Mamoni Dash *Editor*

Biomimetic Biomaterials for Tissue Regeneration and Drug Delivery

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ISBN 978-981-16-4565-5 ISBN 978-981-16-4566-2 (eBook)
https://doi.org/10.1007/978-981-16-4566-2

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Biomimetics is one of the most prevalent topics both in the field of Biomaterials and Materials Science. Having been worked for almost a decade in this field, it was inevitable to edit a book on this topic. Upon invitation to edit a book, I took this opportunity to bring together the knowledge available in the advanced approaches involved in developing biomimetic biomaterials.

The word Biomimetics was introduced and defined for the very first time by Otto Scmitt who very aptly put it as a point of view rather than a subject matter. Biomimetics is thus an approach towards solving problems upon utilizing theories from basic biological sciences. Nature and living beings have a smart and efficient means of repairing damages. The complete understanding and replicating the structure and functioning of the natural phenomenon was once considered a challenge; however, the last decade has greatly evidenced a substantial effort in understanding the biological systems and processes precisely. The comprehensive effort of macromolecular chemistry and understanding of the sophisticated process at the cellular and subcellular level have helped scientists to inspire and mimic these natural procedures for their benefits. The objective of this book is to provide in-depth insights into the novel and effective approaches that have been used to develop such biomimetic biomaterials. The topics covered range from tissue engineering approaches to drug delivery systems to the most recent 3D printing technology. Databases such a PubMed, Scifinder, Google Scholar, and Scopus have been used to search for most recent and relevant literature.

The contributions to the books have been designed in a way that the above mentioned diverse aspects of biomimetic biomaterials are covered. The contributors are chosen experts in their field across the globe. The first three chapters of the book discusses about the two important fields of biomaterials: tissue engineering and drug delivery. These chapters have covered the various biomimetic systems that have contributed to the field of tissue engineering and drug delivery. The highlight of the first chapter is the immunomodulatory aspect of biomimetic biomaterials in addition to the various techniques that are often used to prepare them. The second chapter describes an interesting approach to prepare these systems via exosome mimetics. In line with the second chapter, the third one discusses how membrane-coated systems can be a biomimetic approach. Another important area that is covered in the fourth chapter specifically deals with nanosystems that are targeted essentially towards

regenerative medicine and stem cell biology. Moving from approaches of preparing these biomimetic systems, the last two chapters deal with the characterization of these systems. The focus has mostly been on mechanical characterization of biomimetic biomaterials with a supporting contribution on advances in the field of 3D printing in developing them.

I am sure the book will contribute to the existing knowledge in the field of biomimetic biomaterials, and the readers will benefit by taking forward the information in the book.

The book was mostly prepared during the unfortunate pandemic which has put a constant shadow of doubt and anxiety all over the world. During the preparation of the book, we also lost a revered co-author Dr. Federica Chiellini who left us untimely. With a lot of hiccups the book is completed and thus deserves an acknowledgment to my team members who have been a constant support during this trying times, all the authors who despite the gloomy time kept their word and contributed in the most efficient way, all the reviewers who have done an excellent job in shaping the manuscripts, and the publishing team who have been commendable in their support and patience towards the completion of the book.

Bhubaneswar, Odisha, India Mamoni Dash

Acknowledgment

The editor would like to acknowledge the following reviewers:

Anna Maria Piras Department of Pharmaceutical Technology, University of Pisa, Pisa, Italy

Geert Jan Graulus Department of Biochemistry, Hasselt University, Hasselt, Belgium

Jansper Van Hoorick Xpect-INX, Roosdaal, Belgium

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Dr. Mamoni Dash is a principal Investigator at the Institute of Life Sciences (ILS), Bhubaneswar. Her research team is focusing on the development of rational designs for new biomaterials to regulate repair, regeneration, and drug delivery. Dr. Dash received her Ph.D. in Biomaterials from the University of Pisa, Italy. She pursued postdoctoral research under Prof. Emo Chiellini, BIOLab, University of Pisa, Italy, and subsequently at the Department of Organic Chemistry, Ghent University, Belgium, under Prof. Peter Dubruel. Her research interests lie in the interdisciplinary area of polymers, and her current focus is on the development of polymers as biomaterials for tissue engineering and drug delivery applications. She has published her research articles in peer-reviewed journals of international repute and authored or coauthored numerous book chapters. She holds five patents for commercialized products. Dr. Dash is a member of various international scientific societies and organizations, including the European Society of Biomaterials and the International Association of Advanced Materials.

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Biomimetic Biomaterials in the Tissue Engineering Perspective

1

Debyashreeta Barik, Koustav Kundu, and Mamoni Dash

Abstract

The aim of tissue engineering is to regenerate or replace functional tissues and organs. Biomaterials play a crucial role as scaffolding structures to realize the very aim of tissue engineering. Biomimetic materials are materials that are designed to mimic natural systems. This book chapter reviews the most updated approaches in the development of biomimetic systems aimed towards tissue engineering. Fundamental concepts such as biocompatibility and mechanical properties of such systems are discussed. The development of biomimetic systems involves different architectures such as hydrogels, fibers, and many more, upon using different processing techniques. A major discussion in the chapter involves immunomodulation upon using these biomimetic materials for tissue engineering and regeneration which will further help readers design better materials.

1.1 Introduction

Tissue engineering utilizes engineering fundamentals to fabricate tissues and studying the assembly of functional tissue from native or synthetic sources for its restoration and modification [1]. Each of the tissue-engineered parts has very

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M. Dash (ed.), *Biomimetic Biomaterials for Tissue Regeneration and Drug Delivery*,
https://doi.org/10.1007/978-981-16-4566-2_1

different and specific requirements. Hence, there is a need to understand the properties, principles, and requirements for their use in a particular application. An important approach of tissue engineering is developing biomaterials that can enhance the regeneration processes by delivering therapeutics agents, and also providing structural scaffolding that provides adequate mechanical properties to tissues. Additionally, the biomaterial ought to preferably degrade at an equivalent rate to the growth of new tissue at the site of implantation.

Nature has stimulated the human race for the improvement of technology. Many engrossing biomaterials with unpredicted properties have been apparent within the past few decades. Natural structures such as gecko feet, butterfly wings, lotus leaves, mollusc shells, water repellency of shark skin, the honeycomb structure of the beehive [2], brittle star optics, bird flight, mussel byssus have inspired scientists and led to the development of engineered "smart" biostructures. These organisms produce materials with unique features that go beyond conventional engineering. These characteristic properties are utilized by scientists to create bioinspired biomaterials. The use of these materials has been referred as biomimetic materials since they originate with ingenuity from nature [3].

Biomimetic biomaterials are usually designed and engineered for use in medical biotechnology applications [4, 5]. An important feature of a biomaterial is its biocompatibility, which means it remains inert in the host or it will not initiate a reaction with the host material [6]. For fulfilling this purpose, biomaterials should be non-toxic so that it does not lead to host rejection and also structurally it should have the structural strength and flexibility to fulfill its role in the tissue regeneration process. This book chapter provides an overview of recently developed biomimetic materials and also their biological functionality and their applications in various fields of research.

1.2 Biomimetic Polymers and Their Properties

The term biomimetic means developing materials that can mimic the functions of natural materials, in particular, a biological environment to elicit a desired cellular response, facilitating the fulfillment of their task [7, 8]. Similarly, biomimetic polymers mean designing polymers which can mimic the activities of natural bioactive polymers. Thus, biomimetic polymers imitate the life process in many ways [9].

Biomimetic materials and their design, for example, designing biomimetic composite materials are inspired from the structures already present in nature like the fiber structure of wood, bone structure, spider silk, the honeycomb structure of beehive, etc. Derivation from biology or emulation of nature is referred to as biomimicry and biomimetics is the approach of assimilating concepts that enhances sustainability much like nature.

Biocompatible materials mimicking naturally obtained biomaterial can be differentiated into (1) biological materials and (2) engineered materials [4]. Biological materials consist of complete or just a portion of a living structure

Table 1.1 Examples of polymers involved in biomimetic systems

Materials		References
Natural materials	Gelatin	[10]
	Collagen	[11]
	Hydroxyapatite	[12]
	Chondroitin sulfate	[13]
	Alginate	[14]
	Dextran	[15]
	Agarose	[16]
	Chitosan	[17]
Synthetic and hybrid materials	PEG	[18]
	PLA	[19]
	PEG/PLGA/PGA/PLA	[20]
	Elastin/polypeptide	[21]
	PVA/gelatin	[22]
	PNIPAM/gelatin	[23]
	PEG/chitosan	[24]
	PEG/HA	[25]

that accomplishes a natural function. These materials do not comprise either metals or ceramics or any synthetic polymers. On the other hand, engineered materials comprise of chemically synthesized biomaterials or are derivatives of naturally originated biological materials that mimic the inner biological environment but with enhanced features like increased strength and flexibility that acts as a substratum on which the biological materials can work in collaboration and perform its designated biological functions at the site of tissue regeneration. A biomimetic biomaterial for tissue engineering should be able to mimic certain advantageous features of the natural ECM to facilitate cell recruiting/seeding, adhesion, proliferation, differentiation, and neo tissue genesis. Here, in this section we will be discussing about two basic properties of biomimetic polymers, i.e., biodegradability and mechanical properties in brief (Table 1.1).

1.2.1 Biodegradability of Biomimetic Polymers

The biodegradability property of scaffold materials is an essential property equivalent to the neo tissue formation rate which can further serve the purpose of a template purpose [26]. For example, linear aliphatic polyesters such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers poly(lactic acid-co-glycolic acid) (PLGA) are usually used as polymers for the fabrication of scaffold due to their wide range of biodegradability along with biocompatibility property [19, 27]. But poly(ethylene glycol) (PEG) doesn't show a biodegradability nature rather it is a biocompatible material with related instinctive features to certain restrained tissues

such as cartilage [18, 28]. The best procedure to control this inadequacy is to fabricate copolymers of PEG with PLGA, PGA, or PLA [20].

The alternative techniques to recount biodegradation is the biomimetic way by synthesizing PEG-based biomaterial of polymers demonstrate degradation by specific enzymes, i.e., matrix metalloproteases (MMPs), which mimics the enzymatic biodegradability of collagen as well as other natural ECM components [29]. Degradation of such hydrogels can be done by the cell-secreted MMPs (such as collagenase).

1.2.2 Mechanical Properties of Biomimetic Polymers

Various tissues such as heart valves, blood vessels, and cardiac muscle bear unique elastomeric properties [30–32]. Apart from the elastic materials, those are obtained from the natural ECM [32], poly(ε-caprolactone) (PCL), and polyurethanes (PU) are examples of some of the synthetic polymers. PCL is a semicrystalline polymer that contains a very low glass-transition temperature and therefore is highly elastic at room temperature. PU has not only structural variation to achieve elastomeric properties [33] but it also has a major limitation for biomedical applications in the synthesis. There is a development of biodegradable PU or urethane-based polymers using less toxic diisocyanates [34]. These polymers have been investigated for different tissue engineering applications including vascular tissue engineering [35, 36].

Elastin is an ECM protein synthesized as tropoelastin (~70 kDa), with insoluble, polymeric properties which further provides various tissues in the body with the features of elastic recoil and extensibility [37]. According to, Dan Urry researched on elastin-like polypeptides (ELPs), in which the artificial polypeptides are obtained from the pentapeptide repeat Val-Pro-Gly-Xaa-Gly (VPGXG) found in the hydrophobic domain of tropoelastin [21]. Certain elastin-mimetic have the property of self-assemble into thermoreversible hydrogels by involving triblock (hydrophobic-hydrophilic-hydrophobic) [38]. ELPs are propitious scaffolding materials that can be used in various tissue engineering $(GB1)_8$ and have excellent elastomeric properties [39, 40].

For various tissues, such as ligaments, tensile modulus, and tendons are very crucial. Silk, which is obtained from silkworm cocoon, is a natural fibrous protein and are utilized for surgical sutures and textile production due to its great tensile mechanical properties along with excellent scaffolding material properties for ligament and tendon tissue engineering [41].

Collagen is a naturally derived ECM protein having a triple-helical structure that can be reconstituted into a fibrillar matrix that possesses sufficient mechanical proprieties [11]. Collagen binds to several cellular receptors that can modulate cell's behaviors by increasing the bioactivity of the matrix [42].

1.3 Biomimetic Matrices

Polymers are being used extensively to synthesize potential biomimetic materials in the domain of tissue engineering and regenerative therapy [43]. Hydrogels, scaffolds, and fibers have emerged as the center of attraction due to their mechanical strength to promote the adhesion, growth, proliferation, and differentiation of cells. A major purpose of these materials is to replace or regenerate any damaged tissues or organs.

1.3.1 Hydrogels

Synthesis of hydrogel with natural or synthetic polymers gives the access of incorporating functionalities that resemble the environment of extracellular matrix [44, 45]. To design a hydrogel, there are a few factors that need to be taken care of such as the microenvironment of cells [46], the mechanics of the ECM [47] which will aid the cell adhesion, growth and proliferation, the biocompatibility of the material as well as cytotoxicity. Depending on polymers, the hydrogel matrix can be tuned using different crosslinkers which in turn vary the degree of crosslinking and subsequently dictate various properties like the mechanical strength, water uptake capacity of that material [48–50]. Surface morphology, as well as the microstructure of the hydrogels, plays a crucial role in the cell–environment interaction [51]. The porous structure of the hydrogels has its importance to diffuse oxygen and nutrients, which in turn are responsible for the survival of cells, their growth, and proliferation. Regulation of the interconnectivity and size of the pores dictates the properties of engineered tissue. There are several techniques proposed for this purpose. Methods using a sacrificial crystal have been studied where the hydrogels have been synthesized incorporating the porogens [52], followed by leaching, to generate a continuous network of polymer with uniform 2D or 3D pores. Salt leaching is one such process where NaCl has been used for synthesizing several polymer-based hydrogel systems like poly(ethylene glycol)/poly(ε-caprolactone) [52], alginate-g-poly(*N*-isopropylacrylamide) [53], poly(2-hydroxyethyl methacrylate) [54], and oligo [(PEG) fumarate] [55]. This technique has been studied for a variety of polymers like HA, alginate, chitosan, PEG-diacrylate, those commonly used for tissue engineering applications [52]. The usefulness of this method is that the pore size can be regulated by the size of the crystals, but the distribution of pores and the interconnective nature cannot be controlled in this process [50].

Another widely used method for the generation of pores in polymer-based hydrogel and scaffolds is lyophilization. Here, first the solvent molecules present in the synthesized material are converted to the solid phase by lowering the temperature at −20 °C or −80 °C or sometimes even as low as to −196 °C (using liquid nitrogen) [56], followed by sublimation of the solid phase using a vacuum [57]. For a wide range of natural and synthetic polymer-based hydrogels such as porous collagen/chitosan hydrogel [56], in agarose hydrogel (for generating linear-gradient pores) [58] and so on, lyophilization technique has been used. Scaffolds with

unidirectional pores have been synthesized by this freeze-drying method where the size and direction of the pores have been controlled by controlling the amount of solvent and size of the ice crystal. Glutaraldehyde-crosslinked gelatin scaffolds with microtubule orientation structure has been synthesized by freeze-drying process and uniaxial temperature gradient by liquid nitrogen has been used to generate such pores [59]. Although this process is quite straightforward, the drawback of it lies in controlling the pore size and weak mechanical properties of the fabricated materials [50].

Gas foaming is another useful technique in which gas bubbles, formed either via decomposition of chemicals incorporated with the polymers [60] or by the release of gas from a saturated gas-polymer mixer made at high pressure, are responsible for the generation of porous architecture in hydrogels [61]. Sodium bicarbonate, ammonium carbonate, etc. are a few commonly used foaming/blowing agents that disintegrate chemically to generate gas. Sodium bicarbonate that generates CO_2 in mildly acidic conditions has been employed for macroporous PEG-diacrylate hydrogels [62] and for the system this method has been found to improve the cell uptake, proliferation, and also promoting the mineralization of human mesenchymal stem cells (hMSCs) without any further functionalizing of the material with RGD sequence [62]. Ammonium carbonate has been used in synthesizing carboxymethylcellulose sodium/polyacrylamide hydrogels where the blowing agent decomposed into ammonia and CO_2 upon electron beam irradiation. The use of ammonium carbonate helps to generate interconnected pores and enhance the swelling ratio of the hydrogel [63]. With easy availability as well as being less expensive, the blowing agents are an obvious choice for designing porous materials [51]. Dense gas CO_2 has also been used to generate pores in several synthetic polymer-based hydrogel systems [51].

Another important property of the hydrogel systems is the ability to uptake water molecules. This characteristic is quantified by the swelling ratio of the material. The extent of crosslinking is responsible for this property. It has been found that the attachment and proliferation of cells are dependent on the bound water content of the hydrogels, as higher bound water content facilitates the adsorption of protein molecules of ECM and the cellular infiltration [52]. Hydrogel-based scaffold systems also act as a suitable 3D material to study the progression of a disease, as the properties of the materials can be tuned to study a particular cell line [59]. Photo crosslinked gelatin methacrylate hydrogels have demonstrated as systems to study the invasiveness and chemo-response, as well as some advanced disease state like metastasis of breast cancer cells [64]. In another study, a combination of thiol functionalized hyaluronic acid (HA) and thiol-reactive, protein mimetic hybrid copolymer (poly RGD-AC) has been applied as a biomimetic matrix for studying prostate cancer cells. This type of material is useful to analyze the engineered tumor model and provide information in cancer therapeutics [64]. Hyaluronic acid (HA) and its derivatives are commonly used polysaccharides for the synthesis of hydrogels. Being a naturally occurring component of ECM [65], HA can interact with the receptors present on the surface of the cells and HA binding proteins to facilitate the cell adhesion, migration, and proliferation [66]. Photopolymerized

hydrogels of methacrylated hyaluronic acid using sacrificial crystal are potential material for nerve tissue regeneration [65].

1.3.2 Fibers

A very efficient process for fiber and nanofiber-based scaffold fabrication is electrospinning, which serves as a potential material in tissue engineering application. Fibers having a diameter in nanometers can be produced and the formation of mess-like architecture can facilitate the adhesion, growth, and proliferation of cells seeded on the material. These fiber-based scaffold systems have gained more interest due to their morphological resemblance with the extracellular matrix [67].

A few properties of the fibrous material, like distribution of pores, porosity, and high surface to volume ratio can be controlled precisely by the electrospinning technique (B). Polymers like collagen [68, 69], elastin [68], silk [70], fibrinogen [71], and chitosan [72, 73] have been studied as a suitable material for synthesizing nanofibers.

Type I collagen derived from calfskin and type I and III collagen derived from the human placenta has been used to produce scaffolds of nanofiber having a diameter within 100–250 nm [69]. Collagen is a major component of the natural ECM and that provides structural and biological integrity to the material and makes this scaffold biomimetic which further can be applied in several tissue engineering applications. The effect of solvent and concentration of collagen has also been realized over the final architecture of the material [69]. Human mesenchymal stem cells (hMSCs) seeded on electrospun nanofiber-based scaffolds of silk fibroin [70] has been reported in bone tissue engineering. With the incorporation of HAP and a morphogen, bone morphogenic protein 2 (BMP-2) in the matrix, has been found to enhance the mineralization of bone tissues. Carboxymethyl chitosan (CMCS) nanofibers have been studied for mouse bone marrow stromal cells (mBMSCs). Due to binding tendency of CMCS with Ca^{2+} ions, a biomimetic coating of hydroxyapatite (HAP) can be done with simulated body fluid (5×) and the osteoblastic differentiation was compared between CMCS and CMCS-HA composite nanofibers. The CMCS-HAP composite scaffold has been concluded as a potential material for bone tissue engineering [74].

PCL nanofiber-based scaffold has been studied extensively for various cell lines and found out to be a potential material for various biomedical applications. PCL nanofiber-based scaffolds, seeded with MSCs derived from the bone marrow of neonatal rats, realized as a potential material for bone tissue engineering, as they facilitate the formation of mineralized tissues [75]. A potential design in cartilage tissue engineering has been realized for PCL nanofiber system seeded with human bone marrow-derived MSCs as with the incorporation of transforming growth factor-β (TGF-β), the scaffolds showed enhanced chondrogenesis compared to the cell plate (CP) culture [76]. The system has also been studied for FBCs, and the material was found to support the differentiation state by maintaining the chondrocytic phenotype even after 21 days, due to the biomimetic nature of the

scaffold [76]. Selective differentiation of human mesenchymal stem cells (hMSCs) has also been studied with the PCL system. The nanofibrous scaffold favored the multilineage differentiation, namely, adipogenic, chondrogenic, and osteogenic differentiation from a single cell source [77], which would be beneficial as this was achieved within the same system. In another study, a polymer blend of PCL/gelatin has been used to fabricate the mess of nanofibers, and it has been found to promote nerve cell differentiation and proliferation [78]. A 3D matrix with PCL nanofibers along with collagen nanofibers has been studied in skin tissue engineering as a dermal substitute. The collagen nanofibers here promoted the cell–matrix interaction and hence by facilitating the attachment, migration, and proliferation of human dermal fibroblasts seeded on the scaffold, it was realized as a potential material for wound healing [79].

Aligned and randomly oriented polyurethane nanofibers have been studied for human ligament fibroblasts (HLFs). The aligned orientation serves as a biomimetic structure and has been found out to be a potential material for tissue-engineered ligament due to the increment of ECM production [80]. Another potential material, a biodegradable scaffold of aligned poly(L-lactide-co-ε-caprolactone) in 75:25 ratio copolymer fibers has been studied for directional growth of human coronary artery smooth muscle cells (SMCs) and realized to be applied in blood vessel engineering [81]. Micro- and nanofibers of poly(L-lactic acid) (PLLA) have been prepared for in vitro evaluation of neural stem cells (NSCs). A direction growth of the cells parallel to the alignment of the fiber was found with a higher differentiation rate for the nanofibers than the microfibers [82].

Antibiotic cefazolin-loaded poly(lactide-co-glycolide) (PLAGA) nanofiber matrix has been studied for wound healing purpose [83]. PLAGA nano/microfiber scaffolds with a wide range of diameter have been designed and studied for skin tissue engineering. Fiber matrix with a diameter between 350 and 1100 nm has been found to accelerate the proliferation of seeded human skin fibroblasts (hSFs) significantly and within 28 days multilayers of fibroblasts were formed [84].

1.4 Methods of Processing Biomimetic Materials

In this era, implants have gained significant importance in the biomedical field. Numerous metallic implants are being clinically used for different purposes particularly in the case of bone tissue regeneration. Due to good mechanical properties, wear and corrosion resistance, biocompatibility and osseointegrity, metallic implants made of 316L stainless steel, cobalt-chromium (CoCr) alloys, and Ti-based alloys are being used vastly in orthopedic applications [85]. The biocompatibility of the implants is a very crucial consideration as they stimulate the immunological responses as soon as they come in contact with tissues. The foreign body response system tries to excrete out the material by opsonization [86] followed by a phagocytic activity. For implants, generally, a layer is formed via encapsulating collagen fibers that have been produced by fibroblasts and depending on the nature of the metals and alloys the thickness of the fibrous coating varies [85]. Hence, the implants

Table 1.2 Natural and synthetic polymers used in designing various biomimetic matrices

Materials	Polymer	References
Hydrogels	Poly(ethylene glycol)/poly(ε-caprolactone)	[52]
	Alginate-g-poly(*N*-isopropylacrylamide)	[53]
	Poly(2-hydroxyethyl methacrylate)	[54]
	Oligo [(PEG) fumarate]	[55]
	Collagen	[56]
	Chitosan	[56]
	Agarose	[58]
	Gelatin	[59, 64]
	PEG-diacrylate	[62]
	Carboxymethylcellulose sodium/poly acrylamide	[63]
	Hyaluronic acid	[64, 65]
Fibers	Collagen	[68, 69]
	Elastin	[68]
	Silk	[70]
	Fibrinogen	[71]
	Chitosan	[72, 73]
	Carboxymethyl chitosan	[74]
	Poly(caprolactone)	[75–77]
	PCL/gelatin	[78]
	PCL/collagen	[79]
	Polyurethane	[80]
	Poly(L-lactide-co-ε-caprolactone)	[81]
	Poly(L-lactic acid)	[82]
	Poly(lactide-co-glycolide)	[83, 84]

are kept separated from the target bone; so, for the proper adherent of implants, this biocompatibility barrier is first required to overcome. Osseointegration of the implants is another major factor as primary interaction between bone and implants depend on this [87]. Due to the large difference in elastic modulus of the implants and human bones, the interface is often affected and that hinderss the initial osseointegration [88].

This is why the surface of the implants is needed to be modified or functionalized with some bioactive material that makes it biomimetic and also facilitates the interaction with the natural bones. Hydroxyapatite (HAP) is one of the major components of our bones that provide strength to bones. An extensive study has been done on bioactive ceramics such as hydroxyapatite, bioactive glass, and wollastonite [89] to be coated on the metallic implants to enhance their properties. Coating with HAP has been proven to enhance biocompatibility as well as showing mechanical properties very similar to natural bones [90]. Here are some of the commonly used and commercially available techniques that are being used for the surface coating of the implants (Table 1.2).

1.4.1 Modification Using Different Plasma Techniques

Research on surface modification of materials and commercialization of the products, using different plasma techniques have been extensively done for the past two decades [91]. Modification of substrates to be used for biomimetic application is generally done by non-equilibrium low-temperature plasma as the temperature of equilibrium plasmas range between 4000 and 20,000 K. These temperatures would be destructive for surfaces especially polymeric materials as well as the coatings like HAP that decomposes at such higher temperature. For non-equilibrium plasmas, the ions or atoms in the plasma state can exist at low temperatures such as room temperature, but the processes of modification are done at relatively higher temperature [92].

One of the most common technique is plasma spraying in which the coating molecules are first converted into melted or partially melted forms and then sprayed on the surface of the substrate [93] (Fig. 1.1). Commercially available HAP [94, 95], tricalcium phosphate (TCP) [96], bioactive glass [97, 98], and calcium silicate [89] are a few such coating materials used in this method. Hydroxyapatite being a brittle and relatively weak ceramic decomposes into various phases such as TCP, tetracalcium phosphate (TTCP), and amorphous calcium phosphate (ACP) at higher temperature [90]. Derivatives of HAP-like chlorapatite which melts at a higher temperature without decomposing has been used as a coating material for Ti metal plates [99]. Doping of HAP with Mg and Sr has also been considered as the precursor for better adhesion, growth, and proliferation of cells [93]. Carbon nanotube reinforced HA coating on Ti-6Al-4V surface by plasma spraying method has found out to improve the fracture toughness and the crystallinity [100] (Fig. 1.2).

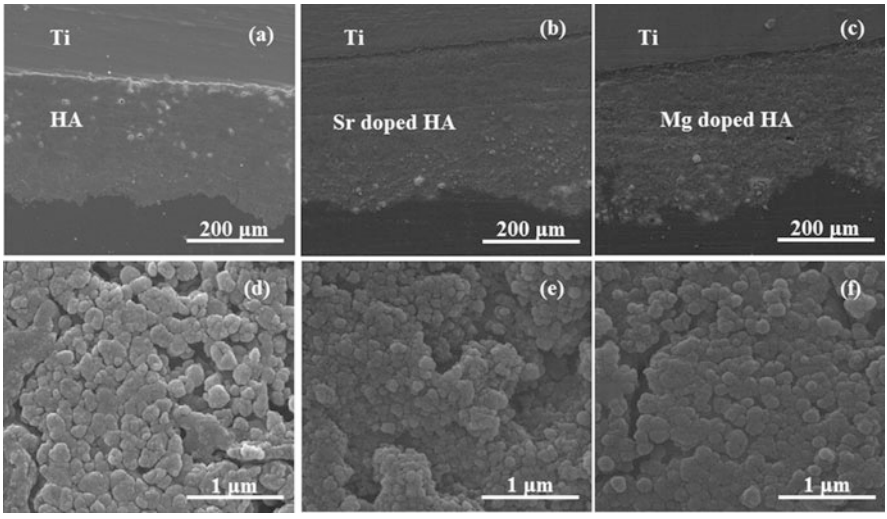

Fig. 1.1 Cross-sectional microstructure of (**a**, **d**) HA coating, (**b**, **e**) Sr-HA coating, and (**c**, **f**) Mg-HA coating. (Reproduced by permission of John Wiley and Sons [93])

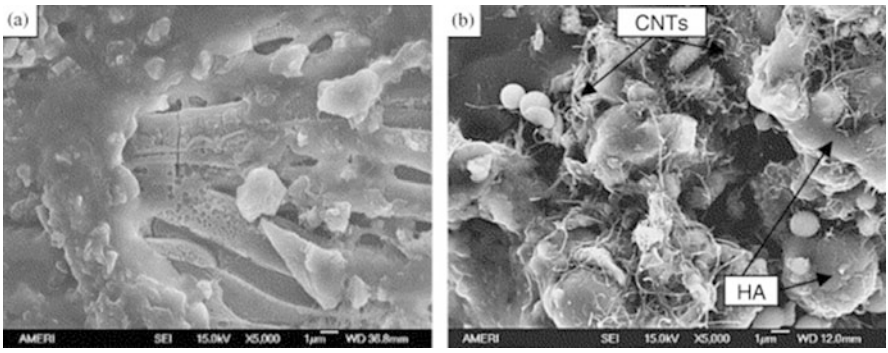

Fig. 1.2 Top surface of plasma-sprayed (**a**) HA coating without CNT, and (**b**) HA–4 wt% CNT coating depicting CNT distribution. (Reproduced by permission of Elsevier [100])

The thickness of the coating is tunable as a range of 50–200 μm has been reported in previous works [94, 95, 101]. Also, from the electron microscope studies of the surface morphology, it is seen that the coating molecules deposit on the surface without making any intermixed layer [93, 101] as in ion beam-assisted deposition (IBAD) technique and hence the adhesion of the coating layer is relatively less than the IBAD [91].

Another method called plasma polymerization has been reported to modify the surface of functional material in the case of bone tissue engineering. Using allylamine, acrylic acid, 1,7-octadiene, and ethanol as a precursor, a homogeneous thin film coating with a various functional group like amine, hydroxyl, carboxylic acid, and methyl can be generated which in turn promote the adhesion, growth, proliferation of cells, as well as promote osteogenic differentiation for human adipose-derived stem cells (hASCs) [102].

1.4.2 Ion Beam-Assisted Deposition (IBAD)

The ion beam-assisted deposition (IBAD) method [91] has widely been used to generate biomimetic coating for many metal-based implants, specifically for bone grafting. This technique is advantageous due to a unique way of adhesion between the substrate interface and the coating molecules [96].

The coating process is done using a polyfunctional IBAD system. For the deposition of hydroxyapatite (HA) and tricalcium phosphate (TCP) on a flat Ti-6Al-4V surface, the substrate is first cleaned with Ar^+ ion bombardment. Next, using an electron beam evaporator [103], the ionized particle-containing HA and TCP is sputtered with Ar^+ ion beam (3.5 keV), and the surface is simultaneously bombarded with another high energetic Ar^+ ion beam (30 keV), to generate a mixed interface containing both the surface molecules and the deposition molecules. After the formation of such intermixed layer, the energy of the Ar^+ beam (200 eV) is significantly lowered for the growth of the coating particles and reinforcing the

compactness of the deposited layer [104]. The temperature of the substrate during the deposition process is measured to be below 100 °C.

1.4.3 Ion Beam Sputtering Deposition (IBSD)

Despite the similarity in its name, this process is different from IBAD and is a type of physical vapor deposition (PVD) technique [105] where the deposition particles are ejected from a targeted material in the form of ions/atoms. In this type of deposition process, the substrate interface is never bombarded with high energy Ar^+ ion beam for intermixed surface formation. Rather after cleaning the surface of the material with an argon ion beam (500 eV and 40.7 mA/cm^2), an energetic Ar^+ ion beam is produced by highly pure argon gas (99.999%) and bombarded on the target material to initiate the sputtering of atoms [105]. The targeted material can be made of hydroxyl-poly-calcium sodium phosphate (HPPA) powder [106], hydroxyapatite (HAP) powder [107], bioactive glass [108], or any other biomimetic material which is the subject of interest of a particular case. The substrate is next subjected to the path of spattered atoms. As reported for the case of monolayer deposition of HA on the surface of pure Ti substrate the high energetic argon ion beam is applied at 900 eV and 20 mA/cm^2 for 3 h, 1200 eV and 40 mA/cm^2 for 90 min and 1500 eV and 60 mA/cm^2 for 60 min, respectively [107] (Fig. 1.3).

This method though has a few disadvantages. The adhesion between the deposited layer and the substrate interface is not very promising for the IBSD method as it has been reported that the adhesive strength of IBAS coatings is almost twice that of IBSD coatings [104]. Another problem is that, for the deposition of calcium

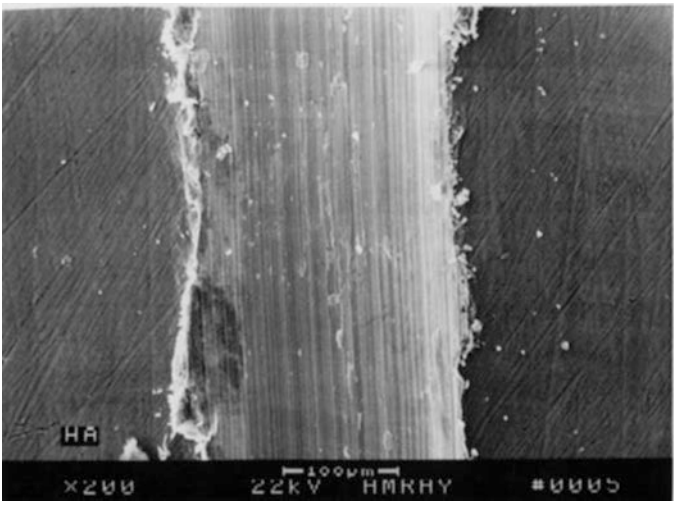

Fig. 1.3 SEM micrographs of scratch in the sputtering deposited Ca-P (Ar^+ beam sputtering deposited monolayer coating). (Figure permission reproduced by Elsevier [107])

phosphate, it has been seen that the coating formed by the IBSD method is amorphous in nature and hence to achieve the crystallinity the post deposited substrates are exposed under high temperature (600 °C for 1 h) to control the rate of dissolution of the coatings [105].

1.4.4 Electrodeposition of HAP/nHAP

Under the influence of an external electric field, charged particles present in a solution tend to move in a particular direction. This fundamental phenomenon influences the process of electrodeposition [109].

The electrophoretic deposition method as introduced almost two decades back is a process where a coating of hydroxyapatite (HAP) with a controllable thickness (in between 1 and 100 μm) can be obtained on metal-based implants like Ti, Ti-based alloys, and stainless steel. Commercially available or pre-synthesized (in the laboratory from its precursors) HAP powder is used as the electrolyte while the material on which deposition is to be done acts as the cathode and copper plate as the anode. A stable voltage is used for a definite time for the controlled deposition of HAP particles. Next for the HAP particles to bond and sinter on the surface of the metals implants, they are heated in a resistance tube furnace at a temperature ranging between 800 and 1000 °C [110]. This has an advantage over the plasma spraying technique in that the temperature is much greater as well as the possibility of decomposition of hydroxyapatite [96].

In another electrodeposition method of HAP or nanohydroxyapatite (nHAP) crystals on the material of our interest requires three-electrode cells coupled to Autolab equipment. The functional material acts as the working electrode, with a platinum electrode as an auxiliary and an Ag/AgCl electrode as a reference electrode. The electrolyte consists of $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$, maintaining the pH at 4.7 and the temperature at 70 °C. A constant potential of −2.0 V for 30 min is then required for the deposition of HAP/nHAP [111]. So, it does not need high voltages like in the case of electrophoretic deposition.

1.4.5 Biomimetic Deposition Using Simulated Body Fluid (SBF)

After the clinical trial of the bioactive ceramic coated implants discussed above, it has been observed that even after coating with these materials the implants can't replace some of the highly loaded bones like femoral and tibial bones present in our body. The reason behind such failure is due to the difference in mechanical properties of the bioactive ceramics and the natural bones. To overcome this problem, the formation of a bone-like apatite layer on the surface of the bioactive ceramics has been proposed [112]; so that proliferation and differentiation of osteoblast cells can happen and with the production of apatite and collagen [113], the surrounding bone interaction takes place with this apatite layer easily without any fibrous tissue formation around the implants. With the same ion concentration as of

the human blood plasma, Kokubo has introduced an acellular simulated body fluid (SBF) in which a homogeneous layer of bone-like apatite can be produced with the advantage of small crystallites of HAP which are easier to be degraded by osteoclasts [114].

In this method, the deposition of HAP/nHAP crystals on the material of interest is done by simply immersing the material in simulated body fluid (SBF). The SBF solution is a combination of NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $Na_2HPO_4 \cdot 2H_2O$, and $NaHCO_3$ [115] with varied concentration. The formation of the apatite layer generally takes between 1 and 4 weeks on metal or ceramic surfaces. So for any functional material that is easily hydrolyzed or not stable in water medium for that long, like scaffolds made of PLLA, it is necessary to use higher strength SBF rather than the normal strength to increase the rate of apatite formation [96]. Generally, SBF solution (5×: 5 times the normal strength) is used for this purpose. Depending upon the surface of the material the time of immersion is widely varied as for PGA fiber meshes and PLLA scaffolds, after 24 h of incubation at 37 °C, a continuous layer of particles (ranging from 200 to 400 nm) are found on the whole surface [116] (Fig. 1.4), whereas for the vertically aligned multi-walled carbon nanotubes films, the incubation period is of 14 days at 36.5 °C for the deposition of nHAP crystals [26]. Also, SBF (10×) has been used for epichlorohydrin (ECH) crosslinked hydroxyethyl cellulose (HEC)/soy protein isolate (SPI) bi-component scaffolds (EHSSs) for the precipitation of HAP [117]. Calcium/magnesium-free Dulbecco's phosphate-buffered saline with $CaCl_2$ dissolved in it has been used for the growth of apatite layer, on the surface of Ca-P thin films that has been already deposited on Ti discs by the IBAD method. Fibronectin has been incorporated to see if they retain their properties even after deposition with the apatite layer. The nucleation and rate of growth of the apatite layer depend on the crystallinity of Ca-P, duration of immersion, as well as the presence of fibronectin which not only retains their biological activity but also influences the morphology of the apatite layer [118].

In this method, the growth of HAP crystals sometimes requires a pre-functionalized or pre-modified surface to facilitate the apatite nucleation. As reported for Al_2O_3/ZrO_2 scaffolds, they are first treated with 5 M H_3PO_4 solution for 4 days at 90 °C before submerging into SBF (5×), to increase the surface area and the surface energy for more adhesion of HAP crystals on the bioinert scaffolds [119] (Fig. 1.5).

1.4.6 Other Methods

Composite-based one-pot synthesis method has been developed using cashew gum (CG) and cerium-doped HAP, in which chemicals similar as of the electrochemical deposition has been taken ($Ca(OH)_2$ and $(NH_4)_2HPO_4$), along with cerium (III) precursor ($Ce(NO_3)_3 \cdot 6H_2O$) for the doping process. A mixture of CG dissolved in an aqueous medium and the other three chemicals are stirred at room temperature for

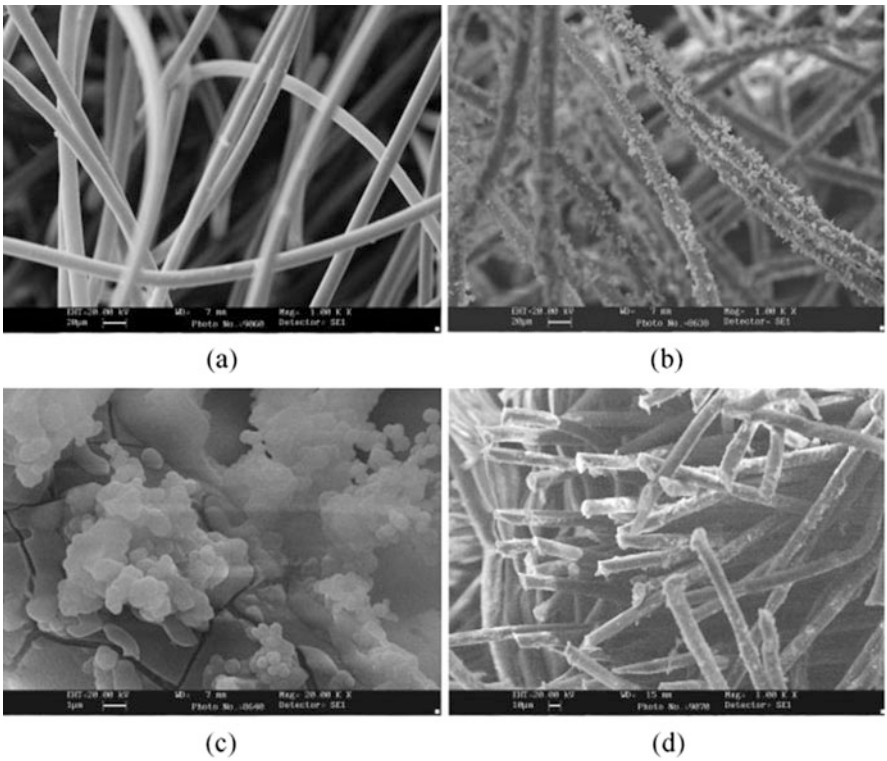

Fig. 1.4 SEM micrographs of PGA scaffolds before and after incubating in 5SBF for (**a**) 0 h, top surface, original magnification 1000; (**b**) 24 h, top surface, original magnification 1000; (**c**) 24 h, top surface, original magnification 20,000; (**d**) 24 h, internal midsection, original magnification 1000. (Reproduced by permission of John Wiley and Sons [116])

4 h followed by 12 h of incubation. The final material is obtained after centrifuging the solutions [120].

Polydopamine (PDA) functionalization can be done on thermoplastic polyurethane (TPU)/cellulose nanofibril (CNF) nanocomposite fibers by immersing and stirring the sample for 30 h, in Tris-HCl buffer solution containing dissolved dopamine. Self-polymerized PDA on the interface of substrate enhances the cell biocompatibility significantly due to the abundance of catechol and amine functional groups [121]. After the PDA coating substrates can be submerged into SBF solution for another coating of HAP, as has been seen for Ti6Al4V scaffolds [122].

Hydrothermal treatment was done for biomimetically coated Ti64 porous scaffolds by immersing the substrate first in H_2O_2, H_3PO_4 followed by $CaCl_2$ solution and subjected to hydrothermal reaction at 220 °C for 24 h and at 120 °C for 8 h, respectively [88].

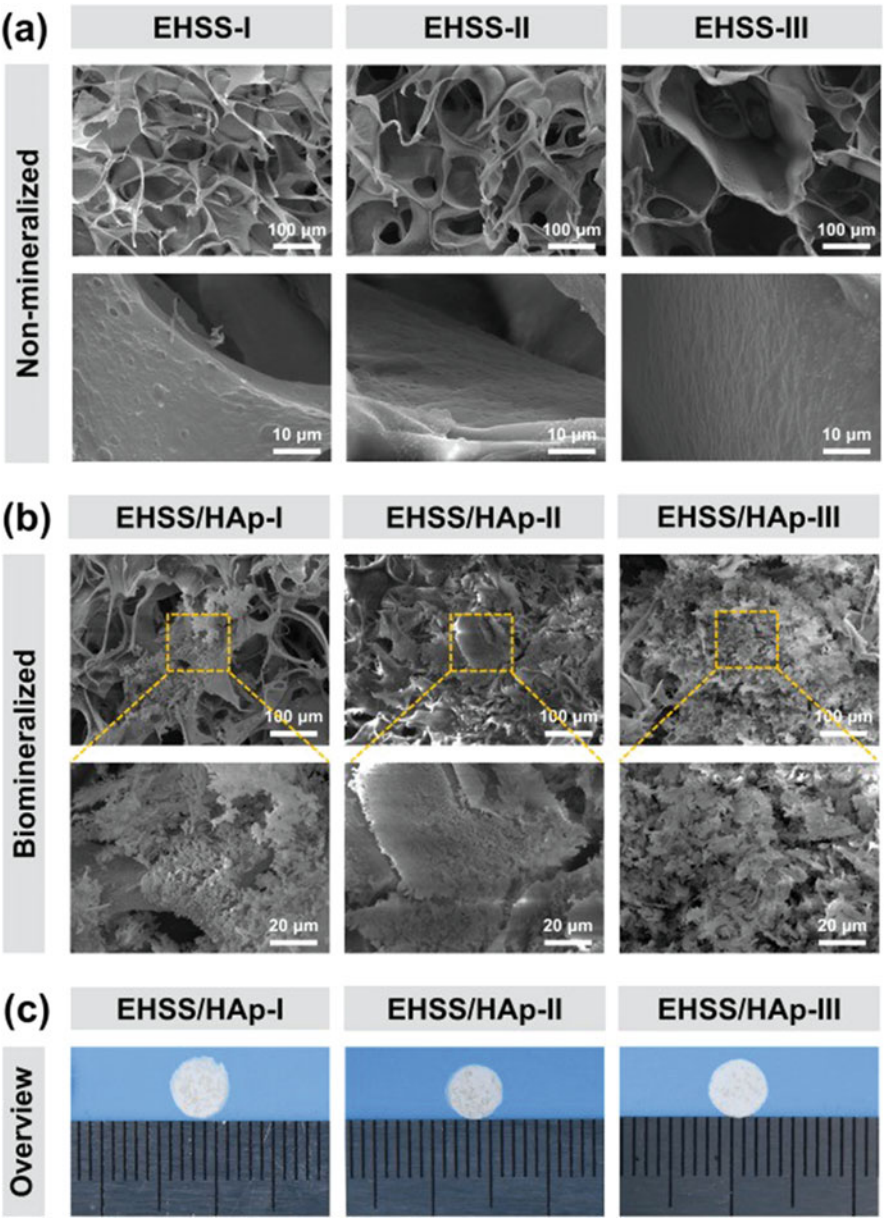

Fig. 1.5 Morphological characteristics of scaffolds before and after soaking in 10× SBF. (**a**) SEM micrographs of EHSS scaffolds prior to biomimetic mineralization. (**b**) SEM micrographs of EHSS/HAp scaffolds post-immersion in 10× SBF for a period of 24 h. (**c**) Digital image of EHSS/HAp scaffolds. (Reproduced by permission of Elsevier [119])

1.5 Immunomodulation Upon Using Biomimetic Biomaterials

An elementary principle of regenerative medicine technologies is creating a base where progenitor cells can develop into functional tissues to replace those lost to trauma or disease using well-designed biomaterials for therapeutic applications [123, 124]. The field of tissue engineering has engrossed the expansion of therapeutic strategies that either help in tissue regeneration by providing a 3D matrix or by sustained drug release at the site of injury [125, 126]. The field of material science has seen an expanding interest in reinforcing biomaterials which is capable of enduring interactions with the complex biological microenvironment of the human body [127, 128]. The immune system plays an important role in securing the human body from detrimental pathogens and foreign materials as well as understating the abnormalities within cells and tissues [129]. The process of conceding these alterations to homeostasis is indicated as inflammation. The inflammatory response is the clue for mobilizing immune cells to the diseased site and thereby initiating the healing process [130]. The fortuitous of implantable biomaterials articulate upon the potential of the chosen biomaterial to confer with the biological barriers in vivo along with maintaining a defined space for tissue engineering and supporting in cell growth. The most consequential of these barriers is the immune system, which is mainly comprised of a highly systematized organization of cells that induce an inflammatory response to the implanted biomaterial. Specific combinations of materials, cells, proteins, and/or genes is necessary to provide required microenvironment to promote tissue regeneration for a variety application Biomimetic platforms have been developed as novel approaches that intent to utilize the principle of biomimicry as a means of immunomodulation [124]. This principle has demonstrated itself in the form of biomimetic biomaterials that emulate the composition and structure of biological cells and tissues. Biomimetic biomaterials utilized in tissue engineering integrate natural components of tissues, including progenitor cells, growth factors, and extracellular matrix (ECM) structural components [131, 132]. Through deeper interpretation of the intrinsic immunogenic features of implantable scaffolds and materials brings advanced visionary chance to control tissue engineering and regenerative medicine applications in better way [133] (Fig. 1.6).

In the field of tissue engineering and regeneration biomimetic and biocompatible biomaterials has been developed since long back to remit biological cues or cells with unambiguous control over the localization, gradients, and sequence of various agents [135]. The biomaterials play an important role as delivery vehicles for in vivo immunomodulation, macroscale transient drug depots, biomaterial niches for in situ modulation of host immune cells, and ex vivo manipulated cellular payloads [136].

The neovascularization process is carried by macrophages. At the time of tube injury and repair, macrophages observe the injury website and contribute neovascularization and promote the recovery of blood flow [137]. The process of vessels maturation is taken care of by M2a macrophages whereas the growth of epithelial tissue cells is stimulated by M1 and M2c macrophages [138]. PEG or PLGA hydrogels deliver 1-phosphate receptor three (S1P3) agonist to induct

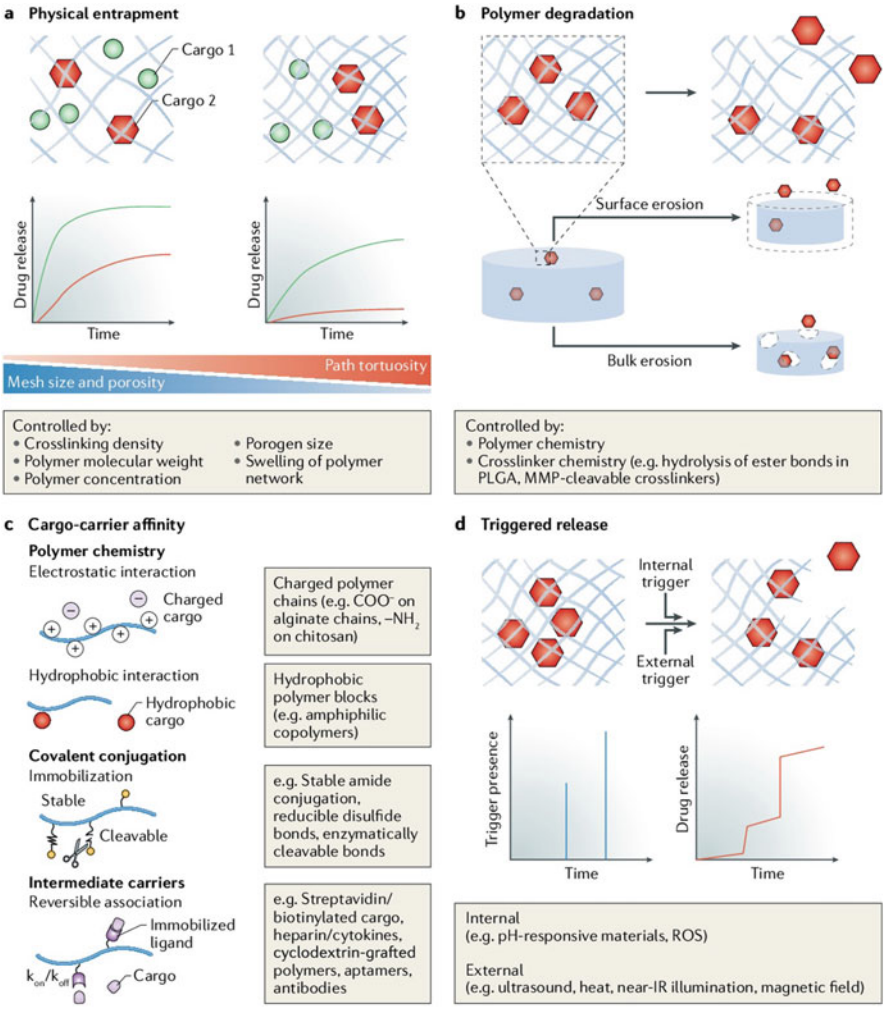

Fig. 1.6 Biomaterial scaffolds as biomolecule carriers. (Reproduced by permission of springer Nature [134])

medicine monocytes, which ends up in a discount in pro-inflammatory protein secretion, a rise in pro-regenerative protein secretion and vessel remodeling in inflamed and ischemic tissue. Immunomodulatory factors can even be enforced to attenuate fibrotic activity [139]. Similarly, glycoprotein, a urinary organ secretion are often delivered mistreatment scleroprotein scaffolds to stop internal organ pathology when infarction [140]. The selection of biomaterials plays a crucial role in delivering factors through biomaterials-based scaffolds as a result of foreign body reactions concerning the planted scaffolds will cause fibrotic capsule formation

encompassing the scaffolds. Decellularized internal organ electronic countermeasures, alginate and chitosan-based scaffolds conjointly help in reducing fibrotic capsule formation around planted scaffolds [141]. The other investigations in this field were, zwitterionic poly(carboxybetaine methacrylate) hydrogels preparation from a carboxybetaine monomer and a carboxybetaine crosslinker that shifted macrophage phenotype to an anti-inflammatory state leading to reduction of protein absorption [142]. This advantage is beneficial for future implanted biomaterial scaffolds designed to reduce fibrotic buildup and rejection due to implants [133].

For locally delivering immunomodulatory factors consisting of proteins (like antibodies, cytokines, etc.), oligonucleotides (like siRNAs and plasmids), and scavengers which helps in eliminating harmful factors like ROS from the site of injury or diseased tissue [143–145].

For using biomimetic scaffolds for delivering immunological factors to be used in tissue regeneration or cancer treatment, several factors and benchmarks need to be considered like the method of delivery, the process of releasing the immunological factors and its reuse or degradation and also the process of removing the materials from the target sites [134]. An extensive result in scaffold designing is the acquisition of less invasive needle or catheter injection and surgical implantation. The mandate thing that needs to be considered is the instinctive features of the materials, different type of polymerization techniques and crosslinkers as well as the size of scaffold and physical availability of the target site, also the in situ formation such as thermal gelling, or in situ crosslinking or injectable scaffolds can be taken into account [146, 147].

The immunogenicity of scaffold materials ought to be fastidiously thought-about to rehabilitate the specificity of therapeutic responses in conjunction with reducing overcritical medical specialty responses. Foreign body reactions (e.g., fibrotic capsule formation) to the surface of biomaterials will be remitted by the activity of the property of the scaffolds to produce associate antiparasitic plating or to intercept nonspecific binding of proteins [148]. Immune-cell-mediated responses to the biomaterial surface can further be modulated by varying the surface charge, topography, and roughness of the materials [149]. Finally, targeting inflammatory and immunomodulatory pathways through material style may also mitigate unfavorable foreign body reactions concomitant pathology [150] (Fig. 1.7).

The purposeful regeneration and repair of tissue is greatly intermediated by pro-inflammatory medication responses that embrace removal of broken tissue trash, revascularization, EW remodeling, and vegetative cell activation and differentiation. Delivering immunomodulatory factors that target these processes became a difficult therapeutic strategy. For example, a poly(lactic-co-glycolic acid) (PLGA) scaffold will be used for the delivery of associate isoform of resolvin D1 (AT-RvD1) to limit WBC accomplishment, recruit medication macrophages, and induce vascularization [151]. Water solubility or retention and delivery rates from scaffolds will be improved by adding with chemicals changed hydrophobic agents with polyacrylic acid or hydrolyzable linkages [152]. The improvement of clearance of pro-inflammatory agents like ROS results in a cut back of inflammation. Injectable hydrogels conjugated with pendant 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl

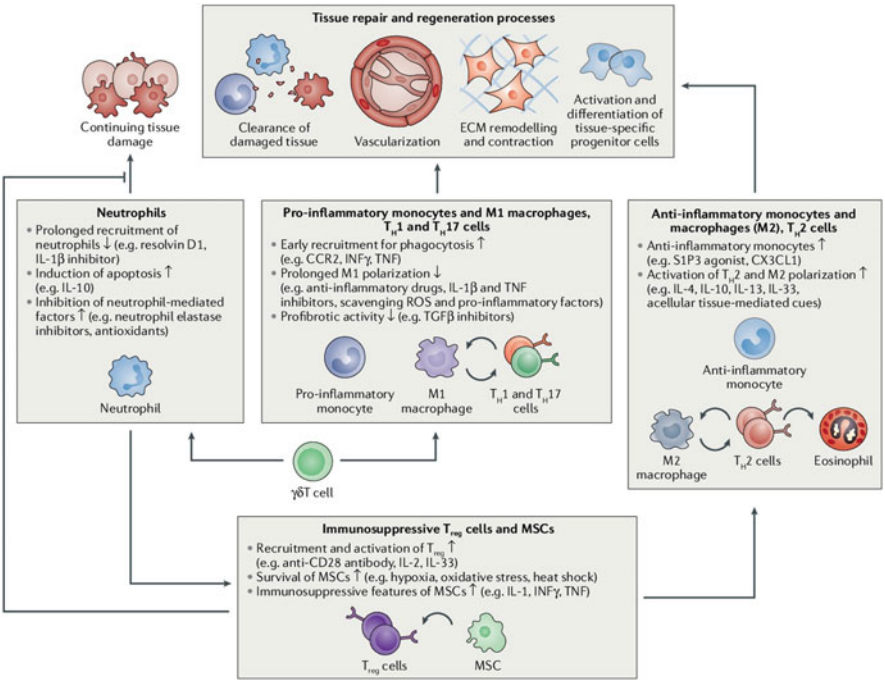

Fig. 1.7 Cellular targets for immunotherapy in tissue repair and regeneration. (Reproduced by permission of Springer nature [134])

(4-amino-TEMPO) teams, cyclohexylamine or reusable nitroxide radicals will be introduced to decrease death in associate infarcted heart muscle [153]. Conjugation of mucopolysaccharide hydrogels with associate antineoplastic mortification issue (anti-TNF) protein will cause a reduction in macrophagic infiltration and also the formation of dead tissue in skin wounds [154]. Similarly, polythene glycol (PEG)–maleimide hydrogels with a bound IL-1 receptor antagonist improve tissue responses to neural electrodes [155].

1.6 Conclusions and Future Perspective

Biomimetic biomaterials in the field of tissue engineering are obtained from materials synthesized and fabricated at the molecular and nanoscale that render specific cellular responses that demonstrate significant potential as analogues to extracellular matrix or as an artificial extracellular matrix for tissue engineering processes. Biomimetic materials are materials that offer good structural performance, biocompatibility, and biodegradability by introducing new biological or biomimetic materials with improved biological properties. In recent years, researchers have put continuous research efforts to overcome the challenges and

also towards the development of new biomimetic materials. The modification of biomaterials with bioactive molecules, coating, self-assembly, and other techniques have been useful to design biomimetic scaffolds that can provide biological cues to elicit specific cellular responses and direct new tissue formation. There are already known facts where physicochemical features alter the immunogenicity of biomaterials. Despite the recent advances towards the development of biomimetic materials for tissue engineering applications, several challenges are remaining including the design of adhesion molecules for specific cell types as needed for guided tissue regeneration and the synthesis of materials exhibiting the mechanical responsiveness of living tissues.

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Biomimetic via Exosome Mimetics in Regenerative Medicine and Therapeutics

2

Sasmita Samal and Mamoni Dash

Abstract

Over the years, the field of regenerative medicine and therapeutics has witnessed several modifications in terms of the delivery vehicle used and their complex engineering to achieve the targeted drug/cargo delivery. The intention to gain enhanced accuracy in the therapeutic usages is the major reason behind the change. The use of synthetic nanomaterials had gained momentum during the past few decades. However, due to the issues involved like poor biocompatibility, cytotoxicity, stability, etc. their use has been limited. That is why the modern day therapeutics has shifted its gear towards a more natural option of delivery system, which is termed as biomimetic. There are several options nowadays for the biomimetic cargo delivery vehicles which are summarized briefly in this chapter with a major focus towards exosomes has been given. Starting from their synthesis methods to their contribution in modern day medicine has been described briefly. Also a comparative analysis has been done among these nanosystems to prove the superiority of exosomes above all. Towards the end, the current challenges involved in these formulations have been depicted.

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M. Dash (ed.), *Biomimetic Biomaterials for Tissue Regeneration and Drug Delivery*,
https://doi.org/10.1007/978-981-16-4566-2_2

2.1 Introduction: Biomimetics and Their Formation

The term biomimetic refers to mimicking the native biological processes occurring inside a living system. With the advancement in biomaterials and therapeutic approaches, a huge number of synthetic nanosystems are being designed to achieve targeted cargo delivery, greater cell penetration ability, and stability inside the host cells [1]. However, due to the rise of a lot of issues regarding biocompatibility, cytotoxicity, bioaccumulation, and host tissue rejection, scientists are trying to design advanced nanosystems which are inspired by natural cell structure [2]. These novel cellular architectures provide us with the benefit of selective targeting as well as better biocompatibility. Some of these efficient biomimetic nanosystems are described below.

2.1.1 Exosomes as an Apt Example of Biomimetic System (How and Why)

Exosomes, the nanosized cell-derived vesicles, with a diameter range of 30–120 nm, originate from multivesicular bodies (MVBs) after fusion with plasma membrane. These lipid bi-layered vesicles are secreted from almost all types of cells and are involved in intercellular communication [3]. For the survival of an organism, intercellular communication and exchange of important information is a vital process which is seen to be taking place in all multicellular organisms. It can be mediated either through direct cell-to-cell contact or by specific receptor–ligand interaction or by the transfer of important information containing small secreted molecules. In the year 1987, Johnstone et al. found that during the maturation of mammalian reticulocytes, selective membrane proteins like transferrin receptors and some other membrane-associated elements are lost in the form of small vesicles (≈50 nm) released from multivesicular bodies (MVB, 0.5–1 μm), which they referred to as exosomes [4, 5]. In 2007, Valadi et al. confirmed that exosomes contain both mRNAs and miRNAs which show their function in the host cells. They called it as exosomal shuttle RNA (esRNA) [6]. After this discovery, considerable investigation has been done to understand the biogenesis and cargo loading into exosomes. Now, there exist huge evidences that exosomes are the natural carriers that are loaded with bioactive molecules, various proteins, lipids, and coding and non-coding RNAs (mRNAs, miRNAs) [7]. Many immune cell types, such as dendritic cells, lymphocytes, platelets, mast cells, epithelial cells, B cells, T cells, and tumor cells secrete exosomes [8]. Most of the body fluids such as blood, urine, saliva, amniotic fluid, breast milk, hydrothoracic fluid as well as cell culture medium of most mammalian cell types also release exosomes [6]. It is established that these nanovesicles play important roles in maintaining healthy cell environment by involving in several biological events like cell–cell communication, transport of various lipids, proteins and nucleic acids, and cellular metabolism. Exosomes also offer new paradigm in the field of therapeutics, disease diagnostics, and treatment [9]. Moreover, due to some favorable features like low immunogenicity,

biodegradability, non-toxicity, strong cargo-loading and cargo-protective capacity, exosomes are being exploited as potential nanocarriers [3, 7]. However, without specific membrane engineering and internal modification according to the target cell of interest, exosomes may not serve as a targeted delivery vehicle in some cases. So, before exosomes can be used in therapeutics, some modifications are necessary. There are two distinct approaches to make the therapeutic use of exosomes: first, therapies that exploit native biological functions of exosomes to mimic natural repair process, and second, drug delivery approaches that use exosomes as vectors to deliver therapeutic entities to the site of repair [10]. The study of exosomes mediated drug delivery system has gained more interest. Looking at the convincing role of exosomes as a therapeutic material for various platforms, we are interested in reviewing the role of exosomes in treating diseases through appropriate chemical modifications. It is being shown that exosomes can also be proved to be effective as drug delivery vehicles towards bone-related disorders and treatment of bone metabolic diseases by appropriate surface designing and drug loading. The existing common practices for bone regeneration are expensive, uncomfortable for the patients, and possess high risks of complications. An extensive amount of research on bone regeneration studies have focused on triggering the function of cells, but the cell-to-cell communication has not yet been completely understood.

2.1.1.1 Exosome Biogenesis

Understanding the pathways of exosome biogenesis and cargo-loading mechanism has opened up new ways to study exosome-mediated bone regeneration more precisely as well as the possibility of using exosomes for therapeutic purpose towards bone has increased. Knowing the factors responsible for exosome release has been proved to be important for altering their expression and modification of certain cargos to get the desired bio-inspired nano-formulation [11]. Out of the three classes of extracellular vesicles, exosomes are the smallest which are released to the extracellular environment by budding off from the plasma membrane after the fusion of late endosomes or multivesicular bodies (MVBs) [12].

In eukaryotic cells, the biogenesis of exosomes starts with the formation of a membrane-bound component called as endosome. At first early endosomes form, which eventually mature into late endosomes, during which ILVs (intraluminal vesicles) are produced by inward folding of endosomal membrane [13]. At this stage, the late endosomes along with ILVs are referred as MVBs (multivesicular bodies). These MVBs can have two fates; either they degrade by fusion with lysosomes, or merge with the cell membrane and release the ILVs to the extracellular environment [14]. These released ILVs are known as exosomes (Fig. 2.1I).

The formation of MVBs may involve a highly monitored pathway including ESCRT (Endosomal sorting complexes required for transport) machinery. ESCRT consists of four different protein complexes: ESCRT-0, -I, -II, -III, and some associated proteins with Vps4 complex [15]. ESCRT-0 forms a large cargo complex with the help of ubiquitin, ESCRT-I and II regulate bud formation and MVB maturation, ESCRT-III induce vesicle dissociation from plasma membrane and recycling of ESCRT machinery. Several Rab-family proteins (Rab27a, Rab27b)

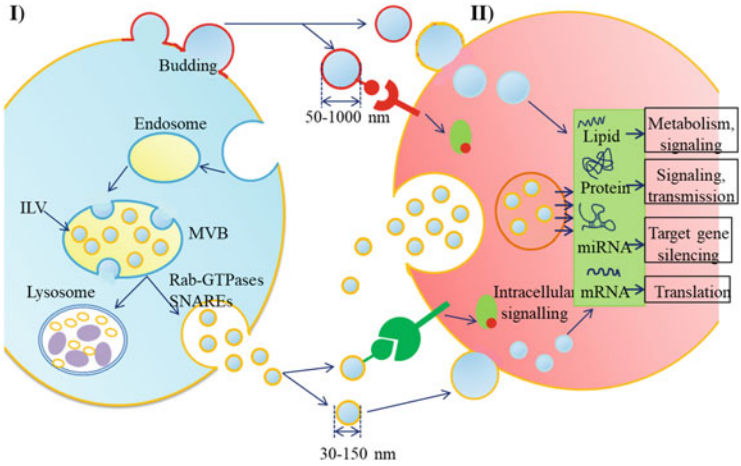

Fig. 2.1 Schematic representation of biogenesis of exosomes and host cell interaction. (I) Exosomes come out of the cells after formation of MVBs via ESCRT machinery. (II) They communicate with the neighboring cells and release their cargo thereby modulating the host cell machinery

also regulate exosome formation [15]. Several studies also suggest a different mechanism of exosome biogenesis which is independent of ESCRT pathway. This pathway involves tetraspanins, lipids, and heat shock proteins. Trans-membrane proteins like CD9, CD82, CD63, Tspan8, and heat shock proteins involve in exosomal release, whereas some lipids like ceramides, phospholipids induce ILV formation and exosome biogenesis [16]. Once the MVBs fuse with plasma membrane, the secretion of these nanovesicles is controlled by Rab-GTPases along with some other parameters like pH, Ca^{2+} concentration, etc. [17]

To induce any physiological changes, these cellular messengers interact with the recipient cells. Exosomes communicate with the neighboring cells mainly by three methods: (1) receptor–ligand interaction, (2) membrane fusion, and (3) receptor-mediated endocytosis. Through this interaction, exosomes hold the ability to transfer vital cellular messages in the form of proteins, transcription factors, genetic materials (e.g., miRNA, mRNA, siRNA), infection-causing agents, oncogenes, etc. [18] The transferred information can regulate host cell metabolism, translation, gene silencing, etc. (Fig. 2.1II).

2.1.2 Liposomes

As the Greek origin of the word suggests, “lipos” meaning fat, and “soma” meaning body, liposomes are spherical vesicles consisting of cholesterol and outer lipid bilayer, due to which they resemble the cell membrane [19]. The ability of liposomes to encapsulate both hydrophilic and hydrophobic drugs has allowed these vesicles to come up as a useful delivery vehicle [20]. The size ranges from very small

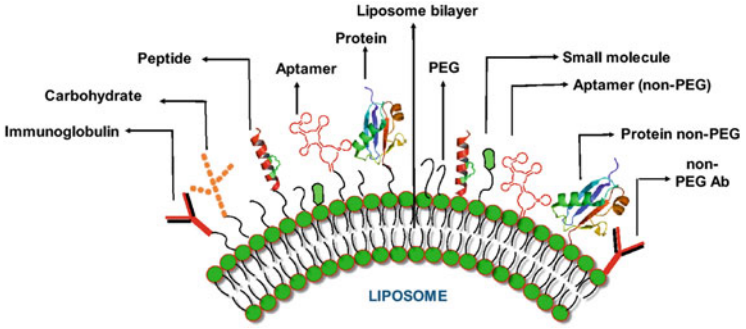

Fig. 2.2 Schematic illustration of liposome with different surface modifications achieved by using peptides, aptamers, small molecules, carbohydrates, proteins either by direct bonding to the phospholipid bilayer or through polyethylene glycol (PEG) [24]

(0.025 μm) to large (2.5 μm). Appropriate size and lamellarity of the vesicle is one of the key factors in determining the drug-loading efficiency and circulation half-life post administration [21]. Liposomes can be differentiated into unilamellar and multiamellar vesicles (MLVs) depending on the number of bilayers. Unilamellar liposomes have a single phospholipid bilayer surrounding the aqueous solution; whereas, multilamellar liposomes have several concentric phospholipid spheres separated by layers of water [22].

Liposomes are generally favorable choice for delivery vehicle as a wide number of therapeutics (both hydrophobic and hydrophilic) can be efficiently encapsulated inside the aqueous core [23]. These can also be surface modified with various targeting moieties for achieving active targeting (Fig. 2.2) [24]. Due to rapid clearance of conventional liposomes from blood stream, surface decoration with hydrophilic polymers has come into play. During the last few decades, various natural and synthetic materials have been developed such as polyethylene glycol (PEG) and poly(ethylene glycol)-linked phospholipids (PEG-PLs) to enhance the stealth property of liposomes [25]. To achieve targeted drug delivery, ligand-specific surface functionalization has been the most promising strategies [26]. Suitable surface engineering for targeting overexpressed receptors on cancer cells such as epidermal growth factor receptors (EGFR), fibroblast growth factor receptors (FGFRs), folate receptors (FRs), and transferrin receptors (TfRs) is a vital approach for actively targeting cancer cells at the tumor site [27].

Liposomes are also very good candidates for the delivery of bioactive agents. Pertaining to their variable structure with easy modification, they are feasible for accommodating hydrophilic molecule in their inner aqueous core, lipophilic molecules within the lipid bilayer and amphiphilic molecules [28]. The encapsulation of bioactive agents can be done passively during the formation of liposomes or actively by encapsulation after liposome formation [29].

Despite the wide use and successful clinical applications, liposomes also have some limitations such as low stability, heterogeneous size distribution, phagocytosis

and renal clearance, drug leakage from the bilayer, low solubility, high production cost, immunogenic issues, and reproducibility. [30]

2.1.2.1 Methods of Preparation of Liposomes

The important physical properties of liposomes can be regulated during their preparation by regulating the key parameters such as temperature, charge, lipid composition, organic solvents, and surfactants. There are both the traditional methods (Fig. 2.3) and recently introduced techniques in liposome preparation. The traditional methods usually include, dissolving the lipids in organic solvents, drying down lipids from these solvents, dispersion of lipid in aqueous medium, purifying the liposomes, and analyzing the final products [31].

(a) **Thin film hydration**
This is the oldest and the simplest liposome preparation method involving three steps. The first step involves dissolving phospholipids and cholesterol using volatile organic solvents like chloroform, methanol, or ether [32]. Then to remove the solvents, the lipids are deposited on glass bottom as a thin film to let the solvents evaporate by a rotary evaporation technique. For hydration, an aqueous buffer is added to the deposited lipids [33]. Despite the easy preparation method, the phospholipids produce heterogeneous MLVs. The used harmful solvents if not completely removed can be toxic affecting the stability of the end product [34].

(b) **Reverse phase evaporation**
In this method, lipid films are prepared by dissolving phospholipids in organic solvents and then the solvents are removed by vacuum evaporation. The thin film is then resuspended in organic phase like diethyl ether followed by addition of water to form reversed vesicles. The preparation is then sonicated for a brief period of time to form homogenous emulsions. The organic solvent is then removed under reduced pressure by placing on a rotating evaporator. As a result, large unilamellar vesicles (LUVs) are formed with high encapsulation efficiency (EE) [28, 35]. The disadvantage is exposure of liposomes to organic solvents and to sonication, which may result in denaturation of some of the products.

(c) **Solvent injection method**
In solvent injection method, lipids are dissolved into organic solvent followed by rapid injection into a buffer where they spontaneously form small unilamellar vesicles (SUVs) with an average diameter of 30–50 nm. Ethanol injection is the earliest method based on the advantage of getting small liposomes by one-step injection without the need of any chemical or physical treatment of lipids [36, 37]. The major limitations of this method are heterogeneous population and difficulty to remove all ethanol. In contrast to ethanol injection, ether injection has the advantage of getting rid of the solvent in the final liposome product [38]. Since ether is immiscible with aqueous phase, after it is injected the aqueous phase is heated above the boiling point of ether to remove the

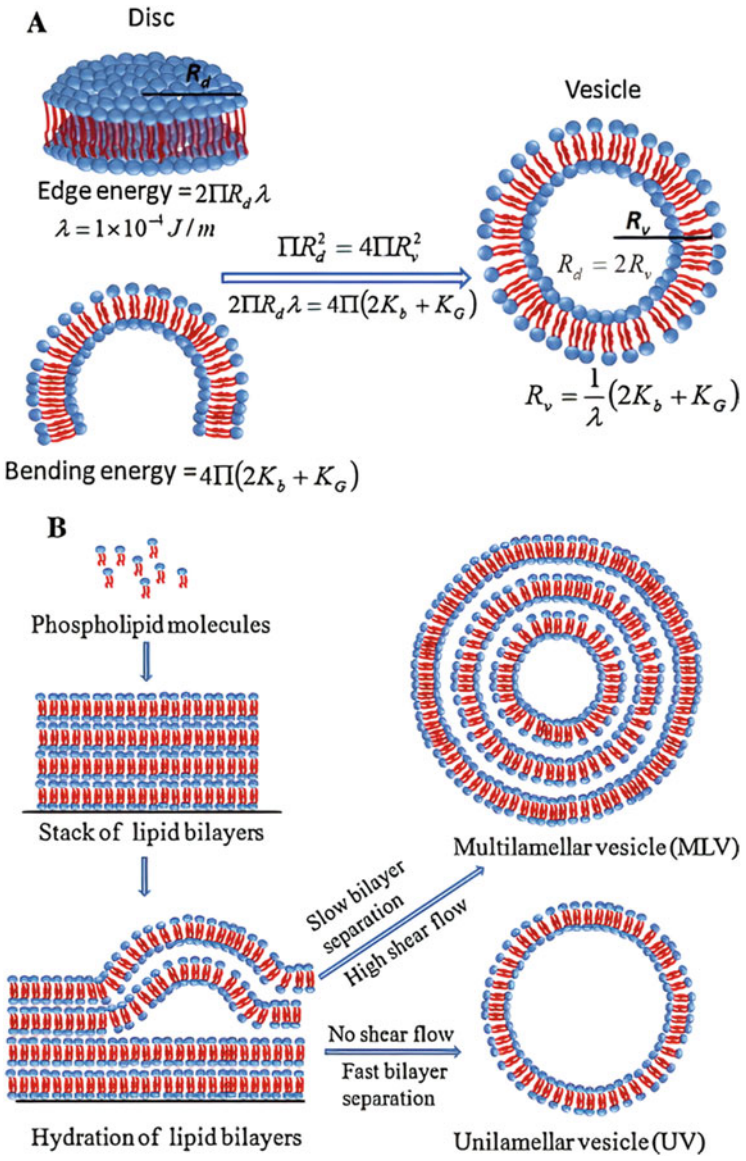

Fig. 2.3 Schematic representation of conventional method of liposome formation by phospholipid self-assembly. (**a**) Exceeding of edge energy to bending energy results in the formation of the smallest vesicle. (**b**) The varied size and lamellarity of liposomes are dependent upon the relative kinetics of bilayer folding and bilayer separation [43]

solvent from the final product. Ether evaporates when comes in contact with water, and the lipids form unilamellar vesicles [39]. This method provides concentrated liposomes with higher encapsulation rate.

(d) **Detergent dialysis**
In this method, phospholipids are dissolved with detergents to prepare mixed micelles. The detergents are then removed sequentially by concentration, washing, dialysis, and subsequent filtration. Homogenous unilamellar vesicles with average diameter ranging from 100 to 200 nm can be obtained which is the major advantage of this method [40, 41]. The rate of dilution and cholesterol content can be adjusted to get the desired vesicle diameter. Also it has been reported to get very large encapsulation volume by this method [42].

Although the conventional methods of liposome preparation are easily accessible, there are some major limitations such as exposure to harmful organic solvents, non-uniform size distribution, and dissociation of vesicles because of which there is a need of direct and advanced techniques [43]. Some of the recent methods include hydration of lipid mixtures in an aqueous medium containing 3% (v/v) glycerol followed by heating at 120 °C. Glycerol is used to enhance liposome stability by preventing coagulation and sedimentation [28]. Microfluidics and extrusion methods are some of the latest techniques used for liposome preparation [44, 45].

2.1.3 Cell or Cell Ghost Membranes

Due to some of the major limitations of exosomes like low production and scalability, low recovery and purity, low immunogenicity, inadequate loading, an effort towards alternative approach has gained momentum. Some of the other lipid bilayer nanoparticles include artificially induced cell ghost membranes or natural cell membranes [46] which are prepared by the method of cell membrane extrusion. A ghost cell is a cell that only contains empty membranes lacking the nucleus. In this method, cells are passed through porous membranes or filters of decreasing size in order to be deconstructed and reconstructed consequentially [47]. The most commonly used cells for this purpose includes Red Blood Cells (RBCs) and immune cells like macrophages [48]. As an example, biomimetic cell-derived vesicles from immune cells (U937 cell line) are prepared through sequential filtering, which they found to encapsulate good amount of doxorubicin [49]. Some studies found similar results from macrophages (RAW264.7 cell line) with a cytotoxic effect on cancer cell model. Similarly, RBCs with many biological advantages have been widely explored as the source of membrane vesicles [50]. By physical extrusion of RBC ghost membranes, the nanoerythrosomes were produced, which were surface linked with doxorubicin (DOX) for achieving targeted drug delivery towards cancer [51]. RBC membrane-derived nanoparticles containing natural lipids, proteins, and glycoproteins were loaded with a hydrophobic drug camptothecin (CPT) which when injected intravenously in mice showed superior stealth and physicochemical properties as compared to other lipid-based nanocarriers [52].

2.1.4 Membrane-Camouflaged Nanoparticles

Another field of biomimetic biomaterials which is rapidly evolving is the use of cell membrane-coated nanoparticles which camouflages the target cell membrane. It connects the link between synthetic nanomaterials and natural cell membrane [53]. The long circulation time and stability is provided by the nanoparticles, while specific ligand–receptor binding is contributed by the surface proteins present on the coated cell membrane. These cell ghost membranes are isolated from the target cells and then coated on the suitable nanomaterials [54]. This method is being tried with various cells like RBCs [55], macrophages, WBCs [56, 57], etc. so as to evade the immune system. Recently, RBC membrane was isolated and coated over PLGA NPs to form RBC-PLGA-alginate scaffold. After 10 days of insertion into C57BL/6J mice, these scaffolds were degraded and showed excellent biocompatibility [58]. This approach holds promising future in therapeutics and regenerative medicine provided the cell membrane of the target cells could be isolated properly. The high cost of manufacturing is the major drawback involved in this method. Also, some issues like improper preparation method, bioaccumulation, and high cost to benefit ratio are making their translational use a difficult task [53].

2.1.4.1 Formation of Cell Membrane-Coated Nanoparticles

Cell membrane-coated nanoparticles based exosome mimetics are synthesized by passing cells through porous membranes of decreasing size in order to be deconstructed and reconstructed simultaneously [59]. After harvesting the cells, the pellet is filtered through 10 μm-filter and centrifuged at high speed. The pellet is usually resuspended in PBS, and the same process is repeated. At last, the pellet is passed through 8 μm filters and centrifuged as before. Again the pellet is resuspended in filtered PBS and run through spin columns for further purification of the solution. This method is known as physical extrusion of cell membranes to get the mimetic vesicles [57]. In some other cases, freeze-thaw method is also applied for the production of exosome mimetics [60].

2.2 Methods of Exosome Isolation, Purification, and Characterization

2.2.1 Techniques for Exosome Isolation

There is a growing demand for simple, cost-effective, and efficient exosome isolation technique for downstream applications. However, due to the lack of proper isolation and characterization methods, many questions still remain unanswered in this field of research. Industrial application of exosomes demands techniques which could provide better purity and recovery rate. Below are some of the conventional methods for exosome isolation [61, 62].

2.2.1.1 Ultracentrifugation

At present, the gold standard protocol for exosomes isolation from a heterogeneous mixture of cell culture supernatants includes ultracentrifugation (UC). This original and most commonly used method for exosome purification [63] involves several centrifugation and ultracentrifugation steps depending upon which particulate constituents in the suspension will sediment according to their density, size, and shape [61]. In some cases, a single filtration step can replace some of the first centrifugation steps [64]. Remaining cells, cellular debris, and large EVs (including microvesicles) are removed from supernatant with varying centrifugation steps, and then exosomes in the supernatant are precipitated by ultracentrifugation steps reaching speeds near 100,000 × *g* [65]. Despite the most commonly used method, it involves several disadvantages like isolated exosomes contain a number of unwanted proteins, a portion of the vesicles is often lost in this method because the pellets are small and fragile [66]. Large amount of starting material; low exosome yields; and the need of extensive training of personnel has made it inefficient for clinical applications [67–69].

2.2.1.2 Density-Gradient Centrifugation

For some downstream applications, it is generally advised to include an extra purification step of density-gradient centrifugation using sucrose cushion after traditional ultracentrifugation [64]. This step eliminates contaminating protein aggregates, apoptotic bodies, or other non-exosomal particles, which are sedimented by centrifugation but do not float on a sucrose gradient as it separates particles using buoyant density [70]. Due to the similar density of exosomes (1.15–1.19 g/mL) to that of sucrose (1.12–1.18 g/mL), a cushion is being produced which maintains the integrity of exosomes and separate protein contaminants of higher density (1.22 g/mL) [64, 70]. An additional discontinuous iodixanol gradient (60%, 40%, 20%, 10%, and 5%), prepared using OptiPrep™ could efficiently enrich the particle concentration (particles/ml) as compared to only ultracentrifugation method [71, 72]. To address the issue of losing some purified exosomes with a two-step isolation method, involving ultracentrifugation followed by 30% sucrose density-gradient, a simple one-step sucrose cushion ultracentrifugation method has been proved to be effective and reproducible technique [73]. However, it fails to separate different members of EV family with similar densities. The complexity, labor-intensive, and time-consuming (up to 2 days) criteria of this technique has made it inefficient for clinical usages [70].

2.2.1.3 Size-Based Exosome Isolation

One of the well-known size-based exosome isolation techniques is ultrafiltration. The principle behind this technique is similar to that of conventional membrane filtration, in which the suspended particles get separated on the basis of their size and molecular weight. Size exclusion chromatography (SEC), another popular technique also uses the same principle [74]. Compared to the conventional ultracentrifugation technique, ultrafiltration-based exosome isolation is faster and efficient approach [75], where the conditioned media is passed through chromatographic columns

composed of sephaex, sepharose, etc. [71] There is no requirement of special equipment handling and man-power, due to which it represents an ideal substitute to the classical ultracentrifugation method [76]. Ultrafiltration in combination with SEC has been seen to be the most efficient and balanced method of getting higher yield and purity of exosomes isolated from clinical urine samples [77, 78] and melanoma cell culture supernatants [79]. However, the major drawbacks involve filter plugging, deformations and breaking up of vesicles due to applied force, small quantity of exosomal protein, which may potentially hamper the results of downstream analysis [70]. Above all, this semi-automated ultrafiltration strategy could maintain functional integrity, therefore holding great potential for exosome-based theranostic translations [80].

2.2.1.4 Exosome Precipitation

Over the past 3 years, the method based on precipitation of exosomes in polymeric solutions has gained second highest popularity after ultracentrifugation, which plays around the size of the particles [65, 81]. The use of commercially available hydrophilic polymers interacts with water molecules surrounding the exosomes to create a hydrophobic micro-environment resulting in exosome precipitation. Among various hydrophilic polymers, polyethylene glycol (PEG), a well-described, non-toxic polymer [82] constitutes the base material for several popular commercially available exosome isolation kits, such as ExoPrep (HansaBioMed, Estonia), ExoQuick (System Biosciences, USA), Total Exosome Isolation Reagent (Invitrogen, USA), Exosome Purification Kit (Norgen Biotek, Canada), miRCURY as well as Exosome Isolation Kit (Exiqon, Denmark) [80]. Exosomes isolated using different kits showed varied physical and molecular characterizations [62]. In most cases, the sole use of PEG-based isolation was proved to be inefficient [53, 83]. Although kit-based precipitation method resulted in production of greater exosomal marker expression [84], in some findings performing UC prior to the use of polymer-based kits was proved to be feasible for translational research [85]. In general, these methods represent quicker and reliable alternatives for exosomes isolation from reduced biofluids, particularly from serum or plasma, validating the use of these kits in clinical research [86, 87].

2.2.1.5 Immunoaffinity-Based Capture

Regardless of their origin, some common proteins are present in all exosomes [88], which offer an excellent opportunity to develop highly specific immunoaffinity-based exosome isolation technique via the receptor-ligand binding, proving it to be the method without the need for ultracentrifugation. Furthermore, this demonstrates colocalization of the protein recognized by the antibody-coated beads with other identified proteins, strengthening the evidence of specific exosome analysis [64]. Exosomal markers for immunoisolation are ideally membrane bound, highly expressed on the surface with no soluble counterparts. Over the past few years, a lot of exosome markers have been recorded including immunoregulators, cytoskeletal proteins, tetraspanins, ribosomal proteins, heat shock proteins, MVB biogenesis proteins, and lipid rafts [11, 12, 17, 89–91]. Among these trans-membrane proteins

such as CD81, CD63, CD9, annexin, Alix have been extensively studied for selective exosome isolation, which has resulted in the availability of various commercialized kits such as exosome isolation and analysis kit (Abcam), exosome-human CD63/CD81/CD9 isolation reagent (Thermofisher) and Exosome Isolation Kit CD81/CD63 (Miltenyi Biotec). Immunocapture-based ELISA, consisting of coating of specific antibody for exosomal antigen, has come out to be a modern technique in the field of disease diagnosis [92]. Recently, it was demonstrated that capturing exosomes directly from cell culture supernatants with magnetic bead-based immunoaffinity method, provided sufficient yield for downstream analysis while demanding minimal hands-on and minimal loss [93]. Recently, EpCAM-based immunoaffinity separation system has been commercially available for cancer research and disease diagnostics [94]. However, high reagent cost and difficulty in removing the antibody-based tags from exosomes lie as some drawbacks of this technique.

A summary of the principle, merits, and demerits of exosome isolation techniques is listed in Table 2.1.

2.2.2 Characterization of Isolated Exosomes

2.2.2.1 Electron Microscopy (EM)

To reveal the size, morphology, and distribution of nanomembrane vesicles like exosomes, accelerated electrons are used as the source of illumination in EM. After fixation with paraformaldehye, contrasting with uranyl acetate, and embedding over grids, concentrated exosomes are visualized under EM (e.g., TEM and SEM) [64, 97]. Immunolabeling with antibodies against known proteins on exosomal membranes can provide information on the presence of specific biomarkers [98]. The advanced use of cryo-electron microscopy has allowed visualizing exosomes without any alterations caused due to electron beam and chemical interventions during sample preparation [99].

2.2.2.2 Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA)

For the detection of single exosomal particles, new approaches based on light scattering such as dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) have emerged. Both these techniques detect the dynamic changes in fluctuations from the light scattering due to Brownian motion [88]. The unsuitability of DLS to measure heterogeneous population of particles has encouraged the use of a more recent alternative image-based method, NTA [100]. With fluorescent-labeled antibodies, specific antigens present on exosomes can be detected with NTA [101]. However, both the techniques fail to distinguish between particles having similar size and similar Brownian motion and hence, isolation and purification steps are needed [102].

Table 2.1 Different methods of exosomes isolation

Sl. No.	Methods	Key principle	Yield	Purity	Merits	Demerits
1	Ultracentrifugation	Sequential separation of particles based on density, size, and shape followed by isolation of smaller exosomes by higher speed ultracentrifugation (100,000 × *g*) from the conditioned media or plasma [61, 64].	Medium	Low	Gold standard protocol for exosome isolation, large volume of conditioned media can be used to improve the yield, reduced cost [65].	Tedious process, damage to exosome integrity, loss of exosomes in several steps [72, 75].
2	Density-gradient centrifugation	Particle enrichment based upon the density of exosomes (e.g., sucrose gradient or OptiPrep density gradient) [76]. It enables separation of subcellular components [64].	Medium	Medium	This step adds extra purity to exosomes after isolation by ultracentrifugation [71], can also be used as a single step of isolation [73].	Higher precision required to separately collect the fraction of purified exosomes, chances of mixing with other fractions [72].
3	Ultrafiltration	Size based isolation of exosomes by using various commercially available filters [71, 95].	Medium	High	Higher particle yield as compared to ultracentrifugation [75, 77].	Filter plugging is a major drawback, damage to membrane integrity due to sheer stress [70, 80].
4	Size exclusion chromatography	Particles separated by size and molecular weight [71]. Conditioned media is passed through chromatographic columns composed of sephaex, sepharose, etc. [65].	Low	High	Exosomes can be isolated from very less volume of plasma [79, 96].	Provides lower recovery rate, long run time, requires dedicated equipment [76].

(continued)

Table 2.1 (continued)

Sl. No.	Methods	Key principle	Yield	Purity	Merits	Demerits
5	Immunoisolation	Capturing exosomes based on specific antigen–antibody interaction between membrane-bound receptors of exosomes and ligands adhered to magnetic beads [92].	Low	High	Highly purified exosomes, isolated particles can be directly used in flow cytometry, much better than other techniques [65, 93].	High reagent cost, tagging needs to be established, difficulty in removing the tags from exosomes after isolation [70].
6	Precipitation	Size-based isolation of exosomes from the conditioned media by using polymers or commercially available kits [81].	High	Low	Simplicity of procedure, no additional large equipment needed, exosomal integrity remains intact [65, 80, 86].	Retention and contamination of polymers, high cost reagents, not suitable for large volume of starting material [53, 83, 85].

2.2.2.3 Flow Cytometry

The important physicochemical characterization of exosomes is being done by this laser-based technology that records both scattering and fluorescence signal generated by individual particles [103]. Due to the inability of conventional flow cytometers to detect vesicles below 300 nm in diameter, using beads of known size and refractive index could provide more accurate size estimation [104]. Nanosize exosomes are conjugated to large beads and labeled with fluorophore-tagged antibodies for better detection by FACS [105]. Flow cytometry is an advanced tool for the analysis of relative size and granulation of single particle as well as for the detection of multiple surface markers.

2.2.2.4 Molecular Characterization

Apart from the nucleic acid content, the overall protein and lipid composition of exosomes has been extensively studied by proteomics and lipidomics analysis respectively [106]. Liquid chromatography coupled with tandem mass spectrometry is being done to identify the proteome of serum exosomes [107]. In addition, lipidomics has provided a clear idea about the lipid composition such as triacylglycerols, diacylglycerols, and phosphatidylcholines [108] Thin layer liquid chromatography (TLC) and MALDI-TOF mass spectroscopy methods are being used for observing several fatty acids, glycerolipids, and phospholipids as the lipidome markers [109]. Molecular characterization of exosomes is important for unraveling novel diagnostic and prognostic markers in therapeutic applications.

2.3 Applications in Tissue Engineering and Targeted Cargo Delivery

The major challenge that still remains in the field of therapeutic approach of a disease is selective targeting. Till now biomimetic nanosystems have been proved to be good carriers of many cargos to the specific diseased sites. But for achieving a successful therapeutic targeting approach, these systems need to be engineered with some targeting moieties so that these can bind and deliver cargos to the desired site. This section of the chapter describes about the different methods of engineering of nanosystems for targeted and sustained delivery of cargos through biomaterials approach. It has been observed that liver and spleen are the primary organ where most of the systemically administered nanomaterials get accumulated, rather than in the target site. Also the issue of short circulation time has to be addressed to achieve sustained drug delivery. Though exosomes are excellent biomimetic systems for drug delivery, still to achieve targeted delivery, surface modification is essential. The engineering is based on the targeting moieties which are expressed on the surface of the target cells or organs. Some of the examples of targeted drug delivery to bone micro-environment have been discussed in this section. In one study, mesenchymal stem cell-derived exosomes (STExos) were modified by specific recognizable ligands to avoid rapid metabolism and clearance (Fig. 2.4IA). Bone marrow mesenchymal stem cell-specific aptamer

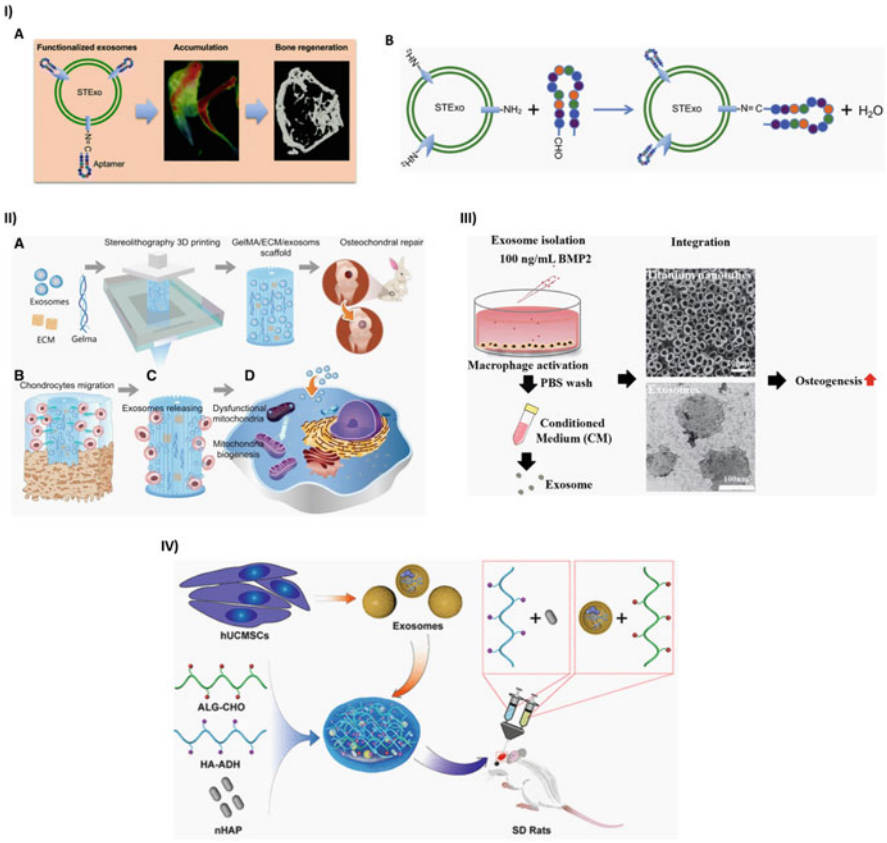

Fig. 2.4 Schematic illustration of (I) (A) aptamer-functionalized exosomes to promote bone regeneration, (B) conjugation procedure between the aptamer and STExos [110], (II) osteochondral defect regeneration by a single pot system [111], (III) integration of exosome in engineered titanium oxide nanotubes for targeted bone regeneration [112], (IV) integration of hUCMSCs-derived exosomes and HA in a hyaluronic acid-alginate hydrogel for bone regeneration [113]

(5′-ACGACGGTGATATGTCAAGGTCGTATGCACGAGTCAGAGG-3′) was designed as a stable structure with an aldehyde group modification at the 5′ end, which could react with the amino group of exosomal membrane proteins forming a stable Schiff base (Fig. 2.4IB). This modified aptamer conjugated to exosomes by incubation (STExo-Apt), facilitated internalization of exosomes in BMSCs with significantly higher distribution in bone region (91.8% positive) (Fig. 2.5I). While investigating the bone regeneration capability of STExo-Apt in postmenopausal osteoporosis mouse model after bilateral ovariectomy (OVX), once per week after 2 months of intravenous injection, higher trabecular volume, trabecular number, and trabecular thickness was observed as compared to vehicle or STExo-treated mice.

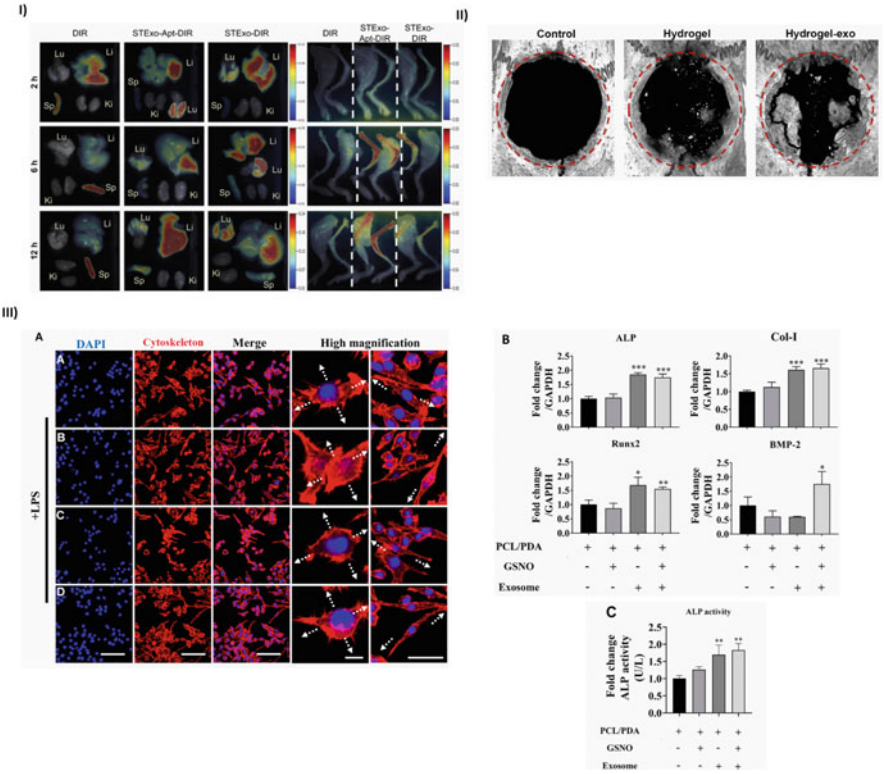

Fig. 2.5 (I) Representative FMT images of FL signals showing significantly more accumulation of STExo or STExo-Aptamer in bone as compared to spleen, lungs, liver, and kidney [110]. (II) Micro-CT analysis of bone regeneration at 8 week post-surgery showing bone formation in hydrogel embedded with exosomes treatment [113]. (III) Changes in macrophage morphology in the scaffold after lipopolysaccharide (LPS) stimulation, (A) images of RAW264.7 cells co-cultured with PCL/PDA (a), PCL/PDA + GSNO (b), PCL/PDA + exosome (c), and PCL/PDA + GSNO + exosome (d) scaffolds. Scale bars 10 μm, (B) Relative expression of osteogenic-related genes 3 day post seeding of hBMSCs with different scaffolds, (C) ALP activity measured after 7 days in culture [115]

An increase in the bone mass was also observed in OVX mice by increased osteogenesis together with increased callus tissue formation and bone mineralization [110]. In another study, MSC-derived exosomes fabricated with 3D printed bio-scaffold were proved to effectively restore osteochondral defects due to mitochondrial dysfunction. A bio-ink composed of photo-crosslinked cartilage extracellular matrix (ECM) and gelatin methacrylate (GelMA) hydrogels with improved mechanical properties was printed based by steriolithography (Fig. 2.4II). This strategy found to be promising for early osteoarthritis treatment caused due to mitochondrial dysfunction and oxidative stress damage. Transwell migration assay showed that the scaffold (ECM/GelMA/exosome) could promote chondrocyte

migration in the defect regions as compared to other groups with or without ECM and exosomes. Due to sustained release of MSC-derived exosomes from the scaffold, these could be internalized effectively by chondrocytes. M2 macrophage polarization was observed by the presence of $CD163^+$ cells upon scaffold implantation in a rabbit osteochondral defect model. According to the ICRS visual histological score system, ECM/GelMA/exosome group had the highest cartilage regeneration score as compared to other groups [111]. In another study, Wei and coworkers in 2019 developed titanium oxide nanotubes integrated with exosomes isolated from BMP-2 stimulated macrophages and observed on osteogenic differentiation and regeneration (Fig. 2.4III). ALP and BMP-2, the two osteoblastic markers had enhanced expression indicating the osteogenic potential of the titanium nanotubes incorporated with BMP-2/macrophage-derived exosomes. ALP was increased after 7 days in human bone marrow mesenchymal stromal cells after cultivating exosomes isolated from RAW264.7 cells activated with BMP-2 and encapsulated in nanotubes, as proven by immunofluorescence staining with rabbit polyclonal antibody against ALP [112]. Human umbilical cord MSCs-derived exosomes combined with hydroxy apatite (HAP) incorporated into an in situ cross-linked hyaluronic acid-alginate (HA-ALG) hydrogel system, significantly enhanced bone regeneration (Fig. 2.4IV). The composite hydrogel system could effectively retain exosomes and exert their reparative effect at the defective sites with a relatively slower rate of release ($71.20 \pm 2.64\%$) than pure hydrogel ($84.81 \pm 4.91\%$) post 14 days. In a calvarial bone defect model in rat, largest extent of new bone formation was detected in hydrogel-exosome group (Fig. 2.5II), with higher expression of osteogenic markers (OCN, Runx2) and CD31-positive cells suggesting angiogenesis, as compared to other groups and control group [113]. Zhang et al. in 2016 combined exosomes isolated from human-induced pluripotent stem cell-derived mesenchymal stem cells (hiPS-MSC-Exos) with tricalcium phosphate (β-TCP) to repair calvarial bone defects. Histological analysis revealed that the exosome/β-TCP complex scaffolds enhanced osteogenesis potential relative to only β-TCP scaffolds [114]. Wang et al. in 2019 designed polycaprolactone (PCL) scaffolds, coated with poly(dopamine) (PDA) to modify the surface, which was then incubated with *S*-nitrosoglutathione (GSNO) and MSC-derived exosomes to get a desired fabricated scaffold (PCL/PDA + GSNO + exosome scaffolds). The PCL/PDA + GSNO + exosome scaffolds, displayed more elongated morphology compared to cells co-cultured with PCL/PDA alone (Fig. 2.5IIIA). Significant down regulation of inflammatory gene expression was noticed in case of scaffold immobilized with exosomes, compared to the control PCL/PDA scaffold, which supported the idea that the modified scaffold with exosomes could regulate inflammation to a larger extent. The gene expression of osteogenic differentiation markers (ALP, Col-I, Runx2, and BMP-2) revealed that these genes were significantly highly expressed in scaffolds with exosomes compared to the control (Fig. 2.5IIIB). ALP activity assay (Fig. 2.5IIIC) also supported the finding, suggesting that incorporation of exosomes onto PCL/PDA scaffolds have profound pro-osteogenic effects [115, 116]. In another study, polylactic acid (PLA), calcium silicate (CaSi), and dicalcium phosphate dehydrate (DCPD) were

used to produce mineral-doped PLA-based porous scaffolds (PLA-10CaSi-10DCPD and PLA-5CaSi-5DCPD) enriched with exosomes to investigate its effect on human adipose mesenchymal stem cells (hAD-MSCs). The developed scaffolds could easily entrap the exosomes, thus enhancing the expression of osteogenic markers such as collagen type I, osteopontin (OPN), osteonectin (ON), osteocalcin (OC), and Runx in case of the PLA-10CaSi-10DCPD scaffolds enriched with exosomes compared to other scaffolds without exosomes and the PLA control scaffold [116].

Liposomes are also being proved to be one of the efficient biomimetic nanosystems to deliver encapsulated therapeutics to the target organ. Due to the controlled synthesis steps, these can be easily modified with targeting moieties during their preparation [23]. Poh et al. synthesized folate-targeted liposome and incorporated DiD fluorophore (Fol-liposome-DiD) and betamethasone (BM) for efficient targeting and imaging. They tried to see the effect of this formulation on rat model of rheumatoid arthritis 8 weeks after intraperitoneal injection. As a result, they found that Fol-liposomes were selectively accumulated in the sites of inflammation in arthritic rat paws to greater extent as compared to the non-targeted control liposomes (NT-liposomes). The incorporated therapeutic agent betamethasone (BM) could successfully exhibit low arthritis score, reduced bone erosion and less paw swelling as that of the control [117]. In another study, Teng et al. co-encapsulated two chemotherapeutic drugs (doxorubicin and ladirubicin) into liposomes during the preparation. The combination of drug loading was found to have high encapsulation efficiency with smaller size of liposome, having good biocompatibility. The formulation showed extremely effective anti-tumor activity, reduced tumor size and tumor lesion [118]. Similarly, Jose et al. in a study analyzed the efficacy of drug delivery via cationic liposomes against skin cancer. They formed a nanocomplex by encapsulating anti-cancer drug curcumin into cationic liposomes and then fixed it with anti-STAT3 (signal transducer and activator of transcription 3) siRNA to suppress melanoma tumor progression in mouse mode of melanoma. This novel formulation could effectively penetrate into skin thereby inhibiting tumor growth as compared to the control [119].

2.4 Current Challenges and Future Perspectives

The biomimetic approach in therapeutics and regenerative medicine is definitely a promising field of research with great advancements. Among all of the enlisted methods, exosomes are proved to be standing out of the crowd because of their excellent cargo-loading and cargo delivery capacity. The striking features include biocompatibility, efficient cellular internalization together with high therapeutic loading capacity. Exosomes, which are naturally occurring nanovesicles secreted by cells, exhibit promise towards regeneration of bone defects as a cell-free therapy. However, despite these advantages a lot of challenges are also associated with this field of research. The major barrier is the tedious isolation method of exosomes. Apart from that proper loading of drugs and their release are still in pilot study.

Nevertheless, some scientists are pressing forward optimistically to get the best out of exosomes.

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Biomimetic Nanosystems in Targeted Drug Delivery

3

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Abstract

Nanotechnology in cancer has been a boon to the translational science bringing advantages to the conventional drug delivery approaches. There are different types of nanoparticles such as liposomes, dendrimers, and mesoporous silica nanoparticles that are being employed to improve the overall biodistribution of the drug; however, this often fails in in vivo model due to the lack of stealth property, ultimately leading to immune rejection. PEG, chitosan, etc. are polymeric coatings that have been used as stealth covering around nanoparticles that prevent the nanoparticles from aggregation of proteins and opsonization. However, synthesis of polymeric coatings requires chemistries for conjugation that are often tedious and labor intensive. In this scenario, biomimetic nanoparticles have become convenient as they can be produced without much use of organic solvents. In addition, they can mediate natural targeting due to the virtue of homotypic interaction with membrane proteins present on the host cell. In addition, they can also prevent immune recognition due to the presence of marker proteins that are often recognized as "self" by the body. There have been several achievements in this field; still there are certain limitations that need to be dealt with. Techniques to produce biomimetic nanoparticles in a cost-effective manner in larger batches can lessen the burden in manufacturing process. Biomimetic

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M. Dash (ed.), *Biomimetic Biomaterials for Tissue Regeneration and Drug Delivery*,
https://doi.org/10.1007/978-981-16-4566-2_3

nanoparticles possess immense benefits with better targeting and stealth property that can reduce the shortcomings of the traditional nanoparticles employed.

3.1 Introduction

Nanotechnology has revolutionized the field of medicine and health sciences. The amenability for wide and tailored functions has made them one of the potent used carriers for therapeutics. Nanosystems are now being recognized worldwide as versatile tools for various applications and have been started getting approved by U.S.F.D.A (Food and Drug Administration). Promising efforts are being continuously made to ensure the biocompatibility of these man-made synthetic nanoparticles. Conventional approaches for drug delivery have faced numerous problems that include renal clearance of the drug leading to its elimination. Although traditional methods like topical, parenteral, intravenous injections have proven to be effective at some point of time, these methods administer drugs systemically and not to the target area specifically with more than 90% of drugs are subjected to renal clearance. Hydrophobic drugs have poor solubility and often face issues in bioavailability when it comes to oral administration. In oral administration, the drugs remain at lower saturation level and are not absorbed properly [1]. In contrast to this, hydrophilic drugs face poor cellular penetration issues [2]. Protein/peptide and gene-based drugs are enzyme and acid sensitive leading to degradation. In such scenario, smart drug delivery approaches are becoming increasingly popular that leads to maximum accumulation of the drug at the target site leading to targeted drug delivery approach. Nanoparticles are usually the first choice for carrier-based drug delivery due to small size and effective cargo loading properties. Nanosystems aim for a controlled and sustained or steady release of the drug leading to maximum deposition in the diseased area. In addition to this, plasma half-life of the therapeutics is also considerably increased when compared to its native counterpart. Nanoparticles (NPs) fall in nanometer range (up to 1000 nm) with various shapes and sizes amenable for diverse purposes. Some of the most frequently used nanoparticles for research, diagnostic, and translation purposes are liposomes, biodegradable polymers, polymeric micelles, gold nanoparticles, mesoporous silica nanoparticles, dendrimers, metallic and carbon-based nanosystems, each serving an unique function of its kind as they have very different structural and functional aspects. Liposomes are primarily synthesized from lipids and proteins and mimic biological cell membranes. Liposomes have the ability to load both water-soluble and -insoluble compounds inside the aqueous core. The major demerit of liposomes is their physical and chemical instability. In some cases, drug leakage has also been reported [3]. Polymeric nanoparticles, on the other hand, include PLA (polylactic acid) and PLGA (polylactic glycolic acid) that have been even approved for human consumption by US FDA. They have improved biodistribution, stability, and biocompatibility as compared to liposomes. Due to small sizes of polymeric nanoparticles, there is scope for increased surface area to volume ratio which allows

for facile conjugation of varieties of ligand and functional groups for specifically targeting the desired site [4]. While these are the advantages of polymeric nanoparticles, the major drawback is faster degradation kinetics and high variation from batch to batch production. Polymeric micelles are formed by spontaneous arrangement of amphiphilic block copolymers in aqueous solutions, basically containing a hydrophobic core and a hydrophilic shell that has the capability to load hydrophobic drugs [5]. Amphiphilic block copolymers self-assemble into stable structures, examples include triblock PCL-b-poly(2,4-dinitrophenylthioethyl ethylene phosphate)b-PEG. Polymeric micelles degrade very slowly in blood thus prolonging the circulation time. However, micelles face issues in loading water-soluble compounds that can be combated by using emulsification method. Still, the usage of organic solvents at industrial level poses a serious health hazard [6]. Dendrimers are monodisperse and highly symmetrical molecule having a perfect symmetry. Polymer brushes come under the high molecular weight dendron and dendrimer [7]. Generally, dendrimers are known for their high loading capacity of the drug by virtue of numerous functional surface groups and internal cavities. The high bioavailability of the attached drug is usually through covalent/non-covalent bonds [8]. Cationic amine dendrimers such as poly(amidoamine) (PAMAM) and poly(propylene imine) (PPI) have the ability to penetrate the negatively charged cell membrane therefore disintegrating the lipid bilayer [9]. However, the toxicity of different dendrimers is a major concern in biomedicine. Magnetic nanoparticle like iron oxide-based NP targets the desired target area via the aid of external magnetic field [10]. Drug localization to the desired area is mainly governed by competition of force generated by the blood compartment and the magnetic force produced from the magnetic core [11]. But, the major demerit of magnetic delivery is the absence of mechanisms for delivery into the depth of the body. Gold nanoparticles are also one of the non-toxic potential theranostic nanocarriers. High surface area to volume ratio enables one to utilize surface conjugation chemistries over the surface for efficient therapeutic purposes. The unique physicochemical properties like unique optics and surface plasmon resonance of the gold core is ideal for thermal ablation and allow for efficient diagnostic applications [12]. However, this field requires a multidisciplinary approach. Mesoporous silica nanoparticles (MSNs) constitute silica matrix that has numerous uniform pores ranging from 2 to 50 nm, each being tuned to the size of the drug to be loaded. MSNs can load multiple drugs at a time to the desired area. The one demerit mesoporous silica nanoparticles have is the speculated hemolysis caused by the interaction of the silanol groups of nanoparticle and the lipid membrane of red blood cell [13].

Although there are many synthetic nanoparticles that can be utilized for drug delivery, biocompatibility and immune acceptance are the major criteria which have led to the emergence of various stealth and targeted nanotherapeutics for evading immune evasion and specific delivery to the diseased area. Passive targeting is the route that guides these stealth-based nanoparticles. Passive targeting is based on EPR effect (Enhanced Permeability and Retention), term given by Matsumura and Maeda in 1908s. Nanoparticles easily pass through the leaky microvasculature and enter into the site of tumor tissue that allows molecules of definite size usually within

600 nm. This phenomenon is termed as the famous EPR effect. However, the major disadvantage of passive targeting is the lack of cell-specific interactions, thus decreasing the chance of the drug concerned to target the diseased area. Coatings of polymer that are most often used can be both natural and semisynthetic. Among natural polymers, polysaccharides originating from nature are used that include dextran, polysialic acid, hyaluronic acid, chitosan (CS), heparin while synthetic polymers include polyvinyl pyrrolidone (PVP), polyacrylamide (Pam), poly(ethylene glycol) (PEG), and PEG-based copolymers such as poloxamers, poloxamines, and polysorbates [14]. PEG-coated NPs evade the mechanism of opsonization and subsequently are rescued from macrophages uptake as studied by Garcia et al. [15]. Despite advancement in stealth coating, PEG-coated nanoparticles are not completely hidden from the immune cells and are engulfed by the mononuclear phagocytes. Anti-PEG antibodies have also been reported in some cases raising immunogenic reactions [16]. In an attempt to have an alternative polymer coating that is more hydrophilic than PEG, polyoxazoline-coated nanomaterials have been developed [17]. However, these polymer-coated nanoparticles require a tedious job of coating and conjugation chemistries that often is not that effective as projected. In addition to this, various other parameters are taken into consideration for increasing the residual time of the nanoparticles in the blood such as molecular weight and conformation of the polymers being used. While passive targeting includes mainly the EPR effect, active targeting aims for increased targeting to a diseased area. Certain moieties are readily employed in active targeting purposes. Antibodies are the most prominent of all the ligands as it utilizes the receptor-mediated endocytosis. It binds to the overexpressed antigens present on certain tumor cells and thus leads to engulfing of the particles that carries the drug and leads to enhanced accumulation of the therapeutics. Trastuzumab (Tmab)-coated lipid-polymer nanoparticles (hybrid nanoparticles) composed of PLGA; PEI (polyethylenimine) and lipids loaded with Docetaxel (DTX) have been developed in which Tmab is surface adsorbed onto the nanoparticle, designed for enhancing targeted delivery to HER-2 receptor-positive breast cancer cells. DTX-loaded-eTmab (e stands for electrostatically adsorbed)-PPLNs have proven to be more cytostatic to BT474 cells as compared to plain PPLNs [18]. Similarly, polyethylene glycol-poly(ε-caprolactone) NPs (PEG-PCL NPs) has been synthesized with conjugated programmed death-ligand 1 (PD-L1) monoclonal antibody. Drug-loaded-PD-L1 antibody-conjugated nanoparticles induce apoptosis arresting G2-M checkpoint, an indication of impairment in microtubule synthesis [19].

Among polysaccharides, hyaluronic acid (HA) is a polymer that is widely used as a targeting moiety in nanoparticulate systems. The concept of using HA as a targeting moiety has been taken from the idea that HA being the main component of ECM (extracellular matrix) beside collagen can bind effectively with CD44 receptors that are highly being overexpressed on tumor cells [20]. Hyaluronic acid nanoparticles are coated with PEG on the nanoparticles to evade RES and maximum retention in the blood. Tumor cells deliberately endocytose these nanoparticles by receptor-mediated endocytosis. Various small molecules have also been exploited as ligands such as folate that are folate receptors specific present on cancer cell

membrane. PLGA NPs have been decorated with DPPC: DSPE–mPEG and folate as ligand encapsulating a photosensitizer, i.e., pheophorbide that kills cancer cells by producing free radicals. Folate-modified PLGA is usually preferably uptaken over the unmodified ones. In vivo MKN28 tumor-bearing mice model also has a higher accumulation of folate-decorated NPs during a period of 24 h after i.v. injection as compared to the unmodified NPs [21]. In the year 2017, Huo and the team improvized the melanoma Trp2 vaccine delivery along with Sunitinib. Sunitinib is a blocking agent for apoptosis and is known to inhibit tumors in melanoma. Huo and colleagues prepared Sunitinib base-loaded polymeric micelles ($SUN_{b\text{-}PM}$) that was functionalized with anisamide. The concept being anisamide will bind to Sigma-2 receptors that are highly expressed on B16F10 skin melanoma cells. B16F10 injected to mice by i.v. injection and treated with Sunitinib-loaded polymeric micelles modified with anisamide to govern the maximum internalization by melanoma cells. Tumor inhibition was maximum for Trp2 and Sunitinib-loaded anisamide decorated polymer micelles. The groups receiving polymeric micelles containing drug and Trp2 showed higher CD8+ T cells, suggesting improvized elicitation of the immune response [22].

Peptides are also the most used targeting ligands for TDDS. They are known to display several advantages: low cost related to productivity, higher stability, and easy conjugable chemistries over NP surface [20]. In the year 2014, Mei et al. showed interest in glioma for gliomas are not that easy to reach because of the blood–brain barrier (BBB), so they developed a dual-targeted nanoparticulate system that can reach and target BBB and glioma, effectively and simultaneously. They designed a cell-penetrating peptide, (E8)-6-aminohexanoyl-PLGLAG-(R8) modified onto PEG-PCL nanoparticle that effectively targets glioma cells as the MMP-2 expression level is quite high in gliomas [23]. In the blood, cationic R8 is usually protected by E8 via ionic bonds because of which the penetration is inhibited as R8 is shielded. PLGLAG is usually used as a linker which is degraded by MMP-2. Thus, E8 is detached from R8 at the site of the glioma, recovering R8 from E8. Low-density lipoprotein-related protein 1 (LRP 1) is a highly expressed receptor on both the BBB and glioma. Angiopep-2, derived from the Kunitz domain of aprotinin (aprotinin also known as bovine pancreatic trypsin inhibitor that breaks down blood clots), binds with a high affinity towards LRP1 [24, 25]. The targeting ability of angiopep-2 and ACP dual-modified NPs (AnACNPs)-loaded Docetaxel(DTX) was investigated in this study (anti-glioma effects). Angiopep-2 was conjugated to the nanoparticle by EDC-NHS(AnNPs) and for the R8 or ACP modification (R8 or ACP modification (CNPs, ACNPs, and AnACNPs), ACPR8 or ACP added to the NP or Angiopep-2-conjugated NP suspension. ACP and R8-modified NPs showed an increased uptake by both BMEC and C6 cells. After 24 h, the distribution of all the NP formulation increased in the brain. AnNPs accumulated to a larger extent in the brain region than that of only NPs and R8/ACP modified NPs, clearly depicting that angiopep-2 effectively crosses BBB and targets glioma interacting with LRP1 which justifies the hypothesis of the study.

Aptamers are short stretches of DNA or RNA oligonucleotides that can selectively bind to specific target molecules and that can fold into 3D structures. They are

employed in TDDS because of their lower immunogenicity, low molecular weights, and effortless availability. For example, AS1411 aptamer-functionalized PEG-PLGA nanoparticles mediated drug delivery systems encapsulating paclitaxel for anti-glioma therapy is a novel approach. AS1411 binds strongly to nucleolin which is highly expressed on the cancer plasma membrane and thus an effective anti-glioma therapy [26]. Aptamer-conjugated NP is found to have a longer residual duration in circulation leading to enhanced paclitaxel accumulation succeeded by tumor inhibition and enhanced longevity of rats bearing C6 glioma xenografts. HPA aptamers on PEGylated PLGA NPs encapsulating paclitaxel are also designed that preferably binds to Heparanase on tumor cells [27].

All these active targeting moieties that mediate the specific targeting to the diseased area, have still some disadvantages as they often fail to target the cell due to the presence of a single targeting moiety that can also be redundant to any other cell. For such reasons, biomimetic nanoparticles have evolved taking the advantage of both active and passive targeting.

3.2 Biomimetic Nanoparticles

Mimicking nature is a powerful tool for the development of newer nanosystems for targeted drug delivery approaches. Nanosystems functionalized with moieties that mimic the structure and chemical nature of the biological entities are somewhat termed Biomimetic NPs. Conventional polymeric stealth coatings and liposomes may serve as biomimetic nanosystems, but they suffer from antigenic responses and stability issues, respectively. The emergence of biomimetic nanosystems was due to the need for such nanosystems that will evade the immune cells and naturally mimic the structural aspects of the certain biological molecule such as protein, protein fragments, and whole cell membrane cloaked onto nanocores. This section will mostly cover the types of biomimetic nanoparticles that are playing a major role in biomedical science.

3.2.1 Protein or Peptide-Based Biomimetic Nanoparticles

Recently, protein/peptide-based biomimetic mineralization is known for their efficient biomimetic property and environment-friendly technology. Biocompatibility, high polarity, and surface area for conjugation of certain chemical groups are the hallmarks of this group of biomimetic nanomaterial. Albumin, ferritin, lipoproteins, enzymes, and peptides are the various biomimetic templates that can be good candidates for being biocompatible nanomaterials. Albumins are universal and robust proteins that can maintain the intact structure at higher (60 °C) temperatures. The nanoparticles from albumin can be easily prepared by emulsion, desolvation, or coacervation method under mild conditions. The low size and controlled release pattern are as good as liposomes and it also provides better patient compliance. Low cost for getting abundant albumin and ease of purification techniques have enabled

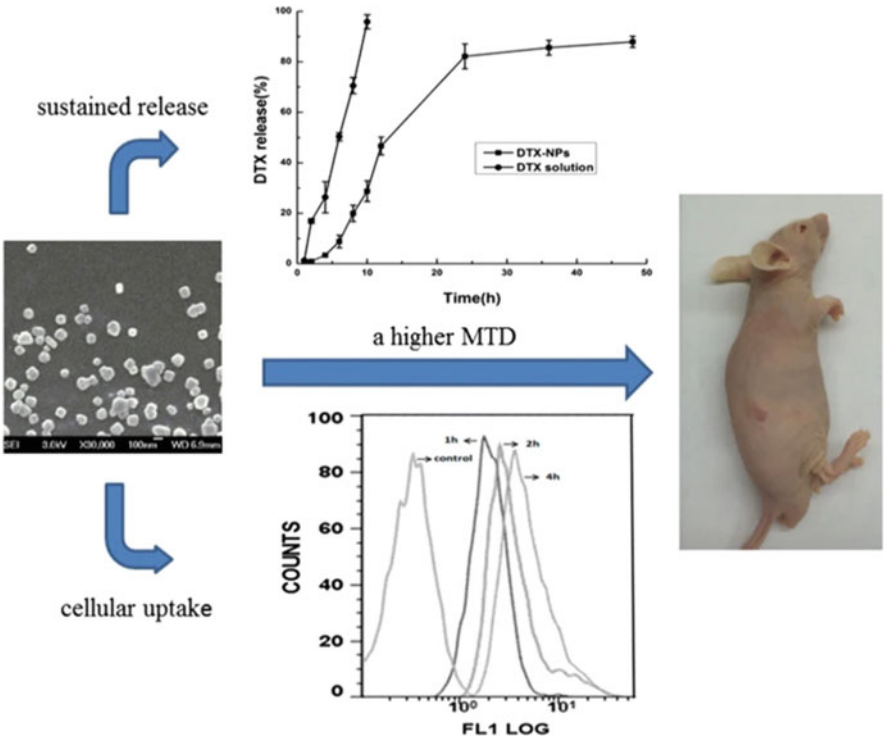

Fig. 3.1 Schematic representation sustained/steady release of Docetaxel (DTX) for prolonged half-life in blood circulation. High cellular uptake and high maximum tolerated dose (MTD) are the cues for anti-tumor activity along with reduced systemic toxicity [29]

Albumin as one of the most used biotemplates for preparing biomimetic nanoparticle. Bovine serum albumin (BSA) biotemplate has been used for making gold (Au) nanoclusters (NCs) via a simple, one-pot, and "green" synthetic route. BSA template-Au NCs are highly stable both in solutions (aqueous or buffer) and in solid form. Also, chloroauric acid ($HAuCl_4 \cdot 3H_2O$) along with hydrazine monohydrate ($N_2H_4 \cdot H_2O$) acts as a reducing agent in the existence of BSA under constant vigorous stirring was prepared to composite gold NPs via one-pot synthesis method [28]. In order to reduce the toxicity profile in vivo while maintaining the stability, human serum albumin is nowadays employed. The albumin paclitaxel (PTX) nanoparticle (Abraxane®) is FDA approved for the treatment of metastatic breast cancer. Use of toxic organic solvents however is the most common demerit of the conventional method for Abraxane® preparation. Therefore, salting-out greener technique is commonly used [29] (Fig. 3.1).

Besides these two categories of albumin, the third category of albumin is also widely used in the nanoparticles preparation, i.e., ovalbumin, also known as egg albumin, a glycoprotein used as vector for drug delivery approaches because of easy availability and cheap production. Ovalbumin forms gel networks and leads to

stabilization of emulsions and foams. Owing to its pH- and temperature-sensitive properties, OVA has a promising potential for being applied as a carrier for sustained drug release [30]. One-pot approach has been used to synthesize OVA-conjugated Ag NPs, in which OVA acted as an active template for the spontaneous reduction of Ag ions. Biomimetic NPs prepared with this facile, cost-effective, and eco-friendly process is proving to be biocompatible through in vitro cell arrays [31].

Ferritin is a ubiquitous intracellular protein that can self-assemble into a cage-like nanostructure with an external diameter of about 12–13 nm, consisting of 12 or 24 subunits which has enabled researchers to synthesize nanomaterials out of it. Ferritin constitutes an efficient protein nanoplatform for in vivo antigen delivery, immune modulation, and antigen presentation. Ferritin NPs can be easily taken up by dendritic cells (DCs) for antigen presentation. Also, ferritin NP elucidates thermal and chemical stability which is amenable for ease of purification process. RGD4C-modified ferritin (RFRT) has been developed as a delivery vehicle to transport a hydrophobic photosensitizer named hexadecafluorophthalocyanine ($ZnF_{16}Pc$) [32]. Drugs like doxorubicin (DOX) can also be encapsulated to RGD-modified apoferritin nanocages having high loading efficiency. Therefore, it is imperative to put forward ferritin as an ideal nanoplatform for drug delivery approaches.

Similarly, lipoproteins such as high density (HDL) and low density lipoproteins (LDL) have an immense application in targeted drug delivery due to their innate mechanism to evade immune system and easy loading capacity for amphiphilic compounds, much like liposomes. A hybrid HDL/polymer NP made up of a polymeric core coated with lipid/apolipoprotein showed not only a typical slow release profile of PLGA NP, but also natural characteristics of HDL, including specific accumulation by macrophages [33]. LDLs have also tendency to accumulate near tumors due to high demand for cholesterol in the tumor area, many of them even have been used to effectively load hydrophobic photosensitizers as these poorly water-soluble compounds interact better with lipoproteins, especially with LDL. Other lipoproteins, such as apolipoprotein A-I, are also in used in research to exploit it as a nanoplatform for pH-responsive drug delivery applications [28].

Inspired by the structure of the natural multi-enzymes, researchers are now also interested in designing enzyme complexes. Diverse enzyme/protein nanoparticles are immobilized onto electrode or a matrix for the fabrication of biosensors. HRP, uricase, cholesterol oxidase, and hemoglobin are some of them that can be used as biosensors. The enzyme-based NP can be produced by desolvation technique. NPs exhibit exceptional properties like optical, electronic, electric, thermal, chemical, mechanical, and catalytic. Trypsin single enzyme nanoparticles have been used to improve stability of enzymes at higher temperature, while chymotrypsin SENs improve cellulose degradation. In addition, the nanocomplexes from the enzymes can be used to specifically target tumors as the surface area is amenable for designing receptors proteins [34].

Peptide NPs are another class of NPs that have the intrinsic capacity to fold into 2D nanostructure that can have wide range applications in biomedical science due to the shape criteria and the design of amino acids that may serve in electrochemical catalysis, nanobiosensor fabrication, and even retroviral transduction. Protein/

peptide-template biomimetic NPs have deeper tissue penetration that can be used for targeting cancer cells with enhanced therapeutic efficacy and in-built biocompatibility. Tryptophan–phenylalanine dipeptide is one such example of a peptide nanoparticle that has the property to diagnose tumor due to their intrinsic criteria of shifting fluorescence spectra from UV to the visible range [35].

3.2.2 Cell Membrane-Coated Nanoparticles (CMCNPs)

The story began when Zhang et al. in 2011 developed the first cell membrane-coated nanoparticle from red blood cell (RBC) membrane. The RBC membrane was coated onto the PLGA polymeric core. Cell membrane-coated NPs offer an autogenous option which is not possible in the case of certain polymeric coatings that can excite the immune system. The cell membrane proteins present on the surface of cell membrane-coated nanoparticles bind to cells expressing the same membrane protein due to the virtue of hemophilic or heterophilic interaction. CMCNPs can be both passively targeted (avoiding mononuclear phagocytes and consequently high retention time) and actively targeted (proteins of CMCNPs binding to the adhesion proteins on the desired cell of interest). By utilizing various nanocores such as polymeric, gold, mesoporous silica NPs, liposomes, and magnetic and coating them with various cell membranes, a variety of functions can be achieved depending on what one needs, targeting, or diagnosis (Fig. 3.2).

Synthesis of cell membrane-coated nanoparticles includes two crucial steps, i.e., isolation of membrane fragments (or vesicles) and vesicle/membrane and

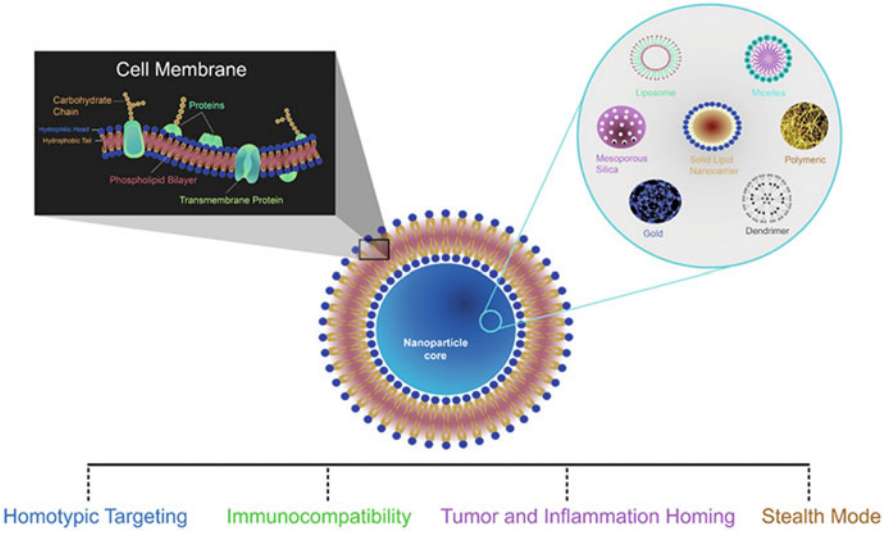

Fig. 3.2 Source cells can be fused to various nanocores to produce cell membrane-coated NPs (CMCNPs) having a broad range of applications [36]

nanoparticle fusion. Coating membrane vesicles to various nanoparticle cores are the most commonly employed method in comparison to membrane fragments as the fusion of fragments to particles do not necessarily facilitate the presence of all the proteins required. The synthesis method differs from cell to cell as in RBCs and platelets nucleus is absent and the isolation of membrane vesicles or fragments is quite straightforward not involving sophisticated steps unlike other eukaryotic cells RBCs are made free from its buffy coat and hemoglobin via employing high-speed centrifugation followed by sonication and then polycarbonate porous membranes (preferably 100 nm) are used to get definite size RBC vesicles which are usually stored at 4 °C for preservation. Throughout the entire process of extraction of membrane fragments, it is made sure that the isolation process is as gentle as can be to assure minimal protein denaturation. Apart from this fact, there is always the use of protease inhibitors for preventing the action of proteases that may act on the membrane proteins. Complex eukaryotic cells like WBCs, cancer cells, stem cells, etc. undergo various complex biochemical processing like hypotonic lysis, ultrasonication along discontinuous sucrose density centrifugation to completely clear the intracellular contents from the cell. The freeze-thaw method and physical homogenization techniques are some other ways of membrane extraction other than hypotonic lysis. In the freeze-thaw method, cells are subjected to cold shock at −80 °C followed by thawing at 37 °C or room temperature as a result of which ice crystals are formed that leads to the disintegration of the membrane [36]. Electroporation enables formation of enough pores in the cell membrane to create flaccidity due to electrical fields [37]. However, this method may also lead to changes or fluctuations in membrane potential. All of these methods have some demerits in them however the most approachable method of cell membrane extraction includes the hypotonic buffer treatment followed by mild sonication for membrane disorientation. Sucrose density centrifugation or ultracentrifugation is usually needed to completely making devoid of nucleus and other intracellular components. For cancer cells, mild lysis followed by ultracentrifugation is needed as compared to RBCs. The difference in the extraction of membrane proteins occurs as a result of the difference in size, granularity, and lipid bilayer of cells that vary from cell to cell. The core nanoparticles can then be fused to membrane vesicles by various approaches to synthesize the cell membrane-coated nanoparticles. The membrane is usually oriented in the right side out position with all the receptor proteins exposed. Most of these used methods depend on the net attraction between the oppositely charged molecules between the inner core particle and the membrane vesicle thus forming a core-shell structure with the proteins of the membrane facing towards right-side-out conformation making it more energetically favorable [38]. The various methods used for fusing nanoparticle cores to membrane vesicles are as follows:

(a) **Co-extrusion approach**: The nanoparticle solution and the vesicle mixture undergo co-extrusion via polycarbonate porous membranes and then sonicated to achieve CMCNPs of tunable sizes. Physical extrusion dictates the principle of strong force to pull off the vesicles to wrap around the nanoparticle core.

(b) **Sonication method**: This is the most reliable method as in the co-extrusion method, large-scale synthesis of nanoparticles remains a challenge. Sonication uses the energy of specific disruptive frequencies to fuse membrane and nanoparticle cores. The frequency, sonication time, and amplitude are the major factors governing the effective fusion process.
(c) **Microfluidic electroporation**: In this method, electromagnetic energy creates pores in the cell membranes creating an imbalance of dielectric field enabling the NPs to be coated by vesicles. This method is a novel approach and is becoming popular among researchers. Also, the stability of particles is unaffected.
(d) **Cell membrane-templated polymerization technique**: This technique relies on the interfacial interactions between the core and the membrane. The polymeric core is grown in situ within the cores unlike in old conventional processes preformed polymers are used which cannot handle the homogeneity of sizes. Acrylamide polymers have been produced within the membrane vesicles with the cell membrane vesicles acting as a nanoreactor containing reaction mixture of polymers, initiators, crosslinkers etc. To prevent any further macrogelation of unencapsulated polymers outside the membrane vesicles, the reaction an is stopped by an inhibitor, i.e., (2,2,6,6-Tetramethylpiperidin-1-yl)oxyl (TEMPO), thus forming cell membrane coated nanogel [39].

The CMCNPs need various characterizations after synthesis that can enable the integrity of membrane coating onto the material cores. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) are the physicochemical procedures that are usually done for the verification of membrane coating on nanoparticles [40]. Morphology of coated nanoparticles shows a halo around the NPs while the uncoated ones do not show such structural features. There is usually an increase in size of the particle due to coating with a shift in zeta potential similar to membrane potential of the cell membrane that confirms the successful coating. Zeta potential of the final CMCNPs formed is similar to membrane vesicles as the membrane vesicles are the ones that are coated onto nanoparticle. Flow cytometric gives the signal fluorescence for antibodies specific to membrane, for example, signal fluorescence signal becomes relatively higher for CD47 when RBCNPs are stained with CD47 antibody. Western blotting also helps for the confirmation of the coating onto particles. Antibodies specific to membrane under consideration are taken for validation of the integrity and right-side-out coating of membrane vesicles. Preparation and characterization procedures are usually more or less generalized that can be taken for confirmation of the membrane fusion to particles [41, 42].

There are various mechanisms by which CMCNPs can be produced; however, the major goal is always to produce intact and stable nanoparticles that can be used for robust functions inside body fluids in vivo and in patient samples. The contribution of each type of membrane-coated particles has an immense effect in biomedical research. In the upcoming sections, there will be discussion on various types of cell membrane-coated nanoparticles including their advantages and disadvantages in the field of targeted drug delivery approaches.

3.3 Cell Membrane-Coated Nanoparticles in the Field of Drug Delivery

3.3.1 Red Blood Cell Membrane-Coated NPs (RBCNPs)

RBCs are blood cells that are predominantly found among all the cells in humans that has role in transporting oxygen to all the body sites via hemoglobin. RBCs are amenable for isolation as they are in the circulatory system. RBC-coated NPs were the first among all to be chosen as a delivery vehicle due to the presence of self-marker proteins such as CD47, CD59, complement factor 1, decay-accelerating factor, and C8 binding protein that avoid the immune system [43].

RBCs and stem cells often fail to specifically target cancer cells specifically as there are no such cell adhesion molecules (CAMs) on the plasma membrane that enhances targeting via homotypic adhesion with like CAMs. For making it more target specific, RBC membranes are decorated with ligand moieties like folate, mannose, transferrin, etc. for entry to desired cells [44]. Certain methods are available that are readily used for conjugating chemical moieties to RBC membrane [45]. Chemical methods interfere with the integrity of intact proteins on the membrane and as a consequence can lead to unsatisfactory results. To rule out such scenario, a non-disruptive lipid insertion approach is usually followed for conjugating ligand or targeting moieties, known as lipid insertion approach. In this method, ligand moieties were incorporated on RBC membranes via lipid tethers and PEG linkers [40].Various conventional chemotherapeutic agents such as doxorubicin, paclitaxel, and cisplatin are encaged inside RBC-coated nanomaterials in both surface modified and unmodified forms. Transferrin, folate, nucleolin-targeting aptamer, AS1411, mannose, etc. are the various receptors that are surface modified on RBC membrane for efficient targeting (Fig. 3.3).

RBC has a long life span of approximately about 120 days and property of evading the immune proteins of body, which can be well implicated in various aspects like targeting, imaging, photodynamic therapy apart from only conventional drug delivery purposes.

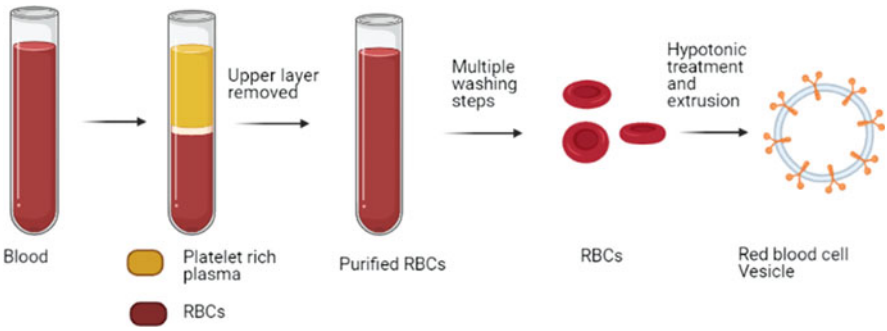

Fig. 3.3 Schematic preparation of red blood cell membrane-derived vesicles (RVs)

3.3.2 White Blood Cell Membrane-Coated NPs (WBCNPs)

WBCNPs are also prominent among various types of biomimetic nanoparticles taking account of inherent homing property to tumor and inflammation prone zones in the body. White blood cells have five major types depending on their granularity and morphology, i.e., monocytes/macrophages, neutrophils, eosinophils, basophils, and lymphocytes. Macrophages have the ability to home to inflamed and hypoxic area due to chemoattractants like CSF-1 (colony-stimulating factor) and chemokine-ligand-2 (CLCL-2) [46]. Receptors such as Tf (Transferrin) can be conjugated to macrophage membrane that is fused to nanoparticle has usually high targeting capability as compared to only membrane coating. Neutrophil-coated NPs have also been developed for targeting cartilages to inhibit synovial fluid inflammation thereby improving the condition of arthritis [47]. Natural killer cells (NK cells) have the ability to target cancer cells releasing granzymes and perforins. NKsome have been produced by fusing cholesterol-liposome with NK cells NK-92 for breast cancer therapy. NKsomes have the inherent ability to be retained in the blood for a longer duration and thus useful in stealth property of coated particles [48]. T cell-coated lipid-PLGA hybrid NPs have also been employed in research as they effectively target Burkitt's lymphoma [49]. Neutrophil membrane-coated nanoparticles (NNPs) have also been developed to overcome the blood–pancreas barrier using poly(ethylene glycol) methyl ether-*block*-poly(lactic-*co*-glycolic acid) (PEG-PLGA) nanoparticles, celastrol being the therapeutic agent. Celastrol-loaded NNPs inhibited tumor as well as liver metastases and overall survivability of tumor-bearing mice [50] (Fig. 3.4).

WBC-coated NPS have the excellent property of targeting tumors due to the intrinsic property of immune; however, it is restricted to certain and not all tumors. The circumvention of these demerits can be countered by other biological-derived cell membrane coating.

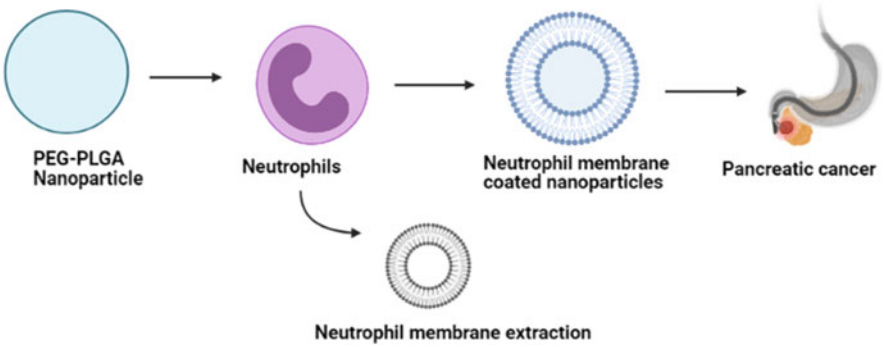

Fig. 3.4 Schematic illustration of action of neutrophil membrane-coated PEG-PLGA NPs against pancreatic cancer

3.3.3 Platelet Membrane-Coated Nanoparticles (PMNPs)

Platelet membrane contains some important cell adhesion molecules like CD47, CD44, and p-selectin. P-selectin binds with higher affinity for circulating tumor cells involving CD44, CD55/59. The adhesive glycoprotein membrane proteins are exploited for coating platelet membrane onto NPs, thus rescuing from macrophages resulting in better targeting ability [51]. NPs coated with platelet membrane bind to CD44+ tumor cells via p-selectin interaction. PLGA NP cloaked by platelet membrane has also been known to reduce the condition of atherosclerosis. The enhanced targeting ability of platelet membrane-coated NPs in joints of collagen-induced arthritis (CIA) model of mice was due to p-selectin and GVPI receptors [52]. Platelet membrane-coated NPs have demonstrated to be a potential biomimetic candidate as they incite low immunogenic response with enhanced biocompatibility that target injuries and inflammation prone area. However, there is one demerit that platelet membrane proteins can also activate immune system that may lead to release of various pro-inflammatory cytokines. To combat the immune evasion, tumor cells that can also be coated onto nanoparticles is discussed in the succeeding Sect. 3.4.

3.3.4 Cancer Cell Membrane-Coated Nanoparticles (CCMNPs)

Tumor cells possess unique property of evading immune cells such as NK cells and macrophage/monocytes, i.e., in simple terms cancer cell membrane-coated NPs does not disturb the immune system of the body. CCM (cancer cell membrane) also possesses certain adhesion glycoproteins. These adhesive anchor proteins help in the attachment of cell to cell. N-cadherin, Epcam, carcinoembryonic antigen, Galectin-3, etc. are the adhesion proteins that facilitate homotypic binding [42]. The CCMNPs therefore can be internalized into cells expressing proteins on the membrane that homotypically target the cells expressing similar protein, thereby releasing the drugs inside the desired cell of interest. The cancer cell membrane can also be decorated on the core nanoparticle with cancer-specific antigens for immunotherapy. The antigens are low immunogenic in nature. Likewise, plethora of research work is accomplished by various scientists to study on cancer cell membrane-camouflaged NPs. There has been successful implementation of therapeutic and imaging agents utilizing homotypic binding to receptors present on the cancer cell membrane in mice model and even in some cases, patient samples. Mesoporous silica NP core cloaked by PEGylated liposome yolk/cancer cell membrane coating have been developed by scientists encapsulating doxorubicin and a PARP inhibitor mefuparib hydrochloride that have potential cytotoxicity than free drug. The higher cytotoxicity is due to higher accumulation of CCMNPs [53].

To summarize, it can be inferred that homotypic targeting of cancer cell membrane-coated NPs can be put in various areas to generate anti-tumor therapeutics be it chemotherapy, PDT, and starvation therapy or immunotherapy.

3.3.5 Stem Cell Membrane-Coated NPs (SCMNPs)

Stem cells have the characteristic property of circulating in the bloodstream for a prolonged duration enabling its stealth property to escape the macrophages of the immune system. MSC-coated NPs circulate for a prolonged duration that impedes the lacuna of tumor cell-coated NPs having property to cross the endothelial barrier. MSCs can be synthesized from a broad range of tissues thus creating opportunities for therapeutic applications. Stem cell-coated gelatin nanogels encapsulating Dox have been produced recently that has displayed higher cytotoxicity and uptake profile than the bare counterpart in Hela cell with higher regression of tumor in mice model. CXCR4 antibody has also been conjugated to stem cell membrane for higher specificity. Stem cell membrane coated NPs however, lack a little specificity towards cancer cells. Compensation for low targeting ability can be achieved by conjugating ligands [54].

Apart from all these cell membranes, others can also be used for various translational approach. As single cell membrane coated NP might lack an advantage, hybrid cell membrane-coated NPs are nowadays synthesized, for example, RBC (for stealth property) and MCF (targeting breast cancer) and RBC (immune evasion—platelet (for tumor homing). Bacterial and viral cell membranes have also been in use as bacteria contains peptide immunogens or epitopes that have the potency to elicit immune response against a specific pathogen. Many of these cell membrane-coated NPs have also been applied for patients as clinical trials have given fruitful result. For convenience, different types of cell membrane-coated NPs are listed in Table 3.1.

3.4 Advances and Limitations

Biomimetic NPs are mostly in the third phase of a clinical trial due to their excellent biocompatibility. Various model drugs as described above are of enhanced therapeutic efficacy. Still, some obstacles such as poor pharmacokinetics and biodistribution are some of the major concerns in this field. Tumor cells most of the time show resistance towards such conventional drugs. Natural targeting mediated by the proteins on the CMCNPs has the potential for both active and passive mode of action. Minimum labor-intensive approach on the preparation of CMCNPs is the biggest advantage as compared to that of a single antibody-conjugated particle (immune-nanoparticle). However, the field is in its pilot stage, and it needs easy scalable and manufacturing practices for better therapeutic translatability in biomedical sciences.

Table 3.1 Cell membrane type with typical characteristics and inner nanoparticles core employed for the delivery of therapeutics

Cell membrane type	Uniqueness	Inner cores	Status
Red blood cell	Enhanced circulation	PLGA	FDA
	time (50 days in mice)	UCNs	Lab test
	Evading immune response	Liposomes	Lab test
		Gold NPs	Lab test
		MSN	Lab test
		Iron oxide	Lab test
		PFCs	Lab test
White blood cell	Homologous targeting	PLGA	Lab test
	To leukocytes and endothelium homing to inflamed zones immune evasion	PLGA-lipid	Lab test
		Liposome	Lab test
Platelets	Homing to damaged prone	PLGA	Lab test
	Immune evasion	PLGA-CS	Lab test
	Long circulation time (10 days)	Polypyrrole	Lab test
		Au nanostars	In vitro
		PLGA	Clinical
Cancer cell	Cancer cell targeting and	PLGA	In vitro
	Cancer antigen presentation	MSNs-PEG-lipid	Lab test
	Or cancer immunotherapy	PBAE NPs	Lab test
		Gelatin	Lab test
		Magnetic NPs	In vitro
		Silica	Lab test
		Porphyrinic-MOFs	In vitro
Mesenchymal cells	Long circulation time	Gelatin	Lab test
	Tumor targeting	PDA-Fe_3O_4	Lab test
		PLGA	Lab test
		MSN-UCNPs	Lab test
Bacterial cell	Recognizes MAMPs and inhibition of pathogen adherence	AuNPs	Lab test
		PLGA	Lab test
Fibroblasts	Homotypic targeting	Semiconducting polymeric NPs	Lab test
	Immune evasion		
Hybrid cells	Dual mode advantage	Polypyrrole	Lab test
		Melanin	Lab test

Table adapted from Dash et al. 2020 [36]

[a]Lab tests = Both in vitro and in vivo work, denoting experiments conducted in cell lines and mice and not involving patient samples or clinical trials

3.5 Conclusion

Biomimetic nanoparticles are a boon to the field of biomaterials as they suffer from minimum resistance in the in vivo system (increased biocompatibility), and the synthesis procedure is also environment friendly. There is no use of organic solvents

and hazardous chemicals in the production process. Sources of cells that are used for cell membrane extraction can be disambiguous and the stability of the biomimetic nanoparticles can be questioned. However, with increasing demands of biomimetic systems and higher number of patients flooding in, it is becoming clear that though this field is naïve, it needs attention because of the immense therapeutic scope.

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Role of Polymeric Nanomaterial in Regenerative Medicine and Stem Cell Biology

4

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Abstract

The combination of nanoparticles (NPs) and stem cells have been extensively investigated in regenerative medicine. Different types of NPs have been designed using various strategies that work very proficiently in controlling the differentiation of stem cells, delivery of therapeutics, and real-time tracking of the transplanted cells, opening new vistas for regenerative medicine. Especially, polymeric NPs have emerged as the game changers, fulfilling the gaps left by many organic and inorganic NPs when it comes to biocompatibility, biodegradability, stability, immune response, invariably enhanced blood circulation time, and economical synthesis. Over the years, novel smart polymeric NPs have also proven themselves to be excellent candidates for targeted delivery of cargo and achieving sustained release. This chapter summarizes the various applicability of polymeric NPs in different areas of stem cells, redefining the regenerative medicine for a better tomorrow.

4.1 Introduction

Since the consent of the first liposomal doxorubicin nano-drug called Doxil, by the Food and Drug Administration (FDA) in 1995, nanotechnology has been extensively employed in medicine and has revolutionized many conventional therapies to

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M. Dash (ed.), *Biomimetic Biomaterials for Tissue Regeneration and Drug Delivery*,
https://doi.org/10.1007/978-981-16-4566-2_4

treat challenging diseased conditions. Nanotechnology manipulates the materials at the nanoscale level, creating nanomaterials which have unique novel properties like large surface area, high strength and stability, optical activity and chemically reactiveness [1].

With the fast progress of nanotechnology in medical research and translational biomedical applications, various multifunctional nanoparticles (NPs) have been developed and are being widely used for the simultaneous diagnosis and therapy of many intimidating and challenging diseases such as neurological disorders [2], different types of malignancies [3], infarctions [4], live-cell tracking [5], and tissue regeneration [6]. There are various types of NPs reported so far that are broadly classified under metallic, ceramic, carbon, and polymeric NPs. One of the fascinating fields where NPs have played an indispensable role is the area of stem cells and regenerative medicine. The role of NPs has been well established in modulating stem cell behavior, directing their differentiation, drug delivery to the stem cells and real-time tracking of the transplanted stem cells [7, 8]. It is well drafted and hypothesized that the heightened understanding of NPs–stem cell interactions ought to improve their application in tissue engineering, stem-cell therapy, and regenerative medicine. However, despite having several superior qualities, the clinical use of NPs made up of metals or carbon is highly restricted due to their inherent toxicity in vivo, eliciting immunological responses, short circulation time, organ homing and accumulation, and fast renal and immunological clearance [9]. To overcome these potholes, recently, the focus has been shifted to NPs synthesized from the polymers considering their advantages over others in terms of biocompatibility, flexible design and synthesis, a variety of structures, and interesting bio-mimetic character. Many of the advantages of nanoparticulate polymeric systems are effective control over particle size, surface characteristics, enhanced permeation, high flexibility, good solubility, efficient loading of drugs, targeted and sustained release of therapeutically active agents, protection of volatile drugs by encapsulating them, and prolonged systemic circulation [10].

The nanoparticles with size from 1 to 100 nm are ideal for biomedical applications. However, owing to high stickiness in the case of soft polymeric material, it has become a challenging task to synthesize smaller NPs. Since two basic synthesis approaches such as top-down and bottom-up, are followed to design 50–300 nm materials [11]. (a) Top-down approach: In this approach, the obtained polymers are dispersed to get nanomaterials. This includes the emulsion evaporation method [12], emulsion diffusion method [13], coacervation method [14], and solvent displacement method [15]. (b) Bottom-up: This approach follows the polymerization of monomers to get a polymeric nanostructure. This includes emulsion polymerization [16], interfacial polymerization [17], interfacial polycondensation and molecular inclusion [18].

Since 1995–2017, 50 pharmaceuticals have received FDA approval for the clinical use of nanoparticle. However, the major challenges of nanoparticle in therapeutics include their pharmacokinetics and biodistribution. Prior reaching to the target site, the nanoparticles has to cross multiple biological barriers such as orally administered nanoparticles must have stability in acidic environment of the

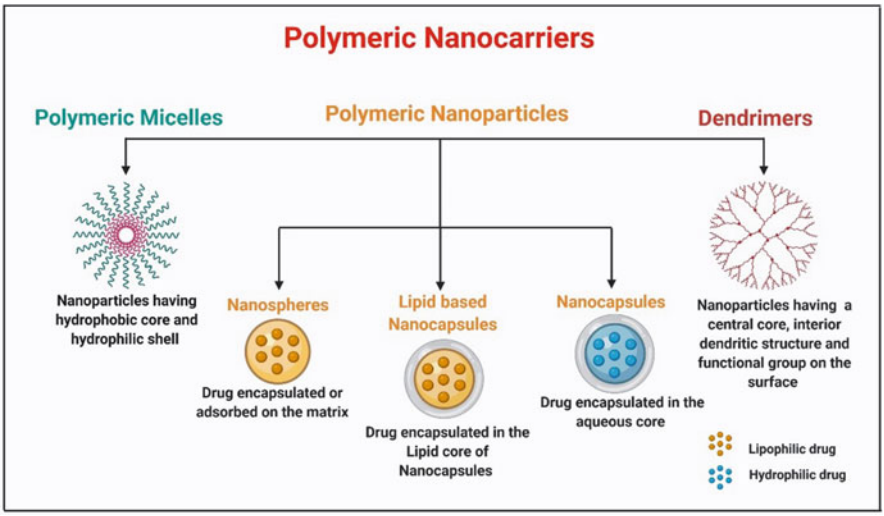

Fig. 4.1 Types of polymeric nanostructures

gastrointestinal tract and should have the ability to penetrate the intestinal epithelium with high systemic bioavailability. In case of IV administration route for central nervous system targeting the nanomaterials have to cross the blood–brain barrier and to achieve that the nanoparticles should be small in size and hydrophobic nature. To reach the target site of brain, the nanoparticles must be carried through the pathways including; paracellular aqueous pathway, transcellular lipophilic pathway, transport protein, receptor-mediated transcytosis, adsorptive transcytosis, and cell-mediated transcytosis depending on their nature. Similarly, in dense tumor stroma with high cancer cell density have poor perfusion thus, nanomaterials face challenges to penetrate the layer. Keeping these challenges in mind, biomaterial-based nanoparticles have gained more attention. Among the various biomaterial-based nanostructures, in this chapter we are focusing on the polymeric nanomaterial owing to their controlled and sustained cargo delivery potential. Additionally, being designed from biomaterial they provide native extracellular matrix environment and good biocompatibility. Hence, due to their favorable characteristics the polymeric nanomaterials are efficient candidates to transport therapeutic to target tissues.

The polymeric NPs can be formed as micelles, dendrimers, nanogels, nanocapsules drug conjugates, and polypeptide and polysaccharide-based NPs (Fig. 4.1). The functionality of a polymeric nanoparticle is determined by its core and corona. However, the polymeric nanoparticle surface functionalization is preferred to enhance their properties such as to increase the nanoparticle residence time in blood and to improve target-specific distribution [19]. More specifically, the NPs are being surface coated with hydrophilic polymers to prevent opsonization, aggregation, and rapid clearance from the body. The polymeric NPs can further be

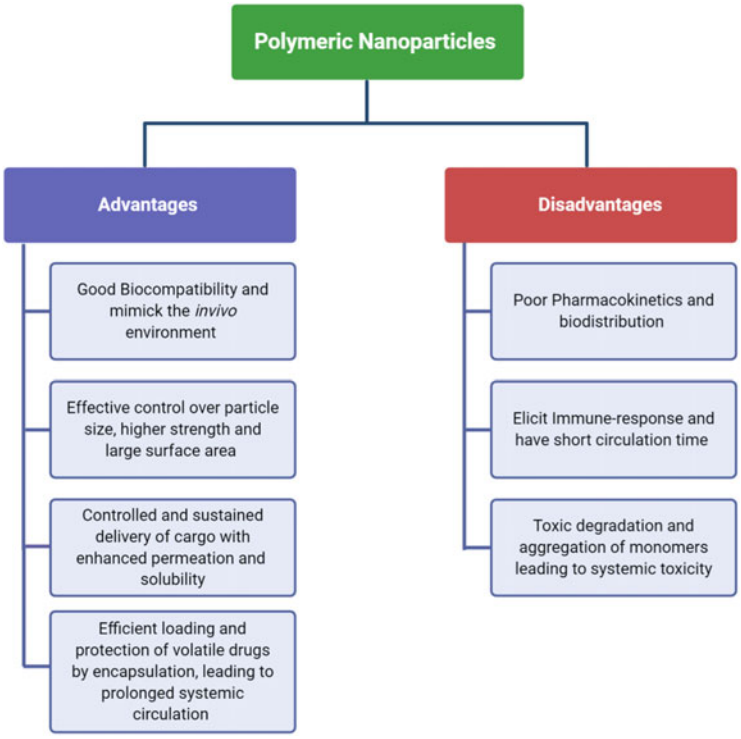

Fig. 4.2 Schematic showing the advantages and disadvantages of polymeric nanoparticles in a tabulated form

conjugated with targeting agents such as folic acid (FA), antibodies (Ab), peptides (e.g., RGD), or aptamers (Ap), and/or imaging agents such as gold NPs (AuNPs), quantum dots (QDs), magnetic NPs (MNPs), and carbon nanotubes (CNTs). Such functionalization makes them multifunctional NPs with great potential in simultaneous targeting, imaging, and therapy of diseases [20]. Despite having the unique characteristic of providing three-dimensional constructs having multiple desired components in nanoscale, the nanomaterials have certain safety and regulatory issues. More specifically, the polymeric nanomaterials show toxic degradation, toxic aggregation of monomers, and toxicity due to associated residual materials. Owing to their complexity, nanomaterials need careful designing, extensive characterization, consistent reproducibility, and in-depth pharmacological profiling (Fig. 4.2).

4.2 Polymeric NPs in Tissue Regeneration

Tissue engineering and regenerative medicine are relative terms where the principles of biological science shake hands with material chemistry and engineering for the repair, restore, and regeneration of living tissues [21]. In tissue regeneration, we discuss about the improvement of tissue (such as bone, cartilage, skin, blood vessels, nerve, cardiac, adipose tissue expression) by the growth and proliferation of cells (may include stem cells or progenitor cells) induced by biomaterial matrices (can be nanoparticles, nanofibers, hydrogels, 2D films, 3D scaffolds, 3D printed materials, etc.) and the addition of bioactive molecules (may be drugs or growth factors, hormones, microRNAs). However, it is evident that an interplay exists between the cell and nanomaterials which encounter many cellular features such as protein folding and collagen banding [22]. Among the various cell types, stem cells are pluripotent cells having the potential to differentiate into three lineages such as osteoblast, chondrocytes, and adipocytes. These are of three categories, i.e., embryonic stem cell, adult stem cell, and induced pluripotent stem cells [8]. In the late 2000s, stem cell enters the field of tissue regeneration giving birth to a new integrated field called regenerative medicine. In 2010, the term was redefined after integrating of material science with the stem cell for the regeneration of complex tissue and organs [23]. To promote stem cell differentiation, many external cues are in use. From a biomaterial perspective the use of polymeric materials to provide appropriate physical and chemical cues for stem cell differentiation is an ideal approach [24]. Human pluripotent stem cells including human embryonic stem cells and induced pluripotent stem cells have gained much attention in tissue engineering applications, due to their ability to generate the desired cell type. For the self-renewal of stem cells, biological cues are required; however, mechanical cues also play a vital role promoting self-renewal of stem cells and directing their differentiation. Keeping in mind the ability of mesenchymal stem cells (MSCs) to differentiate into stromal lineages such as adipogenic, chondrogenic, osteoblastic, myoblastic, and fibroblastic, an extensive study on the role of biocompatible and biodegradable polymeric nanostructure in stem cell modulation is the need of the hour.

In tissue engineering, an ideal biomaterial should support on-site tissue regeneration and should have the ability to degrade in situ and get replaced by the newly formed tissue. The inorganic nanomaterials have achieved global interest in the biomedical field; however, their toxic nature is a major drawback. In this context, the polymeric nanomaterials are the potential biomaterials having protein and polysaccharide backbone mimicking the extracellular matrix. Additionally, the facile synthesis technique, tunable shape and size, low immune reactivity, and in vivo biodegradability make the polymeric nanomaterials suitable for application in tissue engineering. A handful of natural and synthetic polymers are used in biomedical applications. Many natural polymers such as protein based (albumin, collagen, elastin, gelatine, silk, fibrin, keratin) and polysaccharide based (alginic acid, chitosan, cellulose, chondroitin sulfate, hyaluronic acid) are widely in use, due to their biocompatibility and in vivo biodegradability resulting easy elimination

through common metabolic pathways whereas their immunogenicity and poor mechanical property are the major drawbacks. In this regard, the synthetic polymers having better mechanical properties, more control over physicochemical properties, and tunable degradation rates are now being considered more over natural ones in tissue engineering. According to recent articles, commonly used synthetic polymers in regenerative medicine are poly(lactic acid), poly(glycolic acid), poly(lactic-*co*-glycolic acid), poly(hydroxybutyrate), poly(hydroxyvalerate), poly (hydroxybutyrate-valerate), poly(dioxanone), polycaprolactone, polyurethanes, polyphosphazenes, polyanhydrides, polyacetals, poly(propylene fumarate), and poly(ethylene glycol) [25]. Keeping in mind the various pros and cons of natural and synthetic polymers and creating a balance by standardizing the best amalgamation of the two, many natural polymers such as polysaccharides, proteins as well as synthetic polymers such as polyesters, polyamides, polyanhydrides, polyurethanes, and polyacrylates are now being widely used to synthesize polymeric NPs [26–29].

Nowadays, approaches have increased towards developing minimal invasive techniques to achieve specific biological effects on cells. The polymeric nanomaterials enter into cells by endocytosis, passive transport, or phagocytosis depending on the particle size [30]. Thus, they are potential materials to deliver cell impermeable molecules to enhance tissue regeneration. Additionally, their easy availability, facile synthesis, and surface functionalization make them cornerstones in stem cell-based tissue engineering application. The nanostructure influences cell shape, cytoskeleton, and fibronectin deposition by triggering signalling cascades involving Rac, P13K/AKT signalling and upregulation of Nanog and c-Fos [31]. In recent years, the polymeric nanomaterials are mostly used to encapsulate or immobilize bioactive factors or genes for their safe delivery into stem cells to protect the bioactive materials from enzymatic degradation. In this case, the electrostatic interaction of the polymeric nanomaterials becomes advantageous to interact with the biomolecule as well as with the cell.

So far, a detailed study on the effect of polymeric nanomaterials and their degradation rate on stem cell behavior is limited. An extensive study on the mechanism of action of the polymeric nanomaterials on modulating stem cell behavior would provide in-depth knowledge for their use in tissue engineering. Moreover, designing of a multi- and sequential delivery system with specific targeting efficiency will be a smart strategy towards stem cell-based tissue engineering application.

4.2.1 Polymeric Nanomaterials in Bone Tissue Regeneration

Bone defects or injuries due to fracture, traffic accidents, bone tumor resection, old age bone diseases are critical problems in orthopedics. To cure the bone diseases, autologous bone grafting is still considered as the gold standard in clinical settings. However, the autologous bone grafting has many drawbacks including secondary damage, limitation in desired shape, donor site morbidity, insufficiency in autologous bone, and many more. Addressing this issue, the field of bone tissue

engineering has become a hopeful approach which includes regeneration of defect bone at the site of injury without any additional complications [32]. The bone tissue engineering relies on four key factors such as cells that can generate bone tissue, a biocompatible biomaterial that can mimic the extracellular matrix of bone, vascularization for nutrient and waste transport and signals to direct cell behavior [33, 34]. Mesenchymal stem cells have the trilineage differentiation ability, i.e., they can differentiate into osteoblast, chondrocytes, and adipocytes. Each of the cell types is essential for regeneration and repair of musculoskeletal tissue and thus mesenchymal stem cells are considered as potential cell type for bone tissue regeneration. However, to augment their regeneration potential, they need an extracellular matrix-like environment. Moreover, it is evident that micro- and nanosize architecture provide the native bone environment to cells and promote osteogenesis. The microRNAs (miRNAs) play an important role in bone tissue engineering whereas, its application is limited due to its poor stability, unwanted immune response, and low cellular uptake. In this regard, polymeric nanoparticles are used as potential candidates to efficiently deliver non-viral miRNAs in stem cells to enhance osteogenic differentiation [35]. In addition to protected delivery of miRNAs, polymeric nanoparticles also help to preserve the biomolecule from enzymatic degradation by RNase. However, it is evident that the polymeric nanomaterial promotes osteogenic differentiation in stem cells by regulating the Wnt/β-catenin pathway and promotes alkaline phosphatase activity and matrix mineralization [36]. Whereas, extensive study to examine the mechanism of action of different polymeric nanomaterials on various stem cell types is lacking. In bone tissue engineering polymers like chitosan, collagen, alginate, silk, fucoidan, elastin, gelatin, and hyaluronic acid are widely used [32] to mimic the native cellular niche. Among the various natural polymers, chitosan is most promising in osteoregeneration due to the presence of reactive hydroxyl and amino functional groups making the polymer more similar to glycosaminoglycans [37]. These smart polymeric nanomaterials show stimuli-(pH) responsive property owing to protonation and deprotonation in varying pH conditions due to the presence of ionic functional groups. Additionally, the nanomaterial shows potential biological signalling and cell adhesion property and can easily be degraded by the cells. Keeping this in mind the chitosan-based nanomaterials are preferably being used in drug delivery. The modified chitosan-based dendrimer NPs are used to deliver the osteogenic promoting drug such as dexamethasone to pre-program the fate of rat bone marrow stromal cells towards osteoblasts [38]. In another study, the chitosan nanoparticles are used to deliver alendronate to human adipose-derived stem cells for in vitro enhanced osteogenic differentiation [39].

Similarly, collagen is a major component of bone extracellular matrix and thus helps in cell adhesion and proliferation towards bone tissue engineering [40]. The natural polymer silk carries the Arg-Gly-Asp (RGD) sequence to promote cell attachment, proliferation, and integrin binding [41]. Another polymer alginate possesses advantages like the formation of the desired shape, controlled release of growth factors, easy in ligand attachment, and good biocompatibility, and this makes it a very good bone tissue engineering biomaterial [42]. The other natural polymer,

fucoidan has a repeating arrangement of alternate α(1–3) and α(1–4) glycosidic bonds [43]. This facilitates bone cell proliferation and ALP, BMP-2, Runx-2, osteocalcin, osteopontin, and Col-I expression [44]. The BMP activates the MAPK pathway which leads to induction of Runx2 transcription towards bone differentiation. It is known that synthetic polymeric materials provide biocompatibility, adequate mechanical strength, easy reengineering process and have slow degradation rate, which supports sustained release of the desired biomolecule in bone tissue engineering. The synthetic polymers like PCL, PLGA, and PLA are mostly used in osteogenic regeneration [45]. The PLGA nanoparticles are used to deliver bioactive molecules such as BMP-2 to enhance osteogenesis [46].

Other than nanoparticles the nanofibrous mat have also gained attraction in stem cell-based bone tissue engineering due to their nanofiber topography mimicking the extracellular matrix, thus providing good substrate for cell proliferation and bone tissue regeneration [47]. Electrospun PLGA meshes were used to provide constant calcium ion to modulate the fate of adipose-derived stem cells towards osteogenesis [48].

4.2.2 Polymeric Nanomaterials in Cartilage Tissue Regeneration

The cartilage regeneration is a complicated process as the spontaneous healing process is rare and causes osteoarthritic issues. For induced cartilage regeneration, the synthesis of a large amount of extracellular matrix is required. During chondrogenesis, fibrillar proteoglycan layer is predominant in addition to the upregulation of aggrecans and *SOX9* gene [49, 50]. Therefore, delivery of this specific gene helps in cartilage regeneration. The conventional viral vector-mediated gene delivery has many complications like high cost, limited quality, cell damage due to infection and causes adverse immune problems [51]. To overcome this issue, non-viral nanoparticles are now being used in gene delivery for cartilage regeneration. For efficient gene delivery to the target site, few essential aspects are required such as proper conjugation of biomolecules (DNA, siRNA, etc.) to the nanoparticle surface, easy cellular internalization of the nanoparticle, biodegradability, biocompatibility, and mucoadhesiveness. Keeping this in mind, in cartilage tissue engineering cationic polymer NPs are efficiently being used to increase the rate of binding with negatively charged biomolecules by ionic–ionic interaction. Some cationic polymers, namely poly(L-lysine), polyethyleneimine, chitosan, poly(β-amino ester), poly(amidoamine), poly(lactic-*co*-glycolic acid) are used in gene delivery and cartilage tissue regeneration [52–54]. Another complication in cartilage tissue engineering is the cell type for regeneration. The most commonly used cell for cartilage regeneration is the native chondrocyte. However, in in vitro expansion these cells undergo rapid differentiation and thus form fibrocartilage with weak mechanical strength [55]. Hence, the MSCs have come out as an efficient alternative for cartilage regeneration owing to their methodical differentiation in to chondrocyte [56].

4.2.3 Polymeric Nanomaterials in Regeneration of Central Nervous System

The Central Nervous System (CNS) consists of the brain and the spinal cord and co-ordinates major activities of the human body. Disorders in CNS account for major health issue burden worldwide [57]. However, the treatment of CNS-related diseases is more challenging owing to the meticulous trafficking by the Blood–Brain Barrier (BBB) [57]. For the treatment of CNS diseases, various invasive methods such as intraventricular brain infusion, transient BBB disruption by intravenous injection, and intracerebral implantation have been followed. However, these strategies are associated with drawbacks like easy access of pathogen and toxic effect. Thus, in recent years, a less invasive approach for drug delivery has been proposed to enhance safety and efficacy in CNS-related disease treatment. Among the less invasive methods, nanocarrier conjugate drug delivery is a promising method owing to their high surface to volume ratio, polydispersity, and stimuli-responsive properties. Furthermore, the nanocarrier biocompatibility, small size, and rapid biodegradability are important requirements for nanocarrier-mediated drug delivery to brain. In recent years, various non-viral biomaterials, such as the poly(β-amino ester)s nanoparticle, polyethyleneimine nanoparticles, poly(lactic acid) nanoparticles, poly(lactide-co-glycolide) nanoparticles, chitosan nanoparticles, and albumin nanoparticles, have been studied as potential gene delivery vectors with facile synthesis and scale-up [58].

In broader sense the treatment of CNS diseases could be through three pathways such as cessation of a pathological process, enhancement of a protective mechanism, and regeneration of damaged tissue. Stem cell in neuronal disease treatment addresses all the pathways due to its ability of damage cell replacement/repair by merging with endogenous cells, immunomodulatory function, and paracrine effects. However, few extrinsic and intrinsic factors are required to direct the stem cell towards neuronal lineage. Intrinsic factors such as small molecules, mRNAs, growth factors, and other biomolecules play a key role in deciding the cell fate more effectively as they manipulate the cell transcriptional network. In neurogenesis, several transcription factors such as Mash1, neurogenin-2, and NeuroD are required to promote neuronal differentiation [59]. Nowadays, the role of polymeric nanoparticle is not limited to modulate stem cell fate by delivering desired cargo, whereas they are also being used as biocompatible probe for stem cell tracking [60]. Moreover, the field of polymer-based nanotechnology in neuroscience combinedly forming nano-neuroscience is still at the early stage which needs to be explored further.

4.2.4 Polymeric Nanoparticle in Wound Healing

Skin damage and subcutaneous lesions are one of the most common of injuries that people suffer from, and burn wounds get even more challenging to tackle with. Many wound dressings have been used; however, not sufficient satisfaction was

received in terms of infections, scars, and pain, till the introduction of polymeric gels as wound dressings and skin regeneration. Polymeric nanoparticles highly enhance the drug therapeutic efficacy by preventing drug degradation by proteases present at the wound site and facilitating sustained drug release, thereby reducing the administration frequency or need to change the bandage time to time, ensuring a long-term maintenance of effective drug concentration at the wound site. They can also very efficiently deliver certain biomolecules such as antimicrobial agents, growth factors, and genes, without degrading them. Moreover, they create a perfect moist environment for activation and acceleration of wound healing process minimizing the pain that a patient might have to suffer [61].

Among the polymers chitosan is a promising wound dressing material as it has antimicrobial properties and accelerates wound healing [62]. Polyvinyl alcohol is another hydrophilic polymer that is an ideal material for skin regeneration due to its chemical and thermal stability, biodegradability in the physiological environment, and cytocompatibility [63]. Gelatin also has potential use in skin tissue engineering applications [64]. In one of the recent reports, PVA/gelatin/MIP nanoparticle-based wound dressing was shown to be a suitable candidate to accelerate the healing process and improve the tissue regeneration [65]. The molecular imprinted polymeric nanoparticles had a selective binding site for biomolecules and gave controlled release and prolong durability, thus were considered as promising natural polymeric nanomaterial for wound healing. The delivery of growth factors such as VEGF by the polymeric nanoparticle showed promising tissue regeneration in terms of angiogenesis at the site of delivery [66] (Table 4.1).

4.3 Cancer Stem Cells Therapy Using Polymeric NPs

Therapeutic resistance, metastasis, and disease relapse in the case of cancer can be credited to the presence of cancer stem cells (CSCs), which are challenging to eradicate owing to their high resistance to conventional therapies and high plasticity [83]. CSCs have been identified in and isolated from several malignancies, capable of self-renewal, and fuel the propagation and development of tumors. The only way to stop the re-occurrence or prognosis of cancer is by targeting a high drug concentration in the cellular environment of these CSCs.

Polymeric NPs have garnered a lot of attention as the most efficient carriers for drug delivery with regard to having pliable physical properties, various synthesis strategies, and excellent pharmacokinetic properties such as drug loading/release, high stability, long-term circulation of encapsulated drugs, bioinertness, and tunable degradation rates [84, 85]. Polymeric NPs have been employed in both active and passive modes (Fig. 4.3).

The recent research hotspot of polymers utilized for drug-loaded NPs includes poly(D,L-lactic-*co*-glycolic acid) (PLGA), polylactic acid (PLA), poly(ethylene glycol) (PEG), chitosan (CS), and hyaluronic acid (HA), etc.

Table 4.1 List of polymeric nanoparticles helping in stem cell differentiation for various tissue regeneration processes

Polymer	Synthesis method	Structure	Function	Cell source	References
Chitosan	Ionic gelation	Cationic spherical nanoparticle of size <300 nm	Odontogenic differentiation	Stem cell from apical papilla (SCAP)	[67, 68]
Chitosan	Ionic gelation	Cationic spherical nanoparticle of size <300 nm	Osteogenesis	Rat bone marrow-derived mesenchymal stem cell (RBMMSCs)	[35]
Carboxymethyl chitosan and polyamidoamine	Chemical modification	Cationin, spherical, dendrimer of size <250 nm	Osteogenesis	Rat bone marrow stromal cells (RBMSCs)	[30, 38, 69, 70]
Poly(L-lactic acid), poly-benzyl-l-glutamate, collagen	Electrospinning	Nanofibers with fiber diameter <400 nm	Osteogenesis	Adipose-derived stem cells (ADSCs)	[71]
DNA	Complementary base-pairing reaction	Tetrahedral nanomaterials with <20 nm size	Osteogenesis	Adipose-derived stem cells (ADSCs)	[36]
Chitosan, silk fibroin	Electrospinning	Nanofibers with fiber ~500 nm	Osteogenesis	Human bone marrow-derived mesenchymal stem cell (hBMMSCs)	[72]
Poly(lactic-*co*-glycolic acid)	Water-in-oil-in-water solvent evaporation	Nanosphere with size <100 nm	Chondrogenesis	Human MSCs (hMSCs)	[54]
Poly(ethyleneimine), poly(lactic-*co*-glycolic acid)	Water-in-oil-in-water solvent evaporation	NPs with size <100 nm	Chondrogenesis	Human MSCs (hMSCs)	[73]
chitosan, hyluronic acid	Ionic complex assembly	Monodispersed cationic NPs of size ~100 nm	Chondrogenesis	Articular chondrocytes and infrapatellar fat pad-derived MSCs (ACs and IPFA MSCs)	[74, 75]
Poly(β-amino ester)s	Chain polymerization	Nanoparticle of size <150 nm	Neuronal differentiation	Human fetal tissue-derived neural stem cells (hNSCs)	[76, 77]

(continued)

Table 4.1 (continued)

Polymer	Synthesis method	Structure	Function	Cell source	References
Retinoic acid, polyethylenimine, dextran sulfate	Complex assembly	Cationic NPs with size <250 nm	Neuronal regeneration	Subventricular zone neural stem cells (SVZ)	[78]
Poly(*N*-isopropylacrylamide)	Polymerization reaction	Thermo-responsive nanoparticle with phase transition ability of size <400 nm	Neuronal differentiation	Human-induced pluripotent stem cells (hiPSC)	[79]
DNA	Co-assembly	Nanotubes with 15–20 nm diameter and microns in length	Neuron regeneration	Neuronal stem cell (NSCs)	[80]
Chitosan	Ionic gelation	Spherical nanoparticle with size range from 50 to 100 nm	Hepatocyte regeneration	Bone marrow-derived stem cells	[81]
Poly(β-amino esters)	Polymerization reaction	Biodegradable nanoparticle	Angiogenesis	Human mesenchymal stem cell (hMSCs) and human embryonic stem cell-derived cells (hESdCs)	[66]
Chitosan nanoparticle incorporated in PCL, gelatine nanofiber mat	Ionic gelation for nanoparticle and electrospinning for nanofiber	Globular nanoparticle of size <300 nm, and unimodally distributed nanofibers with fiber diameter ~1500 μm	Skin regeneration	Human endometrial stem cells (EnSCs)	[82]

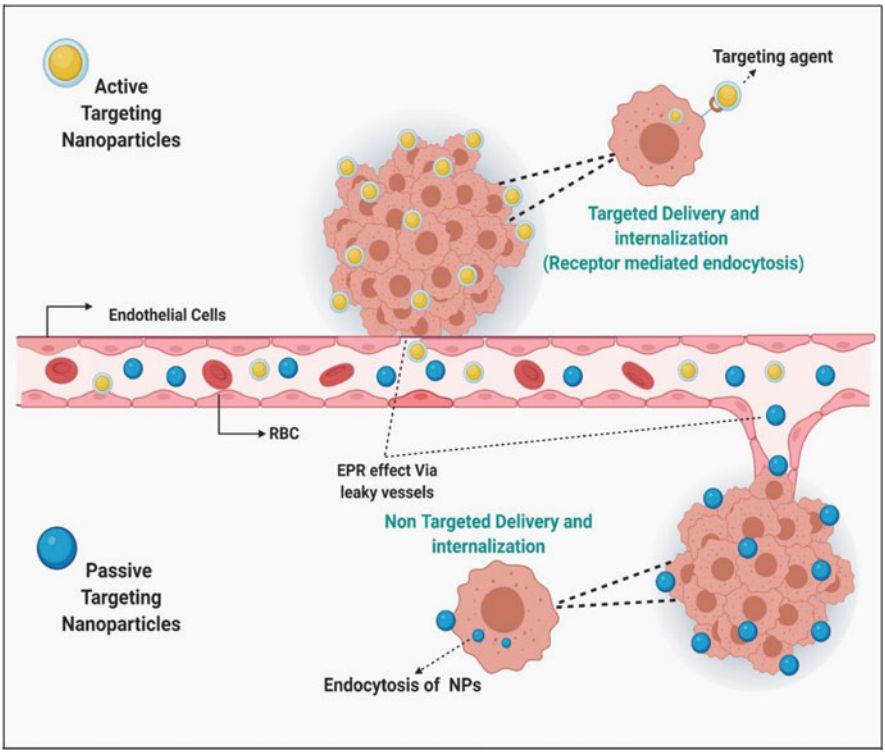

Fig. 4.3 Active and passive mode of drug delivery (legends). *RBC* red blood cells, *EPR* enhanced permeability and retention, *NP* NPs

4.3.1 Polylactic Acid (PLA)

PLA is an FDA-approved polymer extensively being utilized for drug delivery due to its excellent biodegradability, which makes it possible to be wholly excreted after metabolism. PLA NPs loaded with quercetin (Qt) displayed superior sustained release kinetics and inhibited the human breast cancer cells [86]. Yang et al. formulated a CSCs-targeting PLA-encapsulated docetaxel nanoparticle for anti-metastatic therapy in a nude mice model of liver metastasis [87]. Li et al. demonstrated that low dose of decitabine (DAC) encapsulated in PLA NPs (NPDAC) and doxorubicin-encapsulated PLA NPs (NPDOX) could be used to sensitize bulk breast cancer cells and CSCs to significantly replace chemotherapy by overcoming the drug resistance by ALDH(hi) cells [88].

4.3.2 Poly(Lactide-*co*-Glycolide) (PLGA)

PLGA is one of the US FDA-approved polymers for several therapeutical and clinical applications due to its outstanding properties such as biodegradability and biocompatibility [89]. It is the most widely used synthetic polymers in drug-loaded NPs development for cancer therapy [90]. Some other important factors like the crystallinity of PLGA, faster degradation kinetics undergoing hydrolytic degradation in the aqueous environment, glass transition temperature, inherent viscosity, and molecular weight which are very critical factors for modulating a drug delivery system. Gao et al. formulated (PLGA/TPGS) NPs for the co-delivery of docetaxel (DTX), a chemotherapeutic drug and salinomycin (SAL), an anti-CSCs drug, maintaining a synergistic ratio of the drugs in vivo for 24 h to eliminate both CSCs and cancer cells [91]. Wedelolactone has been reported to be effective against breast cancer and ineffective in targeting cancer stem cells; however, the wedelolactone-encapsulated PLGA NPs (nWdl) not only increased its uptake by breast cancer cells and the CSCs, it also enhanced drug retention and sustained release within these cells [92]. Following the same lines, but it was very recently reported that mangostin-encapsulated PLGA NPs (Mang-NPs) could efficiently inhibit pancreatic cancer growth and metastasis by arresting the proliferation of not only pancreatic cells but also destroying the self-renewal capacity of CSCs [93]. Moreover, PLGA-incorporated hyaluronic acid, and PF127-based pH-dependent NPs were prepared for actively targeting CSCs [94]. The study elucidated simultaneous acidic pH-triggered drug release and thermal responsiveness to overcome the drug resistance of the CSCs.

4.3.3 Chitosan

Endowed with excellent properties of biocompatibility, bioinertness, positive surface charges, bioactivity, biodegradability, antimicrobial properties, and mucoadhesion, chitosan qualifies to be a good candidate for diverse biomedical and pharmaceutical applications such as scaffolds for cell growth, tissue engineering, transplantation of encapsulated cells, drug/gene delivery, and wound healing [95]. It has been proven that chitosan NPs of around 20 nm can be efficiently delivered into the tumor to actively target and get internalized in $CD44^+$ cells by EPR effect, wherein, the drugs in NPs would then be released and diffused into the cytosol, eventually accumulating in the nuclei and killing the cancer stem cells [96]. Rao et al. designed chitosan decorated DOX-encapsulated NPs for targeting $CD44^+$ cancer like stem cells (CLSCs) [97]. The doxorubicin encapsulated in these NPs was seen to have increased cytotoxicity by six times compared to the free drug for eliminating CSCs in vitro using 3D mammary tumor spheroids as well as in vivo using an orthotopic xenograft human breast cancer model. Similarly, hyaluronic acid-coated chitosan NPs encapsulated with 5-Fluorouracil were developed to enhance drug accumulation in tumor $CD44^+$ cells thereby improving its antitumor efficiency [98]. Recently, tumor tropism capacity of MSCs (MSCs) and the

controlled release profile of chitosan NPs were exploited for obtaining an inhibitory action on pulmonary melanoma metastasis by designing a cell-nanoparticle hybrid vector where MSCs were used as the targeting cellular carrier, whereas, biotinylated chitosan NPs as the drug depot [98].

4.3.4 Poly(Ethylene Glycol) (PEG)

Excellent, biocompatibility, hydrophilicity, chain mobility, and non-immunogenicity make PEG as one of the most used polymers for increasing water solubility and retention of many hydrophobic drugs. Self-assembled DOX and THZ-loaded PEG-based polymeric micelles were developed for antiproliferative effect on both cancer cells and CSCs [99]. To increase the solubility, a vascular-disrupting agent was bonded to a PEG-based polymer and self-assembled into NPs in the aqueous solution to load DOX, for targeting both breast cancer cells and CSCs in vivo on MCF-7 tumor bearing nude mice demonstrating the enhanced antitumor efficiency [100]. Bortezomib, an anti-cancer hydrophobic drug was loaded in hydrophilic PEG-b-PLA NPs to enter the targeted CSCs, and induce apoptosis and death. This nanoparticulate system kept the drugs in CSCs for a longer time and accumulated the drugs in lesion tissue, inhibiting the proliferation of CSCs, thereby, promoting the therapeutic efficacy in breast cancer therapy [101].

4.3.5 Silk

Silk NPs are known to improve chemotherapeutic drug targeting and delivery to solid tumors by manipulating tumor pathophysiology, exploiting the cellular pharmacokinetics of the drug and ultimately resulting in accumulation in lysosomes that triggers drug release. Silk has been proved to be a biocompatible and biodegradable native biomolecule and has a safe record in vivo [102]. Salinomycin and Paclitaxel-loaded silk fibroin NPs (SF-NPs) were synthesized to simultaneously kill CSCs and non-CSCs and was established as an efficient multi-drug delivery platform for locoregional chemotherapy [103]. A recent study carried out by Totten et al. demonstrated the importance of both pH and lysosomal enzyme activity on doxorubicin release from silk NPs, thereby providing direct evidence for lysosomotropic drug delivery in live cells [104]. Perteghella et al. designed a unique carrier-in-carrier drug delivery system, wherein, silk NPs loaded with curcumin were accumulated in the cytosol around the nuclear membrane in the MSCs [105]. These NPs-loaded MSCs released extracellular vesicles containing curcumin-loaded NPs. This method gave a novel class of drug delivery systems that gave synergistic effects of regenerative cell therapies and drug delivery.

4.4 Polymeric NPs for Stem Cell Tracking

Despite the numerous benefits of stem cells, their use is greatly restricted owing to the inability to accurately study their cellular fate during the regeneration and differentiation processes. Noninvasive, quantitative, and qualitative tracking and monitoring of various functional processes, such as their differentiation process, proliferation dynamics, and homing, is very important to have their in-depth understanding [106].

The most commonly used imaging techniques for stem cell tracking with NPs include magnetic resonance imaging (MRI), fluorescence imaging, nuclear imaging using radioactive isotopes, Positron emission tomography (PET), single-photon emission computed tomography (SPECT), and photoacoustic imaging [107]. Usually, two approaches are used for labelling of stem cells, namely direct labelling, wherein, cells are incubated with appropriate intracellular probes, and indirect labelling, which involves expression of a particular indicator by inserting a reporter gene into the existing genome of the cell. Direct labelling, though easy to achieve is accompanied by a lot of shortcomings with rapid signal decay and in-homogenous distribution of the marker molecules between the cells. On the other hand, indirect labelling involves genetic manipulation of the cells, thereby, raising concerns on their viability and being more time consuming and expensive [108]. Moreover, the different dyes and contrast agents used for tracking produce a lot of interference due to autofluorescence.

With the progress in nanotechnology, such loopholes have been, to some extent, very efficiently filled in enabling successful live-cell tracking and real-time monitoring of various intracellular processes at the biomolecular level [109]. Polymer-based NPs have recently gained a lot of interest in not only differentiating but also tracking different types of stem cells. Organic semiconducting polymer NPs (OSPNs), having excellent optical properties and higher photothermal conversion efficiency, are promising nano-agents for photoacoustic imaging, fluorescence imaging [110, 111], photothermal, and photodynamic therapy [112]. Recently, photoacoustic imaging of embryonic stem cell-derived cardiomyocytes was carried out using OSPNs; however, their excitation wavelength was restricted to the NIR-I region which limited its use in real life [113]. Yin et al., on the other hand, prepared positively charged $OSPNs^{+}$ for stem cells photoacoustic imaging and tracking in the NIR-II region [114]. These $OSPNs^{+}$ had significantly higher signal-to-noise ratio than NIR-I light excited PA imaging, thereby facilitating a better understanding and evaluation of stem cell-based therapies. Many organic and inorganic NPs induce toxicity in vivo due to their accumulation in various regions like liver, spleen, kidneys, etc.; therefore, biodegradable polymeric NPs would be an ideal candidate for bioimaging. Following these lines, Mohseni et al. designed chitosan alginate nanoprobes as an MRI contrast agent for tracking of MSCs derived from Wharton's Jelly [60]. Their in vitro and in vivo results showed the compatibility of these nanoprobes with stem cells with reduced t1 relaxation times, making these polymeric NPs a novel natural nanoprobe for stem cell tracking. Cell transplantation has enabled the repair of injured and damaged tissue functions, and in order to make the

procedure successful, it is essential to not only ensure the safety and efficacy of the treatment, but also to understand their migrational dynamics and regeneration potential better; however, tracking and monitoring of these transplanted cells becomes a daunting task. One such study was carried out by Mahara et al. where they succeeded in tracking millions of transplanted living cells using a polymeric MRI contrast agent [115]. They optimized the labelling conditions for MSCs via a water-mediated sonoporation method and successfully visualized almost the same number of MSCs as routinely used in cell transplantation studies. Similarly, in vivo MRI tracking of transplanted neural stem cells (NSCs) in acute ischemic stroke using the cationic amphipathic polymer of PAsp(DMA), lysine, and hydrophobic CA [116]. These polymeric micelles did not show any adverse effect on cell viability, proliferation, apoptosis, and differentiation. They served as a versatile nanoplatform for in vivo tracking of therapeutic stem cells in regenerative medicine. Real-time monitoring of the transplanted neural stem cells (NSCs) via iridium complexes encapsulated polymeric nanospheres using one- and two-photon imaging properties with stable phosphorescence lasting 72 h.

4.5 Future Prospects and Challenges

Over the course, the field of nanomedicine has been universally transformed, providing promising results due to multidisciplinary and combinatorial approaches. The future of this lies between theoretical and experimental researchers, in addition, the pharmaceutical industry, clinical physicians, and the regulatory agencies may play a critical role and will benefit to correlate and implement the various approaches from bench to bedside. However, in this way, we will be able to initiate the next level of clinical studies. NPs (NPs) made up of polymers clinching recognition because of low cytotoxicity, high biodegradability, and versatility, which opens a vista for broad prospects of materials that could be used at a various or specific application. The drug delivery system is one of the promising areas where polymeric NPs can minimize the risk and drawbacks connected with the old drug administration routes. Whereas, it has become more sophisticated due to better controlled release, therapeutic efficacy, specific site action via active ingredients, therefore avoiding the systemic release of the active molecule. Among the other therapeutic needs, developing novel and efficient mechanisms for ocular therapy is a current need of the hour. However, drug delivery to the eye entails huge dare due to the complicated anatomy and physiology of the organ. Various studies suggest that intravitreal implants can be an alternative for sustained drug delivery in the posterior segment of the ocular globe. Still, it has been reported that it requires various injections or even surgery with successive risks. Therefore, to overcome these obstructions, nanotechnology for ocular therapy has been gaining a high reputation, specifically for biodegradable polymers. The role of these modified designs is to achieve effective dosing at the injury/infected site, either by improved formulation solubility properties, enhanced bioavailability, targeted delivery, more shelf life, and sustained release of medicine.

Oral drug deliveries are the easiest way of administering medicine. On the other hand, oral administration for chemotherapeutic drugs is limited due to an extensive first-pass effect, poor solubility, efflux transport, low intrinsic permeability of drug limits, and poor solubility of drugs. Apart from these drawbacks, it is highly desirable in terms of its ease in synthesis and administration, a vast variety of formulations and most importantly, better patient compliance in terms of chronic ailments. The oral route can be used for systemic as well as localized gastrointestinal tract effects. The major challenge in the delivery of hydrophobic drugs, as they cannot reach the intracellular environment by crossing the cell membrane because of the surrounding aqueous microenvironment around the tissue or organ, whether given orally or IV. On the other hand, nanotechnology engineered nanocarriers with varied sizes from 1 to 1000 nm, modified surface properties such as charge and ligands attached for specific cellular receptors, and shape based upon the features required for carrying a specific molecule to the target site. Drug delivery systems based on NPs have shown promising results and improved the cancer therapy.

In the field of polymer nanocomposites, the majority of the research work has been carried out in polymers/metal oxides systems. The significant improvement of the physical and catalytical properties of the topography has been observed after the modification of metal oxide surfaces with typical polymers. Few studies have been reported stating the benefit of incorporating ZnO to the different polymer matrix, such as increases the modulus and strength, homogenous dispersion of the filler. Moreover, ZnO/polymer nanocomposites are considered as advanced smart material due to its capability of measuring the deformation, flexibility, wearable electronics, no toxicity, and lightweight. Thus, many more explorations must be done towards the industrialization of ZnO/polymer nanocomposites to make them useful for human needs. The future of therapeutics is emerging with polymeric NPs, such as it provides an alternative to lipid NPs for the delivery of RNA therapeutics. This is especially true for extrahepatic delivery-based applications. Various approaches in “intelligent” polymeric design may be used to upregulate cellular uptake, endosomal escape, and the release of nucleic acids from their carriers. These carriers can be designed to disclose sensitivity and specificity towards individual targets diseases/indications, and relevant subcellular compartments, each of which attains their own individual challenges. A “one-size” fits all model cannot be expected for the drug delivery therapeutics. Altogether, polymeric materials are turning up as an alternative polymeric material that might be created by tuning the physiochemical characteristics needed for efficient delivery, while maintaining high loading capacity and low immunogenicity.

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Mechanical Characterization of Additive Manufactured Polymeric Scaffolds for Tissue Engineering

5

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Abstract

In the last decades, tissue engineering has become a promising and important field of research that is opening new perspectives for the treatment of tissue diseases or injuries. Scaffold-guided tissue engineering involves the fabrication of 3D biodegradable polymeric structures with a porous architecture suitable for cell adhesion and proliferation, as well as the regeneration of the damaged tissue. Biodegradable polymers represent the most employed materials for the fabrication of tissue engineering scaffolds, thanks to their versatile physical-chemical properties and relatively easy processing. Additive manufacturing (AM) techniques are attracting growing interest for the fabrication of scaffolds with customized anatomical shape, overall porosity, as well as pores' dimension and geometry. Scaffold's morphological, mechanical, and biological properties optimization is fundamental to obtain structures that precisely mimic the properties of the target tissue and support its regeneration. In particular, scaffold mechanical properties have to be carefully tailored to avoid, for example, the early collapse of the supporting structure or stress-shielding phenomena. This book chapter presents the most important aspects involved in the mechanical characterization of biodegradable polymeric scaffolds fabricated by AM. To this purpose, common strategies employed for enhancing and tuning the mechanical properties of additive manufactured scaffolds are discussed in depth depending on the selected material(s) and the employed AM technique. Relevant experimental approaches, such as the formation of polymeric blends and composites, chemical modification of the starting materials, tailoring scaffold architecture, variation of fabrication parameters, and post-processing treatments, are accordingly overviewed by

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M. Dash (ed.), *Biomimetic Biomaterials for Tissue Regeneration and Drug Delivery*,
https://doi.org/10.1007/978-981-16-4566-2_5

analyzing representative examples reported in literature and focused on biodegradable polymers of either natural or synthetic origin.

5.1 Introduction

Human tissue and organ failure caused by defects, injuries, or other types of damage is one of the most impacting problems in health care system. Relevant surgical strategies include the implantation of total artificial substitutes (e.g., joint prosthesis and artificial kidney) or non-living processed tissues, such as in the case of porcine bioprosthetic heart valves, as well as the transplantation of autogenic or allogenic tissues [1]. All these approaches present major drawbacks, such as risks of infection and lack of biocompatibility, as well as limited durability in the case of non-biological materials. Tissues and organs transplantation is often challenging given the shortage of donors and necessity of lifelong immunosuppression, with relevant complications. In addition, in many cases transplanted tissues do not meet the structural and functional requirements of native tissues, besides the possibility of involving donor-site complications. For these reasons, tissue engineering (TE) is a promising and important field of research that is opening new perspectives for the treatment of tissue diseases [2].

Additive manufacturing (AM) is a technology that is earning great importance in TE because it allows the fabrication of scaffolds personalized on the patient, as well as on a specific tissue defect. Indeed, advanced AM approaches are aimed at tailoring scaffolds composition and anatomical shape, as well as structural and functional properties, to a given tissue regeneration process. Therefore, the characterization of additive manufactured scaffolds physical-chemical, mechanical, and biological properties is of the utmost importance. However, although the fast-growing number of articles involving the mechanical characterization of novel polymeric scaffold prototypes by AM, literature overviewing experimental studies on this aspect is still scarce.

This book chapter is aimed to present the most important aspects involved in the mechanical characterization of biodegradable scaffolds fabricated by AM. In particular, TE is introduced by highlighting physical-chemical, structural, mechanical, and functional requirements of biodegradable scaffolds, in relationship to the most investigated biodegradable polymers in this field. The working principles and application in the TE domain of the different classes of AM techniques so far employed to process biodegradable polymers are also described. Relevant literature on mechanical characterization of additive manufactured scaffolds based on biodegradable polymers of either synthetic or natural origin (e.g., aliphatic polyesters and polysaccharides) is also reviewed to underline the different factors influencing sample behavior under specific mechanical stimuli.

5.1.1 Polymeric Scaffolds for Tissue Engineering

One of the first definitions of TE made in 1993 by Langer and Vacanti was "an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue or organ functions" [3]. TE offers outstanding opportunities for Regenerative Medicine, a field seeking to relieve patients from suffering the consequences of diseases and injuries by restoring the function of their damaged tissues and organs [2].

TE aims at inducing the formation of a specific tissue in a specific location through the selection and manipulation of cells, biomaterials, and/or biologic stimuli. These living tissue constructs should be functionally, structurally, and mechanically comparable to the tissue they must replace [4]. Many efforts have been made to engineer different tissues, including bone [5], cartilage [6], nerves [7], blood vessels [8], skin [9], as well as tissues of the gastrointestinal and urogenital systems [10].

TE has progressed from the use of biomaterials designed to repair or replace diseased or damaged tissues, to the use of three-dimensional (3D) scaffolds [2]. Scaffolds are 3D porous and biodegradable structures that favor cells adhesion and differentiation, mimicking extracellular matrix (ECM) physiological functions, and constitute a structural template that fill the lesion [11]. The scaffold should be non-immunogenic, non-toxic, biocompatible, biodegradable, and easily manufactured. In addition, scaffold macro- and microstructural properties should be properly designed since they affect cells survival, cross-talk, growth, and preservation of native phenotypes, among other activities essential for the tissue regeneration process [11].

Scaffold-guided TE involves seeded cells on a bioresorbable scaffold of synthetic and/or natural origin. The developed tissue engineered construct can be implanted in the targeted site where the defect is repaired by an optimal interaction between the graft and the host tissue. Treatments based on this approach would eliminate the problems of donor-site scarcity, adverse immune reaction and pathogen transfer that characterize conventional treatments, as previously mentioned. Bioactive factors can be employed to stimulate tissue growth and differentiation by two different strategies. In the first approach, exogenous cells used for TE can be treated in vitro with growth and differentiation factors before implantation. The other strategy implies either the loading of the factors within the scaffold polymeric matrix, to provide a favorable environment for the responding hosting cell to populate, or their targeted administration after implantation [11] (Fig. 5.1).

A TE scaffold should meet some key requirements, as summarized in the following [11].

An interconnected and spread porosity with a highly porous surface and microstructure. This would allow in vitro cell adhesion, ingrowth, and reorganization and would provide the necessary space for neovascularization in vivo. Pore

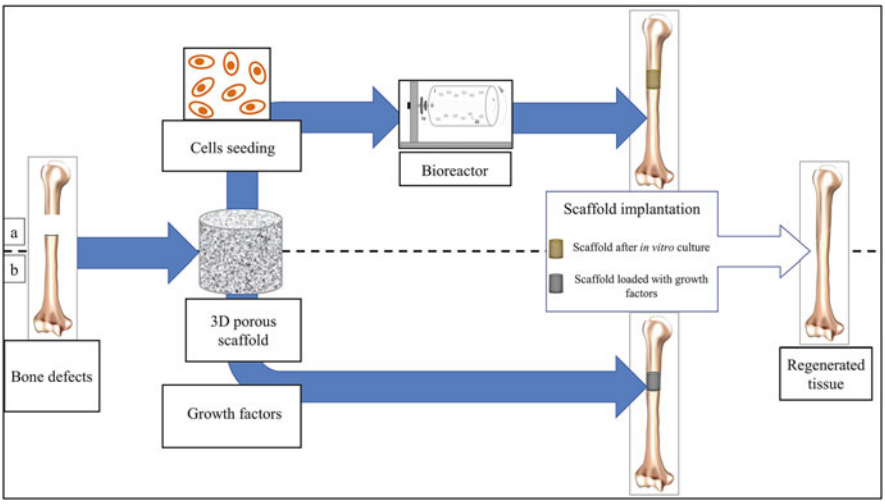


Fig. 5.1 Scaffold-guided TE strategies: (**a**) scaffold implantation after in vitro cells expansion (the image of the bioreactor is modified with permission from [12]); (**b**) implantation of a growth factors-loaded scaffold

interconnectivity directly influences the diffusion of physiological nutrients and gases to cells, as well as the removal of cell metabolic waste and by-products.

Surface properties, such as morphology, wettability, and charge, suitable for in vitro cell adhesion, migration, phenotype maintenance, and intracellular signaling, as well as in vivo cell recruitment and integration at the tissue–scaffold interface.

A biodegradation rate tailored to tissue regeneration kinetics to avoid a critical decrease of the mechanical properties of the tissue engineered construct. Moreover, a fast polymer biodegradation can lead to high concentrations of degradation products that may be harmful for the cells, like in the case of acidic molecules. Although the biodegradation rate is a key element when considering scaffolds for tissue engineering, biostable scaffolds are required in some cases. Indeed, either metallic [13] or polymeric [14] biostable scaffolds are employed as permanent implants or removable space maintainers, in order to solve the problem of limited tissue regeneration, often observed around alloplastic implants.

Mechanical properties suitable to maintain the spaces required for cell ingrowth and matrix formation. Moreover, a scaffold must provide sufficient temporary mechanical support, matching the mechanical properties of the host tissue as closely as possible, to bear in vivo stresses, which play a significant role in homeostasis, remodeling, and repairing of tissues, especially load bearing ones. A gradual transfer of the burden to the regenerating tissue should therefore support and promote the restoration and maintenance of tissue functions. However, in the case of rapidly degrading polymers, physiological stresses may be transferred to the developing tissue prior to sufficient ingrowth and remodeling,

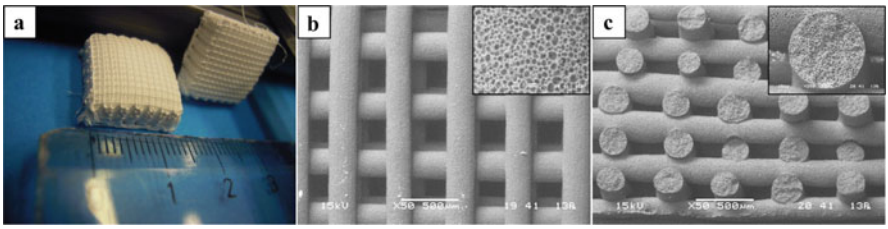


Fig. 5.2 Additive manufactured scaffolds fabricated by Computer-Aided Wet-Spinning (CAWS) (modified with permission from [19]): (**a**) photograph of poly(ε-caprolactone) (PCL) scaffolds; (**b**) SEM micrographs of the top view of a PCL scaffold; (**c**) SEM micrographs of the cross-section of a PCL scaffold. Insert: details of the fibers surface morphology (×1000) (**b**) and single fiber cross-section (×350) (**c**)

compromising the regeneration process and the structural role of the construct. On the other hand, too slowly degrading materials with high mechanical stiffness can shield the regenerating tissue from external stresses preventing cell stimulation.

Favored tissue vascularization to facilitate the diffusion of nutrients, growth factors, and metabolic wastes from the surroundings to cells and vice versa [15]. Tissue vascularization occurs through vasculogenesis and angiogenesis. During vasculogenesis, endothelial progenitor cells migrate to an avascular site, then they proliferate and differentiate to form a primitive capillary vessels network. The formed vessels are remodeled and mature forming a more complex network to satisfy the nutritional needs of the growing tissue, and this occurs through angiogenesis [15]. Angiogenesis consists in forming new blood vessels that sprout from the existing ones. Tissue vascularization can be promoted through materials and scaffold design. For instance, micropatterned biomaterials functionalized with ECM growth factors and peptides are able to promote a guide angiogenesis [16]. As already stated, pore size is a critical determinant in blood-vessel ingrowth; indeed, it has been reported that scaffolds with pores greater than 250 μm present a faster vascularization [17]. Pores interconnectivity is another important factor affecting tissue vascularization because it favors both tissue vasculogenesis and angiogenesis, by allowing cell migration.

The most widely investigated materials for scaffolds fabrication are polymeric materials susceptible to be degraded in physiological environments [18]. As it will discussed in detail in the next section, AM techniques employed to process biodegradable polymers into 3D scaffolds can be divided into five classes, i.e., Vat photopolymerization (VPP) techniques, Selective laser sintering (SLS), Binder Jetting (BJ), Melt-extrusion AM (ME-AM), and Solution-Extrusion AM (SE-AM). A representative example of an additive manufactured poly(ε-caprolactone) (PCL) scaffold is reported in Fig. 5.2, together with SEM micrographs of its top view and cross-section.

Polymers for scaffolds fabrication could be either of natural origin or chemically synthesized. The most exploited biodegradable polymers for TE scaffolds development, with particular emphasis to those employed for AM, are as follows.

5.1.1.1 Natural Polymers for TE Applications

Polysaccharides are widely investigated as biopolymers complying with the concepts of sustainability, eco-efficiency, and green chemistry [20]. Polysaccharides with different chemical structure and physical-chemical properties can be obtained from a variety of vegetable, animal, and microbial sources, with an overall lower environmental and economic impact compared to proteins. In addition, versatile approaches to chemical modification of polysaccharides offer a range of possibilities for tuning scaffold biodegradability, processing properties, and physical behavior.

Alginate (Alg) (Fig. 5.3a) is present as a structural polysaccharide in marine brown algae, and as a capsular polysaccharide in some soil bacteria [21]. It is constituted by (1–4)-linked β-D-mannuronic acid and α-L-guluronic acid units whose sequence changes along the polymer chain. Alg can form insoluble hydrogels in aqueous solutions via ionotropic gelation based on interactions between the carboxylic acid groups along its macromolecular chain and multivalent cations such as Ca^{2+}, Sr^{2+}, and Ba^{2+} [22]. Another approach followed to stabilize Alg in aqueous solutions, exploited also for AM, is based on its complexation with a polycation to form a polyelectrolyte complex. The most exploited AM techniques for the fabrication of Alg hydrogels are SE-AM techniques [20].

Chitosan (Cs) (Fig. 5.3b) is often classified as a semisynthetic polymer since it is obtained by synthetic deacetylation of chitin, a polysaccharide found in the exoskeleton of crustaceans, insects, and some fungi [20, 23]. It is a linear copolymer of β-(1–4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose. Depending on its deacetylation degree, which can range from 55% to >95%, Cs physical-chemical, morphological, and functional properties can vary significantly [24]. For instance, Cs crystallinity degree generally increases by increasing the deacetylation degree [25]. Cs is soluble in aqueous solutions under mild acidic conditions, as a consequence of amino groups protonation conferring the macromolecule with antimicrobial activity [20]. Extrusion of a diluted acetic acid solution in air or directly into a coagulation/crosslinking medium represents the most direct approach to AM of Cs-based hydrogels. Ionic complexation with a polyion is also an effective way to stabilize extruded Cs in a physiological environment.

Cellulose (Cel) (Fig. 5.3c) is a polysaccharide obtainable from different natural sources, including lignocellulosic materials, algae, and bacteria fermentation [20, 26]. From a chemical point of view, it is a linear glucose polymer linked β-1,4. Derivatives of plant Cel in the form of microfibrillated or nanocrystalline polymer are widely investigated for biomedical purposes [27]. In addition, bacterial Cel has found great interest in the TE area, thanks

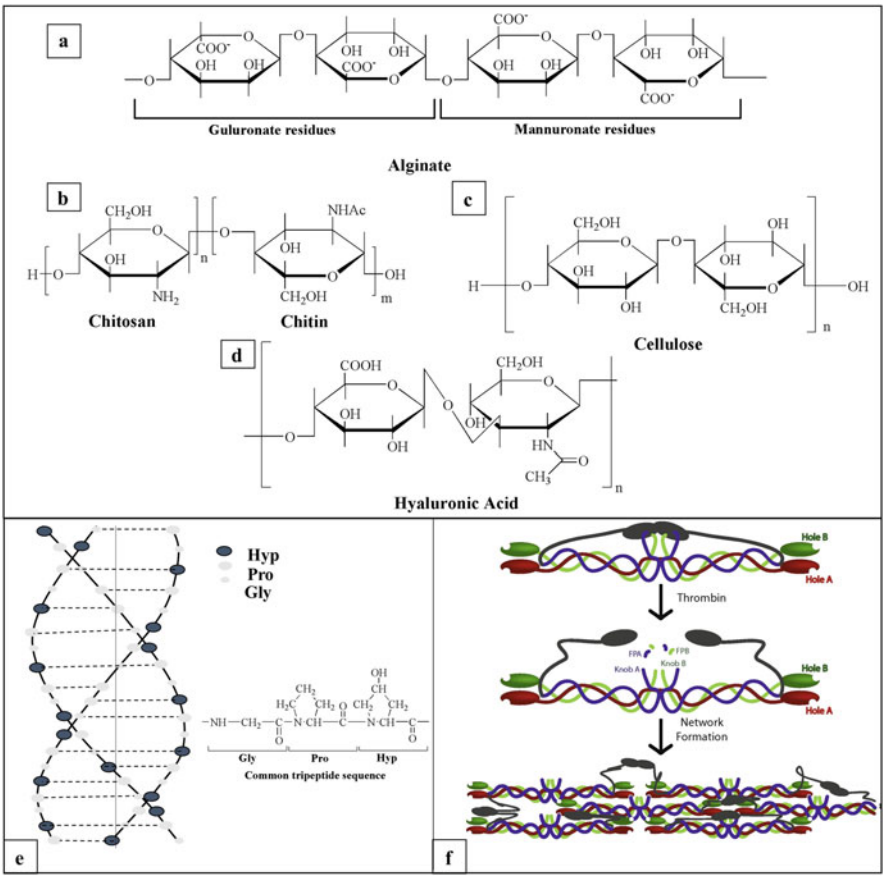


Fig. 5.3 Structure of polysaccharides and proteins most commonly employed in biomedical AM: chemical structure of (**a**) alginate (Alg) (**b**) chitosan (Cs), (**c**) cellulose (Cel), (**d**) hyaluronic acid (Hyal); (**e**) triple chain structure of collagen (Col) fibrils and chemical structure of the most common tripeptide sequence found in Col, composed of glycine (Gly), proline (Pro), and hydroxyproline (Hyp) sequences (modified with permission from [11]); (**f**) fibrin (Fib) polymerization (α chains shown in blue, β chains in green, and γ chains in red; αC domains are in gray) (modified with permission from [32])

to its high purity, superior mechanical properties, and good biological affinity, as a result of a nanofibrillar structure [28]. Cel is typically processed by AM as a blend with other polysaccharides in the form of an aqueous solution or suspension. It is also widely employed as a filler in ME-AM [20].

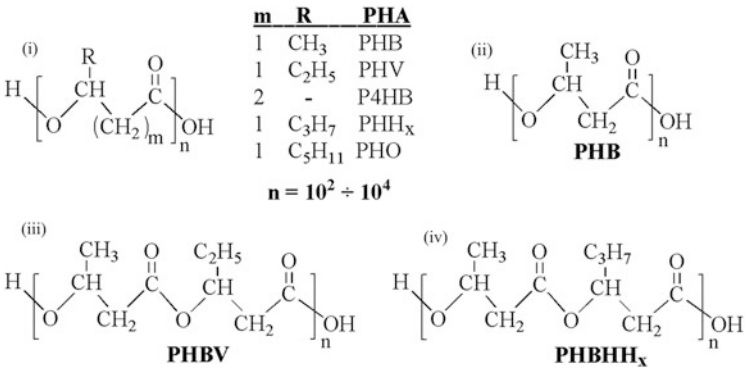
Hyaluronic acid (Hyal) (Fig. 5.3d) is one of the main components of the ECM of different animal connective tissues, and it can be obtained through extraction from animal tissues or by means of microbial fermentation [29]. In native tissues, Hyal plays structural and functional roles by contributing to the water

balance regulation, acting as lubricant and shock absorber, as well as scavenger for free radicals. Hyal macromolecular chain consists of β(1 → 3)-linked disaccharide units of α-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine [30]. It represents one of the most investigated biopolymers in TE, often as a blend with other polysaccharides or proteins to enhance material stability in an aqueous environment or favor cell adhesion, given the hydrophilic, polyanionic nature of Hyal [31]. Indeed, Hyal high molar mass (10^3–10^7 Da) and macromolecular structure typically result in highly viscous aqueous solutions with a slow gelation kinetics not ideal for AM approaches. For this reason, chemical modification, e.g., esterification or methacrylation, represents a powerful tool to widen the possibilities of Hyal processing by means of AM approaches based on solution extrusion or photocrosslinking [20].

Proteins are macromolecules composed by different aminoacids linked together via amide bonds. They are organized on a supramolecular level into complex 3D hierarchical architectures characterized by peculiar physico-chemical, mechanical, electromagnetic, and optical properties [33]. Structural proteins, such as collagen and laminin, include arginine-glycine-aspartic acid (RGD) sequences that act as anchoring sites for a number of different integrins binding receptors that support the attachment of the endogenous cells [34]. Proteins inherent gelation properties can be exploited to fabricate biomedical hydrogels that can be further stabilized in aqueous physiological environment through chemical crosslinking. Bioprinting approaches often involve the use of proteins, such as collagen (Col), gelatin (Gel), and fibrin (Fib), to exploit their high cell affinity. Proteins can also be chemically modified with photopolymerizable pendant groups for Vat photopolymerization (VPP) approaches [20].

Collagen (Col) (Fig. 5.3e) is a structural protein found in native ECM of various biological tissues, such as bone, cartilage, and blood vessels [35]. It is composed of three polypeptide chains that wrap around one another with a right-handed twist forming a packed triple helix. Col has been widely employed as scaffolding material in TE to exploit its cell-binding properties and biomechanical behavior [36]. In order to overcome shortcomings related to Col slow gelation in physiological media, different processing strategies have been followed to optimize relevant AM approaches, such as cryogenic extrusion, chemical crosslinking, or blending with other polymers [20].

Gelatin (Gel) derives from Col denaturation via hydrolysis through acid or basic treatment [37]. Depending on denaturation process conditions, either positively- or negatively charged Gel is obtained. Gel retains the polyaminoacid structure of Col but the denaturation process makes it losing the ternary structure characteristic of Col, thus increasing its solubility in aqueous solutions. Gel shows a temperature-dependent gelation behavior that is exploited in extrusion AM [20]. Gelatin methacryloyl (GelMA) is one of the most investigated natural polymer derivative for photocrosslinking-based AM [38].



m	R	PHA
1	CH_3	PHB
1	C_2H_5	PHV
2	-	P4HB
1	C_3H_7	$PHHH_x$
1	C_5H_{11}	PHO

Fig. 5.4 Chemical structure of the polyhydroxyalkanoates (PHA) most commonly employed in biomedical AM: (i) general structure of PHA; chemical structure of (ii) poly(3-hydroxybutyrate) (PHB), (iii) poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), (iv) poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx)

Fibrin (Fib) (Fig. 5.3f) is a protein naturally polymerized during blood clot formation through a process which starts from thrombin-catalyzed proteolysis of fibrinogen, a protein present in the blood [39]. Plasma-derived formulations based on fibrinogen and thrombin are clinically used to prepare crosslinked Fib products with a variety of applications, such as medical hemostats, adhesives, and sealants [40]. Fibrinogen and thrombin are present in many commercial and experimental bioink formulations for Bioprinting of cell-laden hydrogels by following various processing strategies [20].

Silk fibroin (SF) proteins are produced in the form of fibers by silkworms. Silk fibers are composed by two major fibroin proteins with different molar mass, held together by glue-like proteins called sericin [41]. SF is widely investigated for TE applications, thanks to its ability to form physically crosslinked hydrogels with high mechanical strength and elasticity [42]. This self-assembling behavior is also exploited for tailored SE-AM strategies [43]. In addition, SF can be chemically modified by glycidyl methacrylate to obtain a photocurable formulation [44].

Polyhydroxyalkanoates (PHA) are aliphatic polyesters produced by microbial fermentation, that are finding great interest for biomedical applications, thanks to their biocompatibility and in vivo biodegradability [45]. The physical-chemical, morphological, processing, and mechanical properties of PHA depend on both the length of the pendant groups and the number of methylene groups in the monomeric unit's backbone (Fig. 5.4). Poly(3-hydroxybuytrate) (PHB) has a high degree of crystallization (60–80%) conferring the polymer with a stiff and brittle mechanical behavior. Poly(4-hydroxybutyrate) (P4HB) has a lower degree of crystallization (~35%) and a higher flexibility than PHB, while their copolymers show intermediate morphological and mechanical parameters [46]. Copolymerization of 3HB with 3-hydroxyvalerate (3HV) or 3-hydroxyhexanoate (3HHx) with longer alkyl side groups results in increased

flexibility and ductility, as well as in enhanced thermal processing properties as a consequence of a reduced melting temperature (T_m) [47]. Tailored biotechnological fermentation processes allow tuning PHA molar mass and comonomers percentage with a significant effect on the resulting material mechanical, processing, and biodegradation properties. PHA thermoplastic behavior and solubility in chlorinated solvents to form viscoelastic solutions have been exploited in AM approaches involving material thermal treatment (e.g., SLS) or solution extrusion (e.g., SE-AM) [48].

5.1.1.2 Synthetic Biodegradable Polymers for TE Applications

The main advantage of synthetic polymers is represented by the possibility of precisely tailoring their physical-chemical, mechanical, biodegradation, and functional properties by acting on the synthesis route and relevant experimental conditions [49]. As discussed below, the most investigated synthetic polymers for scaffold fabrication are aliphatic polyesters, thanks to their biocompatibility, as well as suitable processing and mechanical properties.

Poly(ε-caprolactone) (PCL) (Fig. 5.5a) is a semicrystalline aliphatic polyester with thermoplastic behavior, synthesized on an industrial scale by ring-opening polymerization (ROP) of ε-caprolactone using stannous octoate as a catalyst [50]. At the physiological temperature, PCL shows a viscoelastic behavior, being well above its glass transition temperature ($T_g \cong -60$ °C). PCL hydrophobicity and high crystallinity degree justify its use for long-term applications requiring a slow biodegradation, such as in the case of load-bearing tissue regeneration. PCL melt

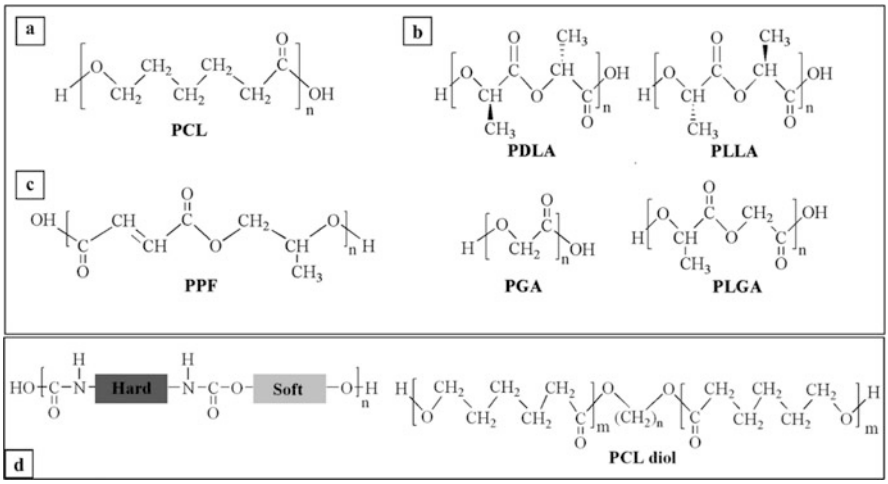


Fig. 5.5 Chemical structure of the biodegradable polyesters most commonly employed in biomedical AM: (**a**) poly(ε-caprolactone) (PCL); (**b**) poly(α-hydroxyacids), i.e., poly(d-lactide) (PDLA), poly(l-lactide) (PLLA), poly(glycolide) (PGA), and poly(lactide-co-glycolide) (PLGA); (**c**) poly (propylene fumarate) (PPF); (**d**) poly(ester urethane)

rheological properties are well suited for AM approaches based on thermal treatment (e.g., FDM and SLS), making it one of the most exploited additive manufactured polymers in clinical applications [51]. In addition, its solubility in different organic solvents to form viscoelastic solutions has been exploited in various studies focused on SE-AM application for scaffolds fabrication [52].

Poly(lactide) (PLA) (Fig. 5.5b) exists in different stereoisomeric forms, e.g., poly(L-lactide) (PLLA), poly(D-lactide) (PDLA), poly(D,L-lactide) (PDLLA), and poly(L-lactide-*co*-D,L-lactide) (PLDLLA). PLA is synthesized on an industrial scale through ring-opening polymerization of lactide, which is obtained by dimerization of lactic acid [53]. PLLA is semicrystalline, showing a thermoplastic behavior, and behaves as a brittle material when employed for clinical use since its amorphous domains are in the glassy state ($T_g \cong 55–70\ ^\circ C$). The broad range of T_g is due to the different crystallinity degree that PLA can assume depending on its thermal history. The increase of T_g with an increase of the polymer crystallinity degree depends on interactions between the amorphous and crystalline phase, as well as on a geometrical constraint imposed by the presence of crystallites to the chain conformational mobility within the amorphous domains [54]. By increasing the D-lactide percentage in PDLLA, the polymer crystallinity degree decreases until above a percentage of 20% the polymer is completely amorphous. Amorphous PDLLA T_g is in the range 50–60 °C, and in some cases a second T_g at about 84 °C is reported [55]. The higher T_g could be attributed to the glass transition of stereocomplexes formed through the interaction between L and D PLA sequences that are present along the polymer backbone [55]. Overall, PDLLA shows higher flexibility and higher biodegradation rate than PLLA, as a consequence of a lower T_g and crystallinity degree. PLA copolymerization with glycolide results in poly (lactide-*co*-glycolide) (PLGA) copolymers with enhanced hydrophilicity and faster biodegradation rate [56]. PLLA and PDLLA are widely employed in ME-AM and SLS, thanks to their versatile melt processing properties. Their solubility in organic solvents is exploited in SE-AM and BJ [20].

Poly(propylene fumarate) (PPF) (Fig. 5.5c) is a linear polyester consisting of repeating units containing one unsaturated bond in trans configuration. It is synthesized on a lab scale through a two-step reaction between diethyl fumarate (DEF) and propylene glycol [57]. Photochemical crosslinking of PPF under controlled conditions allows the obtainment of 3D constructs with tunable mechanical and degradation properties. Macromer, molar mass, crosslinking agent composition and density, scaffold porosity, and the presence of additives are key factors affecting crosslinked PPF properties [58]. Thanks to its versatile photoreactivity, PPF represents one of the most employed biodegradable polymers for photoreactive AM [20].

Polyurethanes (PUs) present urethane groups (–NHCOO–) in their backbone, obtained by reaction of an aliphatic or aromatic diisocyanate, a macrodiol (i.e., a polyether, polyester, or polycarbonate), and a diol or a diamine as a chain extender [59]. Aliphatic polyesters (e.g., PCL PGA, PDLLA) are used as macrodiols for the synthesis of biodegradable PUs, as shown in Fig. 5.5d. As discussed below, PUs with tailored processing properties have been successfully employed for the fabrication of scaffolds by means of FDM or SE-AM.

5.2 Additive Manufacturing (AM) of Scaffolds

According to the American Society for Testing and Materials (ASTM), AM is "the process of joining materials to make objects from three-dimensional (3D) model data, usually layer upon layer, as opposed to subtractive manufacturing methodologies" [60]. Furthermore, volumetric printing (VP) technologies introduced a paradigm shift in respect with AM techniques because they enable the creation of entire objects at once, rather than through the sequential addition of basic building blocks [61]. Indeed, they rely on the projection of a series of 2D patterned optical light fields within a volume of a photopolymer. These 2D patterns act cumulatively producing a 3D distribution that triggers polymerization of the irradiated material into the desired object. A great variety of polymeric, ceramic, and metallic materials can be processed by AM into 3D objects with sizes and shape resembling with good accuracy those of a digital model. This model is directly drawn by means of a software or derived from imaging technique data, and then expressed as layers corresponding to cross-sections of the 3D computer representation of the object [62]. This layered representation is then translated into a computer numerical control (CNC) programming language, providing instructions to an AM machine for physically building up the object.

As described below, a wide range of AM approaches based on computer-aided design (CAD) and manufacturing (CAM) processes have been introduced in the biomedical area for fabricating polymeric scaffolds and other biodegradable devices with customized composition, shape, and porosity [63]. In particular, the possibility of using tomographic images from X-ray and magnetic resonance to model and fabricate anatomically shaped and clinically sized devices, is propelling the employment of AM for personalized medicine. Indeed, clinical use of AM for different applications, such as surgical planning, building prosthetics, dentistry, osteosynthesis devices, and TE, among others, is witnessed by a large body of literature [64]. Personalized 3D-printed tracheal and cardiovascular splints made of bioabsorbable polymers are successful examples of advanced clinical application of AM [65, 66]. In this context, research on biodegradable polymers for biomedical AM is tremendously contributing to medical practice evolution, as well as to advanced materials engineering strategies [20]. Indeed, significant progress has been made on various relevant aspects, such as the synthesis of novel biocompatible photoreactive macromers, the implementation in the AM field of industrial thermoplastic polymers, and the optimization of AM formulations based on natural polymers. In addition, great efforts are continuously made to adapt AM technologies to the fabrication of tailored polymeric scaffolds, as well as to develop new relevant material processing approaches. Indeed, different AM techniques have been optimized to process various biodegradable polymers into 3D scaffolds with predefined porosity and shape (Fig. 5.6). As discussed below, for a given composition and designed porous architecture, each AM approach results in peculiar scaffold structural features/mechanical properties relationships that can be controlled by acting on specific processing parameters.

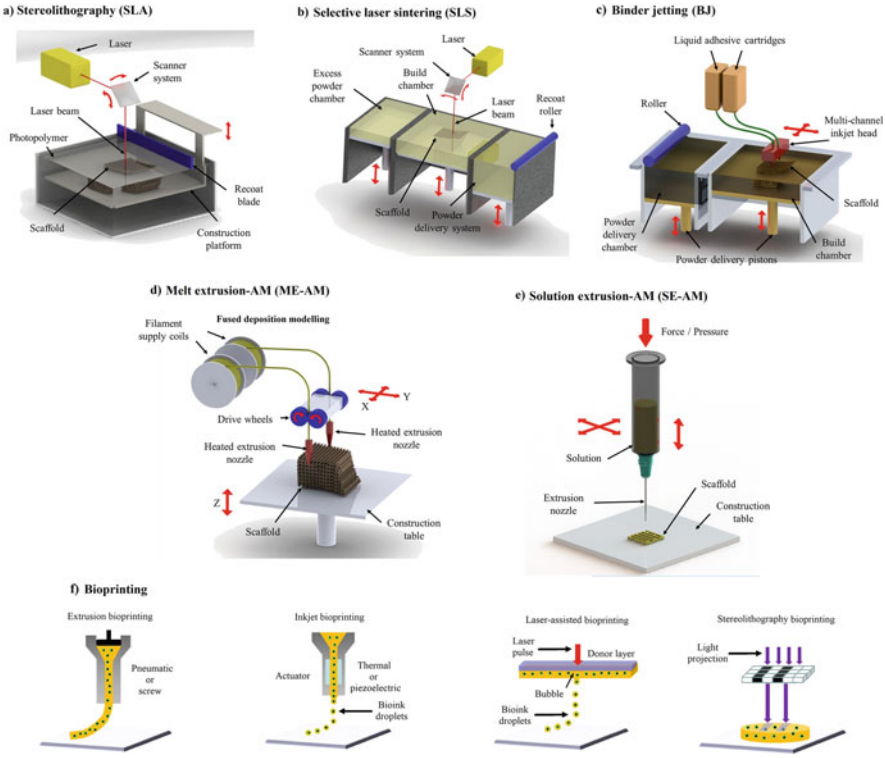


Fig. 5.6 AM techniques employed for biodegradable scaffolds fabrication: (**a**) Stereolithography (SLA); (**b**) Selective laser sintering (SLS); (**c**) Binder jetting (BJ); (**d**) Melt-extrusion AM (ME-AM) (e.g., fused deposition modeling, FDM); (**e**) Solution-extrusion AM (SE-AM); (**f**) Bioprinting based on extrusion, inkjet, laser-assisted process, and SLA. (Modified with permission from [63, 67])

Vat photopolymerization (VPP) involves the patterned polymerization and/or crosslinking under UV or Vis light of a photosensitive resin composed by monomers/macromers, a photoinitiator, and additives [68]. The name is referred to the fact that the light source is irradiated over the surface of a vat filled with the resin to promote its solidification where the laser is scanned. Two of the most important techniques included in this category are Stereolithography (SLA) and Digital Light Processing (DLP). In SLA technique, the photopolymerization proceeds by using a translating laser beam, while in DLP technique it is based on a laser projection. Once cured, a layer is submerged into the liquid resin to cure the next layer on top of the previous one (Fig. 5.6a). Interlayer adhesion and scaffold integrity, and consequently its mechanical behavior, depend on different SLA and DLP parameters, including those related to light source (e.g., laser power), scanning variables (e.g., laser translation speed), and resin composition (e.g., light absorbers). High-resolution μSLA systems based on UV lamps, LEDs, or lasers, enable the fabrication of microstructured devices with overall sizes in

the centimeters scale [69]. In the case of two-photon polymerization (TPP), high spatial resolution at the nanometer scale can be achieved by simultaneous absorption on a photosensitive material of two lower energy photons at high wavelength, typically in the range 780–820 nm [70]. TPP technique represents a valuable way to simulate natural tissue nanostructures and induce significant differences in cellular responses [71]. This technique breaks free from the common paradigm associated to AM techniques. Indeed, it doesn't proceed through a layer-by-layer approach because it utilizes the two-photon absorption of near-infrared light. Since the TPP photopolymer is transparent to this fundamental wavelength, the TPP "tool" is essentially an unsupported floating point that is able to process the material within the photopolymer [72]. However, the time required to fabricate scaffolds with physiologically relevant sizes makes TPP impracticable for this purpose [73].

PPF is the most employed biodegradable polymer for SLA, thanks to the double carbon–carbon bond in its repeating unit [74]. Biodegradable scaffolding materials for SLA can be obtained also starting from oligomers presenting hydrolyzable ester or carbonate linkages through their chemical functionalization with photocurable acrylate, methacrylate, or vinyl functional groups. Functionalized poly(trimethylene carbonate) (PTMC) [75, 76], PCL [77], PCL-*co*-PTMC [78, 79], and PDLLA [80, 81] are successful examples in this context. Natural polymers, such as Gel [82], Col [83], and Cs [84], can also be modified with photopolymerizable pendant groups, even if they usually result in hydrogels with a poorly defined porous structure along their thickness [69].

Selective Laser Sintering (SLS) exploits a computer-controlled translating laser beam to selectively sinter a bed made of polymeric or ceramic particles [85]. After sintering a layer, the construction platform moves downward a given distance, and a new powder bed is spread by means of a translating roller or blade, on top of the previous one. A 3D object is thus built up with a layer-by-layer process based on this powder sintering/spreading cycle (Fig. 5.6b). The energy provided by a high-intensity laser (e.g., CO_2 laser) is absorbed by bed particles that soft and fuse together as a consequence of a local temperature raise. The process can be carried out into a chamber maintained at a temperature close to the melting/softening point. For this reason, in some cases an inert gas atmosphere is required to prevent thermal oxidation. A number of processing parameters can be varied to control scaffold porous structure and topography, such as laser power, scan spacing and speed, particles shape and size, powder bed thickness and temperature, and roller/blade speed. The limited laser energy required to avoid polymer degradation typically results in high surface roughness, as a consequence of an incomplete fusion of the particles.

SLS has been widely employed for fabricating scaffolds made of different aliphatic polyesters with thermoplastic behavior, including PCL [86–90], PDLLA [91–93], and PHBV possibly loaded with calcium phosphates [94–96]. As previously mentioned, different clinical trial studies involved the successful implantation of PCL-based devices fabricated by SLS in infants suffering from tracheobronchomalacia [65, 97, 98].

Binder Jetting (BJ) involves a patterned deposition of a binder that acts as an adhesive on a powder bed. Analogously to the case of SLS, binder deposition is alternated to powder spreading after the platform translation downwards (Fig. 5.6c). The binder is typically deposited in the liquid state, as drops or a jet, to form a continuous solid layer bonded to the previous one [99]. In the case of polymeric particles, a solvent with strong solvating power for the polymer(s) is required as a binder to achieve quick particles fusion. After fabrication, the object is often submitted to post-processing treatments, such as sintering or infiltration of a binder, to optimize material density and mechanical strength [100]. Particles size and binder physical properties (e.g., volatility, surface tension, and viscosity) are critical parameters that together with other fabrication variables, such as deposition speed and specific volume of deposited binder, determine the quality, resolution, and mechanical properties of the printed object [101].

Besides being widely investigated for processing calcium phosphate powders [102–110], BJ is employed to fabricate drug-loaded implants made of aliphatic polyesters, such as PLGA [111], PLLA [112], and PDLLA [113]. Few studies also reported BJ of natural origin polymers, such as starch and Cel derivatives [99, 114].

Melt-Extrusion AM (ME-AM) involves heating and depositing with a controlled pathway a polymeric material extruded as a viscoelastic fluid at a temperature higher than the glass transition temperature (T_g) of the processed polymer (s) [115]. In the case of a semicrystalline polymer, the extrusion temperature is higher than the relevant melting temperature (T_m). The relative motion of the extrusion head and the deposition platform determines the material lay-down pattern to build up 3D scaffolds with a layer-by-layer process (Fig. 5.6d). In the case of the most commercially exploited ME-AM technique, i.e., Fused Deposition Modeling (FDM), the raw material to be extruded is in the form of a continuous polymeric filament which is forced through a heated nozzle. Other technological solutions involve processing polymeric pellets or powders by means of a screw extruder, a pneumatic system, or a plunger [116–118]. As it will be discussed in one of the following paragraphs, polymeric scaffolds porosity and mechanical properties can be tuned in a certain range by acting on processing parameters, such as extrusion temperature, extrusion rate, and deposition velocity [119].

The employment of ME-AM techniques for biodegradable scaffolds fabrication has been reported by many articles focused on thermoplastic aliphatic polyesters, including PCL [120], PDLLA [121], PLGA [121, 122], and PCL/PHBV blends [123]. In particular, different kinds of PCL porous implants by FDM available on the market have been tested in clinical trials [124–126].

Solution-Extrusion AM (SE-AM) techniques involve the controlled deposition of a polymeric solution or suspension during its extrusion through a nozzle. The relative motion of the extruding head and the deposition platform determines the lay-down pattern of the material, which is extruded at a rate controlled by means of a pneumatic or mechanically driven dispensing system (Fig. 5.6e). Depending on different variables, such as polymer chemical structure and

processing approach, the solidification or gelation of the extruded polymeric fluid is obtained through different mechanisms. Solidification of organic solutions of aliphatic polyesters or other hydrophobic polymers can be achieved through solvent casting [127], or physical coagulation by non-solvent-induced phase separation (NIPS) upon direct extrusion into a coagulation bath [52]. The last approach, which is referred to as Computer-Aided Wet-Spinning (CAWS), has been exploited to fabricate a set of scaffolds based on PCL [19] or PHBHHx [128] and endowed with a dual-scale porous structure characterized by a microporosity created in the polymer matrix during the NIPS process [129]. Such microporosity typically results in a lower compressive modulus, but it can be exploited to tailor scaffold properties related to specific surface area, such as biodegradation rate and release kinetics of loaded drugs. A comparison between structural and morphological properties of PCL scaffolds fabricated by FDM and CAWS is shown in Fig. 5.7. Patterning of aqueous solutions of hydrogel-forming natural polymers (i.e., proteins and polysaccharides) is generally achieved through gelation in a liquid medium by means of mechanisms based on physical or chemical crosslinking, such as thermosensitive physical gelation of

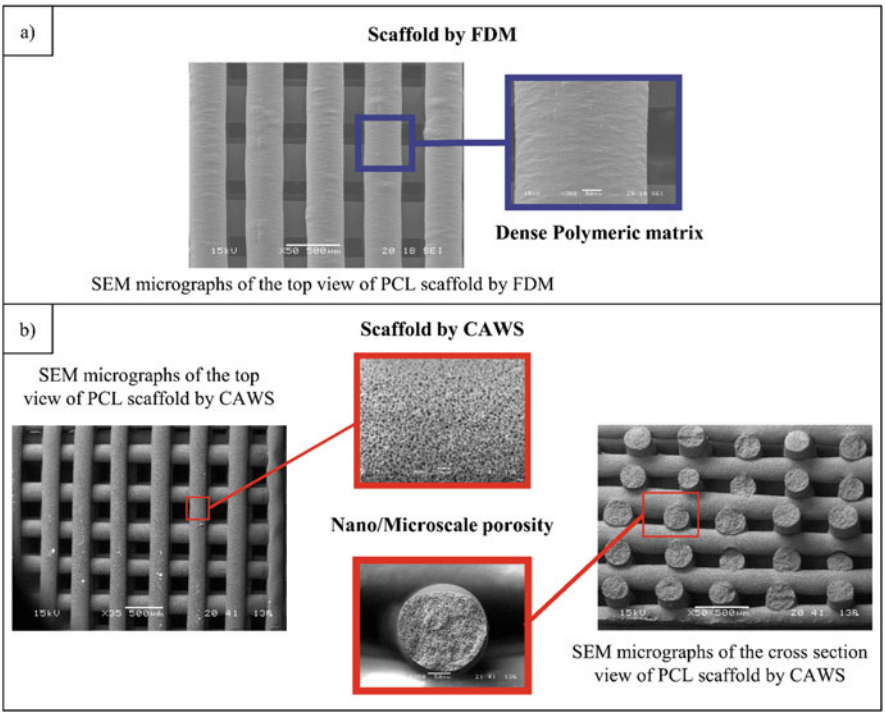


Fig. 5.7 Comparison between structural and morphological properties of PCL scaffolds fabricated by FDM and CAWS: (**a**) SEM micrographs at different magnifications of top view of a PCL scaffold fabricated by FDM; (**b**) SEM micrographs of top view and cross-section of a PCL scaffold fabricated by CAWS

Gel [130], ionotropic gelation of Alg by crosslinking with divalent cations [131], chemical or enzymatic crosslinking of natural polymer derivatives [132, 133], UV photocrosslinking of GelMA [134], and complexation of Cs/poly(γ-glutamic acid) (γ-PGA) polyelectrolytes [135].

Bioprinting is an extended application of various AM approaches, employed to obtain bio-functional materials in a layer-by-layer manner [136]. It typically employs one of the already described techniques but including living cells in the processed materials. For example, a bioink composed by living cells or cells aggregates suspended in a liquid medium, possibly in combination with biomaterials (e.g., a hydrogel-forming polymer) and bioactive molecules, can be deposited in a controlled way [137] by following SE-AM or BJ approaches. The bioink can be delivered by means of pneumatically- or mechanically controlled extrusion through a nozzle, ejection from a nozzle in the form of droplets, or a laser pulse-based system generating an ink droplet (Fig. 5.6f). The possibility of structuring cells-laden hydrogels by means of SLA have been shown by using either UV or V is light depending on the polymer and photoinitiator employed [134, 138]. A wide array of bioinks composed of proteins and/or polysaccharides in combination with different kinds of cells have been developed to fabricate biomimetic tissue-like constructs for in vitro engineering and modeling of health and pathological tissues [139]. While gelation strategies are the same previously described for hydrogel-forming polymers, the bioink and its processing should also meet specific requirements to preserve cell viability, such as reduced liquid viscosity and limited polymer crosslinking degree, with obvious effects on the mechanical properties of the resulting scaffold [140]. In addition, volumetric Bioprinting enables the fabrication of entire cell-laden constructs with arbitrary size and architecture within a time frame of seconds to tens of seconds [61].

5.2.1 Mechanical Properties of Additive Manufactured Scaffolds

Mechanical properties of polymeric systems are typically investigated through an analysis of stress–strain relationship. The stress (σ) is calculated as the ratio between the applied force (F) and the resistant area (A) of the material (Eq. 5.1). The strain (ε) is defined as the ratio between the variation of a sample dimension (e.g., length or thickness for tensile or compression test, respectively) at a defined time (l) and the initial value of that dimension (l_0), and it is usually expressed as a percentage (Eq. 5.2).

$$\sigma = F/A \tag{5.1}$$

$$\varepsilon = (l - l_0)/l_0 \tag{5.2}$$

Mechanical properties of polymeric materials are strongly dependent on temperature [141]. At temperatures below their T_g polymers behave as brittle materials.

This is because the free volume of glassy polymers is too low to permit macromolecular chains to change their relative positions and to deform the material according to the force applied. If the temperature rises above the T_g, the movement of individual chain segments becomes possible, so the polymer behaves as a viscoelastic material. A typical stress–strain graph of a viscoelastic material is generally constituted by three parts. The first part represents the elastic deformation of the material and it is characterized by a linear relationship between the stress and the strain (Eq. 5.3). The slope of this linear region is called elastic modulus (E). The second region is the result of a plastic deformation of the material, in this section the shape of the curve is poorly defined. The last region consists in the failure of the tested material.

$$\sigma = E \cdot \varepsilon \tag{5.3}$$

Mechanical properties of additive manufactured parts can be affected by different factors, such as the raw materials properties, the manufacturing method, and the macrostructure of the fabricated object. An increase in M_W, as well as the presence of polar groups along the macromolecular chains generally increases the mechanical properties of the polymer. Replacing non-polar segments or side groups along the chains with polar ones should increase polymer stiffness, mechanical strength, and viscosity because of an increase of the strength of intermolecular interactions. Indeed, intermolecular interactions between aliphatic polyolefins are prevalently caused by van der Waals forces, while the presence of polar groups (e.g., nitrile groups) increases the stiffness and strength of the polymer through dipole–dipole interactions [141]. In the same manner, hydrogen bonding, ionic interactions, and crosslinking considerably improve the polymer mechanical properties. A schematic depiction of the typical intermolecular interactions or crosslinks between polymer chains is shown in Fig. 5.8.

In the case of solid block copolymers, the different macromolecular blocks usually form separate phases. The material is heterogeneous at a nanoscale, and it can assume different morphologies with peculiar mechanical properties, depending on the molar ratio of the polymer blocks [141]. Taken into account a diblock copolymer, the component present in a lower percentage can form spherical or rod-like aggregates dispersed in a matrix of the other component. If both blocks are about equally distributed in the system, lamellae can form.

Another way to modify the mechanical properties of a polymeric matrix is to load it with a solid organic or inorganic filler, such as graphite, silica gel, glass fibers, aluminum oxide, or calcium phosphate, in order to obtain a composite material. Crosslinking is also one of the most effective ways for the mechanical characteristics of a polymer. It consists in binding polymer chains together via either physical or covalent bonds to form 3D network structures. Crosslinked polymeric networks can be soft, rubber-like, or hard materials, and they can form swollen gels when a solvent is absorbed. The mechanical parameters of a crosslinked polymer, e.g., its elastic modulus, below the T_g are similar to those of the non-crosslinked material. Elastic modulus of a non-crosslinked polymer normally drops during glass transition, while

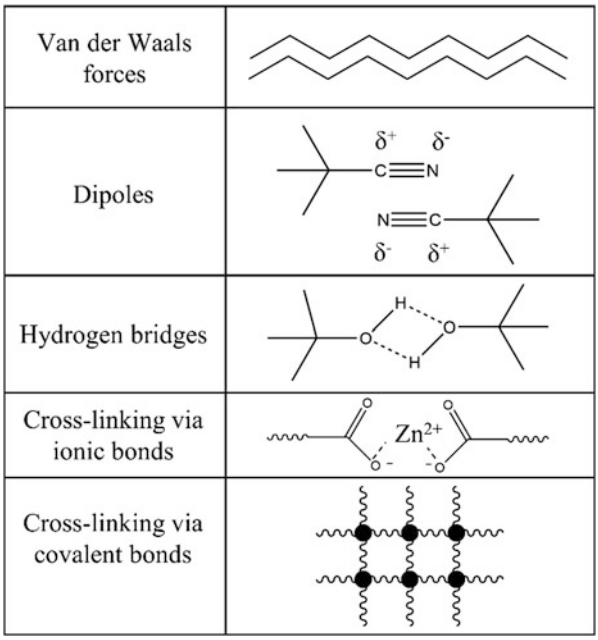


Fig. 5.8 Examples of intermolecular interactions or crosslinks between polymer chains

in the case of a high cross-linking degree it stabilizes at higher level up to the polymer decomposition temperature. The crystallinity degree influences polymers mechanical properties in a similar way. Since only the amorphous phase of the polymer undergoes a glass transition, the decrease of the modulus at T_g is typically lower in semicrystalline polymers compared to amorphous ones. The value of the modulus between T_g and T_m remains constant upon temperature change, and it drops only at T_m [141].

Layered processing of polymeric materials, as in the case of AM, determines more variables that affect the mechanical properties of the manufactured object. The most important ones, among others, are layer thickness, pore size, overall porosity, density, and alignment of fiber–fiber contact points, and filaments orientation [142, 143]. These parameters depend on the materials and fabrication technique employed, as well as on various processing parameters, as discussed below.

5.2.1.1 Mechanical Testing Methods for Additive Manufactured Scaffolds

Mechanical testing is applied to investigate the behavior of a material under the application of a given load. In particular, its aim is the investigation of the relationship between applied forces and the resulting deformation of the sample, as well as the limit stresses that lead to its failure or to the boundary testing conditions. The most common mechanical characterization methods applied for characterizing additive manufactured polymeric scaffolds are introduced below and schematically depicted in Fig. 5.9.

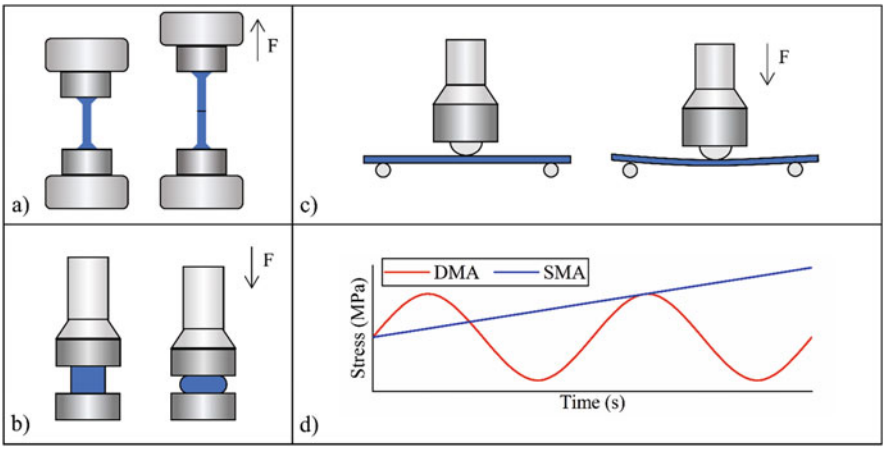


Fig. 5.9 Schematic representation of the most common mechanical testing methods: (**a**) tensile test; (**b**) unconfined compression test; (**c**) three-point bending test; (**d**) comparison of the variation of applied stress as a function of time in the case of static mechanical analysis (SMA) or dynamic mechanical analysis (DMA)

Tensile test: a standardized specimen with a known cross-section area is loaded along its longitudinal direction typically in quasi-static conditions by applying a constant tensile strain rate. The resulting stress–strain diagram shows the tensile mechanical behavior of the material providing the tensile modulus (E), the yield strength, the tensile strength, and the elongation at break. A schematic representation of the tensile test is shown in Fig. 5.9a.

Compression test: a standardized specimen with a known cross-section area is loaded uniformly typically in quasi-static conditions by applying a constant compression strain rate along the longitudinal direction. A uniaxial stress state prevails in the specimen. The resulting stress–strain diagram shows the mechanical behavior of the material providing the compressive modulus (E_c), the yield point, and other useful mechanical parameters, such as the maximum stress at the loading conditions. A schematic representation of a compression test is shown in Fig. 5.9b.

Bending test: the most frequently used test under bending conditions is the three-point bending test. A beam mounted on two supports is bended under a single force applied to the sample center. The bending test demonstrates the relationship between the load of a bending beam and its elastic deformation. A schematic representation of the three-point bending test is shown in Fig. 5.9c.

Dynamic mechanical analysis (DMA): this kind of mechanical characterization is typically carried out by applying a sinusoidal stress or strain to generate a sinusoidal strain or stress, respectively. By measuring both the amplitude of the deformation at the peak of the sine wave and the lag between the stress and strain sine waves, parameters like storage and loss moduli, the viscosity, and the damping can be calculated. The three kinds of mechanical test described above

(i.e., tensile, compression, and bending test), as well as other kinds of test (e.g., shear test) can be carried either with a constant strain rate (static mechanical analysis, SMA) or as DMA. A comparison between the stress variation as a function of time in DMA and SMA is shown in Fig. 5.9d.

5.2.1.2 Mechanical Characterization of Additive Manufactured Scaffolds

Many structural parameters have been studied to characterize and control the mechanical properties of scaffolds constituted by biodegradable polyesters, polysaccharides, and proteins, in order to tailor their suitability for the regeneration of a great variety of biological tissues. As it will be discussed in detail in the following paragraphs, static compressive and tensile characterization have been mainly applied under different testing conditions depending on material chemistry, scaffold geometrical features, and application requirements.

5.2.1.3 Mechanical Properties of Biodegradable Polyester-Based Scaffolds

The mechanical properties of additive manufactured scaffolds constituted by biodegradable polyesters depend on many variables, such as polymer chemical structure, as well as scaffold micro- and macrostructural features. Some of the most studied ways to tune aliphatic polyesters mechanical properties are based on their combination with other materials to obtain polymeric blends or composites (Table 5.1).

Polymeric blends constituted by PCL/PLGA [153], PLA/Cs, PLA/hair-keratin (PLA-H), PLA/feather-keratin (PLA-F) [171], PLA/PCL [159], PCL/PHBV [185], and PHBHHx/PCL [5] have been developed to produce new scaffolding materials for AM with enhanced mechanical properties. As an example, PCL/PLGA scaffolds with a porosity of 66.7% and a pore size of about 300 μm were fabricated using an ME-AM technique. These scaffolds showed a compressive modulus of 51.6 MPa and a compressive strength of 3.2 MPa [153]. Scaffolds based on PLA/PCL blends showed a greater compressive modulus compared with pure PLA scaffolds [159]. PLA-Cs, PLA-H, and PLA-F scaffolds were fabricated by ME-AM employing a polysaccharide or protein reinforcement in the shape of fibers or particles. DMA analysis showed that fibers loading increased the plastic behavior of the material that became less fragile than pure PLA. This behavior was attributed to the presence of a second phase reducing interactions between PLA chains and favoring the molecular slippage at an interface level. Scaffolds made of a particles-loaded matrix showed higher flexibility in a concentration-dependent manner, due to particles agglomeration [171]. In the case of PCL/PHBV scaffolds fabricated by FDM, it was shown that increasing PHBV content in the blend led to an increase in compressive strength. In particular, scaffolds constituted by 75% w/w of PHBV showed a compressive strength value (15.1 MPa) that was nearly threefold greater than that of pure PCL scaffolds (5.8 MPa) [185]. A study on scaffolds fabricated by CAWS and based on PHBHHx/PCL blends revealed that by increasing PCL content the compressive modulus and other mechanical parameters increased significantly [5].

Table 5.1 Mechanical characterization of biodegradable polyester-based scaffolds fabricated by AM

References	Materials	AM technique	Modulus	References	Materials	AM technique	Modulus
[144]	PCL	FDM	20.2–41.9 MPa^{c}	[145]	PLA	FDM	116–207 MPa^{c}
[146]	PCL/TCP	FDM	124 MPa^{c}	[142]	PLA	FDM	27.0–45.6 MPa^{c}
[147]	PCL/PLGA PCL/PLGA/TCP	ME-AM	50.1 MPa^{c} 51.3 MPa^{c}	[148]	PLA/Gel/poly (lysine)	FDM	0.4 GPa^{c}
[149]	PCL PCL/BHA	ME-AM	27 MPa^{c} 30–56 MPa^{c}	[150]	PLA/PEG PLA/PEG/glass	SE-AM	28.4–92.3 MPa^{c} 44.2–99.8 MPa^{c}
[143]	PCL PCL/TCP	SLS	0.07–6.6 MPa^{c} 0.45–8.7 MPa^{c}	[151]	PLA PLA/G5	SE-AM	28.38 MPa^{c} 44.19 MPa^{c}
[121]	PLGA PCL	SE-AM	2.55 MPa^{c} 1.65 MPa^{c}	[152]	TCP/PLA	SLS	~4 MPa^{c}
[153]	PCL/PLGA	ME-AM	n.a.	[154]	PDLLA	SLA	169–224 MPa^{c}
[155]	PCL	ME-AM	~25–35 MPa^{c}	[156]	PDLLA PDLLA/HA	SLA	12.3 MPa^{c} 23.1–30.8 MPa^{c}
[157]	PCL/DCPD/nanoZIF-8	ME-AM	43.5 MPa^{c}	[154]	PDLLA	SLA	47 MPa^{t}
[158]	PCL/NaCl	SLS	81.0–3027 kPa^{c}	[159]	PLA PLA/PCL PLA/PCL/HA PLA/PCL/MWCNT	ME-AM	9.98 MPa^{c} 16.02 MPa^{c} 17.07 MPa^{c} 12.03 MPa^{c}
[160]	PCL PCL/Sr-HA	3DP	~50–55 MPa^{c}	[161]	PLA	FDM	~100–700 MPa^{c}
[162]	PCL PCL/Hyal	FDM	2.1–18.0 MPa^{c} 2.05 MPa^{c}	[163]	PLA	ME-AM	25.3–27.8 MPa^{c}
[164]	PCL/DBM	SLS	0.024–4.33 MPa^{t}	[165]	PLA PLA/Cu PLA/Ag PLA/Bronze	ME-AM	1.26–1.54 MPa^{t} 1.32–1.65 MPa^{t} 1.61–1.70 MPa^{t} 1.43–1.59 MPa^{t}

[166]	PCL PCL/10HA PCL/10SrHA PCL/20HA PCL/20SrHA	ME-AM	~30–55 MPa^{c} ~30–40 MPa^{c} ~25–45 MPa^{c} ~30–60 MPa^{c} ~25–55 MPa^{c}	[167]	PLA	FDM	n.a.
[168]	PCL	SLS	27.3–1944 kPa^{c}	[169]	PLLA PLLA/HNT PLLA/HNT@SiO_2	SLS	420.65 MPa^{t} 548.25 MPa^{t} 637.46 MPa^{t}
[170]	PCL PCL/HA PCL/MWCNT PCL/HA/MWCNT	FDM	~55 MPa^{c} ~85 MPa^{c} ~65 MPa^{c} ~90 MPa^{c}	[171]	PLA/Cs PLA/hair-keratin PLA/feather-keratin	3D Printing	n.a.
[172]	PLLA PCL PCLA	ME-AM	72–124.2 MPa^{c} 12.5–18.8 MPa^{c} 13.3 MPa^{c}	[173]	PLA	FDM	0.95–3.66 GPa^{t}
[174]	PCL annulus/Cel-Col-LMW HA-4S-StarPEG-CNPs	ME-AM	86.3 MPa^{c}	[175]	PLGA/TCP	SE-AM	17.87 MPa^{c}
[176]	PCL	ME-AM	n.a.	[177]	PLGA/pearl powder	SE-AM	12.4–26.6 MPa^{c}
[178]	PCL PCL/HA	FDM	86–207 MPa^{c} 97–220 MPa^{c}	[179]	PLGA/HA	SLS	0.13 GPa^{c}
[180]	PCL PCL/TCP PCL/TCP/COL	SLS	6.77 MPa^{c} 13.66 MPa^{c} 13.74 MPa^{c}	[181]	PLGA/PEG/nHA	ME-AM	0.53–5.2 MPa^{c}
[19]	PCL PCL/HA	CAWS	0.12–0.60 MPa^{c} 0.21–0.90 MPa^{c}	[182]	PHB	SLS	0.4–23.4 MPa^{c}
[183]	*PCL *PCL/HA	CAWS	1.42–3.62 MPa^{c} 7.28 MPa^{c}	[94]	PHBV Ca-P/PHBV PLLA HA/PLLA	SLS	~5.00 MPa^{c} ~6.50 MPa^{c} ~6.00 MPa^{c} ~2.25 MPa^{c}

(continued)

Table 5.1 (continued)

References	Materials	AM technique	Modulus	References	Materials	AM technique	Modulus
[184]	PCL PCL/HA (4:1) PCL/Cs/γ-PGA PCL/HA/Cs/γ-PGA	CAWS	0.2–1.3 MPa[c] 0.3–1.2 MPa[c] 0.10–0.15 MPa[c] 0.03–0.04 MPa[c]	[96]	PHBV Ca-P/PHBV	SLS	2.38 MPa[c] 3.96 MPa[c]
[86]	PCL	SLS	52–67 MPa[c]	[185]	PCL PCL/PHBV	FDM	n.a.
[186]	PCL/HA	SLS	24–102 MPa[c]	[187]	PHBHHx	CAWS	0.71–1.4 MPa[c]
[188]	PLLA	ME-AM	194.96 MPa[c]	[5]	PHBHHx PCL PHBHHx/PCL	CAWS	0.16 MPa[c] 0.6 MPa[c] 0.37–0.39 MPa[c]
[189]	PLLA/TCP	SE-AM	60.11 MPa[c]	[190]	PHBHHx *PCL	CAWS	1.03–1.51 MPa[c] 0.79–0.91 MPa[c]
[191]	PDLLA	FDM	3 MPa[c]	[192]	PPF/DEF	SLA	~15–45 MPa[c]

[c]The marked values were obtained by compression tests
[t]The marked values were obtained by tensile tests

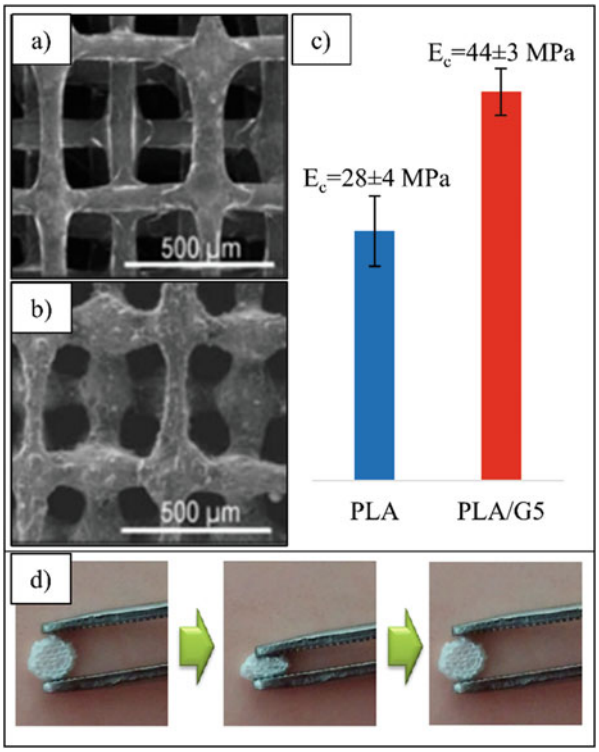


Fig. 5.10 Additive manufactured composite scaffolds: (**a**) SEM micrograph of a PLA scaffold; (**b**) SEM micrograph of a scaffold constituted by a composite based on PLA/phosphate glass (G5); (**c**) comparison of compressive modulus (E_c) of PLA and PLA/G5 scaffolds (modified with permission from [151]); (**d**) representative pictures showing the high flexibility and excellent shape recovery after compression of water-borne PU/HA scaffolds fabricated by SE-AM (modified with permission from [196])

Composite materials have been also widely investigated for the fabrication of additive manufactured scaffolds loaded with osteoconductive calcium phosphate ceramics, e.g., hydroxyapatite (HA) and tricalcium phosphate (TCP). Examples of osteoconductive composite scaffolds fabricated by different AM techniques are, among others, those based on PCL/HA [149, 155, 160, 166, 178, 183, 184, 186, 193, 194], PLA/HA [94, 156], PLGA/HA [179, 181], PPF/DEF/HA and PPD/DEF/SDS/HA [195], PCL/TCP [143, 146], PCL/PLGA/TCP [147], PLA/TCP [152, 189], PLGA/TCP [175], or PLA/PCL/HA [159]. Calcium phosphate loading should result in improved scaffold mechanical properties like compression modulus and compression strength, as it is shown in Fig. 5.10. However, such effect is not always accomplished depending on factors like ceramic particles dispersion in the polymer matrix, particles agglomeration, polymer-ceramic chemical-physical interaction, and scaffold microporosity [160, 166, 178, 193]. As an example, PCL scaffolds fabricated by CAWS showed an improvement of their mechanical properties when loaded with HA only in the case of a designed pore size of 1.5 mm, but not in the case of a pore size of 1 mm. In any case, a great number of articles have described a significant enhancement of compressive mechanical properties for composite scaffolds, such as in the case of PCL/dicalcium phosphate dihydrate (DCPD) [157], PCL/demineralized bone matrix (DBM) [164], PCL/multi-walled carbon nanotubes (MWCNT) [170],

PLA/PCL/MWCNT [159], PLA/bioactive glass [150], PLA/calcium phosphate-based glass (G5) [151], PLA/Cu, PLA/Ag, PLA/bronze (Br) [165], PLA/halloysite nanotubes (HNT), PLA/HNT@SiO_2 [169], PLGA/pearl powder [177], and PHBV/Ca-P [94, 96]. In all these cases, the development of a composite increased the scaffold compressive modulus and strength in comparison with scaffolds made of non-loaded polymers. Moreover, for composite scaffolds based on PLA/metal particles [165], PLA/HNT and PLA/HNT@SiO_2 [169] also the tensile modulus and strength were increased in comparison to analogous non-loaded polymer scaffolds.

Synthesis of copolyesters like poly(D,L-lactide-*co*-ε-caprolactone) P(DLLA-*co*-CL) represents an effective strategy to tune the mechanical properties of the scaffolding material [154]. The main difference between the mechanical properties of additive manufactured scaffolds made of PDLLA and scaffolds made of P(DLLA-*co*-CL) is that the first type yields plastically at 5% strain while the second one can be elastically deformed up to 70% strain [154]. As previously mentioned, aliphatic polyesters can also be employed as soft segments in PUs with tailored processing and mechanical properties [197]. As an example, a poly(ester urethane) based on a PCL-*co*-PDLLA soft segment and a block urethane hard segment, was recently synthesized and processed by FDM into a 3D macroporous scaffold able to support the dynamic compression-relaxation loads physiologically experienced by knee tissues [198]. By incorporating hydrophilic monomers in the chemical structure of the soft segment, e.g., to form a PCL-b-PEG-b-PCL triblock copolymer [199], it is possible to develop thermoplastic PUs suitable for FDM that behave like a tough hydrogel once placed in aqueous environment. In the case of PPF scaffolds by SLA, material crosslinking density significantly affects the resulting mechanical properties. For instance, when DEF was used as diluent and crosslinking agent, an increase in its content resulted in an increase of the compressive modulus of the fabricated scaffolds [192].

Fabrication parameters can significantly affect scaffold mechanical properties. Considering for instance ME-AM, the effect of liquefier temperature, deposition velocity, and screw rotation rate on scaffolds macrostructure and compressive mechanical properties was clearly described for PCL [119]. Increasing the liquefier temperature up to a critical value resulted in a thickening of the extruded filaments and in a decreasing of scaffolds porosity, as a consequence of the decreased viscosity and increased extrusion rate, thus causing an increment in scaffold compressive stiffness and strength. Increasing the deposition velocity, instead, determined the stretching of the filament, resulting in a decrease of its width and in an increase of scaffold porosity. This phenomenon led to a reduction of scaffolds compressive properties. Finally, the increment of the screw rotation velocity raised the amount of material extruded per time unit, resulting in filament thickening and in scaffolds with lower porosity showing higher stiffness and strength [119]. Overall porosity is a parameter that greatly affects scaffold mechanical properties. This is true for scaffolds made of different polymers and fabricated by different techniques [19, 142, 143, 145, 162, 166, 168, 175, 178, 183, 184, 200]. Indeed, increasing porosity and pore size reduces scaffold

stiffness and yield strength because of the reduced density of strands packing and filament–filament contact points. Also the mechanical properties of SLS fabricated scaffolds are dependent on processing parameters like scan spacing (SS) and powder layer thickness (PLT) [182]. For instance, PHB scaffolds obtained with smaller SS and the same PLT showed an increase in mechanical properties due to the higher energy density, resulting in a more compact polymer matrix. Scaffolds fabricated with the same SS but with larger PLT showed a decrease in the mechanical properties because of inhomogeneous sintering along scaffold thickness [182].

Post-processing of fabricated scaffolds can also affect their mechanical properties. For instance, in the case of scaffolds fabricated by SLS a heat treatment after fabrication could increase the mechanical properties of the scaffolds, thanks to increased particles adhesion resulting in improved polymer matrix quality and density [158, 164]. Surface modification by coating [148] or plasma treatment [185] typically does not affect scaffold mechanical properties. Indeed, these treatments usually alter the chemistry of the surface, without affecting the bulk material.

Hybrid AM combines AM principles with conventional scaffold fabrication techniques, such as thermally induced phase separation (TIPS), wet-spinning, or porogen leaching. It can be exploited to fabricate scaffolds integrating predesigned macroscopic pores to guide tissue ingrowth and vascularization, and micropores favoring cell adhesion and differentiation [181]. Such microporosity can result in a significant effect on scaffold mechanical properties, such as a decrease of compressive modulus and strength, as well as increased deformability in the case of elastomeric polymers [129]. Assembling electrospun fibers and additive manufactured structures is a promising approach to obtain multi-scale scaffolds mimicking the complexity and hierarchical structure of biological tissues [201]. Until now no significant differences have been evidenced between FDM-extruded PCL scaffolds and dual-scale scaffolds obtained by combined FDM-electrospinning in terms of compressive modulus and yield stress, even by increasing the electrospun mesh density. This could be attributed to the limited thickness of the electrospun fibers layer and its small volume compared to that of the 3D-printed structure [155].

Inclusion of hydrogels within the pores of an additive manufactured scaffold in some cases represents another way to improve its mechanical properties. It has been demonstrated that upon axial compression the hydrogel phase places PCL fibers under tension with an overall result of increasing scaffold stiffness up to 50-fold [184]. Because of the incompressible nature of highly swollen polymers, each volume of the hydrogel phase confined into a scaffold cell expands in response to the applied stress causing fiber deformation. However, in the case of thicker PCL fibers (>88 μm) mechanical reinforcement was not achieved due to the stronger vertical column formed by the aligned fiber crossings points, causing water to flow out of the scaffold [184]. PCL scaffolds filled with sodium hyaluronate did not show any significant difference in compressive properties in comparison with the unfilled analogous scaffolds, also in this case likely due to

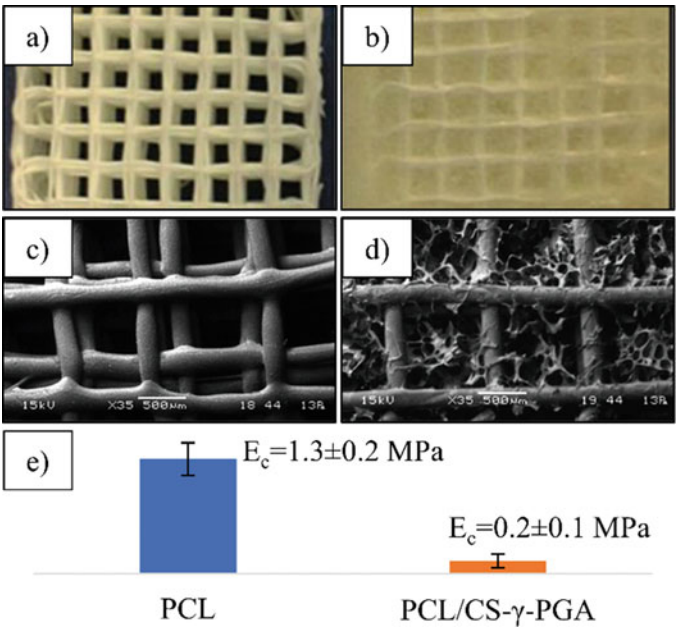


Fig. 5.11 Hydrogel infiltration within additive manufactured PCL pores: (**a**) PCL scaffold; (**b**) PCL/CS-γ-PGA scaffold; (**c**) SEM micrograph of the PCL scaffold; (**d**) SEM micrograph of the PCL/CS-γ-PGA scaffold; (**e**) Comparison of the compressive modulus (E_c) between PCL and PCL/CS-γ-PGA scaffold. (Modified with permission from [184])

filaments width [162]. The inclusion of a cell-laden hydrogel into a PCL annulus scaffold resulted in a biphasic construct with high compressive strength and increased flexibility, more closely resembling that of soft biological tissues [174]. The observed different mechanical behavior of the fabricated annulus in comparison with the natural intervertebral discs could be mainly related to the column-like behavior of the fibers crossing points along vertical direction as well as to a densification of the 3D PCL porous structure during sample compression. Biphasic constructs constituted of a freeze-dried Cs/γ-PGA polyelectrolyte complex hydrogel infiltrated within the pores of a PCL scaffold by CAWS showed a lower compressive strength in respect with pure PCL scaffolds up to 80% strain and a much higher strength in the subsequent region (Fig. 5.11) [184]. Although the fiber size of the biphasic scaffolds reported in that study was relatively large (>200 μm), it is likely that the higher flexibility of microporous wet-spun fibers, in comparison to dense fibers by ME-AM, allowed a uniform deformation of PCL to be achieved with enhancement of the compressive strength of the biphasic construct above 80% strain.

Pore architecture is one of the most important variables affecting scaffold mechanical properties. Confronting porous structures with cube architectures with those with gyroid architecture, it is clear that the first ones are characterized by a more rigid behavior, as a result of the better alignment of the vertical struts with the

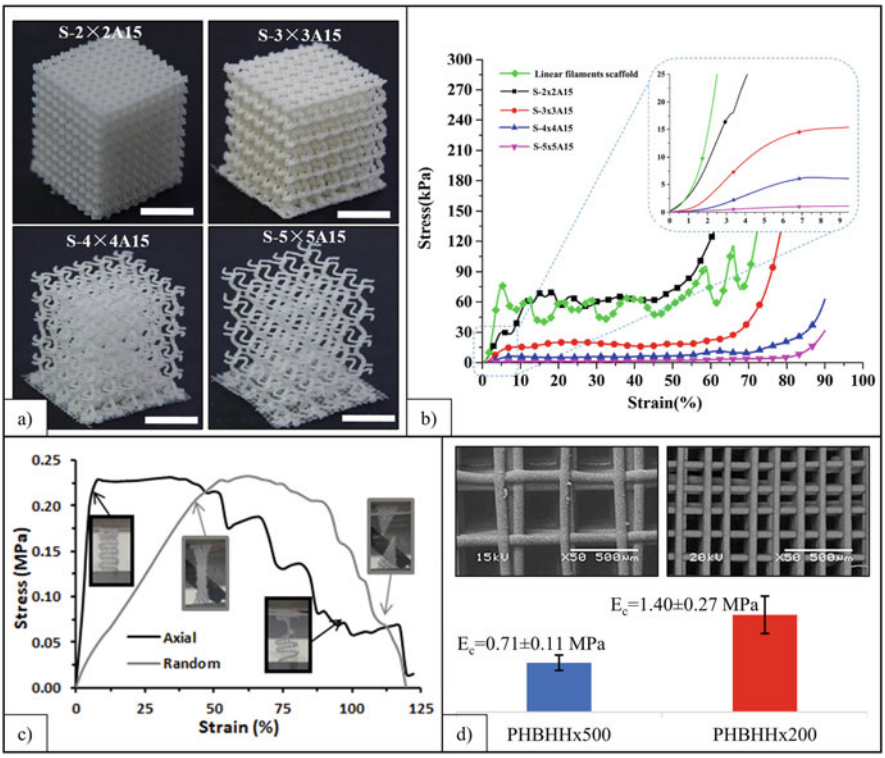


Fig. 5.12 Additive manufactured scaffolds with different pore architecture: (**a**) PCL scaffolds by SLS constituted by sinusoidal filaments with different filament period S-2x2A15 (2 mm), S-3x3A15 (3 mm), S-4x4A15 (4 mm), S-5x5A15 (5 mm); (**b**) compression test on the PCL scaffolds with sinusoidal filaments (modified with permission from [168]). PHBHHx scaffolds by CAWS: (**c**) effect of the random or axial fiber orientation on the tensile properties of PHBHHx scaffolds (modified from [190]); (**d**) SEM micrographs and compressive modulus of PHBHHx scaffolds with a pore size of 500 μm or 200 μm and E_c of the two samples (modified with permission from [187])

compressive force direction [154]. Replacing linear filaments with sinusoidal ones in PCL scaffolds by SLS was reported to reduce scaffold compressive modulus because of a porosity increase (Fig. 5.12a) [168]. Compressive modulus was further reduced by increasing the period of the sinusoidal filaments because of an increase in scaffold porosity, as shown in Fig. 5.12b. Moreover, the compressive modulus of this kind of scaffolds can be tuned from 704.3 kPa to 70.1 kPa by increasing the amplitude to period ratio from 0.05 to 0.25. The unique capability of the sinusoidal scaffolds to regulate their mechanical properties makes it possible to match the specific demands of different types of native tissue.

Lay-down pattern in extrusion-based AM determines pores geometry as well as 3D filament-filament contact points architecture, often with a significant effect on

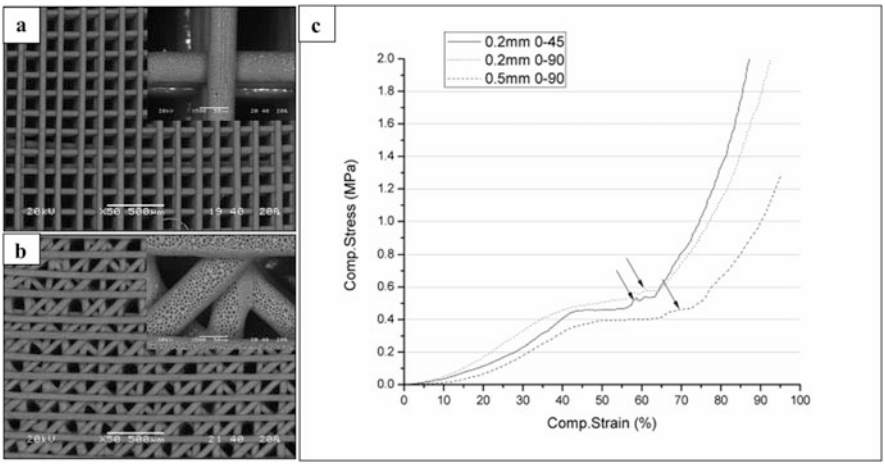


Fig. 5.13 Influence of lay-down pattern and pore size on the mechanical properties of PHBHHx scaffolds fabricated by CAWS: (**a**) SEM micrograph of scaffold with inter-fiber distance of 200 μm and fibers angles of 0°–90°; (**b**) SEM micrograph of scaffold with inter-fiber distance of 200 μm and fibers angles of 0°–45°; (**c**) stress–strain curves of PHBHHx scaffolds with different inter-fiber distance and fibers angles (modified with permission from [187])

scaffold compressive properties. Scaffolds with angles between the fibers of adjacent layers different from 0° to 90° have larger fiber–fiber contact areas. However, as it has been evidenced by articles on PCL and PHBHHx scaffolds [144, 187], angles different from 0° to 90° can reduce the fiber–fiber intersection points alignment along vertical direction, resulting in a lower compression modulus (Fig. 5.13).

Anisotropic mechanical behavior of additive manufactured scaffolds should be taken into account during mechanical characterization. This aspect was highlighted in a recent article on tensile testing of PHBHHx scaffolds by CAWS with a 0°–90° lay-down pattern, showing that the angle between the applied force and scaffold filaments is a parameter that could significantly affect scaffolds mechanical response [190]. In particular, in the case of scaffolds oriented with a random angle between filaments and applied force direction, the tensile modulus was significantly lower and the break strain significantly higher than when the force was applied parallel to fiber axis of alternating layers (Fig. 5.12c). These differences are determined by the different response of the fibrous scaffolds in terms of fiber orientation angle during sample deformation. When scaffolds were oriented with a 0°–90° angle with the applied force, the longitudinally aligned filaments were those mainly involved in load bearing, and their breaking led to sample failure. The filaments oriented perpendicularly to the tensile direction only poorly contributed to load bearing. In the random angle test, all fibers were subjected to deformation and reorientation resulting in sample necking and larger plastic deformation before breaking.

5.2.1.4 Mechanical Properties of Hydrogel Scaffolds

Hydrogel scaffolds are fabricated mainly by SE-AM techniques, by employing Bioprinting approaches in the case of direct fabrication of cell-laden systems. As previously mentioned, most of the studies on AM of hydrogels for TE are based on polysaccharides and proteins, whose gelation can be achieved by different mechanisms, such as ionotropic gelation [131], chemical crosslinking [132], photocrosslinking under UV light [134], and polyelectrolyte complexation [135].

Representative articles on mechanical characterization of polysaccharide- and protein-based hydrogels by AM are summarized in Tables 5.2 and 5.3, respectively, by highlighting the scaffolding material, the fabrication technique, and the resultant elastic modulus.

Table 5.2 Mechanical characterization of polysaccharide-based hydrogels

References	Material(s)	AM technique	Modulus
[202]	Alg	SE-AM	21.65–273.35 kPa[c]
[203]	Alg	Bioprinting	n.a.
[204]	Alg Alg/Gel/HA	Bioprinting	4.13 MPa[c] 8.27 MPa[c]
[205]	Alg/Gel	Bioprinting	30.4–113.64 kPa[c]
[206]	Alg Alg/Gel Alg/Gel/MC Alg/MC	Bioprinting	97.7 kPa[c] 69.4 kPa[c] 82.85 kPa[c] 113.3 kPa[c]
[207]	Alg	Bioprinting	1.5–14.2 kPa[c]
[208]	Alg/MC/HNT/RO	Bioprinting	n.a.
[209]	Alg/Gel	SE-AM	312.6–439.6 kPa[c]
[210]	Alg/NaCl	SE-AM	~1.2–1.5 MPa[c]
[211]	Alg/PCL	Bioprinting	~6 MPa[d]
[212]	MCS-Alg/Col/PCL nanofibers	Bioprinting	4.4 MPa[t]
[213]	ADA/Gel	Bioprinting	~40–25 kPa[c]
[214]	NFC/CMC	SE-AM	1.2–24 MPa[i]
[215]	Cs-HA	SE-AM	~0.7–1.75 GPa[i]
[135]	Cs Cs/γ-PGA	CAWS	9.5 kPa[c] 16.9 kPa[c]
[216]	Cs/Gel/HA Cs/Gel/HA/Gr	Bioprinting	4.2 MPa[c] 5.7–7.6 MPa[c]
[217]	Alg/CSN/OPC	SE-AM	n.a.
[218]	Gellan/Alg/HC Gellan/Alg/HA	SE-AM	116 kPa[t] 230 kPa[t]
[219]	Hyal/Alg Hyal/Alg/Gel	SE-AM	~16 kPa[t] ~6–6.5 kPa[t]

[c]The marked values were obtained by compressive test
[t]The marked values were obtained by tensile test
[d]The marked value was obtained by DMA in compression mode
[i]The marked values were obtained by indentation test

Table 5.3 Mechanical characterization of protein-based hydrogels scaffolds

References	Material(s)	AM technique	Modulus
[220]	Col/heparin sulfate	Bioprinting	3.46 MPa[c]
[221]	GelMA GelMA/CarMA	SE-AM	1.2–2.2 kPa[c] 2.2–2.5 kPa[c]
[222]	Gel/Alg Gel/Alg/GO	Bioprinting	0.69 kPa[c] 1.58–1.63 kPa[c]
[223]	GelMA GelMA/BP	Bioprinting	8.5 kPa[c] 11.07–13.28 kPa[c]
[224]	GelMA	Bioprinting	40–103 kPa[c]
[225]	GelMA GelMA/PCL	SE-AM	0.75 MPa[c] 28 MPa[c]
[226]	GelMA/Gel	Bioprinting	0.5–2.75 kPa[c]
[227]	SF/Gel/BC	Bioprinting	0.32–1.63 MPa[t] 105.9–186.5 kPa[c]
[228]	SF	Bioprinting	0.15–0.32 MPa[c]

[c]The marked values were obtained by compressive test
[t]The marked values were obtained by tensile test

Polymeric blends can be exploited to optimize the mechanical properties of the fabricated hydrogel. For instance, solution-extruded GelMA/methacryloyl carrageenan (CarMA) scaffolds showed better mechanical properties than scaffolds made of GelMA or CarMA alone. This could be due to the higher crosslinking density in hydrogel blend scaffolds that resulted in a lower swelling ratio and thus a higher compressive modulus [221]. Furthermore, bioprinted scaffolds based on Alg/Gel, Alg/methyl cellulose (MC), or Alg/Gel/MC had higher compressive modulus in comparison with Alg scaffolds [206]. Forming a polymeric blend could also be useful to reduce scaffolds stiffness as in the case of bioprinted scaffolds based on Hyal/Alg/Gel. These scaffolds tested at 37 °C showed a decrease of the elastic modulus because of the temperature-dependent gelation property of Gel (i.e., it dissolves in aqueous solutions as a colloidal sol at around 35 °C) leaving behind a porous structure inside the hydrogel [219]. Another study on bioprinted hydrogels for neural regeneration showed that both the compression modulus and strength of a Col/heparin sulfate blend scaffold were significantly higher than those of a Col scaffold [220].

Composite hydrogels composition can be tailored to optimize their mechanical properties, as shown in the case of additive manufactured scaffolds based on Gel/Alg/graphene oxide (GO) [222], GelMA/bone particles (BP) [223], Alg/Gel/HA [204], GelMA/nanosilicate [224], Cs/Gel/HA/graphene (Gr) [216], or Alg/calcium silicate nanowires (CSN)/oligomeric proanthocyanidrine (OPC) [217]. In the case of bioprinted scaffolds constituted by Alg/MC/HNT loaded with Russian olive (RO) powder, an increase in the percentage of Alg and HNT led to an increase in compressive modulus, while an increase in the concentration of MC and RO did not affect the compressive properties of the scaffolds [208]. In addition, inclusion of bacterial cellulose nanofibers is an effective means to

improve the compression and tensile properties of SF/Gel hydrogels by AM [227].

Polyelectrolyte complexation is another direct strategy to stabilize extruded polyions in physiological environments, as well as to tune their mechanical and processing properties. An example is represented by Cs/γ-PGA polyelectrolyte complex scaffolds by SE-AM showing higher compressive modulus than Cs scaffolds with the same designed porous structure [135]. Moreover, scaffolds constituted by Alg/Cs polyelectrolyte complexes were fabricated by SE-AM and the effect of the variation of their composition on hydrogel mechanical properties was evaluated [229]. It was shown that when the concentration of positive charges is different from that of negative charges, the mechanical properties of the scaffolds decreased, so varying the percentage ratio between the two polyions was an effective way to tune scaffold mechanical properties. This phenomenon was associated to the electrostatic repulsion between equal charges that affects the electrostatic interaction between opposite charges.

Cell inclusion in the ink to be bioprinted can also affect the mechanical properties of the resulting hydrogel. Mechanical tests carried out on cell-laden Alg/Gel-printed scaffolds revealed that their compressive strength increased after 3 days of in vitro cell culture (Fig. 5.14a, b). This could be due to the fact that osteoblast cells secreted the ECM inside the hydrogels, strengthening their compressive properties and mimicking the natural tissue formation process [209]. Another work compared the effect of including human micronized cartilage (HC) into a bioink constituted by gellan, Alg, and HA [218]. Tensile dumbbell specimens were printed and tested. The results showed how the acellular constructs presented significantly higher elastic modulus compared to cellular ones, suggesting that the cells increased the compliance of the construct and/or inhibited polymer crosslinking.

Fabrication parameters are one of the main factors affecting the mechanical properties of additive manufactured hydrogels as a direct consequence of a significant effect on their architectural features. As an example, scaffolds made of HA-loaded Cs were fabricated by SE-AM employing different extrusion speeds (0.8–1.2 mm/s) showing that the variation of this processing parameter significantly affected scaffold compression modulus and hardness because of variations of pore size and overall porosity [215]. Furthermore, it was found that scaffolds made of GelMA/Gel showed an increase in compressive modulus with the increase of the flow rate of extruded Gel, caused by a reduction in the pore size creating more dense structures [226]. Other studies demonstrated that an increase in pore size led to a decrease of compressive modulus [205, 221]. A recent article reported a different approach to exploit this porosity/mechanical properties relationship, involving the use of a porogen to create a highly porous structure in printed Alg scaffolds [210]. Increasing the concentration of the porogen (i.e., NaCl) in the solution significantly decreased the scaffold compressive strength and modulus, as a consequence of the increased overall porosity.

Lay-down pattern is a design parameter that can be easily varied to significantly affect both the compressive and tensile properties of hydrogels. As an example,

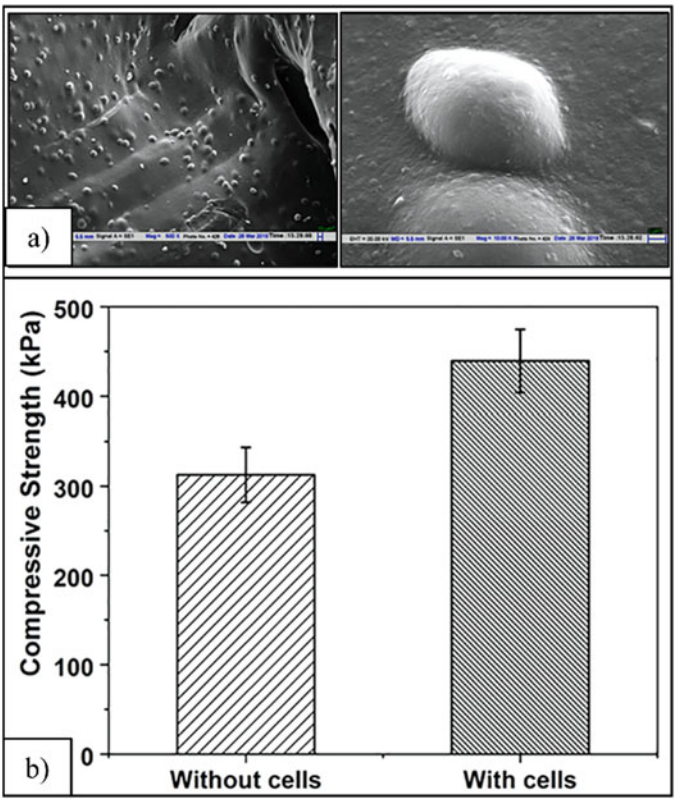


Fig. 5.14 Effect of cells inclusion within the ink: (**a**) SEM micrograph showing well-distributed cells in printed Alg/Gel matrix; (**b**) compressive strength of 3D-printed Alg/Gel hydrogels constructs (modified with permission from [209])

scaffolds constituted by hydrogels based on Alg/Gel were fabricated by SE-AM with different fiber deposition orientation (Fig. 5.15a). Scaffolds with 0/45° lay-down pattern, as shown in Fig. 5.15b, had a higher compressive modulus than those with 0/90° lay-down pattern and the same inter-fiber distance, because of the larger contact area between adjacent layers. Scaffolds with fiber orientation of 0/45/135° showed lower compressive modulus compared with scaffolds with a fiber angle of 0/45° or 0/90° and the same inter-fiber distance because the continuous changing of fiber orientation reduced the contact area between the fibers of adjacent layers [205]. Regarding the effect on scaffold tensile properties, Alg dialdehyde/Gel hydrogels with a 0/90° lay-down pattern showed a higher elastic modulus than those with 0/45/135° and 15/165° pattern [213].

The crosslinking process is one of the most important ways to control the mechanical properties of the fabricated scaffolds. Ionotropic crosslinking is a widely used strategy to reticulate Alg inks employed in AM, through the formation of ionic bonds between the carboxylate groups and divalent metallic ions, in particular

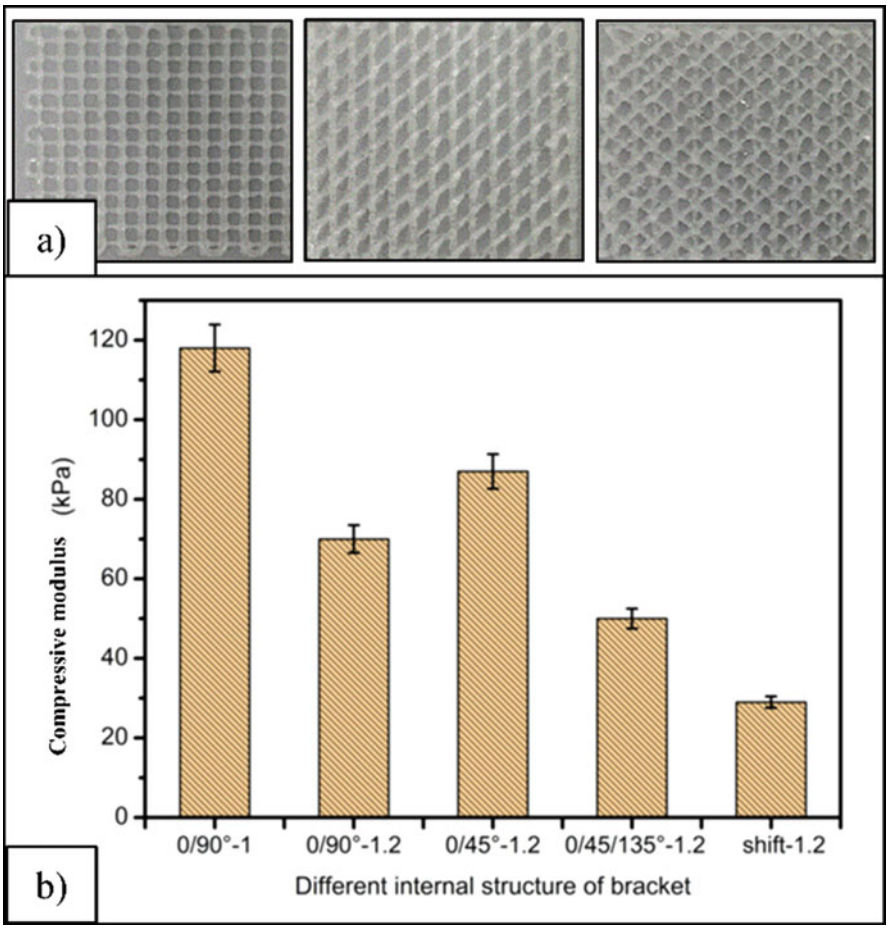


Fig. 5.15 Effect of fiber orientation (lay-down pattern) on scaffold mechanical properties: (**a**) Alg/Gel scaffolds with different fiber orientation angle; (**b**) compressive modulus of Alg/Gel scaffolds with different internal structures (modified with permission from [205])

Ca^{2+}. For instance, Alg scaffolds can be printed directly into a $CaCl_2$ aqueous solution [202]. Increasing the crosslinking time and volume of crosslinking solution usually leads to an increase in compressive modulus. This effect is attributed to the higher number of ions involved in chemical crosslinking of Alg macromolecules. Controlling the crosslinking temperature is another useful tool to optimize the hydrogel mechanical properties. Additive manufactured carboxymethylcellulose (CMC)/nanofibrillated cellulose (NFC) blends were crosslinked using a dehydrothermal treatment [214]. Increasing the temperature of crosslinking treatment from 60 to 120 °C caused a significant increase of the compressive strength. An increase in crosslinking treatment duration also resulted in an increase in hydrogel compressive modulus [214]. Scaffolds made of

regenerated silk fibroin (rSF) and hydroxypropyl methyl cellulose (HPMC) were subjected to ripening treatment to promote and enhance the conformational transition of rSF from random coils to β-sheets [230]. β-sheets domains played the role of physical crosslinkers leading to a great improvement in the mechanical properties of rSF-ripened scaffolds. An increase in sample density and a decrease in pore size were also shown to result in an increase of scaffold compressive modulus. Furthermore, when scaffolds constituted by SF/Gel/HA were subjected to a post-processing treatment in an ethanol solution, the density of hydrogen bonds between SF and HA (Fig. 5.16a) and between SF and ethanol (Fig. 5.16b) increased. This effect led to the rearrangement of peptide chains, causing a transition of SF from a random-coiled structure to an ordered crystallized structure with higher deformability. The treated scaffolds showed an increase in tensile and compressive modulus (Fig. 5.16c,d) by increasing SF concentration [231] because of the increased density of hydrogen bonds.

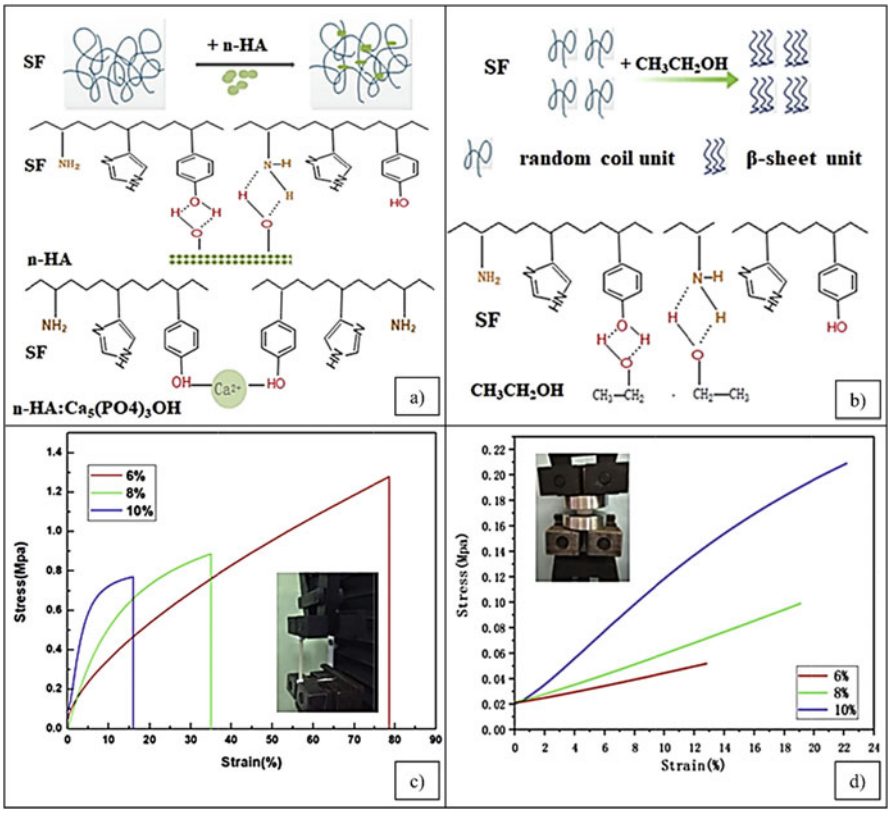


Fig. 5.16 SF mechanical properties enhancement: (**a**) hydrogen bonds between SF and HA; (**b**) hydrogen bonds between SF and ethanol and conformational transition of SF from random coil to β-sheet; (**c**) relevant tensile stress–strain curves; (**d**) relevant compressive stress–strain curves (modified with permission from [231])

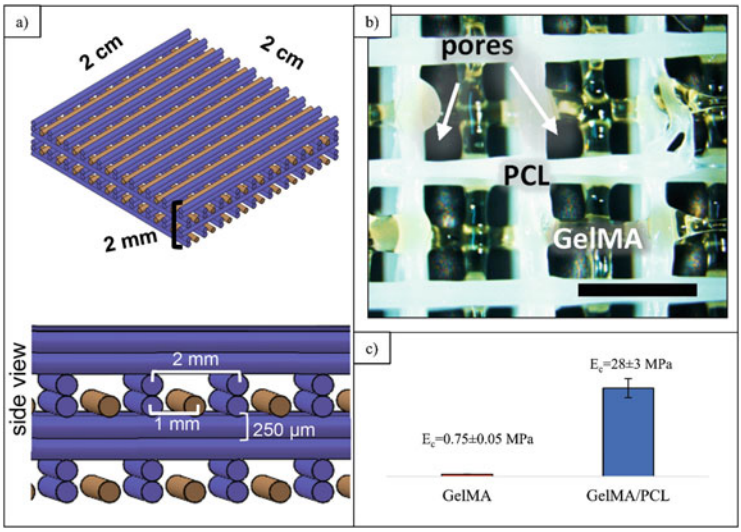


Fig. 5.17 Biphasic scaffolds: (**a**) schematic of the printed structure with PCL (blue) and Gel/MA (orange) strands; (**b**) bright field image of the construct showing the PCL, GelMA, and pores distribution from a top view. Scale bar represents 2 mm; (**c**) Young's modulus of GelMA and GelMA/PCL constructs (modified with permission from [225])

Biphasic scaffolds obtained through simultaneous AM of a crosslinked polysaccharide and a polyester structure represent an effective strategy to significantly increase the mechanical properties of additive manufactured hydrogels. For instance, scaffolds made of PCL/GelMA/chondrocytes were fabricated printing the cell-laden GelMA ink between two strands of PCL, and alternating GelMA layers with two PCL layers (Fig. 5.17a,b). The compression test revealed that the modulus was 37 times higher than the modulus of GelMA scaffolds and similar to that of PCL scaffolds, as shown in Fig. 5.17c [225]. Another article described printing cell-laden Alg between PCL strands to obtain scaffolds with different shape. Even in this case, the compressive modulus of the fabricated constructs was greater than that of Alg samples and similar to that of PCL constructs [211].

5.3 Conclusions

In the last 30 years, TE has become an important research field in biomedicine, but it remains largely at an experimental level for most of the investigated therapeutic applications. Few examples of its clinical translation are opening new perspectives for the treatment of tissue defects by overcoming major drawbacks related to conventional therapeutic approaches (i.e., prosthesis implantation and tissue transplantation).

Biodegradable polymers of natural or synthetic origin are the most employed materials for the fabrication of TE scaffolds, thanks to the versatility offered in terms of physical-chemical and processing properties. Recent progress in the design and development of biodegradable polymers and relevant fabrication approaches is providing unique means for AM of TE scaffolds [20]. Scaffolds fabrication by means of AM techniques allows the development of 3D polymeric structures with well-defined size and shape, as well as a fully interconnected network of pores, possibly personalized to a specific tissue defect.

One of the most important limiting factors that has hindered the progress of a range of TE polymeric scaffolds is the lack of mechanical properties suitable for a specific application. For this reason, many strategies have been followed to tune polymeric scaffolds mechanical behavior. These include, among others, tailoring the starting material composition through the development of polymeric blends or composites, synthesis of copolymers, tuning physical/chemical macromolecular crosslinking, optimizing scaffold fabrication parameters and methods, and developing ad hoc post-processing treatments. For instance, a large body of literature on AM has shown that scaffold structural/mechanical properties relationship can be controlled by acting on various processing variables (e.g., the rate of material deposition in the case of FDM, and the powder layer thickness in the case of SLS) or design parameters (e.g., porosity, pore size, fiber orientation angle, fiber–fiber contact points alignment, and pores architecture). A particular option is constituted by joining soft materials like hydrogels with rigid materials like polyesters, either through inclusion of a hydrogel phase inside the pores of a rigid scaffold, or through the alternated deposition of rigid and soft fiber layers in the polymeric structure. This approach can be employed to obtain scaffolds showing both the mechanical strength of the rigid material and the elasticity and hydrophilicity of the soft material. Scaffolds tailored to the composition and mechanical properties of biphasic tissues, e.g., at the osteochondral interface, can be developed in this way in order to optimize their ability to support the tissue regeneration process.

Although the great progress made on tuning polymeric scaffolds mechanical properties to match those of biological tissues, further research on this aspect is still required to make TE a valuable therapeutic approach for a broader range of applications. In this context, mechanical characterization strategies tailored to novel additive manufactured scaffolds, as well as progress on the analysis of relevant experimental data are of utmost importance. The development of advanced mechanical characterization protocols optimized for testing polymeric scaffolds by AM is therefore expected in the near future to significantly impact the biomedical area.

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3D Bioprinting: A Short Overview and Future Prospects in Healthcare Engineering

6

Sophia Read and Marco Domingos

Abstract

The introduction of 3D Bioprinting in the fields of tissue engineering (TE) and regenerative medicine (RM) has significantly improved our ability to emulate the complex structural and functional organization of human tissues and organs. Through the precise spatial deposition of cells, biomaterials, and other biomolecules, we are now capable of creating 3D tissue surrogates and systematically investigate important mechanisms underlying human organogenesis and disease. Biomaterials, in particular polymeric hydrogels, play a crucial role in TE by providing a 3D extracellular matrix (ECM)-like environment for the cells to adhere, proliferate, and differentiate. When combined with cells to create bioinks for 3D bioprinting, these materials must also display adequate rheological properties to ensure both optimal printability and high cell viability. This is not always easy to achieve and often researchers must find a compromise. However, with the emergence of new bioprinting strategies, such as suspended layer additive manufacturing (SLAM), low viscosity materials can be easily printed into 3D constructs without compromising shape fidelity. In this short review, we cover the most recent advances in terms of automated manufacturing technologies for biofabrication of human tissue models. We briefly discuss the importance of polymeric hydrogels and which requirements are important to consider during their design for 3D bioprinting. We conclude with a short outlook in terms of challenges and prospective developments in this exciting field of biofabrication.

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M. Dash (ed.), *Biomimetic Biomaterials for Tissue Regeneration and Drug Delivery*,
https://doi.org/10.1007/978-981-16-4566-2_6

6.1 Introduction

The origins of 3D Bioprinting can be traced back to the early 1980s and to the pioneering work of Charles Hull on polymeric resins for light-assisted fabrication, commonly termed as stereolithography (SLA) [1]. Although the original system was not designed to work with biological materials, its development marks the beginning of a new era in digital fabrication and lays the foundations for additive manufacturing (AM) as we know it today. Operating in a layer-by-layer fashion, AM systems (also known as 3D printing) allow for the fabrication of 3D objects from the bottom up, mimicking many natural processes and offering unprecedented freedom of design [2]. This and the ability to process multiple materials with high spatial resolution has opened the doors for the introduction of AM technologies in the field of tissue engineering (TE). From 3D scaffolds for skeletal regeneration to hollow conduits for peripheral nerve repair, the number of applications is vast and keeps on rising, placing 3D printing at the forefront of healthcare engineering [3, 4]. However, tissue regeneration is a complex, multifactorial process that requires more than just a 3D physical template (i.e., scaffold) onto which cells can adhere, proliferate, differentiate, and eventually replace with newly synthetized extracellular matrix (ECM). If the aim is to emulate the regeneration processes of the human body, then cells must be combined with engineered materials to create building blocks and assembled into 3D hierarchical constructs thus mimicking the structural and functional organization of native tissues and organs. 3D Bioprinting stems from traditional AM technology and enables the automated processing of cells, biomaterials, and biomolecules (i.e., bioinks) with precise spatial arrangement to create surrogates of human tissues [5]. Falling under the general umbrella of Biofabrication, 3D Bioprinting has been particularly successful in the generation of 3D models for the study of disease mechanisms, regeneration processes, drug testing, and other more conventional TE applications. For a more detailed information on 3D Bioprinting and tissue models, we direct the reader to another recent publication [6]. Herein, we briefly review the most important advances in 3D Bioprinting, focusing on volumetric printing, suspended layer extrusion, and acoustic jetting. We also discuss recent progress on polymeric hydrogels and some of the most important requirements that need to be considered when developing bioinks. We conclude this chapter with a brief perspective in terms of future developments in the area of bioprinting for healthcare engineering.

6.2 3D Bioprinting Technologies

The merging of AM techniques with bioengineering has allowed major advances to take place within the regenerative medicine field. As stated above, perhaps the biggest achievement is the spatiotemporal control it gives to precisely place cells and biomaterials in 3D to mimic their locations in the native tissue. Medical imaging techniques, such as MRI and CT scans, can provide the information required to bioprint these 3D constructs with geometrical, structural, and functional features that

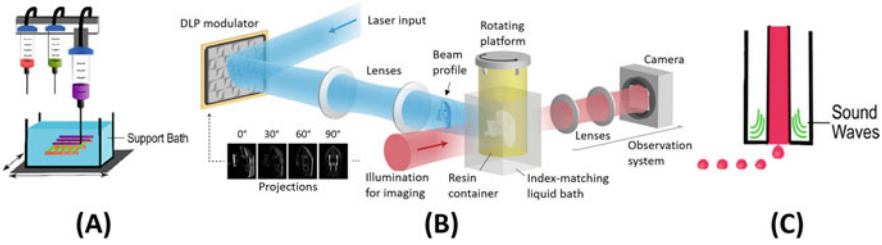

Fig. 6.1 Schematic drawings depicting novel 3D Bioprinting techniques. (**a**) Extrusion-based AM strategies that use a support bath, e.g., FRESH and SLAM, can printing self-supportive overhanging structures. (**b**) Tomographic volumetric AN simultaneously projects multiple visible light images in different directions to rapidly build a 3D construct using a photocurable material. (**c**) Acoustic-based material jetting uses an ultrasound field to eject droplets; it is the only material jetting technique that does not require a nozzle. ((**a**) and (**c**) Reprinted with permission from [6]. Copyright [2020] American Chemical Society. (**b**) Reprinted with permission from [11]. Copyright [2020] Nature Publishing group under CC BY 4.0)

are specific to the patient. Practically, all 3D Bioprinting technologies currently on the market are founded on one or more of the three main concepts: vat photopolymerization, material jetting, and/or extrusion. Each of these techniques has differing advantages and disadvantages to one another, and their choice will mainly depend on the processing material and targeted application (i.e., tissue properties).

At present, extrusion-based systems are the most commonly used bioprinting technique due to their easy handling, cell-friendly processing, and low cost. Its versatility towards a wide range of bioink viscosities makes it highly adaptable to a vast array of polymeric systems. Nevertheless, printing self-supportive overhanging structures using soft materials can be challenging. Techniques devised in the last 5 years such as freeform reversible embedding of suspended hydrogels (FRESH) and suspended layer additive manufacturing (SLAM) that use supportive baths as a sacrificial scaffold (Fig. 6.1a) have aimed to circumvent this issue with relatively good outcomes; the technology behind FRESH has ultimately led to its commercialization [7, 8]. For multimaterial printing, several print heads can be used to deposit bioinks in a discrete manner within a single construct; however, the number of printable materials is usually limited to 3 or 4 (although potentially more) depending on the space available on the structural frame of the printer to which the printheads are attached to. This hindrance has led to microfluidics integration, in which multiple bioink chambers are connected to the same printing nozzle, thus allowing for discrete or continuous gradients of material to be created [9]. In a similar fashion, voxelated printing may also provide an innovative way for scaling-up multimaterial processes although it is yet to find its way into the bioprinting domain [10].

While light-based vat photopolymerization techniques offer fast and omnidirectional printing with no need for support structures, it is unfeasible to create

multimaterial constructs without negatively affecting the overall production time. Moreover, UV light that is commonly used to initiate crosslinking can compromise cell viabilities and cause irreversible DNA damage. Nevertheless, the development of new visible light photoinitiators combined with its potentially swift production time could still offer the best lead in translating bioprinting into a clinically viable technique [12]. Innovative volumetric printing techniques based on volumetric AM, otherwise known as computed axial lithography (CAL) and illustrated in Fig. 6.1b, may just be a good example of this in the future with it being able to generate a centimeter-size cell-laden construct in a matter of tens-of-seconds [11, 13, 14]. Although its current set-up only allows for a single material to be printed, future developments in terms of hardware are expected to integrate multiple materials with CAL.

Inkjet-based bioprinting has the ability to eject small volumes of bioink, thus giving excellent resolution and precision. However, relying on overcoming surface tension for bioink ejection can place restrictions to using low shear viscosity materials, and the dispensing force is often not cell-friendly. Printers harnessing acoustic forces have been developed to attempt to overcome these issues (Fig. 6.1c). In particular, acoustophoretic printing has been shown to confidently print over a wide viscosity range with high cell viability; however, there is yet to be reports on the technique being used to build 3D cell-laden constructs [15].

6.3 Polymer-Based Bioinks

Highly hydrated entangled polymer networks, known as hydrogels, are the most commonly employed materials for fabricating 3D bioprinted constructs. They can be derived from a natural source or synthetically produced, and are ideal for creating the biomimetic environment that is desirable to support encapsulated cells within a 3D space. To formulate the bioink, a biocompatible hydrogel is combined with cells and/or bioactive molecules. Crosslinking the hydrogel results in gelation, which is important for stabilizing the structure post-printing, and can be initiated via reversible interactions, e.g., ionically, hydrogen bonding, or by introducing irreversible covalent bonds. Biodegradability either via enzymatic or hydrolysis mechanisms is an integral bioink prerequisite so to give space for neotissue to be gradually deposited. The general make-up and requirements of a typical bioinks, alongside the chemical structures of a selection of commonly used hydrogels, are shown in Fig. 6.2. Reviews published by Hospodiuk et al. and Fonseca et al. provide comprehensive overviews of the types available and fundamentals of hydrogel-based bioinks [6, 16].

Modern hydrogel-based formulations are aiming towards improving tunability to best meet the required conditions for the printing process and biological application. This could be, for instance, through modulating the hydrogel's rheological properties for improved printability and cell viability, or by introducing bioactivity

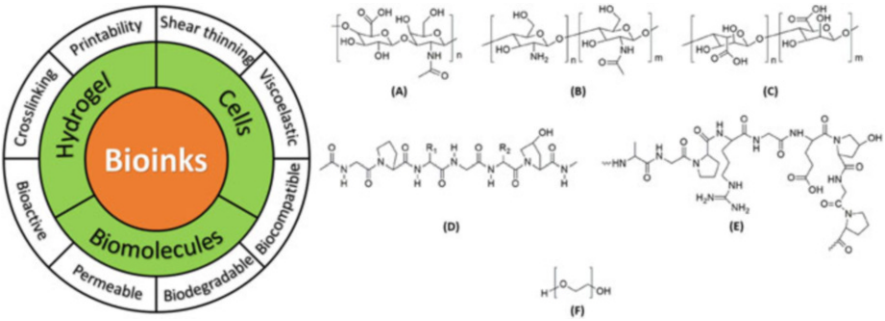

Fig. 6.2 Left—the general composition of bioinks and prerequisites of hydrogels to be used in bioink formulations. Right—the chemical structure of typically used polymers for bioprinting technologies: (A) hyaluronan, (B) chitosan, (C) alginate, (D) primary structure of collagen, (E) gelatin, (F) polyethylene glycol (PEG). (Reprinted with permission from [6]. Copyright [2020] American Chemical Society)

to augment cellular response [17]. Some level of tunability can be achieved by incorporating nanoparticles into the hydrogel. For example, inorganic nanoparticles have been used to improve the stability and printing reliability of mechanically weak natural hydrogels, while also positively influencing cell viabilities in vitro [18–21]. Shear-thinning properties can be imparted onto the bioink by using nanoparticles to improve the extrusion flow and cell viability during printing, illustrated by the use of cellulose nanocrystals and nanosilicates [22, 23]. Aside from nanoparticles, interpenetrating polymer networks can enhance the overall toughness while incorporating the beneficial properties belonging to each entangled polymer [24, 25]. Synthetic hydrogels and some naturally derived hydrogels, such as alginate, lack inherent bioactivity; therefore, it can be important in systems that require cell-matrix adherence to derivatize the polymeric backbone with moieties that promote this. Frequently, this is achieved by a simple Arg-Gly-Asp acid (RGD) peptide functionalization, but more recent developments have used more complex variants of the RGD sequence [26, 27].

4D Bioprinting has emerged within the last decade as a novel approach in integrating materials into a bioink that are able to elicit a response in reaction to an external stimulus. In most cases, it is anticipated that the fourth-dimension is "time." This technique regularly encompasses shape memory polymers (SMPs), which are designed to react to changes in pH, temperature, or electromagnetic forces to return to a pre-defined morphology. This response could be particularly useful in biological applications with dynamic environments, such as in vascular-, neural-, and cardiac-related therapies [28–30]. In another approach, responsive bioinks can also take the form of dynamic hydrogel networks, in which reversible covalent crosslinking and supramolecular assemblies are exploited to circumvent issues related to the printing process and to support tissue remodelling [31].

6.4 Conclusions and Outlook

Over the last 20 years, we have witnessed tremendous advances in the field of TE and regenerative medicine, many of which would have not been possible without 3D Bioprinting. Although still its infancy, compared to other more established technologies 3D Bioprinting is presently recognized by many as the most promising approach to generate human tissues and organs for implantation. But has the technology potential been fully explored? Are we ready to translate bioprinted products from the bench to the bedside? The short answer to both questions is no. Albeit the significant improvements in terms of hardware and software, the available technology still falls short from ideal in many aspects that are crucial for clinical translation. From better strategies to promote angiogenesis in vitro and in vivo to scaled up production of tissues with clinically relevant dimensions, the issues faced by researchers are numerous and varied. If we are to push bioprinted products up in the scale of technology readiness level (TRL), then a convergent approach will be required. This is already happening and there are many examples available in the literature illustrating the integration of multiple technologies (e.g., extrusion with electrospinning) for the production of tissue analogues with imprinted multiscale features or even the combined use of computational modelling tools and bioreactors to scale up the production of cells and tissues [32, 33]. Much of the future success of 3D Bioprinting will also depend on our ability to develop better materials for the formulation of bioinks, incorporating features that mimic the native ECM microenvironment while displaying adequate rheological properties for printing of high shape fidelity constructs. Volumetric printing has definitely improved printing speed but its feasibility and multimaterial capacity is yet to be demonstrated. Last but not least, stem cell technology, in particular induced pluripotent stem cells (iPSCs), offer a unique opportunity to develop patient-specific 3D models of tissues and organs where mechanisms of disease and regeneration, therapies and drugs can be developed and systematically tested under physiological conditions. This will not only improve our knowledge on organogenesis but will also contribute towards the development of personalized medicines.

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