Nanofibers from Polyhydroxyalkanoates and Their Applications in Tissue Engineering

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Sumitra Datta and Gopalakrishnan Menon

Abstract

Nanofibers are one-dimensional nanomaterials with a host of research and commercial applications. They possess exceptional physicochemical characteristics with fiber diameter ranging from tens to hundreds of nanometers. They have large surface area-to-volume ratio and can form highly porous interconnecting network. Natural, synthetic, carbon-based, semiconducting, and composite/blend polymers have been used to synthesize them. By virtue of their special properties like lower acidity and bioactivity, non-toxic degradation, biocompatibility, tunable surface modifications, wide ranging mechano-physico-chemical characteristics, and non-carcinogenicity, polyhydroxyalkanoates (PHAs) have emerged as a potential candidate in the field of tissue engineering and regenerative medicine.

Tissue engineering and regenerative medicine combines biological systems with engineering expertise, to restore the healthy functions at cellular levels. Their system development is based on cells, biomolecules, and biomaterials. Fabrication of nanofibrous 2D and 3D scaffolds have revolutionised the field of tissue engineering with the development of skin grafts, vascular grafts, bone implants and corneal tissue replacements. Research on fabrication of scaffolds and biodegradable polymers/blends required for fabrication is on a rise with their ever-increasing applications.

Keywords

Nanofibers · Electrospinning · Bioprinting · Scaffold · Fabrication · Tissue engineering

S. Datta (✉)
Amity Institute of Biotechnology, Amity University, Kolkata, West Bengal, India
e-mail: sumitra.datta@gmail.com

G. Menon
Vipragen Biosciences Private Limited, Mysore, Karnataka, India

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16.1 Introduction

Nanobiotechnology has essentially become the next-generation technology preferred globally by both science and economy. It has successfully created solutions to a wide range of problems posed by health, environment, energy generation and storage and textile sectors (Haider et al. 2015). The fascinating world of nanomaterials and nanocomposites include the zero-dimensional nanoparticles also known as quantum dots; one-dimensional nanowires, nanorods, nanofibers, and nanotubes; in addition to two-dimensional nanosheets which exhibit extraordinary physicochemical features (Kenry and Lim 2017).

Nanofibers are polymeric fiber whose diameters range from 0.01 to 0.1 μm. At this dimension they exhibit intriguing properties such as high surface to volume ratio, flexibility in surface functionalities, greater porosity and better mechanical performance like stiffness and tensile strength when compared to any other known form of the same polymeric material (Huang et al. 2003). Unlike the conventional rigid porous structures, nanofiberous porous structures form dynamic arrangements where the pore sizes and shapes can be altered by optimizing the start materials and synthesis parameters. When required they can also be converted into rigid structures by linking them across (Ramakrishna et al. 2006).

Polymeric nanofibers have hence become the best suited candidates for multifarious applications. Especially when they are assembled or fabricated into membranes nanofibers they extend wide ranging applications in biomedical (scaffolding used in tissue engineering, wound dressing, drug delivery, artificial organs, vascular grafts), environmental protection (water treatment and ultrafiltration, photocatalysis, chemical and gas sensing), nanosensor, electronic (composites and structures for nano-electronic machines), protective clothing (protective shields and masks in speciality fabrics), separation industry (filter media for submicron particles) and energy generation and storage (batteries, fuel cells, supercapacitors, solar cells, hydrogen storage and generation, piezoelectricity) fields (Frenot and Chronakis 2003; Haider et al. 2015; Kenry and Lim 2017; Thenmozhi et al. 2017). Ligand molecules, biomacromolecules, and cells can either be attached or hybridized with membranous nanofiber systems to be used as affinity membranes for protein purification and waste water treatment; as membrane bioreactors for industrial enzymatic catalysis or synthesis and as biosensors for chemical analysis and diagnostics (Ramakrishna et al. 2006). Furthermore, nanofibers can be aligned to construct unique functional nanostructures such as nanotubes and nanowires whilst their nonwoven composites are used in filters (Frenot and Chronakis 2003).

16.2 Use of Biopolymers for Nanofiber Fabrication

The multifaceted applications of using nanofibers in various fields can be clearly understood from the never-ending list of materials which are used for their synthesis. They can be broadly classified under natural and synthetic polymers, carbon compounds and semiconducting and composite materials (Kenry and Lim 2017).

Biopolymers or green polymers like polysaccharides (cellulose, chitin, chitosan, dextrose, alginate, dextran, hyaluronic acid (HA)), proteins (casein, collagen, zein, egg albumen, human and bovine fibrinogen, gelatin, silk, wool etc.), DNA, PHAs, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and derivatives/composites of two or more biopolymers are also increasingly used as fabrication materials (Schiffman and Schauer 2008; Xuezhu et al. 2012; Haider et al. 2015; Farokhi et al. 2018;) . Biopolymers are preferred owing to their sustainability, cost-effectiveness, eco-efficiency, ease of extraction, antibacterial activity, biodegradability, biocompatibility and renewable nature. However, using biopolymers can be challenging as a number of properties like molecular weight, degree of deacetylation, purity, distribution of charged groups and crystallinity of the same polymer will vary when derived from different sources (Schiffman and Schauer 2008). Lately, attempts have been made to utilize wide ranging blends of natural and synthetic polymers for synthesizing nanofibers (Haider et al. 2015).

16.3 PHA as a Biomaterial

PHAs are linear polyesters synthesized naturally by both Gram positive and negative bacteria under physical or nutritional stress conditions. They are biodegradable, biocompatible with desired mechanical strength and can be easily procured in copious amounts by growing bacterial isolates in optimum physio-chemical parameters. Additionally, a host of different PHAs and their co-polymers can be obtained by either altering the bacterial species used for production or by manipulation of growth conditions. Depending on the number of carbons in monomer, PHAs can be classified as short chain length (scl) PHAs (like Poly(3-hydroxybutyrate) (PHB), poly(3-hydroxyvalerate) (PHV), poly(3-hydroxybutyrate-co-valerate) (PHBV)), medium chain length (mcl) PHAs (like, polyhydroxyoctanoate (PHO), polyhydroxynonanoate (PHN), polyhydroxyhexanoate (PHHx), polyhydroxyheptanoate (PHHp)) and long chain length (lcl) PHAs (Ishii et al. 2009; Gadgil et al. 2017). Amongst all PHAs, PHB and PHBV have been extensively employed for various tissue regeneration applications like sutures, barriers, valves, patches, grafts, scaffolds, pins and guides (Ali and Jamil 2016; Gadgil et al. 2017). Figure 16.1 depicts the various fields of tissue engineering and regeneration that has been revolutionized by the use of PHA nanofibers. Diverse modification methods like epoxidation, carboxylation, chlorination, hydroxylation, and pyrolysis have been used on PHAs to increase their bioactivity, biodegradability, biocompatibility and flexibility while reducing crystallinity and hydrophobicity (Gadgil et al. 2017). It has been found that in comparison to poly lactic acid (PLA) and poly-glycolic acid (PGA), PHAs have lower acidity and bioactivity, thus posing minimal risk when used in vivo (Tang et al. 2010; Ali and Jamil 2016). The monomers of PHA which are either 3-hydroxybutyric acid (3HB) or 4-hydroxybutyric acid (4HB) are less acidic than those of PGA, PLA, and poly(lactic acid-coglycolic acid) (PLGA) (i,e. α-hydroxy acids) and can be effortless expelled from our body in within an hour. Degradation of PHAs in vivo is carried out by hydrolytic enzymes like lipases. Cytotoxicity

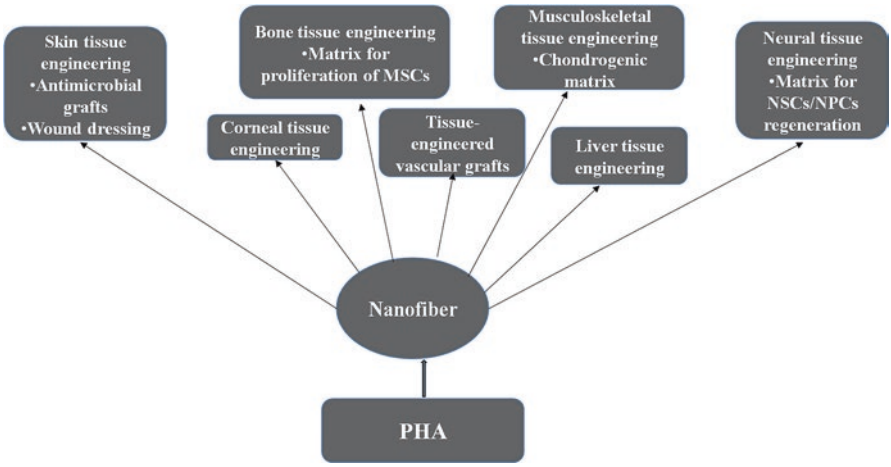

Fig. 16.1 Multifarious applications of PHA in tissue engineering and regeneration

studies of PHAs have shown negligible effects, however, if any exists, they seem to decrease inversely with the length of the oligomeric side chain (Ali and Jamil 2016).

16.4 Nanofiber Fabrication Techniques

In recent years, fabrication of nanofibers has been done by using a few techniques like; drawing, template synthesis, phase separation, self-assembly and electrospinning. While drawing produces single long nanofiber, template synthesis employs nanoporous membrane templates to make solid or hollow tubular nanofibers. Phase separation is a relatively lengthy process that transforms solid polymer into nanoporous foam. In self-assembly, however individual, pre-existing entities organize themselves into patterns. Like phase separation, this technique too is time consuming (Huang et al. 2003). Electrospinning is a simple, but versatile and cost-effective technique based on the principle of "electrostatic attraction" of charges and has been widely used in fabricating non-woven fibers with significant and desirable porosity and surface area. There are certain parameters which critically govern the electrospun nanofiber morphology. They are physiochemical characteristics of polymer (like concentration, viscosity and molecular weight), applied voltage, tip-to-collector distance, and solvent. Electrospinning is preferred over rest of the fabrication techniques as it has been found that electrospun nanofibers possess higher surface area to volume ratio and inter/intra fibrous porosity. This technique was first patented in the US in 1902 by J.F. Cooley (Ramakrishna et al. 2006**;** Haider et al. 2015).

The basic electrospinning setup comprises of one glass syringe containing the polymer solution attached to a metallic needle, power supply and the metallic collector onto which nanofibers deposit or aligns. The process begins as electric charges move into the polymer solution and build up at the tip of the metallic needle causing induction of charges on the polymer droplet. The reciprocal repulsion of charges opposes the surface tension, making the polymer solution to flow along the electric field. This further result in formation of Taylor cone from which nanofibers emerge and are collected on a oppositely charged metallic collector kept at an optimized distance. Finally, the internal and external charge forces cause the whipping of the liquid jet in the direction of the collector which results in the creation of fibers with nanoscale diameters. Currently, attempts are being made to electrospin nanofiber with greater cell infiltration property for production of 3D scaffolds (Haider et al. 2015; Kenry and Lim 2017; Thenmozhi et al. 2017).

Lately, some emerging nanofiber synthesis strategies have also been reported like CO_2 laser supersonic drawing, solution blow spinning, plasma-induced synthesis, centrifugal jet spinning, and electrohydrodynamic direct writing which are capable of fabricating highly porous mesh like network of nanofibers with significant interconnective pores, making them an ideal candidates for a wide ranging applications (Kenry and Lim 2017).

16.5 Nanofibers in Tissue Engineering

Although electrospun nanofibers have broad ranging applications as drug carriers, tissue scaffolds, wound dressings, reinforcement materials, filters, and protective clothing, however, this chapter will principally focus on the multifarious use of nanofibers for fabrication of an array of scaffolds in tissue engineering. As nanofibers mimic structural and functional characteristics of native extracellular matrix (ECM), hence they have been widely used for tissue engineering scaffolds. Apparently, natural and synthetic polymers, biomimetic composites, ceramics, and metals electrospun into nanofibers find hosts of applications in tissue engineering and regenerative medicine (Ingavle and Leach 2014).The success rate of artificial extracellular matrix scaffolds depends on its biocompatibility, osteoconductivity, degradability, high surface to volume ratios and mechanical properties. The porosity of scaffold is a crucial factor as it has direct effects on the number of cell anchoring points and proliferation, wettability, and rate of degradation (Schiffman and Schauer 2008). In addition, with the introduction of the 3D scaffolds profound impacts have been found in the process of mechanical signalling in terms of positive cell receptor ligation, intercellular signalling and cellular migration and differentiation. 3D scaffolds have also been found to control diffusion and adhesion of proteins, growth factors, and enzymes, largely influencing cell viability and function (Nisbet et al. 2009; Pilehvar-Soltanahmadi et al. 2016).

16.6 Current Scenario of PHA-Nanofibrous Scaffolds in Tissue Engineering and Regenerative Medicine

16.6.1 Skin Tissue Engineering

Apart from being the largest organ of our body skin also acts as first line of defence. ECM comprising of collagen, elastin, and glycosaminoglycans, and fibroblasts form the dermis which provides skin with strength and elasticity, besides supporting the vascularisations, neural network and the lymphatic system. Skin is predominantly damaged by burn injuries, chronic wounds, acute trauma, and other dermatological conditions like pigmentation and scar. Skin epidermis has basal layer of epidermal stem cells which helps in damage recovery. However, deep wounds spanning both epidermis and dermis fail to heal by themselves. Wound healing process progresses with hemostasis, inflammation, proliferation, and finally maturation. Non-woven polymeric nanofibrous scaffolds mimic the morphological, fibrillar, and topographical aspects of the native ECM of the skin. Nanofiber scaffolds owing to their small pore size can prevent entry to microbial pathogens while restoring structural integrity and inhibiting wound contraction (Kalia and Purohit 2011; Kalia 2014; Koul et al. 2016; Pilehvar-Soltanahmadi et al. 2016; Wang et al. 2018). Modified PHAs attached with a thioester group in the side chains and PHB co-electrospun with antibiotics have exhibited excellent antibacterial properties against potential pathogen like *Staphylococcus aureus* (Agarwala et al. 2014; Gui et al. 2014; Kalia 2015; Kalia and Kumar 2015; Luef et al. 2015; Ali and Jamil 2016; Jeyanthi and Velusamy 2016; Azman et al. 2017; Kalia et al. 2017). PHB scaffold blends of chitosan have been found to promote cell attachment and proliferation (Pilehvar-Soltanahmadi et al. 2016; Wang et al. 2018). PHB and its copolymers like poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) have been used for skin regeneration (Ahiwale et al. 2017; Grande et al. 2017). In recent times, negative pressure wound therapy (NPWT) or vacuum assisted closure, topical negative pressure therapy or microdeformational wound therapy is employed in healing burns (Wang et al. 2018).

16.7 Bone Tissue Engineering

Bone is complex connective tissue composed of calcified bone matrix, cells and bioactive factors. HAp which is osteoconductive and osteointegrative is the main component of bone matrix formed of collageneous fibers (Jang et al. 2009**;** Wang and Yeung 2017; Zhang et al. 2018a). Osteoblast, osteoclast and progenitor cells are present in the bone matrix. Osteoblasts synthesize organic components of the bone ECM, like type I collagen (90–95% of organic material and serves as a template), vitamin-K dependent proteins, osteocalcin and matrix Gla protein, osteopontin (for cell adhesion) and alkaline phosphatise. Osteoclasts aided with lysosomal secretions help in mineralization and degradation of the organic phase.

Thus, bone ECM should be an organic–inorganic nanocomposite that would perform biomechanical functions. Mesenchymal stem cells (MSCs), contribute to the regeneration and osteogenic differentiation into mesenchymal tissues, like bone, cartilage, muscle, ligament, tendon by acquiring osteoblastic markers and secreting extracellular matrix and calcium crystals (Jang et al. 2009**;** Holzwarth and Ma 2011**;** Wang and Yeung 2017). Electrospun PHA, PHB and PHBV nanofibers have exhibited enhanced cell growth behavior (SaOS-2 cell line) than on their flat film counterparts (Jang et al. 2009). Electrospun PHB scaffolds, with electrosprayed HA nanoparticles (nHA) showed enhanced mechanical properties like porosity and direct biceramic exposure to the human MSCs. This assisted them to proliferate till reaching confluence (Ramier et al. 2014). Elevated biomineralization rate is also reported in a similar study where nHA-sprayed PHA scaffold was used facilitating osteoblastic differentiation. Moreover, it was found that epoxydation of PHA scaffolds immobilizes vital enzymes and proteins promoting cell adhesion and proliferation (Grande et al. 2017).

16.8 Musculoskeletal Tissue Engineering

As bone tissue engineering has been previously discussed, hence this section focuses on skeletal muscle, tendon, cartilage, and ligament tissue engineering. When compared to fracture, large segmental defects are difficult to heal. This is because the articular cartilage lacks self-reparative potential (i.e., unavailability of chondrocytes and progenitor cells) and although tendons and ligaments heal slowly but is impaired to a certain degree. Thus, erosion of articular cartilage and meniscus tissue might lead to degenerative osteoarthritis (Vasita and Katti 2006; Lin et al. 2018). Most of the tendon injury reports are associated with tearing of the interdigitating tendons of the rotator cuff. In general, MSCs derived from bone marrow, umbilical cord, muscle satellite cells and induced pluripotent stem cells embedded in self-assembled or 3D nanofibrous scaffolds help in regeneration of musculoskeletal tissue by mimicking the native ECM with growth factor sequestering nanofibers (Vasita and Katti 2006; Lin et al. 2018; Loebel and Burdick 2018). It has been reported that planar P(3HB-co-3HHx) films exhibit noteworthy cellular response interms of chondrogenic initiation of bone marrow MSCs. PHA binding protein combined with an mcl-PHA might also prove an excellent scaffold for these cells (Ali and Jamil 2016). Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) is yet another member of PHA family that can be tailor made to possess excellent mechanical and thermal properties required for both bone and soft tissue regeneration (Chang et al. 2014).

16.9 Neural Tissue Engineering

Electrospun and self assembled nanofibrous scaffolds have been used both in vitro and in vivo for nerve repair and regeneration. Even though polymers like collagen, gelatin, laminin, chitosan, PGA, poly (L-lactic acid) (PLLA), polycaprolactone

(PCL) and their copolymers/blends have been electrospun into scaffolds for growing neural stem cells/neural progenitor cells (NSCs/NPCs), PHB is mostly preferred owing to its biocompatibility and low cytotoxic nature (Cao et al. 2009; Lu et al. 2013). However, reduction in cell adhesion and proliferation has been found due to poor hydrophilicity of PHA. Hence, various approaches like grafting, ultraviolet exposure, plasma treatment and surface hydrolysis have been applied to PHA for improving its hydrophilicity resulting in stronger cell attachment (Lu et al. 2013). In another study, it was found that three different PHAs like PHB, copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate (P3HB4HB), and PHBHHx when fabricated into 2D and 3D nanofibrous scaffolds using a modified phase separation method supported NSC growth and differentiation mimicking the natural ECM (Xu et al. 2010).

16.10 Tissue-Engineered Vascular Grafts (TEVGs)

TEVGs have successfully substituted damaged vessels. They have the required mechanical properties, blood compatibility, endothelium friendliness and biodegradability. Unlike conventional vascular grafts made of polytetrafluoroethylene (PTFE) and polyurethane (PU), cells seeded on the TEVGs can give rise to a newly regenerated blood vessel as the newly formed ECM with slowly replace the biodegradable scaffold. Fabrication techniques involve 3D printing, solvent casting, phase separation and electrospinning (Sell et al. 2009**;** Wu et al. 2018). PHA treated by salt leaching had been used for heart valve scaffold fabrication which had three valve leaflets (Sodian et al. 2000). The valve is a complex structure made out of single spongy layer sandwiched between laminar anisotropic fibrous layers on either side. PHA-polymers, like poly-3-hydroxybutyrate (poly(3HB)), poly-3-hydroxybutyrate-co3-hydroxyvalerate (poly(3HB-co-3 HV), poly-R-3-hydrooxyoctanoate-co-R-3-hydroxyhexanoate (poly(3-HO-co-3HH)), poly-4- hydroxybutyrate (poly(4HB)) and poly-R-3-hydroxybutyrate-co4-hydroxybutyrate (poly(3-HO-co-4HB)) are being used by companies including Metabolix and Tapha for fabrication of TEVGs (Jana et al. 2014).

16.11 Corneal Tissue Engineering

Stroma comprises 90% of the cornea and is made up of collagen types I, V and VI. Of late stromal defects are treated by either corneal transplantation or artificial corneas. Hence, tissue engineering approaches are being sought after (Matthyssen et al. 2018). (PHBV)/gelatine nanofirbrous scaffolds have facilitated attachment, proliferation and formation of corneal epithelium with improved transparency (Kong and Mi 2016).

16.12 Liver Tissue Engineering

Primary hepatocytes cultured on 2D collagen coated matrix have been found to produce honeycomb like morphology with increased gene expression. Enhanced hepatocyte aggregation, spreading, and metabolism were exhibited when primary hepatocytes were seeded in 3D scaffold. Mesenchymal, embryonic, and induced pluripotent stem cells have been widely used (Zhang et al. 2018b). A recent study reported the recovery of injured mice liver when a Poly (3-Hydroxybutyrate-Co-3-Hydroxyvalerate-Co3-Hydroxyhexanoate) (PHBVHHx) scaffold loaded with human umbilical cord Wharton's jelly (WJ) MSCs was transplanted (Li et al. 2015).

16.13 Future Prospects

The inherent property of the natural polymers to be tailor made for consumer specific usage has created a unique niche for them as compared to their counterparts (Schiffman and Schauer 2008). Currently most of the nanofibrous scaffolds are dependent on electrospinning for their fabrication. It should be noted that this technique also has some bottlenecks, like less throughput, cumbersome equipment setup, high voltage, and conducting collectors, and complex in situ nanofiber deposition. Sometimes, electrospun nanofibrous systems exhibit low mechanical strength owing to low crystallinity and random alignment. Therefore, it is quintessential to develop high novel throughput nanofiber fabrication methods integrating the current and the emerging ones. New variations such as multi-needle and needleless electrospinning maybe employed. For acquiring usage specific functionalities in nanofibers appropriate start materials coupled with novel fabrication techniques might help in manipulating nanofiber in terms of smaller diameter, interfiber adhesion and surface functionalization (Kenry and Lim 2017). Approaches like 'click chemistry' and co-axial electrospinning show promising future for introducing surface functionality modulation and variable configurations like core-shell, multilayer, and multicomponent to nanofibers respectively (Konwarh et al. 2013; Kenry and Lim 2017). In addition, it is imperative to study the degradation rates, tissue regeneration and mechano-spatio-geometric requirements of the new class of PHA like PHBHHx and interactions of other copolymers with different bioceramics (Misra et al. 2006; Yang et al. 2014). In conclusion, research has to be focussed on improving PHA yield in the cells, so that large scale production of the polymer could be done from cheap substrates (Luef et al. 2015). As a matter of fact, researchers are strongly focusing on development of innovative fabrication technologies using biomaterials to reduce reliance on fossil fuels (Schiffman and Schauer 2008).

Despite the relentless progress in engineered constructs, they fail to offer the growing cells a holistic cell-cell and cell-ECM interaction due to loads of exogenous signals. This issue has been addressed by bioprinting which constructs

reproducible and flexible models with accurately arranged live cells. However, there still exit several drawbacks like printing resolution, advanced cell/printing permissive bioink, spatiotemporal control with proper supply of nutrients and oxygen that are to be addressed (Varsha et al. 2016; Zhuang et al. 2018).

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