-based core–shells with diameters of 316 ± 110 nm and nanofibers by electrospinning with diameters of 350 ± 112 nm. The proliferation of Schwann cell was comparatively higher on core–shell scaffolds than on blended nanofiber scaffolds [105]. Koh et al. demonstrated that Schwann cells could proliferate and migrate along the axons of neurons, but differentiation into the myelinating phenotype was not observed in the absence of laminin. Thus, *in vitro* studies showed that neurite outgrowth of neurons was enhanced on nano-scaffolds with laminin [106]. In addition, carbon substances as nanomaterials have been used to transmit signals between neuron cells because of its high electrical conductivity [107].

Taken together, neurons and neuroglia are sensitive to nanotopographical features (e.g., aligned and random nanofibrous scaffold for enhancing cell adhesion, neurite length, axonal growth, and proliferation [108]). The nanopatterned scaffolds may be major factors in the development of neural tissue engineering strategies using neural cells.

22.3.2 Nanopatterned Scaffolds for Stem Cells

Stem cells have the potential to differentiate into various mature cells. For example, NSCs have self-renewal capacity and can form neural cells (i.e., neurons and glia) [109] in the nervous system of all mammalian organisms [110]. NSCs are derived from three different sources: direct extraction from CNS tissues, differentiation from pluripotent stem cells (i.e., embryonic stem cells and induced-pluripotent stem cells), and transdifferentiation from somatic cells [111]. In a previous study, medium containing basic fibroblast

growth factor and epidermal growth factor was used to proliferate NSCs *in vitro*. After basic fibroblast growth factor and epidermal growth factor were removed, the medium was replaced with neurobasal medium containing brain-derived neurotrophic factor and glial cell-derived neurotrophic factor to differentiate the neural stem cells into neural cells [112, 113]. Neural cells are typically generated from NSCs during CNS development, during which NSCs undergo symmetric division into two NSCs (differentiation into neurons) or two progenitor cells (differentiation into astrocytes and oligodendrocytes). Progenitor cells can only differentiate into a specific cell type and undergo asymmetric division, which can self-renew and yield NSCs and pro-

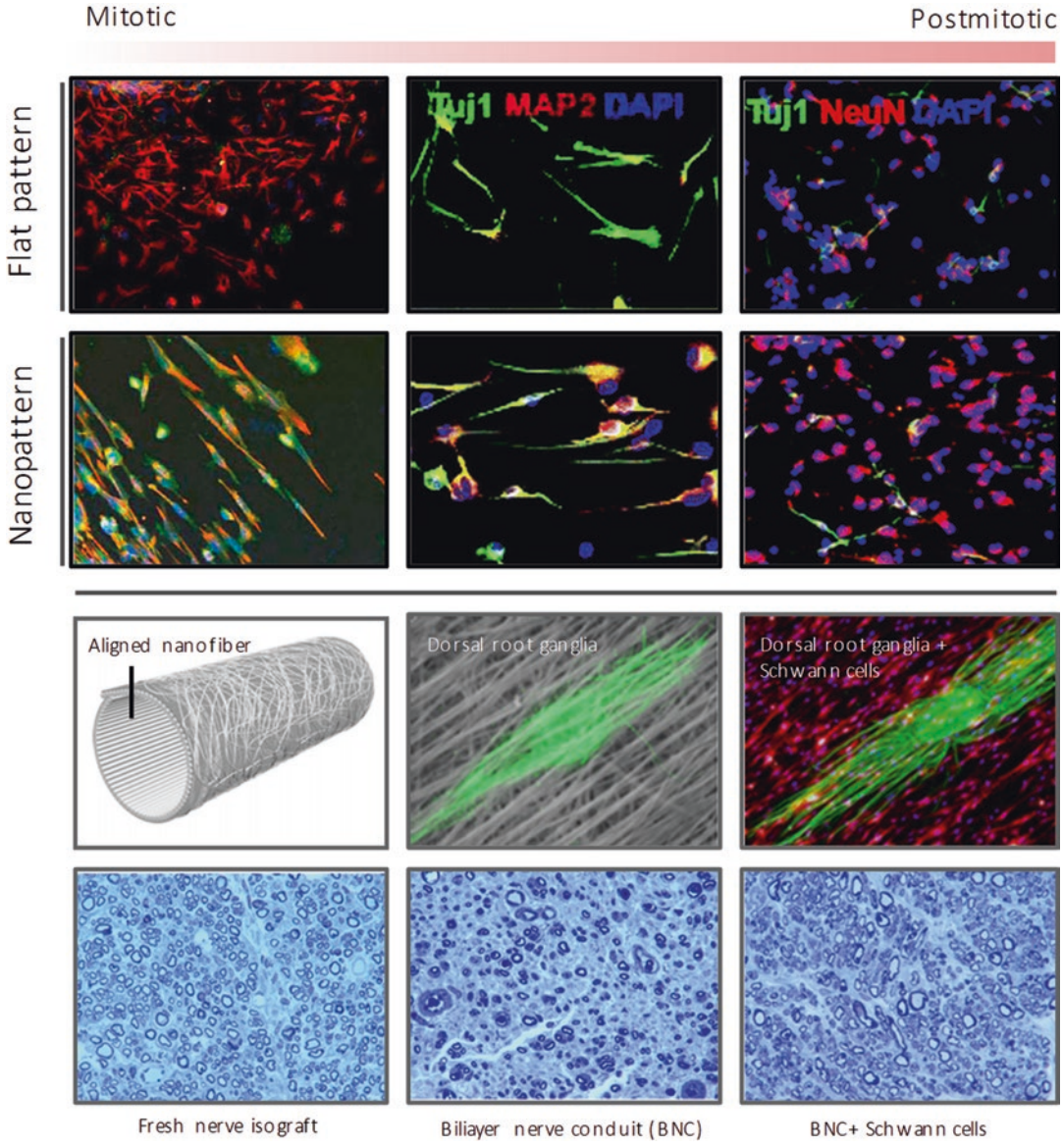

Fig. 22.3 Effects of nanopatterned scaffolds for neural tissue engineering. The neuronal differentiation of stem cells was controlled by nanotopographical cues, and mature neurons were formed on the nanoscale structure [84, 96]. In an *in vivo* study, implantation of a nanoaligned scaffold with neuroglia enhanced nerve regeneration [97]

genitor cells [109, 114–116]. NSCs can be transplanted into the brain and spinal cord after injury or to treat neurodegenerative diseases. However, transplantation of neural cells and tissues is limited by the low survival rate of grafted cells and lack of tissue donors [117]. Thus, various platforms for neural tissue engineering have been studied to overcome these limitations.

Recent studies have demonstrated the importance of nanotopographical cues in enhancing the neuronal differentiation of stem cells [118]. Among studies using embryonic stem cells, Lee et al. reported that the use of gelatin-coated PUA with a 350-nm ridge/groove patterned scaffold induced the differentiation of hESCs into neuronal lineages without the need for a differentiation-inducing agent. The gene expression levels of NeuroD1 (neuronal differentiation marker) were up-regulated on the nanopatterned scaffolds compared to on the flat surface, while gene expression levels of GATA6 (endoderm gene marker) and DCN (mesoderm marker) were lower on the nanopatterned scaffolds than on the flat surface. Furthermore, hESCs on the nanopatterned scaffolds were positively stained for β-tubulin III (Tuj1, early neuronal marker), brachyury (mesoderm marker), and Pdx1 (endoderm marker) to further analyze the differentiation of hESCs into neuronal lineages. As a result, neurite extension along the direction of the pattern arrays and differentiation of hESCs into neuronal lineages was induced by nanopatterned scaffolds without adding biochemical or biological inducers of differentiation [84]. In Fig. 22.3, the neuronal differentiation of stem cells was higher on the nanopatterned scaffolds than on the flat surface by increasing the expression of Tuj1 and neuronal nuclei (NeuN, later neuronal marker) [84, 96]. Yim et al. studied the topographical effects of anisotropic and isotropic micro- and nanostructures on stem cell differentiation. In this study, hESCs were cultured on a multiscale PDMS substrate, such as 1-μm micropillars, 250-nm nanopatterned scaffolds, and anisotropic 2-μm and 250-nm hierarchical scaffolds. Poly-L-ornithine and laminin (extracellular matrix materials) were also coated on the substrate for hESC attachment. The differentiation of hESCs into neuronal lineages was analyzed by immunofluorescence staining of Tuj1 and glial fibrillary acidic protein (GFAP, astrocyte marker). hESCs on the anisotropic micro- and nanopatterned scaffolds showed a higher percentage of Tuj1-positive cells compared to the GFAP-positive cells population. In contrast, hESCs on isotropic scaffolds showed a larger GFAP-positive population compared to the Tuj1-positive population. The N:A (neuron:astrocyte) ratio, which is defined as the ratio of Tuj1-positive to GFAP-positive cells, was higher following nanografting. In addition, neurons on the nanopatterned substrate were significantly more elongated than those derived from the flat-patterned substrate. The researchers speculated that physical cues from the topographical substrate affected changes in gene expression, which determined the neural differentiation of hESCs [86]. Mahairaki et al. reported that hESCs on the aligned fibrous PCL substrate fabricated by electrospinning showed a high propensity for neuronal differentiation with axonal outgrowth along the direction of aligned fibers. In this study, the researchers compared the effects of four scaffolds (i.e., aligned micro-/aligned nano-/random micro- and random nano-fiber scaffolds) on the differentiation of hESCs. The diameters of fibers were ~250 nm and ~1 μm, and the surfaces were coated with poly-L-ornithine and laminin. Immunofluorescence staining of Tuj1 was conducted to evaluate the effects of fiber diameter and alignment on the differentiation of hESCs. The cells cultured on the aligned micro- and nanofiber substrates showed higher levels of Tuj1 immunoreactivity compared to those on the random fiber substrate and tissue culture plate surface [80].

As with embryonic stem cells, nanotopographical cues promote the differentiation of adult stem cells including NSCs, MSCs, and induced pluripotent stem cells (iPSCs) into neuronal lineages [119]. Yang et al. reported that NSCs on PUA nanogroove and pillar patterned scaffolds showed greater differentiation into neural cells compared to those on flat-patterned scaffolds. The scaffolds were fabricated by the nano-imprinting method, showing 300-nm

ridges, 300-nm heights, and 300–1500-nm grooves. The hNSCs showed elongated morphology along the axis of the groove on the nanopatterned scaffolds. Immunofluorescent straining of Tuj1 and GFAP were conducted to detect the elongated neurite extension and enhanced differentiation of hNSCs into neuronal lineages and astrocytes. The expressions of Tuj1 and GFAP was much higher on nanopatterned substrates with small gaps (groove: 300 nm, ridge: 300 nm) than on the flat substrate and large gap patterned substrate. This study demonstrated that the differentiation of hNSCs into neural cells (i.e., neurons and astrocytes) was promoted on the nanogroove and pillar-patterned scaffolds [85]. Bucaro et al. used silicone nanopillar arrays with dedicated pitches of 1, 2, or 4 μm fabricated to have a 100-nm radius and be 5 μm in height and evaluated the response of hMSCs [120]. Representative SEM images of cells on the nanopillar showed different morphologies as a function of pillar density. At a high-aspect ratio, the growth and polarization of cells were increased, while the expression of Tuj1 was higher than on other substrates. The author determined the cell spreading mechanism according to the nanopillar arrays by controlling the morphology and alignment of the cells. The nanopillar array was not inspired by the human tissue environment, but this finding showed that stem cells sensitively reacted to the nanotopography and their morphology and functions were controlled. In another study, hMSCs on a 550-nm PUA nanopatterned anisotropic substrate showed greater differentiation into neuronal cells compared to those on flat substrates based on immunostaining of neural cell markers (Tuj1 and NeuN) [99].

iPSCs, which generated from human somatic cells through transduction of defined reprogramming transcription factors, can differentiate into pluripotent stem cells such as embryonic stem cells [121]. Fewer studies have examined iPSCs with nanotopographical cues compared to other cell types because this type of tissue engineering was only recently developed and the transduction process is complex. In 2013, Pan et al. confirmed the effect of the nanotopography on iPSC differentiation into neural cells [90]. Using 350-nm, 2-μm, and 5-μm PDMS substrates, cell direction analysis showed that more cells were aligned on the 350-nm substrate. On the flat PDMS substrates, the cells aggregated and could not properly differentiate into neural cells. Gene expression using real-time reverse transcription-PCR and immunostaining revealed up-regulation of neuronal marker expression on the 350-nm substrate. Song et al. evaluated neuronal differentiation of human iPSCs on various nanotopographies [91]. Six types of nanotopographies were prepared (aligned pattern: width of 500 and 1000 nm, height of 560 nm and 150 nm/nanopillars: width of 500 nm, height 560 nm and 150 nm). The authors investigated cell spreading, proliferation, and neural differentiation, among other factors, and found that the nanotopography influenced neuronal differentiation. The neuronal differentiation of iPSCs was significantly promoted on the PDMS nanograting substrate with a height of 560 nm compared to that on the flat substrate. However, nanotopography-modulated Yes-associated protein nucleocytoplasmic localization alone was not sufficient to induce neural differentiation from hiPSCs in immunofluorescence images. These results suggested that the nanotopography can deliver the signal to promote the differentiation of stem cells into the neuronal lineage and that Yes-associated protein cytoplasmic localization was essential for increasing the neuronal differentiation of iPSCs. These results demonstrated that nanotopography affects stem cell differentiation into neuronal lineages, which is crucial for forming environments that enhance cell functions.

22.4 Nanopatterned Scaffolds for Neural Tissue Repair and Regeneration

Approaches for neural tissue repair and regeneration can provide guidance to neurons and neuroglia and supplement the gaps between damaged neural tissue and undamaged neural tissue in the CNS and PNS. In clinical treatments of damaged nerves, a cylinder-shaped conduit is the most

widely used shape for nerve regeneration because it can: (i) surround the aligned nerve, (ii) bridge the gap of the damaged nerve, and (iii) create a real nerve system. Thus, the nerve conduit is an alternative nerve graft for connecting small nerve gaps to regenerate neural tissue. Over the past few decades, silicone has been widely used for fabricating nerve conduits, but must be removed by revision surgeries after nerve regeneration. Therefore, various biodegradable materials were introduced for developing alternative nerve conduits, particularly PCL and PLGA, among others [122, 123]. Since then, the ideal properties have been determined for the design and manipulation of artificial nerve conduits and essential factors were evaluated (i.e., biocompatibility, porosity, anisotropy, protein control, mechanical properties, relationship with support cells, and electrical signal transmission) [124]. To add directionality such as in the ECM of neural tissue in existing nerve conduits, a biomaterial-based nanotopography matrix was introduced and showed enhanced functions as a nerve conduit. Various nanoengineering approaches such as aligned nanopatterns and electrospun nanofiber were also developed. Bini et al. produced the first biodegradable polymer nanofiber tubes using the electrospinning technique for peripheral nerve regeneration [125]. Miller et al. showed that Schwann cells cultured on laminin-coated and micropatterned biodegradable substrates and the incorporation of physical, chemical, and cellular guidance factors enhances neurite alignment, outgrowth, and axonal regeneration [126, 127]. Subsequently, many studies using nanopatterned nerve conduits have been conducted for neural tissue engineering.

To fabricate nerve conduit-like human nerve tissues, hierarchical nanofibrous cylinder scaffolds using electrospinning technique are mainly used. Madduri's group demonstrated that the aligned nanofibrous substrates provide guidance to Schwann cells and glia, unlike random nanofibers. Additionally, the axonal length of the chicken embryonic dorsal root ganglion and spinal cord on the aligned nanofibrous scaffold is much longer than the random nanofibrous scaffold [128]. Huang et al. compared cell functions between aligned nanofibrous scaffolds and random nanofibrous scaffolds [129]. The proliferation of the porcine iliac artery endothelial cells showed no significant differences on the two surfaces, while the proliferation on random nanofiber scaffolds was higher than on aligned nanofiber scaffolds. The morphologies of the cells controlled by the aligned surface were observed by SEM imaging and hematoxylin and eosin staining. Ouyang et al. evaluated collagen/PLGA-based aligned and random nanofibers for nerve regeneration and rat Schwann cells were cultured on the prepared substrates. The morphologies of Schwann cells were aligned on the electrospun nanofibers and proliferation showed a greater increase on the aligned nanofibers [130]. As such, various studies confirmed that the cells reacted on the aligned nanofiber substrates and morphologies and functions were controlled by the substrate structures [131–133]. Recently, tissue-like nanofibrous nerve conduits were implanted into the animal's nerve to confirm the effects in tissue. Chang et al. emphasized the need to guide axonal outgrowth and neurite elongation and fabricated a multichanneled nerve guidance conduit with aligned nanofibrous substrates using gelatin [134]. The fabricated nerve conduit showed tunable mechanical properties, excellent degradation rates, and easy release in *in vitro* and *in vivo* experiments. Overall, the rate of functional recovery of the neural tissue was higher for the gelatin-based aligned nanofibrous nerve conduit than for the controls including the autograft group and sham. The biomimetic nerve conduit using RGD, polyurea, and PCL was implanted into damaged rat nerves by Lee et al. [135]. The functional recovery and reduction in muscle atrophy and fibrotic tissue formation were similar to those in the autograft, but the recovery of electrophysiological activity showed greater improvement than that in the autograft. The electrospun nanopatterned nerve conduit was suggested as

the best alternative strategy for nerve conduit regeneration.

In addition, more sophisticated scaffolds using lithography and 3D printing techniques were gradually introduced. Huang et al. fabricated a PLLA based functional nanopatterned nerve conduit, which consisted of linearly patterned grooves as guided channels for nutrient transport [136]. Schwann cells on the fabricated platform formed a spherical myelin structure and linear bundle by interacting with PC12 axons, and the structure of the Schwann cells extended along the surface direction. A PLGA bundle with a nanopatterned PLGA membrane as a nerve guidance conduit was developed by Peng [10]. Degradation of the PLGA membrane was performed after 14 days, and neuron progenitor KT 98 cells were cultured on the fabricated PLGA membrane. The cell migration rate was higher on the hierarchical micro/nanostructured membrane than on the flat membrane, and proliferation increased on the hierarchical PLGA membrane. Recently, nerve conduit regeneration using the bio 3D conduit was studied by Yurie. The vertical cylinder of the homogeneous multicellular spheroids consisted of human normal dermal fibroblasts [11]. The prepared nerve conduit was inserted into the nerve defect of a rat; the regenerated nerve using the 3D-printed nerve conduit was thicker than the silicon nerve conduit after 8 weeks. Immunohistochemistry and histological assay results revealed neural tissue formation and numerous myelinated axons were formed in the 3D-printed nerve conduit.

As described above, the nanoaligned nerve conduit prepared using various techniques such as electrospinning, lithography, and 3D bioprinting technique was developed to provide guidance and a tissue-inspired environment to neuron and stem cells. The cell morphology including neurite outgrowth and neurite length was changed on the nonaligned substrates and functions including proliferation and differentiation were enhanced by structural stimulation. Accordingly, the short recovery time was confirmed *in vivo* and the nanopatterned scaffold-implanted neural tissue was successfully formed compared to that on control platforms.

22.5 Summary

Neural tissue engineering is an essential approach for promoting human health care. Previously, chemical cues were regarded as crucial factors for regenerating neural tissue. However, it was recently found that topographical cues are important factors for repairing and regenerating neural tissues. The neural tissue consists of nanofibrils of collagen bundles, which form hierarchical micro- and nanostructures. Neural cells including neurons and neuroglia were exposed to different chemical and physical environments, which affected their morphologies and functions.

To provide more suitable nanostructures to damaged neural tissue, various nanoscale-based approaches have been suggested. In neural tissue engineering, electrospinning, lithography, and 3D bioprinting are efficient methods for fabricating nanostructured scaffolds. Electrospinning is a general tool used to fabricate a nanofiber structure on varying scales by controlling the fabrication process. Electrospun nanofibrous scaffolds are very similar to the ECM structures, and the morphology and function of cells are controlled on the nanofibrous surface. Lithography is advantageous for developing various nanosurface-like nanoaligned patterns, nanodots, and nanopillars, and optimization for fabricating ECM-inspired scaffolds can be achieved easily and quickly. 3D bioprinting is effective for forming a 3D scaffold, although current techniques show limitations in precisely controlling the nanoscale structures.

In the early stage of neural tissue engineering using nanotopography-based scaffolds, various polymers including synthetic and natural polymers were used to provide nanotopographical cues to neural cells. Studies demonstrated that the cells were sensitive to the nanotopography because the direction of the neurite was con-

trolled by nanotopographical cues, and the functions of the cells were enhanced. The role of neurons and neuroglia are notably different; neurons transmit electrical and chemical signals as information, whereas neuroglia support the neurons in signaling and metabolite transmission. Therefore, optimal scaffolds according to different cell types should be developed for neural tissue engineering. From aligned nanopattern to nanopillar-based scaffolds, various nanostructures have been proposed to promote neural cell function. Stem cells were also affected by the nanostructures (e.g., enhanced differentiation into mature neurons). Stem cells including NSCs, MSCs, ESCs, and ASCs were cultured on nanopatterned scaffolds with enhanced differentiation and functions.

Biodegradable polymer-based nanotopographical scaffolds have emerged as suitable implant platforms for enhancing neural tissue repair and regeneration. The nerve conduit is an alternative nerve graft for connecting small gaps from damaged to undamaged nerves. It is important to create an aligned nanostructure on the nerve conduit that mimics native neural tissues.

In this chapter, we summarized representative nanotechniques such as electrospinning, lithography, and 3D bioprinting for designing and fabricating nanopatterned scaffolds for neural tissue engineering and regenerative medicine. Nanotopographical cues in combination with other cues (e.g., chemical cues) are crucial for neural tissue repair and regeneration using cells, including various stem cells (Fig. 22.4). Biologically inspired nanopatterned scaffolds may encourage the next revolution and provide a foundation for advanced neural tissue engineering and regenerative medicine.

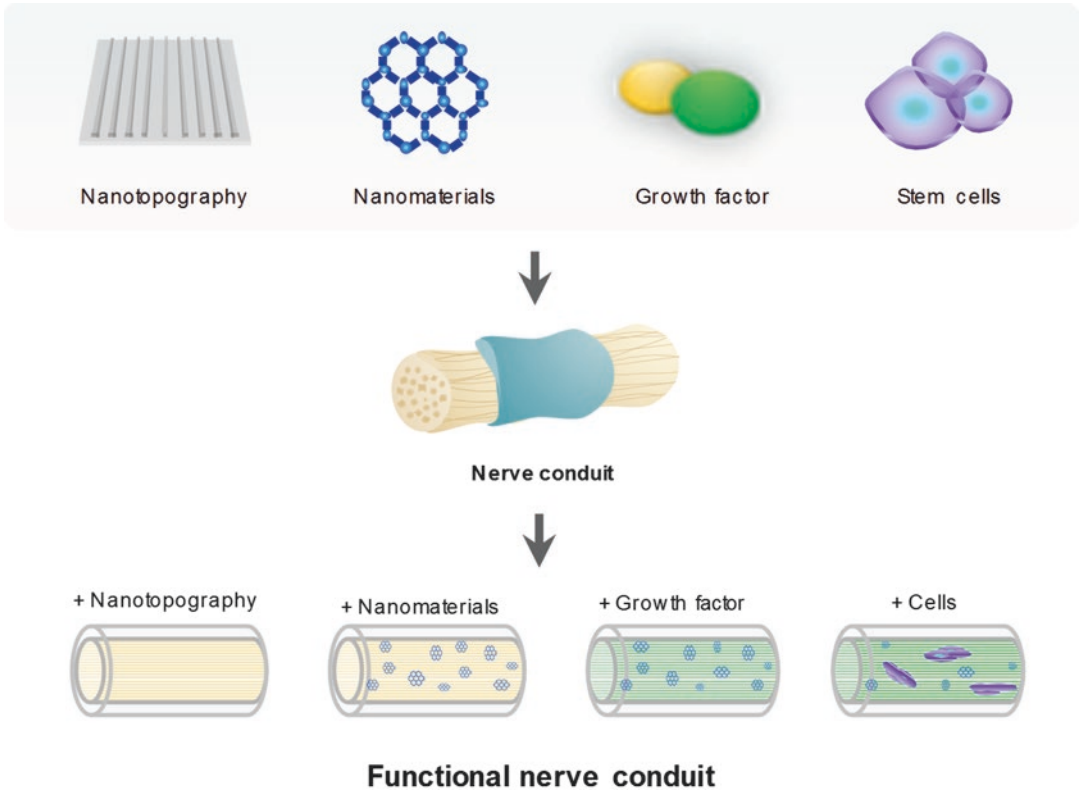

Fig. 22.4 Proposed nanopatterned scaffolds in combination with ultra-short nanomaterials (including drugs and genes), growth factors, and stem cells for neural tissue repair and regeneration

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23 Process System Engineering Methodologies Applied to Tissue Development and Regenerative Medicine

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Abstract

Tissue engineering and the manufacturing of regenerative medicine products demand strict control over the production process and product quality monitoring. In this chapter, the application of process systems engineering (PSE) approaches in the production of cell-based products has been discussed. Mechanistic, empirical, continuum and discrete models are compared and their use in describing cellular phenomena is reviewed. In addition, model-based optimization strategies employed in the field of tissue engineering and regenerative medicine are discussed. An introduction to process control theory is given and the main applications of classical and advanced methods in cellular production processes are described. Finally, new nondestructive and noninvasive monitoring techniques have been reviewed, focusing on large-scale manufacturing systems for cell-based constructs and therapeutic products. The application of the PSE methodologies presented here offers a promising alternative to overcome the main challenges in manufacturing engineered tissue and regeneration products.

Keywords

PSE · Tissue engineering · Regenerative medicine · Mathematical modeling · Process control · Optimization · Biomaterials

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23.1 Introduction

Tissue engineering and regenerative medicine encompass different areas of knowledge, such as chemical, medical, pharmacological, engineering, and material sciences, to develop disease treatment alternatives and strategies for tissue and organ repair or replacement [17].

The main approaches for developing tissue engineering and regenerative medicine therapies require allogeneic or autologous cell manufacture, with the necessity of the former being compatible with the patients. Some tissue and organs

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require a support with mechanical strength and/or the availability of chemical and topological cues to enhance cell response on the injury site, which can be provided by decellularized and synthetic scaffolds. Different cellular and tissue-based products and gene therapies have been developed in this field, and some have already been implanted in patients to treat tissue and organ damage in bladder, urethra, trachea, blood vessels and vaginal organs [17].

However, several challenges, such as the unpredictability of cellular behavior, contamination risks and specific cell function loss, are involved in maintaining the quality of these products in large-scale manufacturing processes. Along with cell viability, secreted physiologically-active substances and cell-derived undesirable molecules can be measured to characterize the product in terms of efficacy and potency. To determine product purity, process-related materials and contaminating cells must be quantified. Moreover, the variability of the manufacturing processes and of the manufactured products should be controlled to decrease production risks [44]. The demand for monitoring, modeling and control strategies to successfully translate laboratory processes into cost-effective large-scale manufacturing processes for cell based therapies have been widely reported [17, 25, 40, 44]. In addition, the development of analytical techniques is required to allow for nondestructive and noninvasive measurements and, thus, expansion of the monitoring process and control alternatives in the field [25, 44].

Process systems engineering (PSE) is an interdisciplinary field that applies modeling, monitoring, optimization, and control strategies to processes and product design, planning, scheduling, and operation [49]. In Fig. 23.1, a scheme of how PSE methodologies can be applied to these different tasks is presented.

PSE approaches have been successfully employed in diverse fields of knowledge, such as chemical engineering and environmental and pharmaceutical sciences. De Araújo and collaborators [10] used model and control approaches to design an entire control structure of a large-scale plant for the production of benzene. Regulatory and supervisory control layers were designed and steady-state and dynamic models were used to validate the proposed configuration. The simulations showed that the control structure developed was sufficiently robust for the considered disturbances and resulted in good dynamic performance. Miller and collaborators [34] used a wide range of PSE methodologies to study solid-sorbent-based and liquid-solvent-based postcombustion capture of carbon dioxide. Initially, models based on one-dimension partial differential equations and computational fluid dynamic models were developed. Following this, a derivative free optimization algorithm was used to optimize the design and operating conditions costs and a Bayesian technique was used to quantify efficiency and cost uncertainties. To regulate capture rates, linear-model predictive, proportional-integral-differential and feedback-feedforward controllers were compared. It was demonstrated that modeling, optimization and control strategies can be employed in the development of different technologies for carbon dioxide capture. Cervera-Padrell and collaborators [6] applied PSE techniques in a batch-wise process of production of clopenthixol, an active pharmaceutical ingredient. Through a retrofit analysis of the process, using design and optimization tools, some batch operations were exchanged by continuous reactors, enhancing the process yields and allowing for process simplification. In addition, material and environmental footprints and separation steps were significantly reduced with the proposed design. The continuous reactor was implemented at H. Lundbeck A/S, demonstrating that PSE techniques can be successfully applied for retrofitting in the pharmaceutical industry. These results show the potential of the application of design, control and modeling methods in a complete chemical plant.

The necessity to monitor and control quality indicators and process parameters (dissolved oxygen, pH, biomass, nutrients, and metabolites) and to access the operational performance in the large-scale production of cell-based products and tissue engineered constructs could be achieved with the employment of PSE approaches. While process control could be used to regulate process

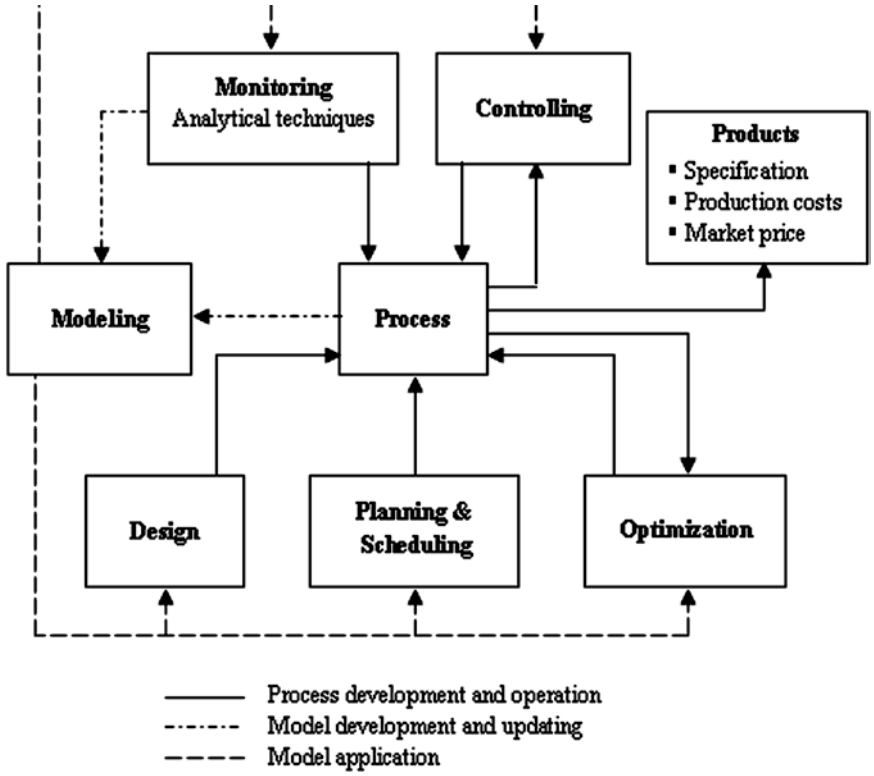

Fig. 23.1 PSE methodologies scheme

and product variability, features such as purity, potency, and viability could be monitored and optimized through optimization strategies. Nevertheless, mathematical models could be used to design the process with optimal conditions or in combination with control and optimization algorithms to develop advanced strategies and account for fault detection and process diagnosis. This chapter will discuss modeling, control, optimization and monitoring strategies that could and have been applied in the field of tissue engineering and regenerative medicine.

23.2 Mathematical Modeling and Simulation

Modeling approaches can be categorized according to several criteria, with the main ones being the relationship with the mathematical representation of the population and the basis of the equations used in the model. Based on the first criterion, the model can be classified into continuum and discrete, while the second divides the models into empirical and mechanistic. Continuum and discrete models differ on the population description, while the former uses a set of partial differential equations to treat a population of cells with an average property (cellular aggregates given by densities or concentrations), while discrete models use a discrete set of individual agents to treat each cell, interacting with neighbor particles separately [46]. On the other hand, the main difference between empirical and mechanistic models lies in the fact that one is based on the fit of experimental data using an arbitrary functional form and the other on the theoretical description to explain chemical, physical or biological processes [15]. Mathematical models can also be classified based on their observation scale, as presented in Fig. 23.2. It is important to observe that a specified modeling strategy may include more than one of these classes of models, for example, cellular

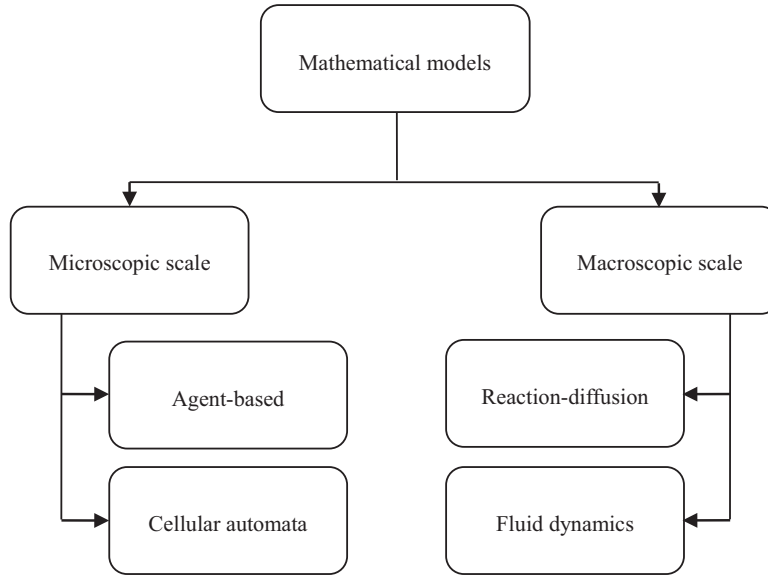

Fig. 23.2 Micro and macroscale mathematical models

automaton is a mechanistic, discrete and microscale approach.

In the last decade, mathematical modeling techniques have been widely explored in the field of tissue engineering and regenerative medicine. Mechanistic and continuum models with different levels of detail and complexity have been used to predict cell activity (growth, differentiation, adhesion, and molecules secretion) and/or mass transport. In general, computational data are generated to design and assess the performance of three-dimensional scaffolds and surrounding tissue, or to elucidate a specific feature of cell behavior under a particular condition [37].

Peng and collaborators [37] developed a continuous model capable of predicting how mechanical stimuli and memory affect cell fate transitions during mesenchymal stem cell differentiation. The model considers that the cells relate with the substrate stiffness through adhesion, triggering transcriptional changes involved in cell differentiation by regulating transcriptional factors Tafazzin (TAZ) and Yes-associated protein (YAP) signaling. Neurogenic (tubulin beta-3 gene, TUBB3), an adipogenic (peroxisome proliferator-activated receptor gamma, PPARG), a muscle (myogenic differentiation protein 1, MYOD1), and osteogenic (runt-related transcription factor 2, RUNX2) genes were used to describe four distinct cell fates. Simulation of two consecutive cultures with different durations and on substrates of different stiffness indicated the occurrence of a mechanical memory, i.e., influence of past mechanical dosing in cell fate. The effect of cell fate regulation was reduced with a shorter first culture and a more flexible substrate in the second culture, allowing for a larger number of cell fates.

Fu and collaborators [13] developed a hybrid mechanistic-empirical model to describe chondrogenic differentiation and glycosaminoglycan (GAG) production by human mesenchymal stem cells derived from bone marrow along with drug release and uptake in gelatin microspheres loaded with transforming growth factor (TGF)-β1. The model considered a differentiation rate coefficient dependent on growth factor concentration and, based on experimental DNA quantification, neglectful cell division and death. The remodeling of the extracellular matrix, which contains the produced GAG, was assumed to occur concurrently with the polymer degradation, while the rate of growth factor release was dependent on microsphere degradation. Several growth factor loading and microsphere degradation conditions were studied to evaluate their effects on GAG production. Both at fast or slow degradation rates, lower growth factor loading resulted in less

GAG production. While very high rates increased GAG production, very slow degradation rates led to reduced GAG production due to growth factor release limitation. This model could be used to design microspheres with controllable production of extracellular matrix and drug release.

Coy and collaborators [8] used a model describing neuronal regeneration in a cylindrical construct to evaluate the effect of porosity and spatial arrangements of the construct on cell growth. They observed an optimal value of porosity, which maximizes the degree of neurite growth within the construct. Concerning the spatial arrangement of the construct, designs with larger surface area resulted in greater cell growth. In order to evaluate the hypotheses of enhancing nerve ingrowth into the construct (consisting of several aligned rods), a relationship to calculate the number or size of the rods as a function of the porosity and diameter of the construct was proposed. The optimal porosity value provided by the model was used and the obtained dimensions could be validated to determine the proper construct design for clinical application with minimized costs and experimental efforts.

Lubowiecka [30] developed a mathematical model of abdominal hernia regeneration to study biomaterial behavior after implantation. The junction forces between the biomaterial and the surrounding tissue were simulated under pressure loads corresponding to a human post-operative cough. The model was validated based on experimental data obtained with a commercial polyvinylidene fluoride and polypropylene biomaterial (DynaMesh IPOM®) disposed over a polygonal elastic material (representing the abdominal wall) with a central orifice (representing the removed hernia). With this model, the recurrence of an abdominal hernia caused by coughing after implantation could be predicted, based on the maximum values of the junction forces.

Vieira and collaborators [50] used a bilinear isotropic material model and Bergstrom–Boyce model to predict the mechanical behavior of polydioxone (PDO) and polylactic acid and polycaprolactone blend (PLA–PCL) biodegradable suture fibers. The mechanical behavior was considered dependent on degradation by calibrating the linear hardening rate of the bilinear isotropic material model and the shear modulus and flow resistance of the Bergstrom–Boyce with experimental hydrolysis data. The simulations were performed for different strain levels (until rupture) and degradation steps, and the plastic strain accumulation at each loading cycle was determined. Plastic strain accumulation could be observed in both models, but only the Bergstrom–Boyce model was able to simulate the time-dependant response of PLA-PCL.

Discrete modeling approaches, such as agent-based and cellular automata models have also been applied to study cellular processes. In agent-based models, cell distribution and population size evolution are given as a function of the interaction of one cell with the neighbor cells and particles. Sun and Wang [46] used a multicellular 3D lattice agent-based model of cell-cell and cell-media interactions. The kinetic Monte Carlo (KMC) method was used to simulate the time evolution of fusing cellular aggregates in post printed tissue structures with different geometries, considering identical cells. Cellular aggregate fusion was also simulated, considering cells with different adhesive properties in order to study the biomanufacture of a thyroid gland with two blood vessels composed of tissue spheroids of smooth muscle cells and endothelial cells.

Cellular automata (CA) models use a mechanistic discrete approach that accounts for the spatial, temporal and stochastic features of the system. Ibrahim and collaborators [18] used a CA model and probabilistic rules to study lung tissue recovery during strain/stretch induced inflammation. The model describes the processes of fibrosis and apoptosis through the interaction between strain, cells (macrophages, fibroblasts) and cytokines – TNF, Transforming growth factor (TGF). Low, medium and high elastic field conditions were simulated under mechanical ventilation. The predicted data suggest that there is a strain threshold for innate tissue recovery under which the tissue structure can adapt and the inflammation can be suppressed.

With the advances in imaging processing and sensor development, mathematical models can be validated with *in vivo* data obtained from sensor

measurements and thus be used to predict the behavior of scaffolds and cells *in vivo,* reducing the number of preclinical and clinical experiments.

Sweeney et al [47] developed a model of cerebral blood flow and oxygen transport and validated the computational results with *in vivo* imaging data of mouse cortical vasculature acquired with a two-photon microscope. The blood vessel and smooth muscle regions from the imaging data were processed to be used as inputs for the model. Different scenarios of vessel constriction were studied and it was verified that, in most cases, vessel constriction led to an increase in blood velocity. Only the case of uni-directional propagation upstream towards the cortical surface in constricted precapillary arterioles led to the decrease in blood velocity, as observed experimentally. The effect of flow constriction on oxygen transport was also studied. It was observed that both nano-scale capillary vasoconstriction and precapillary arteriole constriction provoked reductions in oxygen partial pressure. In addition, simulations revealed that local smooth muscle actin constriction cascade, provoked by precapillary arterioles, is capable to regulate intravascular and interstitial oxygen transport, even with no pericyte capillary constriction. The model has also predicted that arteriolar dilatation, alongside erythrocyte deformation, augmented the reduction of capillary oxygen partial pressure under constriction. Therefore, complex interactions observed *in vivo* could be examined with this model, elucidating important features of physiological processes involved in the cortical vasculature.

Namas and collaborators [35] have used mechanistic models to evaluate bacterial endotoxin aging effect on inflammation *in vivo*. Several ordinary differential equations were used to describe cells (neutrophils, macrophages), cytokines –Tumor necrosis factor (TNF), Interleukin 6 (IL-6), Interleukin 10 (IL-10), Interleukin 12 (IL-12) –, nitric oxide synthases and reaction products (nitrate ion), blood pressure, and tissue damage dynamics. The model was first calibrated for 2–3 month old mice and then re-calibrated with experimental data of cytokines and nitrate ion from 6 to 8 months old mice. Five sets which best fit the data of aged mice were selected and the parameters with significant modification in the re-parametrization process were identified. When comparing the results obtained with the aged mice parameter sets with the ones with young mice, it was observed an increase in cell death, expression of IL-6 and production of pro-inflammatory mediators. These observations were supported by results from experimental *in vivo* studies in mice.

Ribeiro and collaborators [39] proposed a mechano-chemical model of bone morphogenetic protein-2 (BMP-2) delivery in alginate hydrogels and validated it with *in vivo* healing data from femoral defects in mice. The growth factor release was described as a function of hydrogel hydrolysis and cell-based degradation. Meanwhile, cell behavior (proliferation, migration, differentiation, maturation, and matrix production) was considered to depend on mechanical stimulus and to modulate growth factor dynamics. Simulations were performed to predict bone healing with hydrogel alone and with hydrogel soaked with BMP-2. Computational results were in agreement with *in vivo* data of bone formation, distribution and amount of bone within the defect.

As exemplified by the above models, mathematical modeling techniques can be used to develop tissue substitutes and generate hypotheses by simulating different conditions, scenarios and designs. In addition, these approaches can help in identifying the required design modifications for different clinical and preclinical scenarios in a consistent, systematic, rapid and not expensive way [8]. On the other hand, models can also be associated with sensor measurements to capture tissue dynamics from *in vivo* response [22]. These features are summarized in Fig. 23.3.

The model applications presented above exemplify how continuum models are more suited for systems in which the measurements of the particles of interest are given in terms of concentration (metabolites, nutrients, cytokines, and growth factors), density (cells), or when architectural and mechanical properties are being considered. The different modeling strategies are summarized in Table 23.1. In general, when there

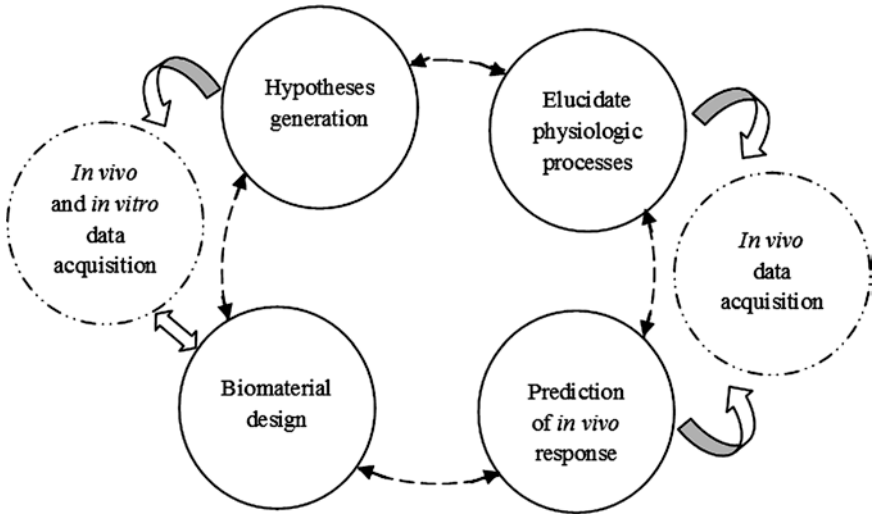

Fig. 23.3 Mathematical models applications in tissue engineering and regenerative medicine

Table 23.1 Mathematical model types features

Model	Advantage	Disadvantage
Continuum	Allows for the derivation of expressions based on variables related to measurable quantities	May require a complex structure to describe heterogenic populations
Discrete	Easy language, deals well with heterogeneity and subpopulations	Difficult parameter estimation and model validation
Mechanistic	Can be used for different data ranges once the parameters are estimated	Difficult validation for complex models
Empirical	Easy parameter estimation	Can only be used withinrestricted operating conditions range

is a theoretical description corroborated by large experimental data to explain a determined phenomenon, mechanistic models are a very interesting choice for modeling biological processes. However, these models can have a high number of parameters and variables, which are not always measurable due to limitations in instrumentation and sensor technology. Thus, lack of *in vivo* validation is one of the major drawbacks of mechanistic models in the field of tissue engineering and regenerative medicine. In this context, empirical models can help to evaluate the relationship between variables and to study a particular experimental system. In these models, the behavior of the experimental process can be described through curve fitting and the parameters of the model do not necessarily have a physical or biological meaning. Thus, empirical models can, strictly speaking, only be used within the range of data with which the parameters were fitted, what can hinder their application in systems with different operating conditions. On the other hand, discrete models are very interesting in cases where cellular interactions with other cells and with the environment have to be studied within a heterogenic population. These models can be applied to test and generate hypotheses regarding different cell-cell or cell-matrix mechanisms of communication. Discrete approaches are also attractive for the study of *in vivo* response to elucidate a particular phenomenon of tissue regeneration. Although the structure of discrete algorithms can be straightforward, the elements of each equation must be populated with data from actual populations, and accessing this infor-

mation for model estimation and validation is one of the major limitations of the technique [5].

23.3 Optimization

With the development and validation of new mathematical models, optimization can be applied to maximize or minimize biological variables and enhance the applicability of pioneering protocols, biomaterials and cell therapies. Until recently, the complexity of biological models caused optimization to be a fairly neglected subject in the areas of tissue engineering and regenerative medicine, mainly due to the high computational effort required. However, this situation is changing fast due to the increasing technological development in computational science and the enhanced processing power that can be achieved with high performance clusters [41].

In general, an optimization problem is defined by decision variables and an objective function, while in some cases there can be constraints, according to Fig. 23.4.

Different mathematical methods can be used to solve optimization problems, such as linear programming, non linear programming,– which includes sequential quadratic programming and the method of moving asymptotes –, mixed-integer optimization, dynamic optimization and stochastic search methods',– including genetic algorithms and Bayesian networks. In Table 23.2, the main features of each method are summarized.

One of the main applications of optimization approaches is parameter estimation or curve fitting. In these cases, the objective function is

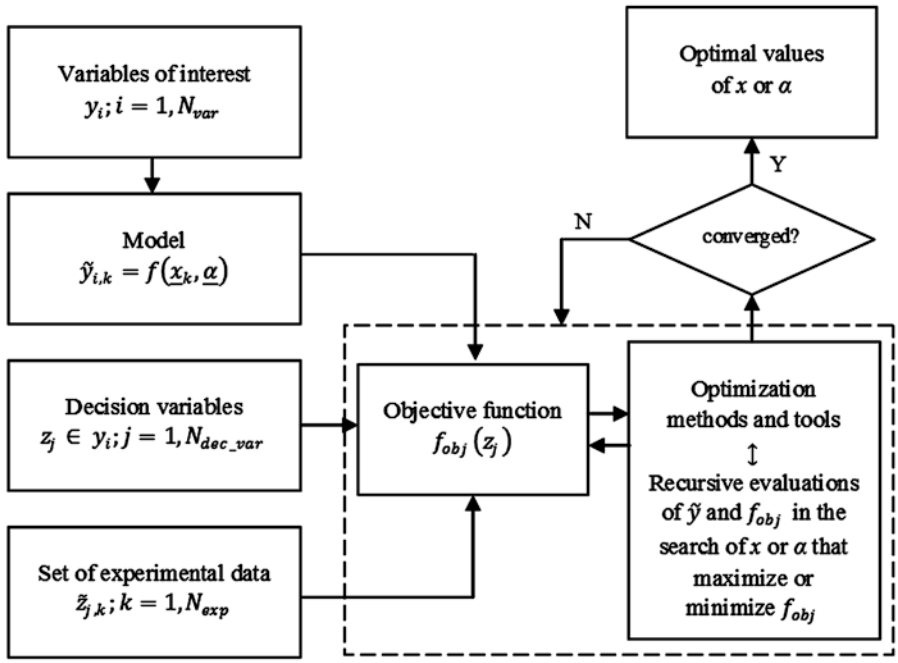

Fig. 23.4 Optimization problem scheme

Table 23.2 Optimization methods

Method	Description	Advantages	Limitations
Linear programming	The objective function and the constraints are linear	Simple	Requires function linearization in non-linear systems; inadequate for highly nonlinear; the solution may be a local optimum
Non-linear programming	The objective function and/or some of the constraints are non-linear	Does not require linearization; allows constraint flexibility	The solution may be a local optimum; requires more robust numerical algorithms
Mixed-integer optimization	The optimization problem has discrete and continuous variables	Can use linear or non-linear, continuous or discrete approaches	Large size of the optimization problem; requires great caution due to multiple possibilities of formulation; rather mathematically complex when applied to nonlinear systems
Dynamic optimization	Based on a dynamic model (non-null time derivatives)	Applicable to linear and non-linear models, discrete and continuous variables, deterministic or stochastic problems	Computationally expensive with a large number of state variables
Stochastic methods	The objective function and/or the constraints are random	Does not require discrete or continuous functions	Expensive processing time

defined in order to minimize the differences between the experimental and model predicted values of the output variables. The optimization result is a set of optimal values for the parameters, allowing the model to fit the experimental data with the higher possible accuracy. Kochaki [23] has used a genetic algorithm to identify key and optimal values for the parameters of a vascularization model. The model considered endothelial mutation, proliferation, death, extracellular polymeric production, chemotactic secretion, cell-cell adhesion and chemotaxis. Regarding the process of chemotaxis, optimal values of chemotactic secretion and decay rates, Monod kinetic coefficient (chemotactic concentration at which the secretion rate is half of its maximum value) and magnitude of the responses to the chemical gradient were obtained. Vessel development, given by the result of attraction and repulsion forces between the cells, was also optimized by targeting tight junction formation and breakage parameters. The proposed methodology was successfully applied for the development of a robust vascular system. This application exemplifies the use of stochastic algorithms, contrasting with well established and commonly used methods, such as linear and non-linear programming, in estimating complex models parameters in the field of tissue engineering and regenerative medicine.

Another application of optimization methods is the minimization or maximization of a specific process parameter, such as cell density, to obtain optimal values of scaffold properties or culture conditions.

Optimization approaches have been widely used along with mathematical models to estimate optimal scaffold properties for tissue engineering. Dias and collaborators [11] used a gradient-based topology optimization algorithm (method of moving asymptotes) to design scaffolds with optimal values of stiffness and permeability. The optimal micro-structures designs were used with selective laser sintering for polycaprolactone-4%hydroxyapatite scaffold manufacture for validation. The experimentally obtained constructs presented similar pore interconnection, strut size and porosity than the optimally designed ones.

Boccaccio and collaborators [3] and Boccaccio and collaborators [4] have used a nonlinear optimization method (sequential quadratic programming) to optimize the microstructure design of

functionally graded scaffolds (with a porosity gradient). Different mechanical conditions were studied to identify the optimal design which maximizes volumetric bone formation. Boccaccio and collaborators [3] studied different porosity distribution laws, loading conditions and scaffold Young's modulus values with this mechano-regulated optimization algorithm. In compression, a small increase in bone formation was obtained with the optimal case when compared to the homogeneous porosity case, due to almost no variation of pore dimensions through the scaffold. In shear conditions, a significant increase was observed in the volume occupied by bone, when compared to the homogeneous case.

Instead of the porosity distribution, Boccaccio and collaborators [4] optimized the shape, spatial distribution and number of pores per unit area, and also studied different values of compression loading and scaffold Young's modulus. While oriented (rectangular and elliptic) pores led to increased bone formation, when compared to square and circular shapes, respectively. On the other hand, the number of pores per unit area did not presented a significant effect on bone volume. And regarding the mechanical effects, bone formation were maximized with the combination of high compressing loads, Young's modulus values, small and oriented pores, or low loading and large pores.

Another field of application of optimization techniques is identification of optimal culture conditions for cell culture inside tissue constructs. Couet and Mantovani [7] used the Markov decision process to optimize culture conditions in a bioreactor for vascular tissue engineering. Scaffold internal pressure and external diameter, culture medium flow and pressure frequency were measured and used in regression models to estimate the variation of elastic modulus as a function of circumferential strain, elastic modulus, longitudinal shear stress, pressure frequency and maturation time. Using genetic programming, the model with the smallest value of variance of the regression parameters was obtained. The model was then formulated as a Markov decision process and solved by dynamic programming to optimize vascular growth or information gathering. Numerical simulations were performed with a non-linear model of mechano-regulated growth and maturation of vascular smooth muscle cells on a collagen scaffold inside a bioreactor to validate the culture parameters obtained by the Markov decision process. A maturation path was observed in the bioreactor where actions regarding the use of different growth factors were applied to drive the scaffold toward a synthetic phenotype at initial times, while a contractile phenotype was favored at the final culture period. However, these computational results were not validated with *in vitro* data, which could lead to further analysis.

Mehrian and collaborators [32] used a Bayesian method to determine optimal values of frequency and percentage of medium exchange for a perfusion bioreactor to maximize tissue growth within regular 3D scaffolds. At first, a detailed mechanistic model of tissue development under perfusion, accounting for scaffold geometry, shear stress, medium pH, oxygen, glucose and lactate concentrations, was reparametrized with a genetic algorithm technique. The resultant reduced model was used in Bayesian optimization to obtain the optimal culture medium exchange approach. Maximum tissue growth was obtained for a high frequency and percentage of exchange, as observed in the experimental data.

Pang and collaborators [36] applied mixed-integer linear programming to find an optimal drug combination. In therapeutic treatments, a certain drug is designed to interact with an on-target protein. However, this drug can also interact with other proteins (off-target) that are not associated with the disease that is being treated. In order to evaluate the algorithm performance to maximize the interaction of multiple drugs with on-target proteins and minimize their effect on off-target proteins, six disease sets were optimized. It was shown that the developed optimization tool could predict optimal well-known drug combinations and suggest alternative applications for some drugs.

Optimization tools have also been used to obtain information from cell imaging data, such as in model-based cell tracking algorithms, which

aim to optimize the probability of cell detection. Kachouie and collaborators [20] used a linear programming optimization algorithm to determine local maxima for a probability map of cell center localization, which is obtained by associating the phase contrast microscopic images with a statistical cell model. By thresholding the optimal map and describing cell motion by random walking, it was possible to track hematopoietic stem cells over time.

In the applications mentioned above, it was possible to observe the use of different optimization approaches in parameter estimation, scaffold design and process design (culture conditions and feeding strategies). However, hardly any information is given concerning the comparative performance of the applied methods to the considered models and systems. The identification of the most appropriate strategy for a defined optimization problem remains a challenge in the field of tissue engineering, especially when more complex models are considered. The different models validated with *in vivo* data presented in the previous section could also be used with an optimization algorithm, but the chosen strategy should require small computational effort, due to the model complexity. An alternative for using computationally expensive optimization methods is to first simplify the model, identifying key parameters and mechanisms with a low computational cost optimization tool and then applying the chosen optimization algorithm to a reduced model. With these strategies, optimal *in vivo* response could be researched with the formerly mentioned models to provide appropriate biomaterial design and cell therapy protocol, enhancing the probability of success of clinical and preclinical trials.

23.4 Control

In process control, manipulated variables are adjusted (receive the correction action from the controller) to regulate control variables that must be maintained near a desired value, called setpoint, despite variations in disturbance variables that can affect the controlled variables. There are several process control strategies available to apply in tissue engineering and regenerative medicine. They can be classified in open-loop and closed-loop, feedback and feedforward, conventional and advanced. In general, feedback and feedforward methods are classified as conventional strategies, the former being closed-loop and the latter open-loop. Optimal control, multivariate statistical process control and adaptive control methods are classified as advanced methods.

Open-loop control is based on enforcing manipulated variable trajectories, predefined offline, to obtain expected state variables profiles (Fig. 23.5a). In general, this approach can be accurate with simple models but complex models and constraints usually make the optimal problem solution computationally impractical [14]. On the other hand, in closed-loop control (Fig. 23.5b, c), the trajectory enforced on the process state can be maintained or periodically modified. The majority of applications of process control are based on a closed-loop structure.

In the feedback control, the controlled variable changes are measured and fed as input variables to the controller, as shown in Fig. 23.5b, reducing the impact of disturbances on the controlled variable. As no correction is enforced before a disturbance affects the system (until the controlled variable differs from the setpoint), this type of control can be very oscillatory or even unstable. Nevertheless, this is the most applied strategy in process control and is mainly employed with the classical Proportional-Integral-Derivative (PID) algorithm [14]. Feedforward control enforces a correction action on the manipulated variables based on disturbance variable measurements. However, for this type of control, all the disturbances would need to be measured on-line and, with no measurement of the output variables, there is no information regarding the accuracy of the corrections made. Due to these shortcomings, feedforward strategy is often combined with feedback structures to be applied in process control [14].

Despite the simplicity of implementation of conventional controllers, the solution for the control problem often relies on linearization strate-

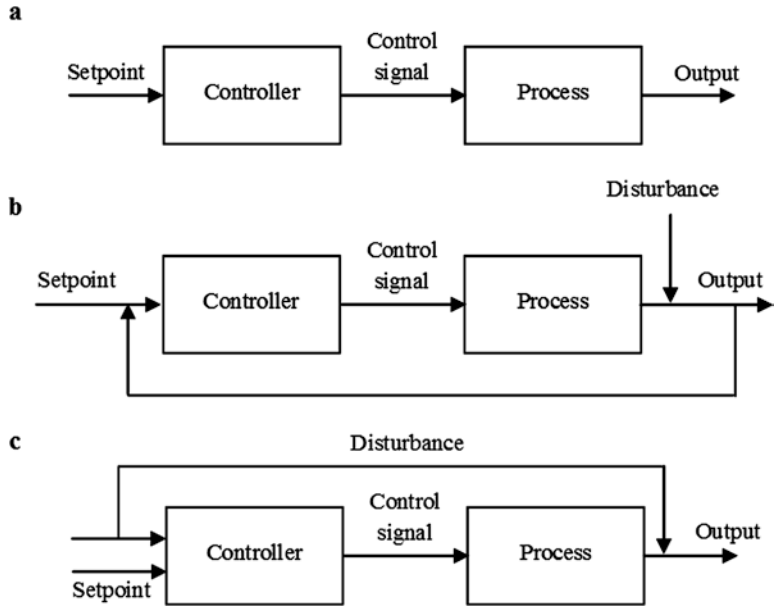

Fig. 23.5 Block diagram of conventional control strategies: open-loop (**a**), feedback (**b**) and feedforward (**c**)

gies. However, more complex systems are usually highly non-linear, hindering the use of linearization strategies, and require advanced approaches to maintain process stability. Advanced methods use mathematical models to develop control algorithms and can be based on statistical methods, optimization or adaptive routines.

Multivariate statistical process control is an advanced monitoring and control technique for process performance. In this strategy, information from all process variables is reduced to a few composite metrics using statistical modeling techniques (Fig. 23.6a). These metrics can be monitored in real time and can be applied for fault detection and diagnosis. However, this approach may not be straightforward in a multivariate control problem, or even in general problems due to the need of identifying which of the monitored variables is provoking the out-of-control signals [1, 2, 42].

In adaptive control, the control parameters can be adjusted to handle systemic variations and external disturbances (Fig. 23.6b). The model can be embedded in the controller to adjust the control parameters concurrently with system variation or updated with on-line data to be used in the controller. It is an interesting strategy for dynamic and nonlinear systems with large disturbances [48]. However, the adaptive signals in the transient state can be very oscillatory [45].

Optimal control is based on an objective oriented trajectory obtained by a constrained optimization of a function of state and control variables (Fig. 23.6c) [16]. Depending on the solution method chosen to solve the optimal control problem, it may be difficult to perform model linearization of complex models to obtain an analytical solution, or to prove that the discretized nonlinear programming problem obtained is equivalent to the original one [38].

Several control approaches have been applied to bioreactor systems to maintain cellular culture conditions within an appropriate range. Konakovsky and collaborators [24] proposed an automatic control strategy for a glucose-limited fed-batch culture of Chinese hamster ovary (CHO) cells. The aim of the approach was to control the process metabolic state by using an adaptive feed rate setpoint in real-time to achieve low lactic acid levels and pH stability. The control of the feed rate setpoint was performed by a pump which received the adaptive feed rate setpoint in real time. Biomass values were estimated from real time signals obtained with a capacitance probe and used to calculate the adaptive feed rate setpoint. It was observed that by setting the pH to

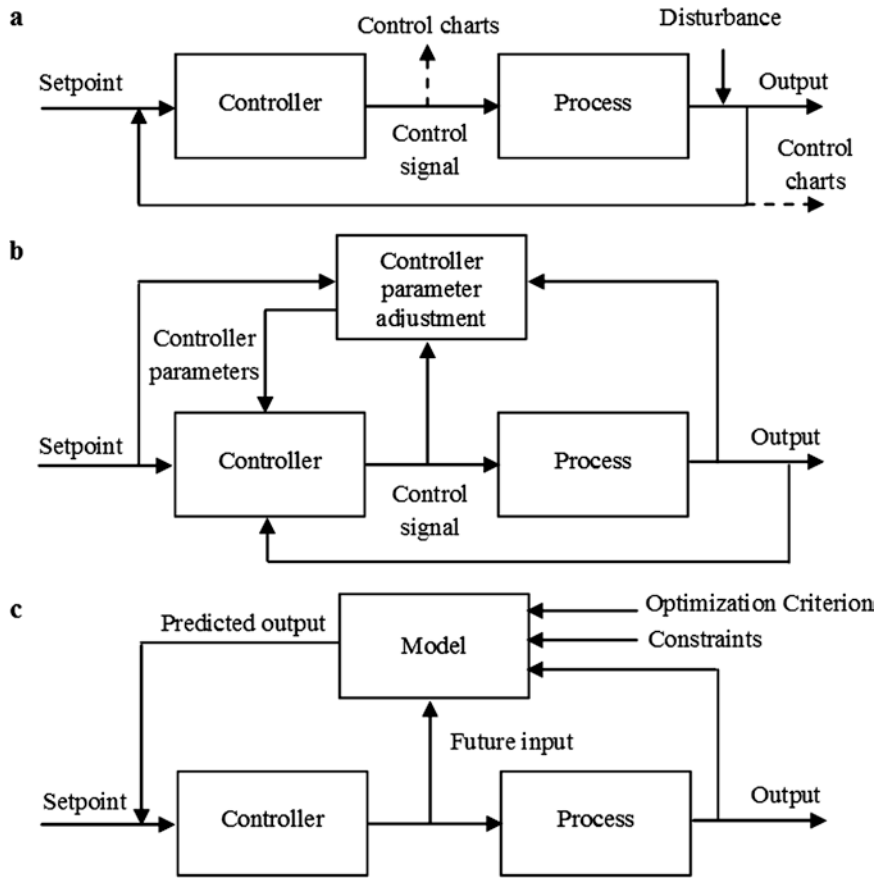

Fig. 23.6 Block diagram of advanced control strategies: multivariate statistical process control (**a**), adaptive control (**b**), and optimal control (**c**)

a higher value than the physiological range (6.9–7.1), the glucose flux was increased and, as a consequence, the yield of lactic acid/glucose was reduced. In this metabolic state, lactic acid was consumed and not produced. This strategy could be used to control dynamic cell culture in three-dimensional biomaterials, which can present lactic acid build-up inside the cellularized structure.

Liu and collaborators [29] used a multivariate statistical process control (MSPC) approach to detect early contamination in Immunoglobulin G antibody production by CHO cells cultivated in a stirred tank bioreactor. The data acquisition was performed by an in-line Raman spectrometer, which allows for simultaneous measurement of nutrients (glucose, glutamate and glutamine), metabolites (lactate and ammonia), and total and viable cell densities. The MSPC was used to diagnose batches out of the control range based on synchronized datasets derived from Raman spectrums (with multiple variables). The control range was defined by the confidence intervals of Hotelling's statistic – Mahalanobis distance to the origin of the principal component – and of the squared predicted error – error between the projected and the original models– of the normal operation condition batches. The proposed controller was able to predict the variability of the process and to detect early contamination of the cultures, which could not be achieved by traditional diagnosis approaches. This type of control can be an interesting approach for dynamic cultures of stem cells and cellularized constructs.

Schuerlein and collaborators [43] developed a modular bioreactor for tissue engineering with a bag pump (pressure-controlled air system), a heat and a gas exchanger and a control unit. At first, fluid dynamic models were used to simulate and design heat and gas exchangers. The gas exchanger unit was developed to maintain pH stability in the bicarbonate-buffered culture medium through controlled carbon dioxide and oxygen transfer. A proportional/integral/differential (PID) controller, which is a control loop feedback mechanism, was used to control the valves of the pump. This controller calculates an error value between the setpoint and the measured variable, being the correction applied through proportional, integral, and derivative terms. The feasibility of the system was verified with cultures of native carotid arteries, blood vessels and intestinal tissue constructs. In the carotid arteries, endothelial and smooth muscle layers were maintained after submission to the dynamic culture. This indicates that the controller strategy proposed for the modules was capable of providing enough stability for the process.

Li and collaborators [28] introduced an axial-stress bioreactor system with pulsatile pumps, substance exchanger, dissolved oxygen, pH sensors, linear servomotor (to deliver axial deformation to the construct), control system, integrated laminar flow hood, chamber temperature controller and ozonizer. The data obtained from the sensors were transferred to a microprocessor control unit, programmed to control the servomotor loading, the culture medium pH, dissolved oxygen and temperature. The servomotor loading was described by frequency, compression, strain, and deformation-time waveforms. The pH and dissolved oxygen were corrected with a feedback control mechanism. The system performance was demonstrated on the culture of mouse bone-marrow mesenchymal stem cells seeded in decalcified bone matrix. The proposed control approach was able to maintain the pH and dissolved oxygen within a defined range with small fluctuations along with steady levels of nutrients and metabolic waste. In addition, higher cell density, proliferation (cell cycle analysis), metabolic activity, alkaline phosphatase activity and calcium content were observed in the constructs cultivated in the bioreactor in comparison with the static culture results. Thus, with an environment steadily controlled, it was possible to maintain the appropriate conditions for tissue development under mechanical stimuli.

Optimal control has also been used to regulate molecular conditions inside tissue constructs for tissue engineering purposes. Kishida and collaborators [21] compared optimal control approaches to regulate growth factor uptake at a specified distance from where its molecular release occurs within a tissue construct with fluid flow. The controllers were designed to minimize the difference between the desired and the measured cellular uptake rates, subject to a reaction–diffusion–convection equation for the growth factor (which can be replaced for drugs, hormones, and DNA) as a constraint, and the concentration of growth factor as the control input. The growth factor uptake kinetics and desired growth factor uptake rate were determined in order to generate a cellular response (i.e. differentiation). Four approaches – basis function expansion (BFE), method of moments, internal model control (IMC) and model predictive control (MPC) – were used to solve the one dimension optimal control problem to evaluate which one would be more appropriate to solve a three-dimensional problem. The BFE method was computationally efficient but the authors remark that basis functions and reference trajectory choice should be made in such a way that non-negativity constraint violations on the control input can be avoided. The “method of moments” was computationally efficient and enforced a non-negativity constraint on the control trajectory. However, there is no generalization of the method to account for state constraints or reference trajectories with discontinuities. The IMC method could not consider the constraints explicitly in the optimization and presented a slow performance compared with the method of moments. The MPC approach was computationally expensive but was the most flexible as it can handle control and state constraints. An alternative strategy was suggested based on the combination of different approaches, using a simple one (method of moments) for initialization of the

problem and a more complex one (MPC) to refine the search.

Another application of control methods is the study of biological mechanisms which present similar strategies of regulation as those proposed in a control problem, such as genetic and epigenetic regulation. Lei and collaborators [27] developed a model to describe the adult stem cell regeneration and the cross-talk between genetic and epigenetic regulation. According to the model, during each cell cycle, stem cells leave the resting phase due to differentiation or enter the proliferating phase, from which the cells leave to undergo either mitosis or apoptosis, with a defined probability. The stem cell distribution was described as a function of epigenetic states, while physiological cell performance depended on cell proliferation, apoptosis and differentiation. Three optimal control strategies were studied: homogeneous proliferation (proliferation is independent of the epigenetic state), heterogeneous proliferation (different proliferation probabilities) and negative feedback (independent of epigenetics). When the cell population was decreased to simulate stem cell tissue damage, homogeneous and heterogeneous proliferations strategies, which include complex feedback and epigenetic states, revealed faster recovery dynamic. When differentiation probability was increased and the level of differentiated cells was decreased, the heterogeneous mechanism led to a smaller variation in the cell population and presented a faster recovery. Thus, the cross-talk between genetic and epigenetic regulation and the proposed performance function has shown to be able to control a stem cell population with a heterogeneous distribution of epigenetic states.

According to the above applications of process control methods, it is possible to conclude that the feedback approach can be efficiently used to regulate bioreactor culture medium conditions, such as pH and dissolved oxygen. However, more advanced approaches are required to maintain process stability when other control variables are considered. While mathematical models can be used to estimate variables of state, more flexible control structures are required to compute such complex interactions. Optimal control strategies could be well applied in this field, but previous model validation with extensive experimental data is required.

23.5 Monitoring

Process performance and product quality in tissue engineering and the manufacturing of regenerative medicine products can be accessed by online monitoring of process parameters to collect process data for controller and mathematical models. Dielectric spectroscopy measures the capacitance of cells, which represents the cell membrane capacity to store electrical charges. This measurement is converted into permittivity, a constant that relates the electric field to the electric displacement and can be used to measure viable cell density. However, measurements can be disturbed by media turbidity and cellular debris buildup [31]. Near-infrared spectroscopy can also be used to measure total or viable cell density, and at the same time predict several analyte concentrations, such as glucose, glutamine, glutamate, lactate and ammonia. The obtained absorbance measurements gather information regarding vibrations of O-H, N-H and C-H bonds in spectra with overlapping absorption bands and light scattering differences provoked by cell concentration variations [12]. The spectral variations can be correlated to cell and analyte concentration changes through calibration with regression models. Mercier and collaborators [33] studied different calibration strategies for dielectric and near-infrared spectra to monitor a perfusion cultivation of PER.C6®. It was observed that dielectric spectroscopy was more accurate in measuring viable cell density and that an online strategy for near-infrared spectroscopy was appropriate for monitoring nutrients and metabolites in the perfusion system. As the bounds stretched by near-infrared spectroscopy are highly common in organic molecules, the obtained spectra for different compounds can be very similar [52]. Fourier transform infrared spectroscopy can yield higher molecular selectivity through the identification of molecule structural bonds based on their infrared absorption. Wu and collaborators

[52] used this technique to monitor several analytes (glucose, glutamine, lactate, and ammonia) simultaneously and the protein yield in a bioreactor for the production of the monoclonal antibody immunoglobulin 3. Despite the technical feasibility of the method, such as limitations regarding equipment configuration (samples must be similar, can only handle a small number of samples, and require reference light paths) should be considered before its application in large scale manufacturing.

In order to predict the performance of tissue regeneration *in vivo*, stem cell function, phenotype and migration could be monitored by live imaging to collect data for mathematical models. Two-photon excitation microscopy can be used to detect second harmonic generation. This technique is based on the flow of two near-infrared photons through a noncentrosymmetric material, from which only one photon with higher energy comes out and can be visualized. Cell function can be monitored with this approach by detecting extracellular matrix components [26]. Phase contrast microscopy has also been reported as an alternative to monitor cell number and morphological state. Smith and collaborators [44] used an automated live cell imaging platform based on phase contrast microscopy and Machine Vision technology to measure and quantify morphological features in pluripotent stem cells cultures. It was possible to verify a correlation between morphologic features visualized in immunostaining images and the biomarkers Oct3/4, Nanog, and Sox-2, measured by flow cytometry. This approach could be used to obtain quantitative phenotype information from image analysis to validate mathematical models. Bioluminescent imaging uses genetic engineering of cells to allow for the expression of luciferase, which can react with a substrate and catalyze the oxidation of luciferin, releasing photons of light [19, 26]. With this technique, gene expression, signaling, metabolism, luciferase-expressing cells distribution, migration and fusion with other cell types can be monitored during the process of tissue regeneration. However, the application of this approach can be hindered by resolution limitations in most internal tissue [26].

Non-invasive imaging techniques have also been applied to evaluate tissue regeneration. Hyperspectral imaging obtains an object spectral reflectance (after light irradiation) in several wavebands, which can be analyzed by machine learning or data mining tools to provide information on cell quantity, wound depth and tissue vitality (metabolic activity) [51]. However, the feasibility of this method has only been tested in skin models and it is therefore important to verify if it could be applied to thicker and more internal tissue. Molecular ultrasound imaging using targeted microbubbles and two-photon laser scanning microscopy imaging has been used to measure vascular cell adhesion molecule (VCAM)-1 and monitor vessel reendothelialization [9]. While the former approach is based on microbubble binding with the targeted molecule and build-up in high expression sites, depicted as bright signals areas, the latter is based on immunofluorescence staining of the targeted molecules [9, 53]. These approaches are limited to the choice and number of the targeted molecules that may be necessary to obtain relevant information regarding the state of the tissue for regeneration. Nevertheless, these approaches can be employed for therapeutic post-interventional follow-up to collect data that could be used in mathematical models to optimize product design.

Even though well-established pH, temperature and dissolved oxygen sensors are available for the control of culture conditions, analytical methods and sensors for online monitoring of concentrations of cells, nutrients, metabolites and other relevant analytes are still in development. Imaging techniques are also being developed to quantify extracellular matrix components and bimolecular expression and to access cell number, phenotype, distribution and migration. The reliable application of these methods in cell-based manufacturing process will still depend on the accuracy level and validation of the results with large experimental data sets.

23.6 Conclusions and Perspectives

Process control and product quality regulation requirements in manufacturing cell-based constructs and therapeutic products have motivated the development of monitoring, modeling, optimization and control strategies in the field of tissue engineering and regenerative medicine. In this chapter, several PSE strategies were discussed in terms of their applicability in large-scale production of cell-based products. To describe the process and evaluate product quality outcome, different mathematical modeling approaches can be used. Once *in vivo* data is obtained to corroborate computational results and validate these models, they can be applied in advanced process control strategies and optimization algorithms to account for disturbances and enhance process design. However, one of the major challenges in mathematical modeling is the lack of available measuring techniques for obtaining proper *in vivo* data for model validation. While some approaches have been well established to monitor and control process parameters such as dissolved oxygen, temperature and pH, monitoring technologies for other key analytes (concentrations of cells, nutrients, and metabolites) and main product quality indicators (cell phenotype, function, distribution, and migration) they still require validation for large-scale production. Non-destructive and non-invasive imaging techniques have been applied to measure several cellular features and are a promissing approach for online monitoring in cell-based manufacturing.

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Biomimetic Extracellular Matrices and Scaffolds Prepared from Cultured Cells

24

Guoping Chen and Naoki Kawazoe

Abstract

Extracellular matrix (ECM) interacts with cells and provides important signals to control cell functions and to maintain homeostasis of living organisms. Composition of ECM in each tissue is dependent on cell type and cell phenotype. ECM also dynamically changes its composition during stem cell differentiation and tissue development. Various ECM substrates and scaffolds have been prepared for stem cells culture and tissue engineering. They can be reconstructed by using isolated ECM components or acellular matrices from different tissues and organs. In recent years, cultured cells have been used as a useful source to prepare biomimetic ECM substrates and scaffolds. ECM derived from different cell type can be prepared by culturing the respective cells to allow the cells to secrete desirable ECM components. Furthermore, dynamically changing ECM can be prepared by controlling the stepwise differentiation of stem cells. The composition of the biomimetic ECM substrates and scaffolds changes with cell type and has different effects on differentiation of stem cells. The latest progress on biomimetic ECM substrates and scaffolds derived from cultured cells is summarized and highlighted.

Keywords

Extracellular matrix · Biomimetic · Scaffolds · Acellular matrix · Scaffold · Cell culture · Stem cells · Differentiation · Tissue engineering

24.1 Introduction

In tissue and organs, cells are surrounded with their specific extracellular matrix (ECM) that regulates cell functions such as cell adhesion, survival, proliferation, migration and differentiation [1, 2]. ECM is a complex network composed of variety of proteins and proteoglycans. Development of biomaterials and scaffolds that mimic in vivo ECM microenvironments has been a primary strategy for stem cell research and tissue engineering applications [3, 4].

Many methods have been used to prepare biomimetic ECM biomaterials and scaffolds. One of the frequently used methods is usage of isolated ECM components. The isolated ECM components can be coated or immobilized on the surfaces of biomaterials and implants. They are also used to reconstruct biomimetic scaffolds. ECM components such as collagens, fibronectin, and laminins have been used for *in vitro* cell culture

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[5–9]. The coating and immobilization of ECM components has some advantages on controlling of specific ECM components and simplicity. However, it is difficult to fabricate *in vitro* models mimicking in vivo ECM complexity by physicochemical methods. Another method is decellularization of tissues and organs to prepare acellular ECM. ECM derived from decellularized tissues and organs has been widely explored as a source of biomimetic scaffolds for tissue engineering applications. Acellular ECM has been prepared by decellularization of small intestinal submucosa, heart valve, blood vessel, skin, nerve, tendon, ligament, urinary bladder, vocal fold, amniotic membrane, heart, liver and lung [10]. The ECM scaffolds obtained from decellularized tissues and organs offer the advantage of maintaining the structures of the respective tissues and organs. However they suffer from problems of autologous tissue/organ scarcity, host responses and pathogen transfer when allogenic and xenogeneic tissues and organs are used.

ECM preparation by using cell culture method has been used as an alternative way to prepare biomimetic ECM substrates and scaffolds. Cell-formed decellularized matrices have been reported since the 1970s [11, 12]. A cell-free substratum containing matrices has been prepared from culture of chick embryo fibroblasts by decellularization treatment with non-ionic detergent, NP-40, at pH 9.6 [11]. Pericellular matrices of human fibroblast have been isolated by decellularization [10]. These studies indicate that the decellularized matrices can be used as a new cell culture substrate. Cell-derived ECM has been deposited on the surfaces of Ti alloys to promote cell adhesion and osteoconductivity of Ti alloys [13]. Osteoblast-like SAOS-2 cells were cultured on Ti alloys and allowed to secrete and deposit their ECM on Ti surfaces. After the deposition of ECM proteins, the cell/Ti complex was decellularized to obtain ECM-modified Ti surfaces. Cell culture method has the advantage of unlimited cell sources, culture condition controlling and versatility. Furthermore, cell culture method can be used to prepare ECM that mimic the dynamic ECM change during stem cell differentiation and tissue development [10]. In the chapter, the latest development of the biomimetic ECM substrates and scaffolds from culture cells will be summarized and highlighted.

24.2 ECM Prepared from Cultured Somatic Cells

Somatic cells isolated from various tissues and organs have been used for preparation of cultured cells-derived ECM. During in vitro culture, somatic cells secrete their own ECM depositing beneath the cells to cover the cell culture substrate surfaces (Fig. 24.1). After the cells secrete enough amount of ECM, cellular components including cell membrane and nucleus are removed by decellularization treatment. The ECM deposited on the substrates can be used for cell culture to investigate ECM-cell interaction and to control cell functions.

A basement membrane model has been formed by alveolar epithelial cells [14–17]. Alveolar epithelial cells were cultured on air-dried collagen gels with fibroblasts or Matrigel. After cell culture, lamina densa was formed beneath the cells and was used as a new cell culture substrate. On this basement membrane model, primary hepatocytes could survive longer than on a type I collagen matrix and Matrigel. In addition, the cells could maintain their differentiated functions such as albumin secretion and cytochrome P450 gene expression on the decellularized basement membrane model at higher

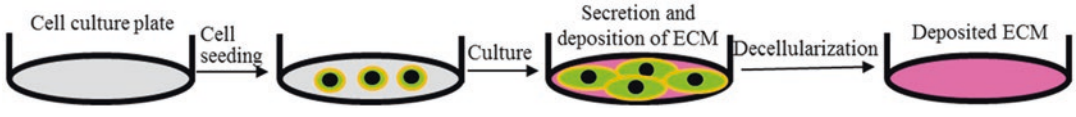

Fig. 24.1 Preparation scheme of extracellular matrices from cultured cells. Pink color represents ECM secreted by cultured cells and deposited on the surface of cell culture plate

levels than cells on a type I collagen matrix and Matrigel [16].

Cells are surrounded by their specific ECM *in vivo* that elicits the need to elucidate the effects of ECM derived from different cell sources on cell functions. Three types of ECM have been prepared from human articular chondrocytes, human dermal fibroblasts and human mesenchymal stem cells (MSC). They have been used for culture of chondrocytes to compare their effects on chondrocyte adhesion, proliferation, and differentiation [18]. ECM derived from chondrocytes, fibroblasts, and MSC were prepared by culturing these cells on tissue-culture polystyrene plates and decellularization of the cells after 1 week culture. Cellular components were selectively removed by treatment with a hypotonic solution (10 mM Tris-HCl, pH 8.0, and 5 mM EDTA) containing 0.1% Triton X-100 for 6 h at 4 °C, followed by a treatment with 100 μg/ml DNase I and 100 μg/ml RNase A for 3 h at 37 °C. Two ECM proteins, fibronectin and type I collagen, were examined by immunostaining to confirm their existence in the cell-derived ECM because they are two major ECM proteins affecting cell functions. The chondrocyte-, fibroblast- and MSC-derived ECM contained the same levels of type I collagen whereas the chondrocyte-derived ECM contained less fibronectin than did the fibroblast- and MSC-derived ECM.

Chondrocytes were cultured on these ECM and their effects on the adhesion, proliferation and differentiation of chondrocytes were compared. Chondrocyte adhesion to the chondrocyte-derived ECM was greater than to the fibroblast- and MSC-derived ECM. ECM obtained from the same cell source might contain more favorable molecules and structure for cell adhesion. In contrast, chondrocyte proliferation was lower on the chondrocyte-derived ECM than on the fibroblast- and MSC-derived ECM, which might be due to the less fibronectin deposition in the chondrocyte-derived ECM. No difference in the effects among these ECM on the chondrocyte differentiation was evident. The different effects of the three types of ECM on chondrocyte adhesion and proliferation might be explained by the difference in composition among the ECM. The effect of ECM on chondrocyte functions was dependent on the cell source used to prepare the ECM.

Articular chondrocytes may lose their cartilaginous phenotype or dedifferentiate during in vitro cell culture. Maintenance of cartilaginous phenotype during in vitro expansion culture is a great challenge to achieve sufficient cell number for transplantation. Culture system mimicking the ECM microenvironments surrounding chondrocytes *in vivo* is an effective method to promote cell proliferation and maintain their functions. To investigate the effect of chondrocyte-derived ECM on the chondrocytes functions, ECM has been prepared from serially passaged bovine chondrocytes and used for culture of bovine chondrocytes [19].

Primary bovine chondrocytes (P0 BAc) were isolated from bovine articular cartilage and subcultured serially. Freshly isolated primary chondrocytes (P0) were used as fully differentiated chondrocytes. Chondrocytes after six passages (P6) were used as completely dedifferentiated chondrocytes. Chondrocytes after two passages (P2) were used as the intermediate. P0, P2, and P6 BAc were cultured on tissue-culture polystyrene (TCPS) plates in basal DMEM. After the P0, P2 and P6 chondrocytes were cultured in TCPS plates in basal DMEM for 10 days, cellular components were selectively removed from the ECM by decellularization to obtain the ECM derived from P0, P2, and P6 BAc, which were named as P0-ECM, P2-ECM and P6-ECM, respectively. The P0-, P2- and P6 ECM had different composition patterns. The production of type II collagen and aggrecan was confirmed for P0 BAc, decreased for P2 BAc and lost for P6 BAc. In contrast to type II collagen and aggrecan, type I collagen production in P2 and P6 BAc was increased compared with P0 BAc. Fibronectin production was increased in P2 BAc and slightly decreased in P6 BAc.

The P0-ECM, P2-ECM and P6-ECM were used for culture of chondrocytes to compare their effects on chondrocyte adhesion, proliferation and differentiation. Bovine chondrocytes could adhere to and proliferated on the P0-, P2-, and P6-ECM. The dedifferentiation of chondrocytes

during *in vitro* passage was partially suppressed by the P0-ECM. However, the suppressive effect on the dedifferentiation of chondrocytes was not observed with the P2- and P6-ECM. Therefore, the suppression effect of the ECM on the dedifferentiation of chondrocytes during in vitro passage culture was completely different depending on the phenotype of chondrocytes used to produce the ECM. The primary chondrocytes-derived ECM suppressed the dedifferentiation of chondrocytes during in vitro passage culture and will be a good candidate for chondrocyte subculture for tissue engineering.

ECM has also been prepared from tumor cells and their effects have been compared with ECM from normal cells [20]. ECM were deposited on the surfaces of TCPS plates by culturing human bone marrow-derived MSCs, normal human dermal fibroblasts, normal human osteoblasts and MG63 human osteosarcoma cells in TCPS plates. After the cells deposited sufficient amount of ECM, cellular components were removed by decellularization. The ECM from different cell types had different components. The MSC-derived ECM contained type I collagen, fibronectin, biglycan and versican. The fibroblasts-derived ECM was rich in fibronectin and versican. The osteoblasts-derived ECM was composed of type I collagen, fibronectin, biglycan, decorin and versican. The MG63-derived ECM contained type I collagen, fibronectin, decorin and versican.

The ECM from different cell types showed different effects on the morphology, adhesion and proliferation of MSCs and MG63 cells. The ECM derived from normal cells promoted the adhesion and spreading of both MSCs and MG63 cells more strongly than did the ECM derived from MG63 osteosarcoma cells. Proliferation of MSCs and MG63 cells on the ECM-deposited surfaces was dependent on both the ECM and cell type. ECM promoted proliferation of MSCs but inhibited proliferation of MG63 osteosarcoma cells. When the ECMs derived from normal cells (MSCs, FBs, and OBs) and osteosarcoma cells were compared, the ECMs from normal cells inhibited proliferation of MG63 cells more strongly than did the ECMs derived from osteosarcoma cells.

24.3 Stepwise Development-Mimicking Matrices Prepared from Culture Cells

During tissue development, stem cells differentiate into somatic cells, which pass through stepwise stages of maturation [21, 22]. Accompanying with the stepwise differentiation of stem cells, extracellular microenvironments, especially ECM composition, dynamically change to regulate the process [23–25]. It is desirable to prepare ECM that can mimic the dynamic ECM change during stem cell differentiation and tissue development to investigate the interaction between ECM and stem cells. To prepare such dynamically changing ECM, a coating method needs identification and isolation of the matrices at the different stages of stem cell maturation, which has many difficulties. It is also unrealistic to use tissue-derived acellular matrices as *in vitro* models mimicking ECM dynamics during human tissue development because of lack of tissue sources. Recently, a method by controlling the stepwise differentiation of stem cells *in vitro* has been developed to prepare ECM that mimics the dynamically changing ECM (Fig. 24.2) [26, 27].

Matrices mimicking *in vivo* ECM remodeling during the osteogenesis of MSCs, which is referred to as stepwise osteogenesis-mimicking matrices, have been prepared from MSCs controlled at different stages of osteogenesis [26]. ALP and calcium deposition were used as early stage and late stage marker of osteogenesis, respectively. When human MSCs were cultured in proliferation medium, MSCs maintained their stemness without differentiation. When MSCs were cultured in osteogenic induction culture for 1 week, ALP activity was high while no calcium deposition was detected. After 3 weeks culture in osteogenic induction, both ALP activity and calcium deposition were detected. Therefore, MSCs after 1 and 3 weeks of osteogenic induction culture were defined as cells at the early and late stages of osteogenesis, respectively. The MSCs cultured in proliferation medium were defined as being in an undifferentiated stem cell stage. The different stages MSCs during osteogenesis were also confirmed by real-time PCR analysis of gene expressions of osteogenic genes such as *ALP* and *IBS*.

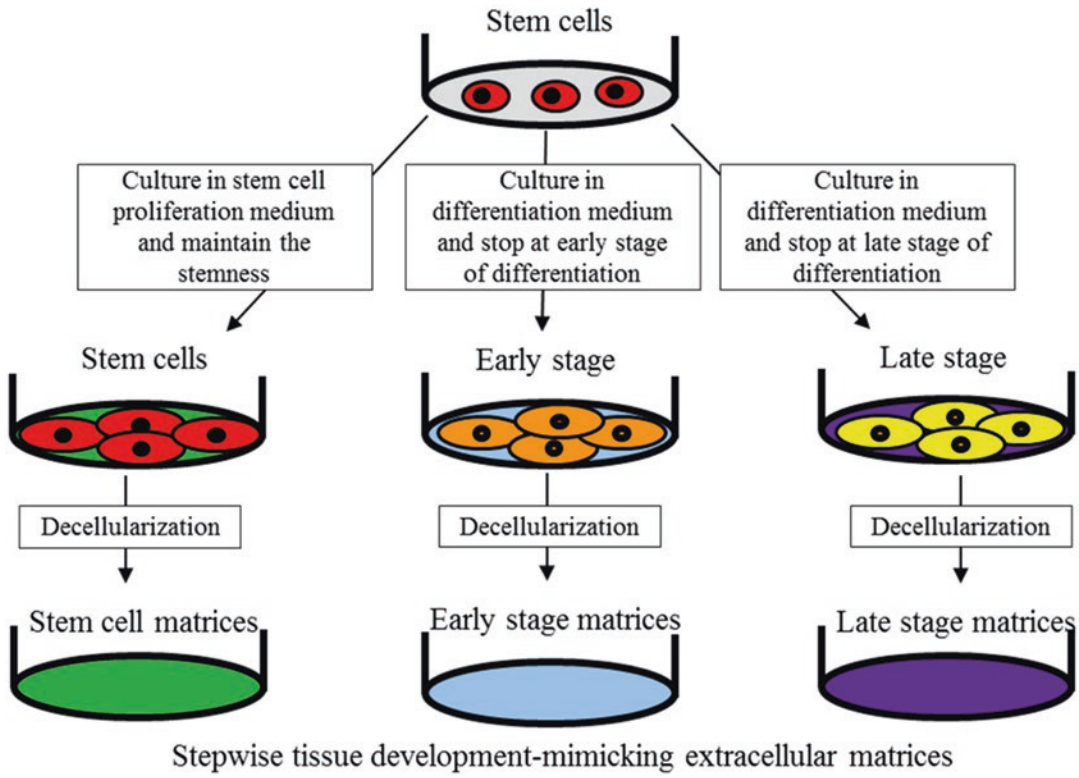

Fig. 24.2 Preparation scheme of stepwise tissue development-mimicking matrices. Green, blue and purple colors represent the ECM derived from stem cells, cells at early or late stage of differentiation, respectively

The ECM composition deposited by cells at different stage of stem cells, early and late stages of osteogenesis was dependent on the stage of osteogenesis. Immunocytochemical staining showed that fibronectin and type I collagen were positively stained in all the matrices produced by the undifferentiated and osteogenic differentiated cells in both the early and late stages. Versican was strongly stained in the matrices of early stage osteogenic cells and undifferentiated MSCs but only weakly stained in the matrices of late stage osteogenic MSCs. Biglycan was detected in the matrices of undifferentiated MSCs but only weakly detected in the matrices of early and late stage osteogenic MSCs. Decorin was strongly detected in the matrices of early stage osteogenic cells and weakly detected in the matrices of late stage osteogenic cells and undifferentiated MSCs. The results indicated that the components of ECM changed during the osteogenesis of MSCs *in vitro*.

After removal of cellular components by decellularization, extracellular proteins were remained on the cell culture plate. The matrices derived from the MSCs at early stage of osteogenesis and late stage of osteogenesis and stem cell stage were defined as osteogenic early stage matrices, osteogenic late stage matrices and stem cell matrices, respectively. The osteogenesis-mimicking matrices were used for culture of human MSCs. They supported the adhesion and proliferation of MSCs. Their effects on osteogenesis of MSCs were different. On osteogenic early stage matrices, osteogenesis occurred more rapidly than did that on the osteogenic late stage matrices and stem cell matrices. The results demonstrated that the osteogenesis-mimicking matrices had different effects on the osteogenesis of MSCs and the early stage matrices provided a favorable microenvironment for osteogenesis.

Stepwise adipogenesis-mimicking matrices have been prepared by the same method [27].

The matrices were prepared from cultured MSCs controlled at different stages of adipogenesis. Human MSCs were cultured in adipogenic medium for 3, 7 or 10 days. Lipid droplets, a late stage marker of adipogenesis, were detected by Nile red staining in the cells after 7 days of adipogenic induction. On the other hand, no lipid droplets were observed in the cells that were cultured in adipogenic induction medium for 3 days and in the cells without adipogenic induction. Lipoprotein lipase (*LPL*) gene expression, an early stage marker of adipogenesis, increased after 2 days of adipogenic induction. These results confirmed stepwise adipogenesis of the MSCs. Therefore, MSCs after 3 and 10 days of adipogenic induction culture were defined as cells at the early stage and late stage of adipogenesis, respectively. MSCs cultured without adipogenic induction kept the property of stem cells.

MSCs at the stages of stem cells, early and late stages of adipogenesis secreted different matrices. Immunocytochemical staining showed that fibronectin, type I collagen and versican were detected in the matrices of undifferentiated MSCs but only weakly detected in the matrices of early and late stage adipogenic MSCs. In contrast, laminin α4 chain was detected in the matrices of early and late stage adipogenic MSCs but was not detected in the matrices of undifferentiated MSCs. Biglycan was strongly detected in the matrices of undifferentiated MSCs and its presence gradually decreased through the progression of adipogenesis. Decorin was weakly detected in the matrices of undifferentiated MSCs and hardly detected in the matrices of the early and late stage adipogenic MSCs.

After removal of cellular components, stepwise adipogenesis-mimicking matrices were obtained. The matrices derived from MSCs after 10 days of culture in proliferation medium and MSCs after 3 and 10 days of adipogenic induction culture were referred to as stem cell matrices, early stage matrices, and late stage matrices, respectively. The matrices were used for culture of MSCs to investigate their effects on proliferation and adipogenic differentiation of MSCs. Although all the stepwise adipogenesis-mimicking matrices supported MSCs proliferation, the early stage matrices suppressed the proliferation of MSCs compared to stem cell matrices and late stage matrices. The early stage matrices were more favorable to the adipogenesis of MSCs compared with the stem cell and late stage matrices.

The stepwise osteogenesis-mimicking matrices and stepwise adipogenesis-mimicking matrices were compared in terms of their effects on their roles in controlling the balance between osteogenesis and adipogenesis of MSCs [28]. Osteogenic early stage matrices upregulated *RUNX2* expression, suppressed *PPARG* expression and therefore promoted osteogenesis of MSCs, while adipogenic early stage matrices suppressed expression of *RUNX2* and *MSX2* and facilitated adipogenesis of MSCs. The matrices showed tissue- and stage-specific effects on osteogenic and adipogenic differentiation of MSCs.

Furthermore, ECM mimicking the simultaneous osteogenesis and adipogenesis of MSCs has been prepared [29]. The simultaneous osteogenic and adipogenic differentiation of MSCs was induced by controlling the ratio of osteogenic induction medium and adipogenic induction medium (O/A ratio) in the mixture induction medium and induction culture time. The progress of simultaneous osteogenic and adipogenic differentiation of MSCs was confirmed by histological staining and related gene expression. MSCs cultured in the mixture induction medium showed simultaneous osteogenesis and adipogenesis at different stages. MSCs cultured in a mixture induction medium with an O/A ratio of 85:15 for 1 week showed early osteogenesis and early adipogenesis (EOEA). MSCs cultured in a mixture induction medium with an O/A ratio of 50:50 for 2 weeks showed early osteogenesis and late adipogenesis (EOLA) stage. MSCs cultured in a mixture induction medium with an O/A ratio of 95:5 for 3 weeks were at a stage of late osteogenesis and early adipogenesis (LOEA). MSCs cultured in a mixture induction medium with an O/A ratio of 70:30 for 3 weeks were at a stage of late osteogenesis and late adipogenesis (LOLA). MSCs cultured in proliferation medium for 1 week were defined as cells at the undifferentiated stem cell stage (SC). The stepwise osteogenesis-co-adipogenesis-mimicking

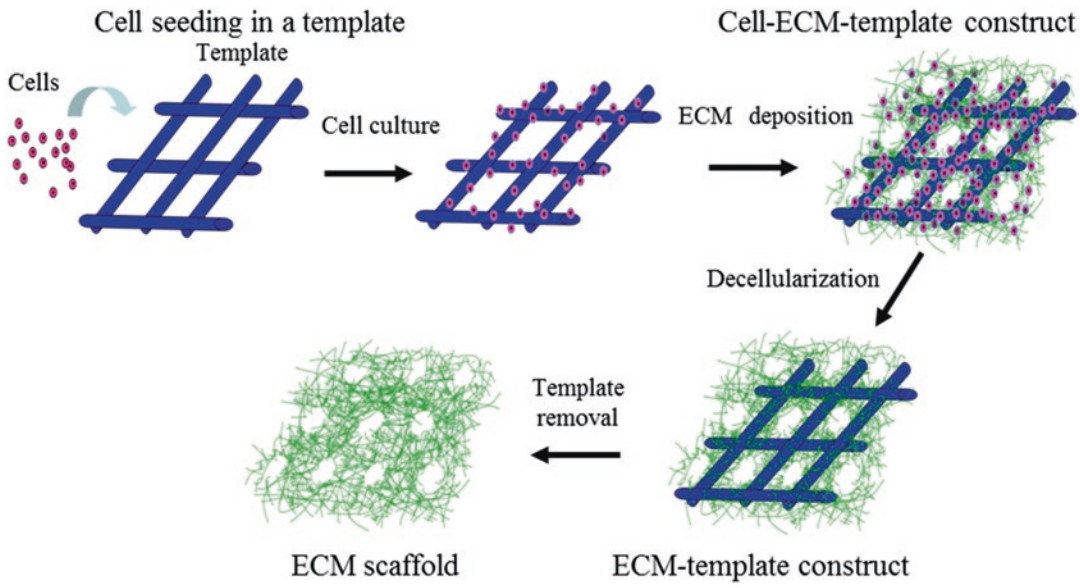

Fig. 24.3 Preparation scheme of ECM scaffolds by combination of 3D culture of cells in a template, decellularization and template removal. (Adapted from Ref. [30] with a permission from Elsevier)

matrices (OEAE, OLAE, OEAL, OLAL and SC) were obtained after decellularization. Immunocytochemical staining showed that the composition of the matrices changed depending on the stepwise stage of osteogenic and adipogenic differentiation.

The stepwise osteogenesis-co-adipogenesis-mimicking matrices were used for culture of MSCs to investigate their effect on adhesion, proliferation and differentiation of MSCs. The results indicated that all the matrices supported cell adhesion and promoted cell proliferation. They had different effects on differentiation of MSCs. LOEA and LOLA matrices promoted osteogenic differentiation but not adipogenic differentiation of MSCs. EOEA matrices promoted adipogenic differentiation but not osteogenic differentiation of MSCs. EOLA did not promote either osteogenic or adipogenic differentiation of MSCs.

24.4 ECM Scaffolds Prepared from Cultured Cells

Not only ECM from cultured cells can be deposited on the surfaces of cell culture substrates or implants, ECM scaffolds can also be prepared from cultured cells. By culturing cells in a temporary template, ECM scaffolds can be prepared after decellularization and removal of template. When cells are cultured in a three-dimensional template, cells adhere and distribute in the template. The cells proliferate and excrete their own extracellular matrices. After three-dimensional culture to allow secretion of sufficient amount of ECM, the cellular components are removed by decellularization. Selective removal of the template leaves formation of the ECM scaffolds (Fig. 24.3).

The method has been used to prepare ECM scaffolds from human bone-marrow MSCs, human articular chondrocytes and human dermal fibroblasts by using PLGA mesh as a template [30, 31]. The ECM scaffolds from MSCs (ECM-M), chondrocytes (ECM-C) and fibroblasts (ECM-F) had similar porous structure as that of the template (Fig. 24.4). The composition of the ECM scaffolds was dependent on the cell type and phenotype used to prepare the ECM scaffolds. The method can be used to prepare autologous ECM scaffolds if autologous cells are used during scaffold preparation.

If stem cells and development-mimicking cell types are used for the scaffold preparation, development-mimicking ECM scaffolds can be

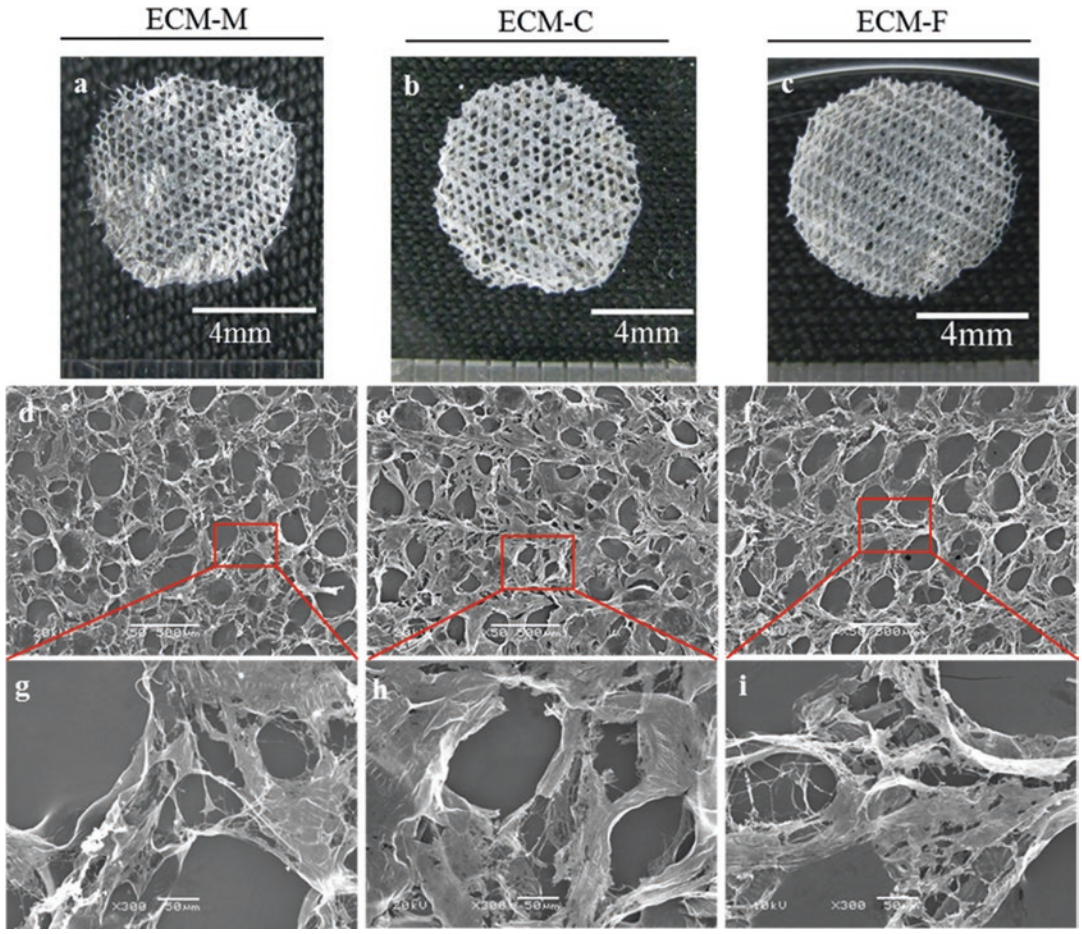

Fig. 24.4 Gross appearance of ECM-M (**a**), ECM-C (**b**) and ECM-F (**c**) prepared from human bone marrow-derived mesenchymal stem cells, human articular chondrocytes and human dermal fibroblasts. And their SEM images at low (**d–f**) and high (**g–i**) magnifications. (Adapted from Ref. [30] with a permission from Elsevier)

obtained. The method has been used to prepare chondrogenesis-mimicking ECM scaffolds [32]. The cells at stem cell stage, early stage and late stage of chondrogenesis were used to prepare stem cell ECM scaffold (SC-ECM scaffold), early stage chondrogenesis-mimicking ECM scaffold (CE-ECM scaffold) and late stage chondrogenesis-mimicking ECM scaffold (CL-ECM scaffold), respectively.

Human bone marrow-derived MSCs were seeded in a knitted PLGA mesh disk and cultured in proliferation medium for 1 week, which was used to prepare SC-ECM. MSCs cultured in PLGA mesh disks with chondrogenic induction medium for 1 and 3 weeks were used to prepare the CE-ECM scaffold and CL-ECM scaffold, respectively.

After the induction culture for 1 w, the content of sGAG was at the same level as that in the MSCs-PLGA constructs cultured in the growth medium, although the related genes expression indicated the initiation of chondrogenic differentiation. Both the high expression level of chondrogenic genes and the high content of cartilage-specific matrix indicated that the cells differentiated into more mature chondrocytes after culture for 3 weeks in the presence of chondrogenic medium. The level of MSCs chondro-

genesis progression was completely different when MSCs were cultured after 1 and 3 weeks, which suggested the different stages of stepwise chondrogenesis. Therefore, MSCs cultured in chondrogenic medium for 1 and 3 w were defined as the early stage (CE) and late stage (CL) of chondrogenesis, respectively. The ECM scaffolds had different compositions depending on the stage of chondrogenic differentiation and showed different effects on the chondrogenic differentiation of MSCs. The ECM scaffold mimicking early stage of chondrogenesis enhanced the chondrogenic differentiation of MSCs while the ECM scaffold mimicking late stage of chondrogenesis had an inhibition effect.

24.5 Conclusions

Various types of biomimetic ECM have been prepared from cultured cells. Normal somatic cells such as chondrocytes and fibroblasts and tumor cells have been cultured to prepare their respective ECM. Mesenchymal stem cells have been cultured at different induction conditions to prepare stepwise osteogenesis-mimicking ECM, adipogenesis-mimicking ECM and stepwise osteogenesis-co-adipogenesis-mimicking matrices. By using 3D cell culture in a removable template, ECM scaffolds from different cell types and stepwise chondrogenesiss-mimicking ECM scaffolds have been prepared. All the ECMs and scaffolds support cell adhesion and proliferation. They have shown different effects on the maintenance of chondrocyte phenotype, osteogenic and adipogenic differentiation of mesenchymal stem cells. These ECMs and scaffolds will provide useful tools for investigation of ECM-cell interaction and the effect of ECM on stem cells function. They will also provide useful guideline for the design and preparation of the functional scaffolds for tissue engineering.

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Tissue Scaffolds As a Local Drug Delivery System for Bone Regeneration

25

Elif Sarigol-Calamak and Canan Hascicek

Abstract

Healing fractures resulting from bone disorders such as osteoporosis, osteoarthritis, osteomyelitis, and osteosarcoma remain a significant clinical challenge. In this chapter, we focus on scaffold based local drug delivery applications for promoting bone regeneration. For this purpose, we first review bone disorders, which require drug treatment and current fabrication techniques for bone tissue scaffold as a drug carrier. Next, we address the role of antimicrobial agents, anti-inflammatory drugs, anti-cancer drugs and bisphosphonates in promoting vascularized bone regeneration and discuss various local therapeutic delivery strategies for controlled and sustained drug delivery. Specifically, this review addresses the concept of drug loaded scaffold design and local drug release effects on bone regeneration. We conclude this review with a discussion of local drug delivery approaches to bone regeneration and discuss why it has the potential to be more efficient than traditional bone treatment methods.

Keywords

Bone disorders · Local drug delivery · Bone regeneration · Tissue scaffold · Pharmacological treatment · Scaffold fabrication

25.1 Introduction

25.1.1 Bone Tissue

Bone tissue provides structural support to the body, as well as many other physiological and metabolic tasks including calcium and phosphate storage, blood cell production, energy storage (by harboring of bone marrow) etc. Bone is a very dynamic and specialized tissue that consists of many cell types (support cells: osteoblasts and osteocytes originated from mesenchymal stem cells; and remodeling cells: osteoclasts originated from hematopoietic stem cells) and extracellular matrix (ECM) with mineral crystals (crystalline hydroxyapatite), and organic matrix (structural proteins: collagen fibrils, non-collagenous proteins-osteoid; bioactive regulatory peptides: growth factors, cytokines etc.) [6, 17, 28, 32, 58, 97, 121] (Fig. 25.1).

While the bones are constantly restructured throughout life to adapt changing biomechanical forces, they are in a continuous cycle to replace the old bone. It is a lifetime process, removing old bone by replacing of new bone, and bone

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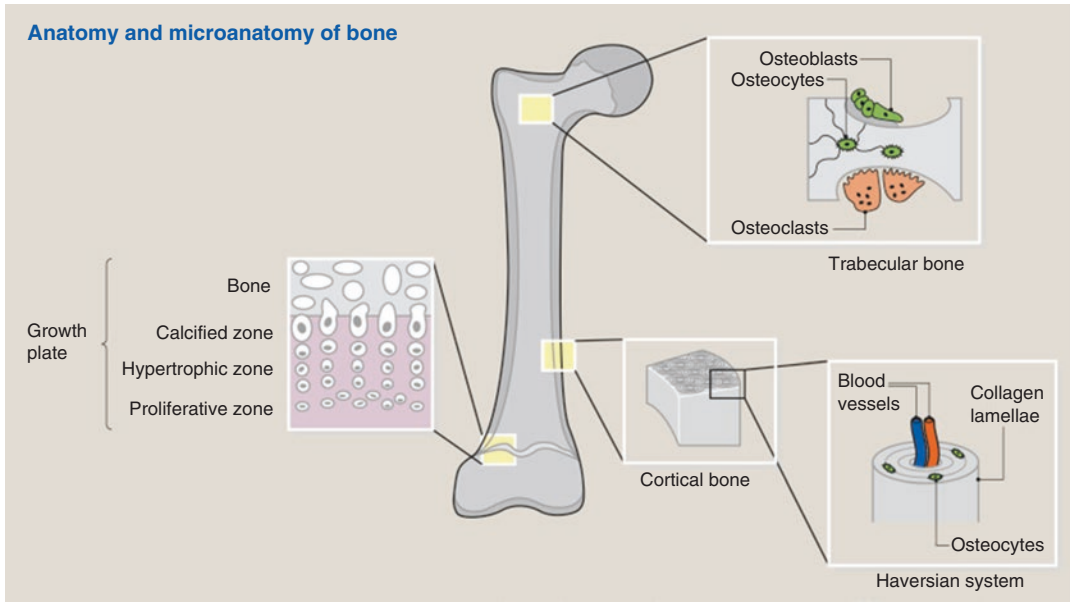

Fig. 25.1 Macro and micro-architecture of bone [125]

remodeling cycle consists of three steps; osteoclast-mediated bone resorption, the transition from resorption to the new bone formation and osteoblast-mediated bone formation. Owing to this continuous cycle up to critical sizes bone defects and fractures are easily repaired, but larger defects need external intervention [6, 34, 58]. Besides that, there must be a balance between resorption and formation to ensure the mechanically strong and healthy bone after remodeling cycle. If the inadequate healing process occurs, remodeling process causes pathological conditions which lead to developing bone disorders [32, 34].

Bone organic matrix is strengthened by the accumulation of calcium phosphate crystals, which are affected by genetic, metabolic and mechanical factors [6]. The bone metabolism can be affected by levels of some hormones; e.g. parathyroid hormone (PTH – while low levels increases osteoblastic bone formation; high levels increases osteoclastic bone resorption), calcitonin (CT – inhibits bone resorption), gonadal steroids (maintain skeletal mass), glucocorticoids (inhibit bone formation), insulin, growth hormone, androgens (promote bone growth), estrogen (deficiency leads to bone remodeling with decreased bone mass) and vitamin D (promotes the mineralization of osteoid and causes indirectly bone resorption) [58, 90]. Since a disorder can inhibit secretion of these hormones, bone formation process may be negatively affected. Bone tissue can replenish itself effectively due to the high recovery potential of the bone structure. Especially in young patients, many of the bone injuries can heal without serious intervention. However, large damages caused by the removal of tumors or damages due to the union of broken bone tips from unboned bones cannot heal without surgical intervention.

25.1.1.1 Bone Tissue Disorders Requiring Drug Treatment

Bone regeneration is crucial for the treatment of some diseases that cause loss of bone mass, segmental bone defects and fractures. Genetic diseases, osteoporosis and trauma cause approximately 6 million fractures in the United States annually. An ideal bone regeneration requires comprehensive biological processes, which include mineralization, cell proliferation and transport of signaling molecules (growth

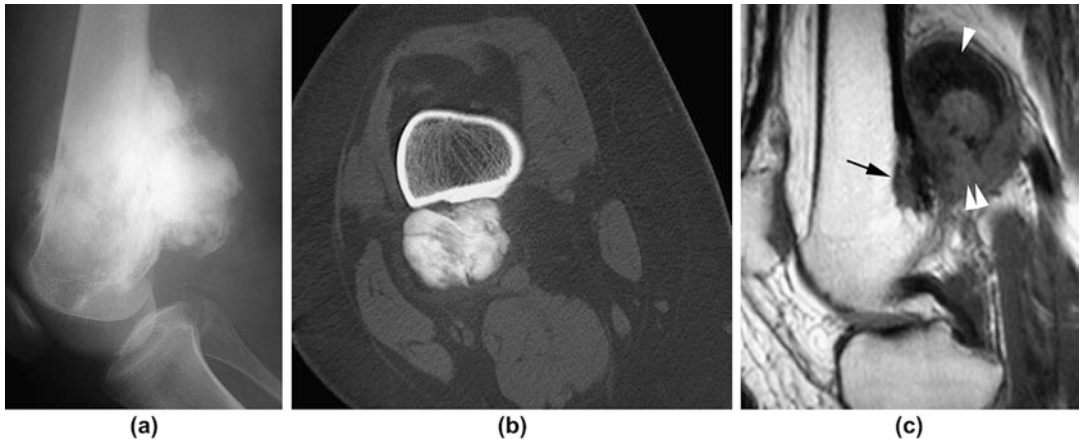

Fig. 25.2 Graphy of parosteal osteosarcoma in the same patient Lateral radiograph (**a**), Axial CT (**b**) and Sagittal T1W SE MRI (**c**) [110]

factors). To mimic this complex processes, regenerative approaches generally utilize cell transplantation and local delivery of bioactive agents including drugs and growth factors. Although regenerative strategies have yielded clinically successful results in some parts, there are still significant challenges in bone regeneration. Bone diseases can start with a movement disorder and lead to deaths over time. In bone tissue treatments, one of the most popular approach is to replace the bone tissue by tissue engineering after a loss caused by trauma or consciously removal and to provide effective treatment with the therapeutically active molecules by local or targeted drug delivery strategies. Most common types of bone disorders can be examined as osteosarcoma, osteoporosis, osteoarthritis, infections, regeneration problems and bone damage/deficiency [39, 49].

Osteosarcoma

Osteosarcoma is the most common type of bone cancers and occurs most likely in children and adolescents. In osteosarcoma, the osteoid structure produced by malignant mesenchymal cells causes immature bone growth, especially in rapidly growing bone tissue. These tumors are usually locally aggressive and tend to undergo systemic metastases usually to the lungs or other bones [92, 95]. The graphy of osteosarcoma is shown in Fig. 25.2.

Although it is the most commonly diagnosed malignant bone tumor type among children and adolescents, its incidence among all cancer types is very rare, less than 1% in the United States. The etiology still remains not fully understood which makes the diagnosis quite difficult. Therapy requires complete resection of the tumor site by surgery and treatment with more than one antineoplastic agents preoperatively (neoadjuvant) and postoperatively (adjuvant). In diagnosed patients, treatment includes radiation therapy [72, 95]. In case of replacement is needed after removing the tumoral site by surgery replacement with the endoprosthesis, allografts, and autografts are the options. Chemotherapy is usually administered systemically via intraarterial or intravenous routes. The metastasis number or reoccurrence can be reduced or delayed with proved chemotherapy regimens [111]. Most commonly used chemotherapeutic regimens include doxorubicin (ADM), cisplatin (CDP), ifosfamide (IFO) and high dose methotrexate (MTX) with leucovorin rescue [72, 95]. But systemic chemotherapy for osteosarcoma treatment provokes the occurrence of early toxicity such as hematologic toxicity, acute liver (MTX)/renal toxicity (CDP and IFO) and late toxicity in prolonged survival such as cardiac, second tumor, sterility, chronic renal failure and neurological toxicity (especially ototoxicity due to CDP) [71].

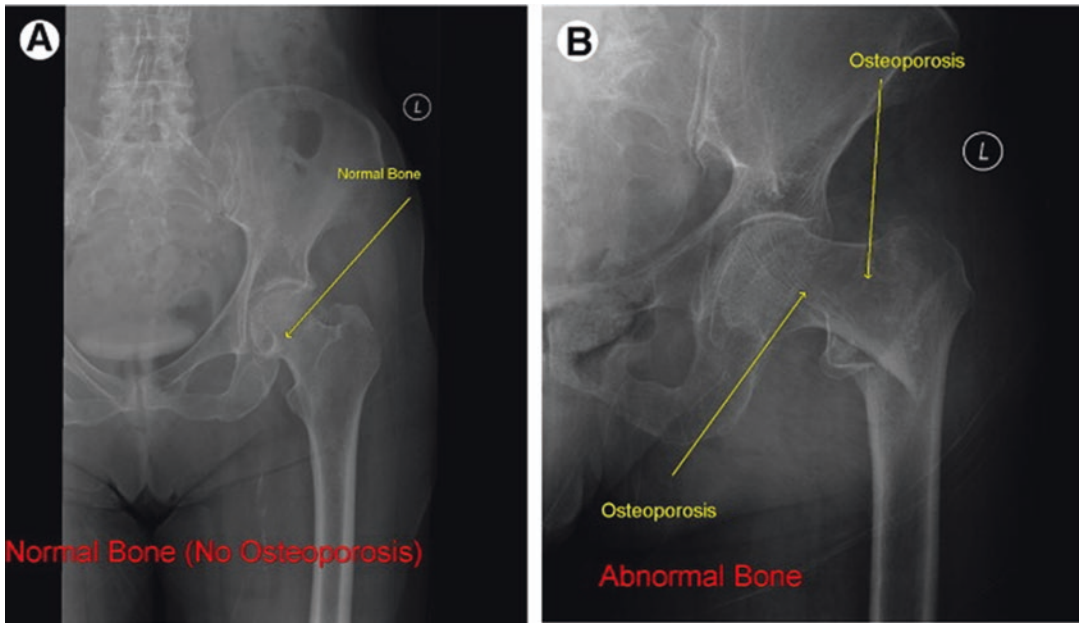

Fig. 25.3 Normal bone (**a**) and osteoporosis (**b**) [48]

Osteoporosis

Osteoporosis or bone erosion is a disorder that occurs as a result of dilution of the protein matrix in the bone and decrease of mineral density. Especially, lack of estrogen level caused osteoporosis in postmenopausal women and decreased hormone level makes bone very fragile (Fig. 25.3). The metabolic dysfunction could arise from multifactorial problems like hereditary, mechanical, nutritional and autoimmune factors. Increased fragility, can cause fractures at any skeletal zone, which can occur even with a low force in osteoporosis, especially around the vertebrae, hip, and distal radius regions [5, 51]. Treatment of osteoporotic defects is challenging due to lack of healing capacity. In this regard, osteoporosis fractures mainly require orthopedic surgery.

In the case of osteoporotic bone fracture, the first management of osteoporosis requires treatment with orthopedic surgery. The drug treatment aims to relieve the fracture symptoms with analgesics and reduce the risk of further fractures with drugs inhibiting bone resorption or stimulating bone formation. The antiresorptive drugs can be classified as bisphosphonates, receptor activator of nuclear factor kappa-B ligand (RANKL) antibody, and selective estrogen receptor modulators (SERM) and they inhibit the osteoclast function with different mechanisms of actions. Teriparatide, a recombinant DNA form of parathyroid hormone is the only anabolic therapeutic molecule for osteoporosis [18].

Osteoarthritis

Osteoarthritis is a progressive joint disease results from complex and multifunctional factors, including genetic, biological, and biomechanical processes. This disorder is characterized by inflammation of the joints, deterioration of cartilage tissue, and loss of synovial fluid [23, 37]. The loss of cartilage tissue begins with the joint surface and can spread to the subchondral bone matrix, periarticular muscles, and peripheral nerve [23] (Fig. 25.4).

There are three types of treatment approaches, non-pharmacological (lifestyle modification, exercise, weight reduction), pharmacological and surgical. In early stages, treatment should require reduction of pain and stiffness and prevention of progression of joint damage. Paracetamol is the first line oral analgesic. Additionally, nonsteroidal

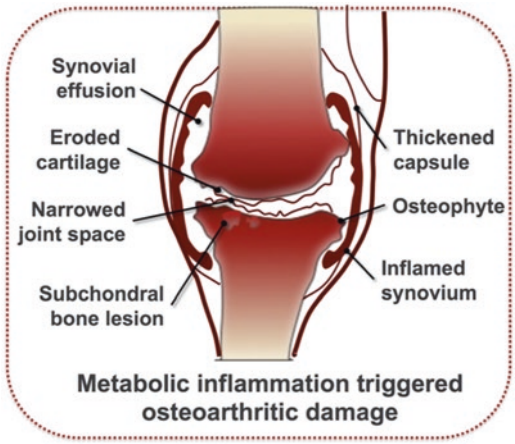

Fig. 25.4 Osteoarthritis [119]

anti-inflammatory drug (NSAID) is mainly used. If only these drugs are ineffective, weak opioids and narcotic analgesics can be used as an alternative way. However, oral therapy with all of these analgesics is limited because of the cardiovascular and gastrointestinal side effect risks. Intra-articular injection of long-acting glucocorticoids and hyaluronic acid is another effective treatment approach. In the case of advanced disease, joint replacement can be an effective treatment despite its drawbacks like poor functional outcomes and limited lifespan prostheses [12, 37].

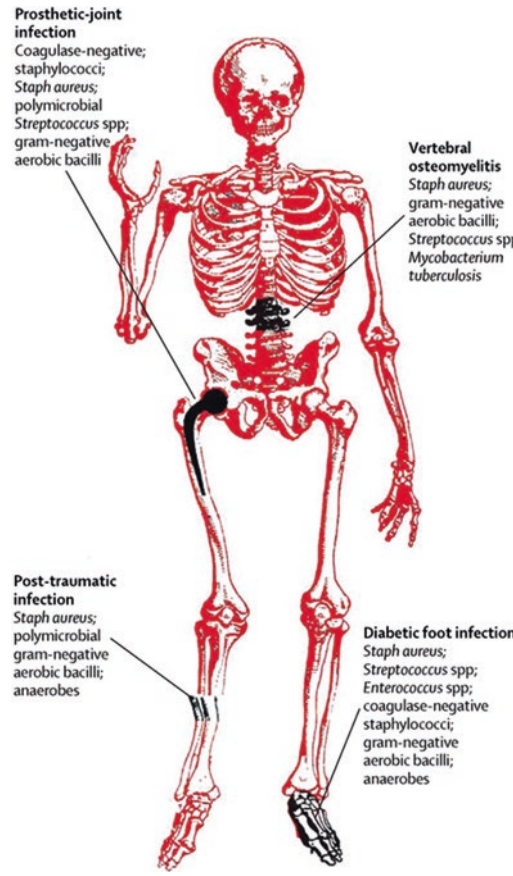

Fig. 25.5 Infection origins in osteomyelitis [64]

Osteomyelitis

Osteomyelitis is a progressive inflammatory disease which occurs because of bacterial infection and it causes bone destruction. The infection is mostly bacterial origin and may occur in two basic ways: hematologic spread or local infection (after surgery or trauma, etc.) [64]. Generally, a bacterium leads to hematogenous osteomyelitis. The hematogenous osteomyelitis usually occurs in pubertal children. In adult patients, osteomyelitis is usually seen as post-traumatic [67]. Infection origins in osteomyelitis are summarized in Fig. 25.5.

Both in acute and chronic osteomyelitis bone vascularization becomes compressed because of the inflammation, which causes poor blood supply and bone necrosis. Therefore, the antibiotic delivery to the infected bone via oral or intravenous route turns into almost ineffective. Especially in chronic osteomyelitis, the debridement of the infected tissue with surgery and antibiotic treatment is needed for eradication [36, 67].

25.1.1.2 Challenges and Advantages of Local Drug Delivery to Bone

The drug administration routes can be classified mainly into two groups as systemic and local routes. Especially in conventional systemic route, drug delivery occurs via the circulatory system which may result in many disadvantages like systemic toxicity, side effects, renal and liver complications, drug interactions, poor distribution to the targeted tissue and decreased patient compliance [86]. However, local drug delivery systems, overcome these restrictions with limited side effects, high concentrations in the targeted tissue and little systemic uptake [105]. Also, a sustained

and controlled local release system improves the drug release profiles by releasing the desired amount of drug at a controlled rate and time (from hours to years) with protecting from surrounding factors such as degradation and increases drug safety and efficacy [10]. On the contrary, these systems have some disadvantages such as limited implantation to hard-to-reach tissues, infection and inflammation risk on the surrounding tissue and in some cases difficulties in drug penetration into the targeted area [73, 105]. To the best of our knowledge, drug-loaded bone tissue scaffolds that provide local drug delivery to the disease region after removal of the damaged bone, allow the new bone regeneration. It is an important milestone in the treatment of bone diseases which are difficult to treat with conventional methods.

25.2 Bone Scaffolds

Bone fracture defects or bone resections because of a disorder results in serious skeletal deficits especially if the scarred area are above a critical size. Reconstruction surgery with autografts (bone from the patient) or allografts (bone from a human cadaver) has been widely used for a long time. However, these scaffolds have many disadvantages like limitations in finding the suitable donor and immunological response and infections risks. To overcome these limitations, synthetic or natural biomaterials based scaffolds are widely studied by researchers as an alternative route [65, 84].

Bone tissue scaffolds are based on four basic components taking the natural structure of bone as a model: matrix, cells, cell signaling for tissue formation, blood circulation for oxygen and nutrition supply and waste removal [98]. The primary aim of tissue engineering is to provide the physiochemical biomimetic three-dimensional microenvironment for cells by mimicking the ECM. The bone ECM consists of mineralized fibrous collagen protein mainly. The fibrous structure allows transport of vital molecules like nutrients, oxygen, growth factors and waste products for cell growth and facilitates the cell-cell interaction, cell migration into the scaffold and vascularization [47].

A great variety of approaches have been studied to increase the adaptation of bone scaffolds into host bone tissue during the bone regeneration process. One of them is to modify the surface composition of the scaffold via surface modification of intrinsic osteoinductive and osteoconductive factors through grafting, patterning and coating techniques [2, 86]. Besides, the incorporation of biological signal molecules into scaffolds enables cell-mediated bone integration to achieve satisfactory bone regeneration [53, 66].

The optimal biomaterials for tissue engineering should integrate with biological molecules and cells. Also, these materials should promote migration, proliferation and differentiation of cells and provide mechanical support to regenerate tissues. Further, for bone tissue engineering scaffolds should have osteoinductive (promoting pluripotential cells differentiation to osteoblastic cells) and osteoconductive (supporting bone growth by enhancing cellular activity) properties [108]. In bone tissue engineering, scaffold properties such as porosity and pore size have crucial roles in osteogenic signal expression. Porosity and pore size mainly affect cell density, distribution and migration by affecting cell attachment to the scaffold. In addition, inter-connected pore structure enhances bone tissue development by increasing intercellular signaling pathways and indirectly contributes to developing bone morphology. Recent studies have shown that, interconnected pores with a mean diameter (or width) of 100 μm or greater, and the open porosity of >50% are generally considered to be the minimum requirements to allow cell filtration and migration into scaffold and tissue ingrowth [89]. The porous structure of the bone scaffolds also serves satisfactory oxygen and nutrient diffusion. Mechanical properties are also important for the load-bearing capability of natural bone tissue. Mechanical stiffness and physical microenvironmental parameters such as hydrostatic pressure, compression, tension, fluid shear and interstitial flow influence cell behavior by mechanical transduction and cell-scaffold interactions. [57, 94].

In the light of these properties, an ideal bone tissue scaffold should provide mechanical resistance to adhered bone cells, controllable degradation profile with non-toxic and non-immunological degradation products [87]. Cell-free and cell-laden constructs with various chemical and biological structures act as a scaffold to support bone regeneration process. A great variety of the studies have shown that osteoblast and mesenchymal stem cell-laden bone tissue scaffolds indicated differentiation, vascularization, real bone like tissues under proper conditions [24, 76, 79, 93]. Providing the adequate biological, chemical and physical extracellular environmental features like presence of calcium or local growth factors (transforming growth factor beta-TGF-β, bone morphogenetic proteins-BMPs etc.) to promote differentiation toward the osteoblastic lineage has critical importance [43, 98]. All of these architectural, chemical and bio-functional properties, which are mentioned above, have great importance in the development of a successful bone tissue scaffold design.

The adaptation of bone tissue with surrounding scaffold microenvironment is one of the prerequisites to generate functional bone regeneration. During bone regenerations process, bone scaffolds can not only provide cell migration and tissue ingrowth, but also serve osteoinductive bio-signals to contact with the surrounding host networks including vasculature and nerve system [118]. Bone scaffolds with appropriate bio-chemical composition and porous microarchitecture support vascular formation and stabilization [96].

25.2.1 Scaffold Materials, Fabrication Methods and Characterizations

A scaffold is an important component in bone tissue engineering providing as a three-dimensional microarchitecture for cell interactions. Moreover, it provides optimum functional and structural hubs for the newly formed tissue. As a gold standard, bone scaffold for tissue regeneration should have the degradation profile as the same rate as bone regeneration. In order to meet this certain criteria, metals, ceramics, polymers (natural and synthetic) and their composites are mainly used in bone tissue engineering applications. Materials of the bone tissue scaffolds can be divided into three fundamental groups as inorganic, polymeric and composite materials. Each material type has advantages or disadvantages. The sections are organized by the group of scaffold materials with the discussion of processing techniques and characterization methods.

25.2.1.1 Inorganic Scaffolds (Metals, Bioactive Ceramics and Glasses)

Metallic implants have some significant disadvantages such as non-degradability and limited processability in the biological environment and metals and alloys have a long history of the application as bone implants [120]. In the literature, titanium (Ti), cobalt (Co) based alloys (CoCrMo) and stainless steels are widely studied due to their excellent mechanical strength, satisfactory corrosion resistance and biocompatibility. The elastic modulus of the cortical bone ranges from 3 to 30 GPa since cancellous bone has significantly lower elastic moduli of 0.02–2 GPa. Most of the metallic implants have greater elastic modulus than the bone (Ti6Al4V:110 GPa and CoCrMo: 210 GPa) [70, 80]. Thus, equivalent elastic and Young's modulus have to be adjusted when using these bulk materials.

As inorganic biomaterials, the calcium derivatives such, bioceramics (hydroxyapatite (HA), tricalcium phosphate (TCP), dicalcium phosphate dihydrate (DCPD), octacalcium phosphate (OCP), etc.), bioactive glasses (silicate, borate, phosphate and borosilicate glasses) and their composites have been widely used for damaged bone repair owing to their structural and chemical similarity with the inorganic component of bone [108]. These materials are capable of activating formation, deposition and precipitation of CaP and forming a direct bond between implants and native bone [41, 59]. The use of calcium salts as resorbable bone void filler device is approved by FDA (U.S. Food & Drug Administration). Calcium phosphate bioceramics emerge as the natural deg-

radation product of bone resorption circle and the calcium and phosphate ions are naturally metabolized. They show promising biological properties as bone scaffold material with their biocompatible, resorbable, osteoconductive and osteoinductive features. They can be obtained by synthesis after sintering or aqueous precipitation, or by natural sources like coral hydroxyapatite. Even if, they are highly biocompatible, they show a deficiency in load-bearing functions. Physiologic stability of calcium phosphates depends on the particle size, crystallographic features, and density. Naturally porous bioceramics are convenient bone scaffold candidates. The ionic exchange occurring on the surface of bioceramics promotes physicochemical and mechanical cohesion between the scaffold and bone [6, 123].

Bioactive glasses which is another important class of bioceramics have also widely used for bone tissue engineering applications. Bioactive glasses have an amorphous structure. The carbonate-substituted hydroxyapatite-like (HCA) layer occurring on the silicate bioactive glass (45S5 glass) surface bonds enhances bonding with living bone tissue. Sodium and calcium ions, HCA molecules and silicon occur as the biodegradation products. The difficulties in fabrication of porous scaffolds, slow degradation rates and the uncertainty about long-term effects of *in-vivo* SiO_2 restricts its use as a tissue scaffold. Borate-based bioglasses both enhances the bone formation more than the silica-based bioglasses and also their degradation rates are more closely to the rate of new bone formation [89].

25.2.1.2 Polymeric Scaffolds

Tissue adaptation of the biomaterials is very important to design optimal bone tissue scaffolds. Polymers that can recapitulate natural bone ECM microenvironment with the required biochemical and mechanical properties. Natural and synthetic polymers including chitosan, collagen, alginate, silk, poly lacticacid (PLA), polycaprolactone (PCL) and poly (lactic-co-glycolic acid (PLGA) have been produced as a bone scaffold in various morphologies for bone tissue engineering applications. Among these, synthetic polymers have easily adjustable physicochemical and mechanical properties as well as biodegradation rates. PLA is used as an orthopedic device, and medical implants (in the form of screws, pins, rods and mesh) [63]. PLA is also used as a biodegradable and biocompatible scaffold material in bone tissue engineering applications. Lin et al. prepared hydroxyapatite (HA) mineralized on chitosan (CS)-coated polylactic acid (PLA) nanofibers as a bone tissue engineering scaffold. Their results show that the nanocomposite scaffolds HA/CS/PLA with nanofibrous surface roughness, biocompatibility and similar structural similarity can help as a good candidate biomaterial for bone regeneration [68]. The polymeric nanofibers are excellent materials as a tissue scaffold for proliferation and differentiation of the cells [13–16]. As noted above, Lin et al. used PLA to produce nanofibers containing HA, which promotes bone mineralization. Nanofibers based scaffolds, which are modified ceramics, can be a good choice for bone regeneration. In another study in the field of bone tissue engineering, Hao-Yang Mi et al. produced composed thermoplastic polyurethane (TPU) and polylactic acid (PLA) tissue engineering scaffolds at various ratios. Their results show that PLA/TPU composite material has similar structural and biological functions of natural bone that have the possibility to be used as synthetic scaffolds in tissue engineering science [75].

Polycaprolactone (PCL) is a biocompatible and biodegradable polyester, which is mostly used for bone regenerations due to its excellent mechanical properties and processability. Recent studies have shown that PCL is a biocompatible scaffold to be used in regeneration of bone and cartilage. Uma Maheshwari et al. designed a polymer–ceramic bilayer nanocomposite scaffold based on electrospun polycaprolactone (PCL)/polyvinyl alcohol (PVA) bilayer nanofibers incorporated with hydroxyapatite nanoparticles. The results demonstrated that PVA/HA/PCL composite nanofiber based scaffolds have excellent host tissue adaptation and have a great potential for bone tissue engineering applications.

Poly (lactic-co-glycolic acid (PLGA) is another biocompatible and biodegradable poly-

mer used in tissue engineering and drug delivery systems. Also, PLGA is an FDA approved polymer for bone regeneration. Sidney et al. fabricated diclofenac sodium loaded porous scaffolds composed of PLGA and polyethylene glycol (PEG). Diclofenac sodium is known as anti-inflammatory drug and stimulates bone regeneration. The produced scaffold was loaded with the various concentration of diclofenac sodium. The release studies carried out to adjust the optimum drug concentration for bone regeneration. The results demonstrate that PLGA/PEG scaffolds provide controlled release of anti-inflammatory drugs and activate bone regeneration [104].

25.2.1.3 Composite Scaffolds

Due to the load bearing and strong mechanical requirements of the scaffold design for bone tissue engineering, composite materials have been widely used to merge the advantages of two or more materials together to meet these needs. Ceramics have the low tensile strength and are very fragile. For this reason, they can not be used in damages where flexural areas. One important type of composite materials in bone tissue engineering is inorganic-polymer composites that merge the elasticity of a polymer phase and strength and stiffness of an inorganic phase. These composites provide advanced mechanical properties and desirable degradation profiles. Also, incorporation of polymers into the ceramics allows better processability and control over the composite uniformity and structure. For instance, the addition of hydroxyapatite into biodegradable synthetic polymers such as PLA, PCL and PLGA led to the formation of composite materials possessing both excellent mechanical properties and osteoactivity [29, 54, 55, 78]. Laurencin et al. produced 3D composites of PLGA (50:50) and HA using a solvent leaching/particle leaching method. Long-term osteoblast culture *in-vitro* showed that the PLGA-HA scaffolds supported cell proliferation, differentiation, and mineral formation. Tunable degradability of PLGA and the strong mechanical properties of HA together demonstrated great promise as a synthetic scaffold for bone healing. The results indicated the excellent potential of calcium phosphate/polymer composites in the healing of critical size bone defects [27]. HA was also incorporated into biodegradable polymers with adjustable degradability and biochemical properties. Bhattacharyya et al. fabricated HA containing electrospun poly [bis(ethylalanato) phosphazene] (PNEA) nanofiber meshes as scaffolds for bone tissue regeneration. The results showed HA crystals were uniformly dispersed in the nanofibers and uniformity results were confirmed by calcium mapping. Also, electrospun PNEA/HA nanofibers closely mimic bone ECM microarchitecture and demonstrated excellent osteoinductivity and osteoconductivity [8, 9].

Addition of bioglass particles into a polymeric matrix can enable both osteoconductivity and osteoinductivity [83]. In the literature, it was reported that ionic components of bioglass such as Na^{+}, Si^{+} and Ca^{2+} released into body fluid could trigger osteoblast attachment and protein adsorption[81, 126]. These tremendous properties of bioglass composite scaffolds can activate to new bone formation and adaptation between host bone and composite implants. Addition of bioglass into polymeric matrix has launched to the successful commercially product which is called Vitoss® in 2008. It is also one of the best-selling synthetic bone substitutes. Processing and integration of the various bone compatible materials would be further beneficial for bone healing and bone related defects.

25.2.2 Fabrication Methods for Bone Tissue Scaffolds

Another critical step for bone tissue engineering is to design of bone scaffolds and implants, which recapitulate the biological and biomechanical properties of the host tissue. Traditional methods for fabricating interconnected porous scaffolds based on metals, ceramics and composites include solid state processing (powder metallurgy, sintering of powders and fibers, etc.), liquid state processing (direct foaming and spray foaming), vapor deposition and electrodeposition. The methods based on sintering are widely used in the fabrication of inorganic scaf-

folds due to their strong particle bonding ability. The sintering techniques require heating at high temperatures by gradual temperature raising excessing 1200 °C up to several hours [60]. The exposing heat at high temperatures causes some side effects especially in bioglasses like crystallization because of the sintering above the glass transition temperature, which results in low strength. In addition to that, sintering steps cause decomposition of incorporated therapeutic drugs or growth factor [89]. Denry et al. have conducted a recent study to overcome these obstacles by sintering under vacuum [26]. Using a mold with desired properties is the simplest sintering production approach [25, 26, 85]. Mixing the bioceramics or bioglasses with porogen agents (NaCl, Mg particles or organic solvents etc.) is another approach to increase the porosity but obtaining an interconnected pore-network still remains a challenge [4, 11, 117].

Recent approaches combine sintering procedures with three-dimensional printing techniques via computer-aided design (CAD) (solid free-form fabrication (SFF) or rapid prototyping methods), which ensures a pre-designed controllable external and internal architecture. With these methods pore sizes, numbers and interconnectivity can be tailored allowing healthy tissue ingrowth and nutrient-waste exchange [103, 115, 116]. Solid state processing for metal manufacturing such as electron beam melting (EBM), selective laser melting (SLM) are computer aided production methods which based on a layer by layer assembling. SLM and EBM are widely used for the production of porous ceramics, metals and their composites with complex architecture [33, 122]. Recent advances in computer aided bone scaffold production methods have promised an exciting future for individual bone healing.

New generation computer aided approaches have the ability to fabricate bone scaffolds that mimic the microarchitectures of bone tissue, and a print layer thickness as small as 10 μm, which allows bone regeneration. Stereolithography (SLA) is a versatile method that is desirable for fabricating bone scaffolds due to its precision and the increasing availability of biocompatible photopolymers. Cooke et al. used SLA to produce 3D scaffolds for bone tissue engineering using biodegradable polymers, including diethyl fumarate and poly (propylene fumarate). In addition, a photocurable ceramic acrylate suspension was used to form a cancellous bone construct [22, 62, 102]. As another technique, printing resolution of the selective laser sintering (SLS) printing technique is dependent on the spot size of the laser and size of the powder particles. Both systems have the minimum printing resolution of about 400 μm and minimum void size of about 50 μm [102, 127]. The world's first computer aided manufactured mandible was implanted in a patient by Dr. Jules Poukens in 2012 in Belgium. In 2014, Choong et al. successfully implanted the world's first 3D printed titanium heel bone into a patient. CAD provides extraordinary opportunities for fabricating tailor made bone tissue scaffolds as this technology can fabricate structures of complex external shapes and complex interconnected architectures.

Complex and customized structures, which were fabricated by a variety of processing methods are shown in Fig. 25.6.

25.3 Drug Loaded Bone Scaffolds

As aforementioned, bone regeneration process needs some bioactive molecules besides the susceptible environmental conditions. In addition to these regular regeneration processes, the delivery of therapeutic agents into the damaged bone tissue has a great importance. Although there has been significant progress in developing of tissue targeted drug delivery systems which reach drugs to bone tissue in therapeutic dose remains as a challenge because of the unique anatomical features of bone [46]. To overcome this drawback, development of local drug delivery systems offers great advantages for treatment of bone disorders. The localized drug delivery systems facilitate high drug concentrations at the diseased site, prolonged and controlled drug delivery, enhanced drug stability, improved efficacy, little systemic

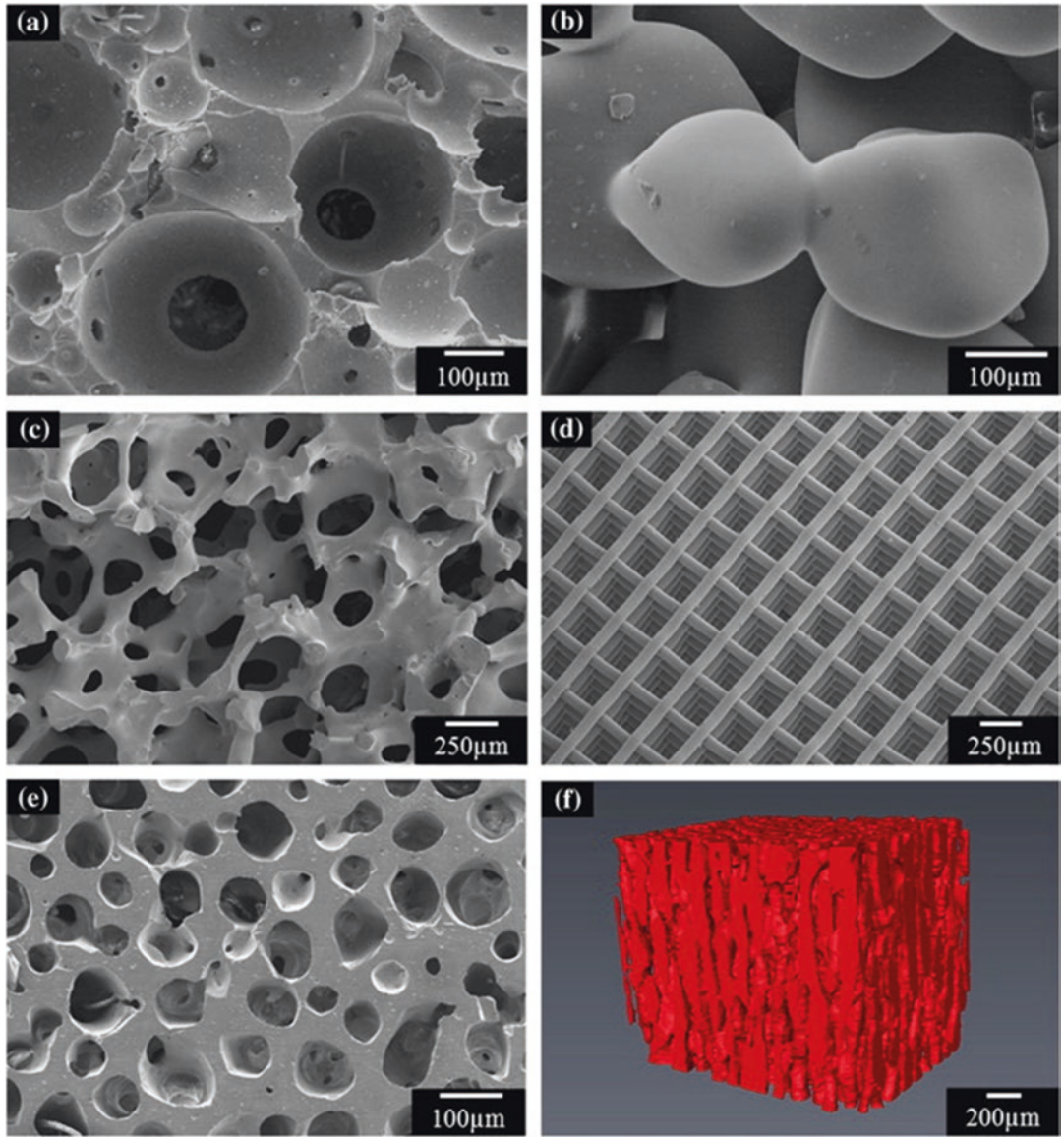

Fig. 25.6 Microstructures of bioactive glass scaffolds created by a variety of processing methods: (**a**) sol–gel; (**b**) thermal bonding (sintering) of particles (microspheres); (**c**) 'trabecular' microstructure prepared by a polymer foam replication technique; (**d**) grid-like microstructure prepared by Robocasting; (**e**) oriented microstructure prepared by unidirectional freezing of suspensions (plane perpendicular to the orientation direction); (**f**) Micro-computed tomography image of the oriented scaffolds in (**e**) [35]

uptake and reduced systemic toxicity risk [124]. Up to now, non-biodegradable and biodegradable materials have been widely researched as drug delivery implants [91, 101, 106]. Current techniques for bone tissue engineering fabrication enable the incorporation of drugs into tissue scaffolds and the regeneration of new bone tissue in the damaged area, as well as controlled and sustained drug release. Drug loaded bone scaffold should provide a suitable structure for bone regeneration and support and protect the drugs from environmental decomposition factors [107]. The drug loading can be obtained by direct mixing with scaffold material before fabrication [74,

112], by entrapping with a basic drug carrier and adding this system to scaffold material ([7, 82]), by coating the fabricated scaffold in a polymer or composite solution [54–56] and by impregnating on the fabricated scaffold [44, 61].

After fabrication, the drug loading efficacy, drug release patterns, drug activity and stability should be determined [77]. The drug-loading efficacy can be measured mainly by a convenient quantity measurement method for loaded drug and released drug. The amount of the scaffold should be precisely weighed before the determination of drug content [38, 88]. Although there is no gold standard for *in-vitro* investigating of drug-release profile yet, most of the methods are based on simulating the *in-vivo* conditions like body temperature, blood circulation, physiologic pH conditions. In literature, low volume of phosphate buffer saline (PBS) is used for dissolution media due to its similarity to body fluids. It would be preferred if the sink conditions and mimicking low blood flow speed around bone with low shaking speeds are ensured [52, 88, 99]. In order to trace the drug stability and drug-scaffold interaction after production procedures, glass transition temperatures (Tg) with differential scanning calorimetry (DSC), Fourier transform-infrared (FTIR) spectroscopy, nuclear magnetic resonance spectroscopy (NMR) and X-ray diffraction (XRD) analysis can be conducted before and after fabrication ([54, 55], Kim [52, 56, 88]).

The most commonly developed drug-loaded bone scaffolds usually contain antibiotics, anti-inflammatory drugs, bisphosphonates and anticancer drugs.

25.3.1 Anticancer Drugs

Osteosarcoma treatment is still challenging despite the improved technology in drug delivery, radiotherapy, and surgery. Most osteosarcomas still need both chemotherapy and surgical resection of the tumor site due to the spread or recurrence risk. Systemic chemotherapy may cause irreversible toxicity in some organs and inadequate treatment of osteosarcoma because of the difficulties in reaching therapeutic drug concentration at the tumor site. Therefore localized controlled drug delivery systems have increasing importance in osteosarcoma treatment. Anticancer drug loaded bone scaffolds offer the facility both treatment of tumor and also replacement of the resected bone tissue. That's why designing of these systems have a great interest recently.

Doxorubicin is a widely preferred chemotherapeutic agent in osteosarcoma treatment. Sun et al. produced doxorubicin loaded composite scaffold. At first stage, they fabricated PCL scaffolds with rapid prototyping technique. After that, they coated this scaffolds by immersing into doxorubicin loaded chitosan/nanoclay/β--tricalcium phosphate composite. They implanted the scaffolds and injected of the same dose of doxorubicin subcutaneously to the mice to evaluate the *in-vivo* release behavior of doxorubicin from scaffolds. The fluorescence imaging of the implanted areas showed prolonged release from drug-loaded scaffolds. The determination of plasma concentrations showed three-fold lower peak plasma concentrations from scaffold system compared to injection group. These results indicated that the systemic side effects decreased with scaffold system while the local concentrations enhanced [109].

Cisplatin is one of the most effective anticancer agents. However, its utilization is limited due to its poor water solubility and stability. Andronescu et al. prepared cisplatin loaded collagen/HA composite scaffolds by the freeze-drying method. A controlled release of cisplatin occurred after a burst release *in-vitro* release studies. To determine the cytotoxicity and anticancer effects they used G292 human osteosarcoma cell line. The results proved the cytotoxic, anti-proliferative and anti-invasive activities of the drug-loaded composite scaffold system [1].

Hess et al. developed a combined anticancer drug-loaded bone scaffold to enhance the anticancer efficacy. They decided to combine doxorubicin and cisplatin because of their common use as combination osteosarcoma therapy. After preparation of cisplatin and doxorubicin loaded TCP beads one by one they incorporated the beads in HA matrix by freeze gelation technique.

The drug release from scaffolds was monitored over 40 days, and CDDP showed a short burst release while DOX had a continuous release. *In-vitro* cell viability tests conducted with MG-63 osteosarcoma cell line. The results showed enhanced cell toxicity with co-loaded scaffolds compared to single drug loaded scaffolds which indicate that combined drug loaded scaffolds are promising thanks to their synergistic effect [45]. All these studies show that in the near future, replacement of bone tissue after resection can be provided in addition to effective local anticancer therapy in osteosarcoma.

25.3.2 Bisphosphonates

Bisphosphonates, stabilized analogs of pyrophosphates, inhibit bone resorption. These class of drugs enhances osteocalsification by reducing the activity and potency of osteoclasts and increasing their apoptosis. Bisphosphonates are the primary preferred treatment option for osteoporosis, but their oral bioavailability is poor, which decreases their bone concentration in therapy compared to oral given doses [30]. Bisphosphonates loaded bone scaffold systems are promising in case of increased local bone concentrations.

Alendronate is one of the most commonly used drugs for osteoporosis. Tarafder and Bose et al. fabricated TCP scaffolds by 3D printing and coated this scaffolds with alendronate dispersed PCL solution. *In-vitro* release studies showed sustained and prolonged release over 168 h. They also conducted *in-vivo* studies on Sprague-Dawley rats. The scaffolds were implanted into the femoral defect sites. After 6 weeks bone formation enhanced in drug loaded scaffold implanted rats compared to blank scaffold group, which proved by histomorphology and histomorphometric analysis [114].

Zoledronic acid is another widely used bisphosphonate class of drug. Locs et al. prepared bioglass based scaffolds by foam replication method. Then, they impregnated the scaffolds into zoledronic acid dissolution in water and coated this system with PLLA by immersing in the polymer solution. *In-vitro* release, studies showed the extended release of zoledronic acid with the PLLA coating. The PLLA coating also contributed to the mechanical functions of the composite scaffold [69].

In another study, Guo et al. developed ibandronate loaded collagen sponges by lyophilization and obtained controlled short-term release profile over 150 h. After *in-vitro* studies they implanted the scaffolds into femoral fracture sites of Sprague-Dawley rats. The rats were euthanized after 4 and 12 weeks to evaluate the bone regeneration. The radiographs and histopathological evaluations demonstrated that the ibandronate loaded scaffolds improved the fracture healing [40]. The recent studies in the literature about bisphosphonate loaded bone scaffolds have supporting results in enhanced bone regeneration.

25.3.3 Anti–inflammatory Drugs

Osteoarthritis is a progressive joint disease characterized by inflammation of the joints, deterioration of cartilage tissue, and loss of synovial fluid. The disruption of the environmental tissue can result in surgical resection of the damaged area. The main therapeutic agents for the pharmacologic treatment of osteoarthritis include oral and topical NSAIDs, and intraarticular glucocorticoids [12, 23, 37]. Oral therapy with NSAIDs is limited because of the cardiovascular and gastrointestinal side effect risks. To overcome these side effects and provide support for new bone tissue formation anti-inflammatory drug loaded bone scaffold are of great attention.

Dexamethasone is a preferred NSAID in designing bone scaffold because of its osteogenic differentiation-enhancing ability addition to its anti-inflammatory effects. Chen et al. prepared physically loaded dexamethasone biphasic calcium phosphate nanoparticles and fabricated the composite scaffolds by freeze-drying method after the dispersion of the nanoparticles in collagen solution. They added to the composite solution ice particles to obtain the porous structure. *In-vitro*, dexamethasone release studies was con-

ducted and sustained and prolonged release was obtained over 35 days. *In-vitro* cell culture analysis proved the scaffolds good biocompatibility and osteogenic differentiation ability for human mesenchymal stem cells. *In- vivo* studies was conducted by subcutaneous implantation of scaffolds to mice and resulted in increased cell attachment and proliferation [19].

Farooq et al. presented piroxicam loaded electrospun chitosan/PVA/HA nanocomposite scaffold for periodontal regeneration. The scaffold swelled after submersion in the PBS solution which is a necessary property for physiological conditions. Piroxicam released from scaffold in a controlled manner in 24 h. *In-vitro* cell culture results showed that the scaffold had biocompatible and proliferative feature [31].

25.3.4 Antimicrobial Agents

Osteomyelitis has an increasing incidence because of the predisposing conditions like diabetes mellitus and peripheral vascular diseases [42]. In addition to these entire disorders, medical device related osteomyelitis (MDRO) after surgical operations remains still as a drawback. MDRO results in the removal of the implants because of the difficulties in treating the infection due to poor vascular perfusion, bone necrosis, and increased antibiotic drug resistance [113]. Local antibiotic-loaded systems are getting more attention in recent years thanks to their localized antibiotic release ability in high concentration around the infected area without systemic side effects and toxicity [50, 99, 100]. Preparing antibiotic-loaded bone tissue scaffolds to prevent infection and scaffold rejection due to infection is also being extensively investigated nowadays.

Antibiotic-resistant biofilm formation caused by methicillin-resistant *Staphylococcus aureus* (MRSA) infection on scaffold after implantation is a prevalent and difficult to treat infectious disease [100]. Vancomycin is an effective antibacterial agent against MRSA. Cheng et al. developed vancomycin loaded mesoporous bioactive glass (MBG)/PLGA scaffold via freeze-drying fabrication. The vancomycin release from scaffold lasted more than 8 weeks at *in- vitro* conditions in a controlled manner and showed strong antibacterial activity against MRSA and biofilm formation. In addition to antibacterial activity, the scaffold system was found cytocompatible and efficient in osteoblastic differentiation due to MBG related enhanced osteogenic activity [20].

Levofloxacin is a quinolone antibiotic with broad spectrum and can penetrate into both trabecular and cortical bone. Cicuéndez et al. prepared a levofloxacin-loaded mesostructured SiO_2–CaO–P_2O_5 glass wall based nanocomposite scaffold using rapid prototyping technique with the aim of destroying emerged biofilm and allowing bone regeneration at the same time. Levofloxacin was incorporated to the scaffold by impregnation method. This scaffold system showed pH-depended sustained levofloxacin release, which increased from physiological pH (pH 7.4) to acidic pH (pH 6.7 and pH 5.5) around the infected area. The antibiotic release pattern is founded able to inhibit *S. aureus* growth and to destroy the biofilm. Also *in-vitro* cell culture studies demonstrated adequate cell colonization without toxicity [21].

Gentamycin is a widely used antibiotic in the treatment of osteomyelitis due to its broad spectrum. Sezer et al. produced gentamicin-loaded β-tricalcium phosphate (β-TCP)/gelatin microspheres and dispersed them into PCL solution. The scaffolds were prepared via freeze drying-lyophilization method after pouring into cylindrical molds. The amount of gentamycin loaded microspheres in the scaffold affected on the mechanical properties. The mechanical resistance decreased as the number of microspheres increased because of the increased pore sizes, but it also led to a faster release of gentamicin. *In-vitro* release studies showed sustained gentamycin release behavior. Furthermore, the composite scaffold increased the cell proliferation [3].

25.4 Conclusions and Future Perspectives

As discussed in this chapter, the new bone tissue formation after an injury is a difficult process, especially if the bone tissue lost occurs as a result of disorders which are mentioned above. Although there has been significant progress in developing of tissue targeted drug delivery systems reaching to bone tissue is still a challenge because of the low blood circulation and anatomical properties of bone. The local drug delivery systems offer higher drug concentration at damaged area and controlled release of drugs. These favorable features facilitate protection of drug stability and reduce systemic side effects and toxicity thanks to the little systemic uptake. Novel tissue engineering approaches offer combining drug delivery strategies with tissue regenerative scaffolds. To the best of our knowledge, recent scaffold production techniques will have the great potential to be more efficient than traditional bone treatment methods.

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