Journal of Applied Polymer Science

REVIEW

Electrospun biodegradable nanofibers scaffolds for bone tissue engineering

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ABSTRACT: Many polymeric materials have been developed and introduced for bone regeneration. Especially, their nanofibrous forms are mostly applied for artificial extracellular matrices. Polymeric materials in their nanofibrous form show some potent properties such as high surface-to-volume ratio, tunable porosity, and ease of surface functionalization. Benefiting from the properties of their main polymer and additives, they can provide new opportunities for cell seeding, proliferation, and new 3D-tissue formation. This article focuses on most cited polymeric nanofibrous scaffolds fabricated by electrospinning and recent achievements. They were divided into two main categories: natural (collagen, silk, keratin, gelatin, chitosan, and alginate) and synthetic (e.g., polycaprolactone, polylactic acid, and polyglycolic acid) polymers. The role of several additives like hydroxyapatite, bone morphogenetic proteins (BMPs), tricalcium phosphate, and collagen type I in improving the adhesion, differentiation, and tissue formation of stem cells were discussed. Finally, the osteogenic capacity and ability of nanofibrous scaffolds to support the growth of clinically relevant bone tissue were briefly studied. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 42883.

KEYWORDS: bioengineering; biomaterials; biomedical applications; electrospinning; fibers

Received 6 March 2015; accepted 24 August 2015
DOI: 10.1002/app.42883

INTRODUCTION

Bone is a hierarchical structure [Figure 1(a)]. It consists of a dense compact shell called cortical bone and a porous core called spongiosa or trabecular bone. The major components of bone tissue are collagen fibrils, hydroxyapatite particles, and proteoglycans. Collagen has a helical shape with a length of 10 nm; it can self-assemble into fibrous structures with varying diameter ranging from 50 to 500 nm and plays a critical role in the mechanical and biological properties of bones.[1] Bone defects and injuries pose significant medical challenges. However, bone tissue engineering presents a versatile way for bone tissue regeneration and repair.[1–3] As a complicated and dynamic process, this technique regulates bone cell migration, proliferation, and differentiation and accelerates bone matrix formation, resulting in shorter healing time compared to traditional procedures (e.g., autograft and allograft).[4–12] Unlike permanent implants, scaffolds are first supposed to provide temporary support for cells adhesion.[4–8] Figure 1(b) shows the degradation in growing cells. Scaffolds deliver bioactive agents that promote tissue regeneration, and they are able to mimic the intricate fibrillar architecture of natural extracellular matrix (ECM) components. Biomimetic scaffolds provide a synthetic osteogenic microenvironment to facilitate the ossification process and improve clinical therapy.[8–12]

Some criteria such as ability to deliver cells, osteoconductivity, biodegradability, mechanical properties, porosity, and fabrication capability should be considered for selecting biomaterials in bone tissue engineering. Selected materials should have excellent chemistry to facilitate cell attachment, migration, differentiation, and proliferation. Osteoconductivity has an important effect on bonding between the scaffold and host bone. Another important factor which must be noticed when materials are going to be selected is biodegradability, which controls biodegradation at sufficient rates for tissue regeneration. In addition, the mechanical strength of scaffold should be appropriate to provide mechanical stability for constructs in load-bearing sites prior to synthesis of new ECM by cells. Moreover, the material should possess desired fabrication capability, so that it can form into irregular shapes in order to match the defects of bone.

Different methods such as solvent casting, particulate-leaching techniques, gas foaming, phase separation, porogen leaching, fiber mesh, fiber bonding, self-assembly, rapid prototyping, melt molding, membrane lamination, freeze drying, and

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electrospinning are employed to fabricate scaffold. Nanofibrous scaffolds are ideal for bone regeneration due to their intrinsic properties[1–5] and, so far, many different methods have been presented for their fabrication. In the last decade, electrospinning technique as a versatile method is developed to produce nanofibers with an architecture similar to natural fibrillar ECM. Nanofibers are produced through an electrically charged jet of polymer solution or polymer melts in this method.[4–15] A wide range of natural polymers such as collagen,[16] chitosan,[17] and silk fibroin[18] and synthetic polymers such as polylactide acid,[19–21] polyglycolide,[22] and poly (ε-caprolactone) (PCL)[20,23–26] have been studied for this purpose so far. One of their distinctive properties is their highly porous structure. It not only can provide enough and better support for cell adhesion but also provides an ideal environment for cells migration and proliferation.[14]

This study aims to present a brief review on new achievements in electrospun nanofibrous scaffolds, especially those mimicking the natural ECM structure in bone. This study describes the two categories of most commonly used polymers, namely, natural (collagen, silk, keratin, gelatin, chitosan, and alginate) and synthetic (polycaprolactone, polylactic acid, and polyglycolic acid) polymers. Next, the role of some additives such as hydroxyapatite, bone morphogenic protein (BMP-2), tricalcium phosphate (β-TCP), and collagen type I in improving the adhesion, differentiation, and tissue formation of stem cells are discussed.

MOST ELECTROSPUN POLYMERS USED FOR BONE REGENERATION

Most electrospun polymers for the purpose can be divided into two categories: natural and synthetic polymers.

Natural Polymers

The major examples of natural polymers used in scaffold fabrication are proteins such as collagen,[26–36] silk fibroin,[37–42] keratin,[43–47] gelatin,[48–58] and polysaccharides such as chitosan[59–64] and alginate.[65–72]

Collagen. The native bone tissue itself contains a high level of type I collagen and it has been used as one of the major fibrous components of the ECM.[73,74] Collagen is easily degraded and absorbed by human body, leading to good attachment to cells, although it has less mechanical strength ($E \sim 100$ MPa) than bone ($E \sim 2$ GPa).[75,76] Therefore, collagen is often utilized as a composite material and blended with other materials such as ceramics (hydroxypatite, β-tricalcium phosphate)[77–82] and various synthetic polymers (polycaprolactone, polylactic acid, and polyglycolic acid).[28,83–88] Yeo *et al.*[80] fabricated a hierarchical collagen/PCL/β-TCP scaffold with combined melt-plotting and electrospinning technique. PCL/β-TCP is melt-plotted on electrospun collagen nanofibers (diameter = 160 nm). The cell (MG63) attachment and proliferation rate was 2.2 and 2 times higher for collagen/PCL/β-TCP scaffold than PCL/β-TCP.

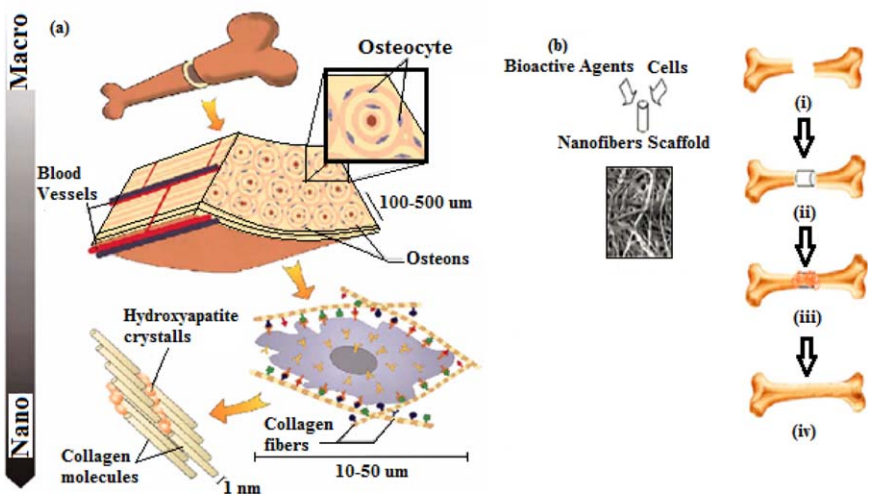

Figure 1. (a) Hierarchical organization of bone over different length scales.[1] (Copyright 2013, reproduced with permission from Elsevier Ltd.) (b) Concept of tissue engineering: (i) damaged bone, (ii) scaffold implanted into bone, (iii) new bone tissue formation on the scaffold, and (iv) degradation of scaffold and complete regeneration of bone tissue.[3] (Copyright 2013, reproduced with permission from Springer Ltd.) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Using type I collagen nanofibers prepared by electrospin technology, Shih *et al.* (2005) investigated the effect of nanofiber diameter on cellular activities such as proliferation, cell adhesion, cell migration, and alkaline phosphate activity (ALP).[89] They compared the results obtained from collagen nanofibers with those on tissue culture polystyrene (TCP). The findings revealed an increase in cell adhesion and a decrease in cell migration when fiber diameters are increased. After 3 days in culture, the cells on nanofibers had more polygonal and flattened cell morphology on 500–1000 nm [Figure 2(A,B)] compared to those on polystyrene tissue culture [Figure 2(C,D)]. Further, cells on the nanofibers displayed higher numbers of filopodia-like thin protrusions, and the contour of cells extended toward and along the lengths of nanofibers.

There are two main approaches to producing collagen/nanohydroxyapatite (nHA) nanofibers: for the first, an nHA suspension is electrosprayed on electrospun collagen nanofibers; whereas, in the second type, the collagen/nHA nanocomposite fibers can be manufactured via precipitation process. Through elctrospraying/electrospinning collagen/nHA scaffolds for the purpose of mimicking bone ECM, Ribeiro *et al.* (2014) addressed the effect of collagen/nHA scaffolds on metabolic activity, distribution, and morphology of cells *in vitro*.[90] The collagen was dissolved in acetic acid: ethyl acetate: water (40 : 30 : 30) for electrospinning. Both collagen and biocomposite constructs were found to be noncytotoxic and have the ability to support MC3T3-E1 osteoblast adhesion. However, osteoblasts cultured on pure electrospun collagen nanofibers have less metabolic activity values

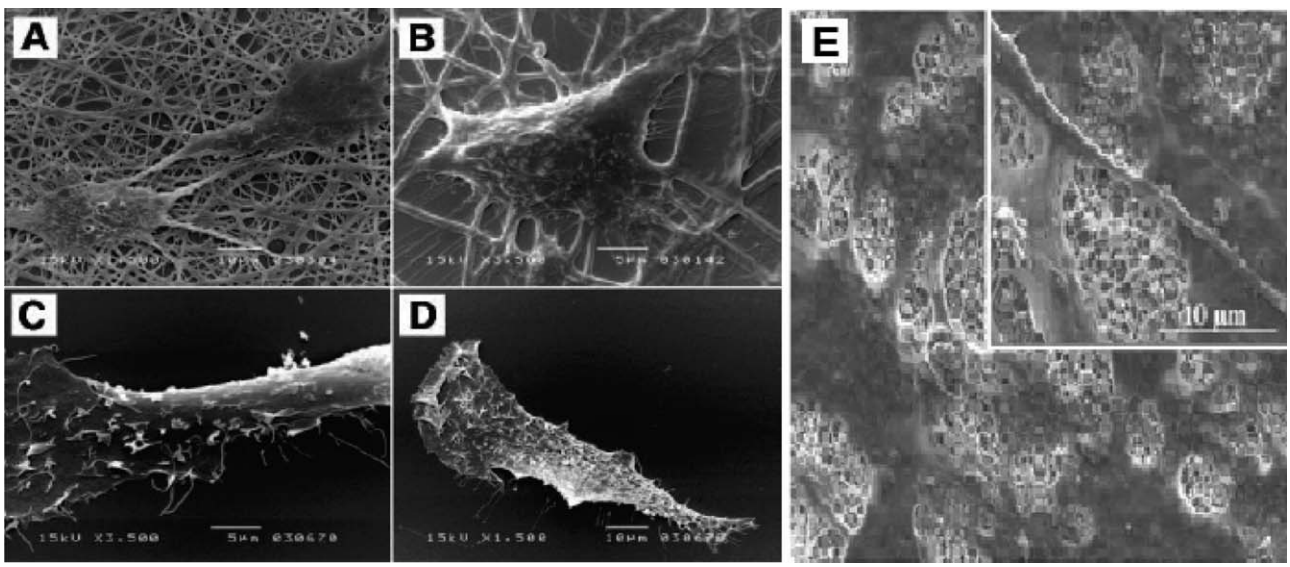

Figure 2. Cell distribution and morphology of mesenchymal osteoblast of (A, B) 500–1000 nm electrospun collagen nanofibers, (C, D) polystyrene tissue culture, and (E) electrospun/electrosprayed collagen/nHA.[89] (Copyright 2006, reproduced with permission from Wiley & Son Ltd.)

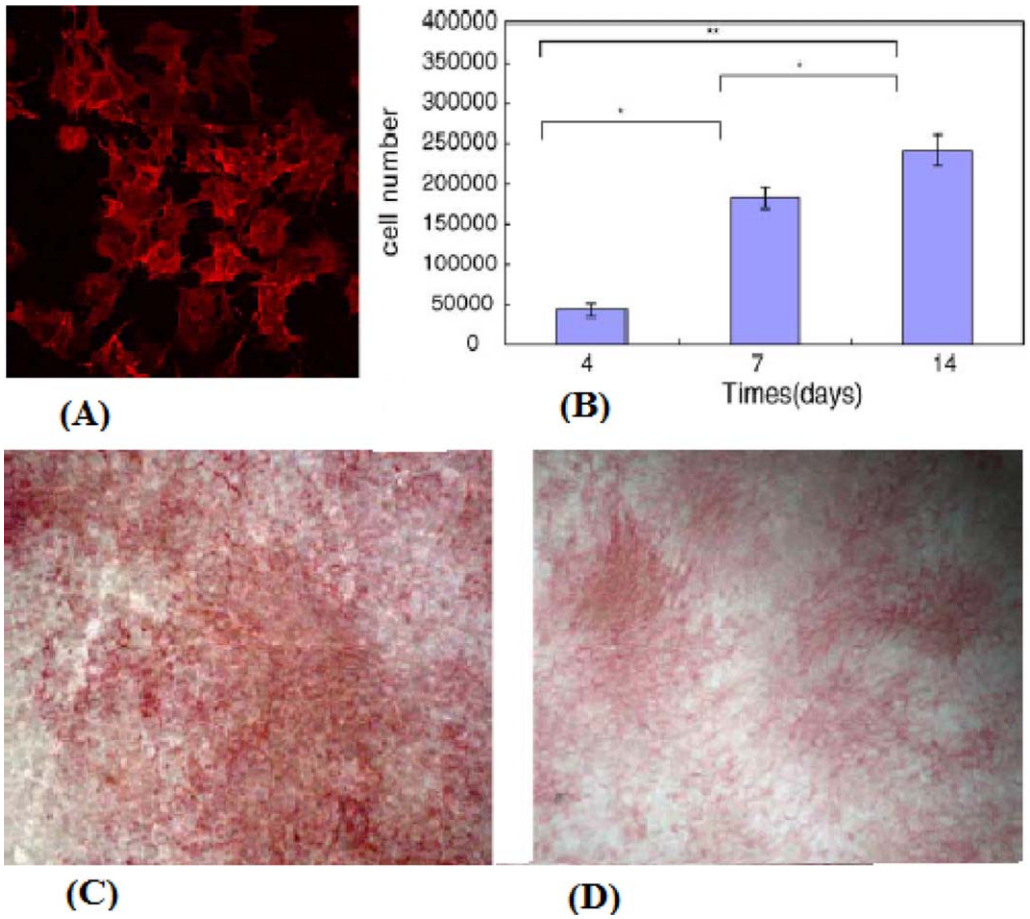

Figure 3. (A) Confocal laser-scanning microscope cells attached onto silk nanofibers incubated after 4 h using rhodamine–phalloidin (magnification: 300×), (B) the proliferation of cells grown on the silk nanofiber membranes after different culture periods and ALP activity of cells. (C) Results from cells grown on silk nanofibers and (D) those from cells grown on culture dishes (magnification: 40×).[90] (Copyright 2005, reproduced with permission from Elsevier Ltd.) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

compared to biocomposite constructs cultured at the latter time points.[90] The electrospun scaffold exhibited elastic moduli between 0.3 and 2 GPa with fiber diameter of 30 nm. The inclusion of nHA on type I collagen scaffold led proliferation of MC3T3-E1 osteoblasts after 4 days of cell culture. It was found that osteoblast cells were attached and spread out across the surface with elongated shape and fusion fibroblastic appearance. Thomas *et al.*[78] dissolved electrospun collagen/nHA in 1,1,1,3,3,3,-hexafluoro-2-propanol (HFP) to mimic physical nanofeatures and composition of bone. Depending on nHA content, electrospun collagen scaffold exhibited 93–82% porosity. The optimized spinning condition to obtain uniform nanofibers was as follows: electric potential: 25 kV; flow rate: 5 mL/h; collagen concentration in HFP: 10% (wt/v); and distance between needle and collector: 12.5 cm. The fabricated scaffold had diameters ranging from 50 to 500 nm. However, the pure collagen nanofiber had diameter in the range of 100 and 500 nm. It was found that the fiber would acquire greater diameter if nHA content is increased. Mechanical properties of the ECM environment can influence intracellular signaling and cell response. The local nanomechanical properties of scaffolds in contact with cytoskeleton likely have important implications for cell differentiation and regenerative functions. It has been reported that compared to microsized HA, cell adhesion, differentiation, proliferation, osteointegration, and deposition of calcium mineral of the surface are improved when nHA is added. The pure collagen fibrous matrix showed a tensile strength of 1.68 ± 0.10 MPa and modulus of 6.21 ± 0.8 MPa with strain to failure value 5 ± 10%. It has been seen that the strength increased to 5 ± 0.5 MPa and the modulus increased to 230 ± 30 MPa via increasing nHA content. Similar results were obtained with composite scaffolds of collagen/perlecan domain I (PlnDI) and heparin–BSA,[91] collagen/gelatin/HA,[92] collagen/alginate/chitosan,[93] collagen/PCL,[83–85] and collagen/PLGA.[87,88]

Vozzi *et al.*[92] seeded a human primary osteoblast on collagen/gelatin/genipin and investigated cellular adhesion, proliferation, ALP, osteopontin (OPN), and osteocalcin (OC) after 3, 7, 15, and 21 days. The cellular proliferation, ALP, OPN, and OC increased over time in culture (maximum at 21 days). It was concluded that primary osteoblast cells are disposed as monolayer while their cellular shape is very flattened on collagen nanofibers. Moreover, the cells could penetrate on collagen/gelatin scaffold which cross-linked with genipin and HA (10, 20, and 30%) and they started to colonize inside the scaffold.[92] Yu *et al.*[93] investigated the degree of disintegration of collagen from

electrospun collagen/alginate/chitosan/HA in collagenase solution. The 35% disintegration is seen after 10 days. Casper *et al.*[91] fabricated collagen scaffold cross-linked with PlnDI (2.6 μm in diameter) and heparin–BSA to improve binding of basic fibroblast growth factor (FGF-2) for optimizing cell growth, differentiation, migration, and survival. PlnDI and heparin–BSA–biotin protein were attached to the collagen fibers via coupling a reaction with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxy sulfosuccinimide. It was seen the cells (MG63) could attach and infiltrate on electrospun matrix over a short time period and remained alive and proliferated over 10 days.[91]

Silk. Following collagen, it is considered to be the most promising natural protein replacement in bone tissue engineering owning to its biocompatibility and excellent mechanical properties. In the past decade, a number of natural sources of silk, such as silkworms (*Bombyx mori*) and dragline silks of spiders (golden orb-web spider; *Nephila clavipes*) have been used to produce nanofiber scaffolds using electrospinning technique.[94–98] Further, due to their excellent biocompatibility, a variety of biomaterial forms such as sponges, gels, and films are used for tissue engineering.[99–104] High oxygen and water vapor permeability, as well as minimal inflammatory reactions *in vivo* have made silks become one of the best materials of regenerating skeletal tissues.[105,106] Kim *et al.*[107] observed that the cell numbers (MC3T3-E1 of rabbit calvarial model) with stellate shape (Figure 3) and OC production increased after 4, 7, and 14 days on silk fibroin membrane. It was seen that complete bony union was produced after 8 weeks and the defects had completely healed with new bone after 12 weeks.[107] Ninety percent of surface was covered with MC3T3-E1 cells after 7 days.

In order to improve cell differentiation and proliferation capacity, silk fiber can be usually spun in combination with other biopolymers such as Aloe Vera,[108] chitosan,[109] and collagen.[110,111] Several researchers have also combined silk fibroin with other polymers as to enhance its processability. For example, Jin *et al.* (2002) blended an aqueous silk fibroin with a polyethylene oxide (PEO).[112] In this study, the resultant electrospun silk/PEO mats were washed to remove PEO and then treated with methanol to induce water-soluble β-sheet conformation. Silk scaffolds demonstrated the ability to support the attachment, growth, and differentiation of adult human progenitor bone marrow stromal cells (BMSCs).[107] Lai *et al.*[109] used a mixture solvent system (trifluoroacetic acid/dicholoro methane) to prepare silk/chitosan spinning solution. The osteogenic differentiation ability of silk fibroin/chitosan scaffold was determined considering the alizarin red staining, ALP activity, and expression of osteogenic marker gens. Blending chitosan with silk enhanced osteogenic differentiation and proliferation of human bone marrow mesenchymal stem cells (hMSCs).[109]

It was found that 3D electrospun fibroin nanofibrous scaffolds offer high porosity and high bone regeneration ability, which are important for cell adhesion and proliferation.[113,114] In comparison with 2D nanofiber sheets, the seeded cells adhered and proliferated well on produced 3D nanofibers because of its high porosity.[115,116] Western immunoblotting for activated paxillin, FAK, AKT, C-Src, and ERK1/2 antibodies were significantly increased in 3-D nanofibrous fibroin scaffold (diameter = 411 ± 98 and porosity = 94 ± 2).[114]

Recently, silk-based composite nanofibers incorporated in HA nanoparticles[117–121] and bone morphogenic protein 2 (BMP-2) have been fabricated as to enhance bone formation.[122] As expected, the addition of HA and BMP-2 in electrospun scaffolds caused higher rate of calcium deposition than that on pristine samples.[117–122] Human mesenchymal stem cells were cultured on eri silk fibroin (ESF) (diameter = 800 m) and ESF/HA (diameter = 1000–1200 nm) scaffolds; then, cytocompatibity, blood compatibility, cells attachment, and growth were studied.[118] The average tensile stress of the pure ESF and ESF/HA scaffold were found to be 1.84 and 0.378 MPa, respectively. The hemolysis percentage of ESF and ESF/HA were <5% which indicate their good blood compatibility. It was seen that crystallinity and thermal stability of the ESF/Ha were better than pure ESF scaffold.[118] Moreover, it has been found that ceramics such as titanium oxide can improve the growth rate and bone formation ability.[123–127] Anodized titanium dioxidenanotubes (TiO_2 NTs) also exhibit enhanced growth and accelerated osteogenic differentiation of human MSCs.[128–131] Recently, Bayram *et al.* (2014) found that silk/titanium nanotubes fabricated through electrospinning had 92% ALP activity and 86% calcium content after 14 days.[132]

Keratin. Keratin has been investigated as a biomaterial in various forms such as gels, films, and scaffolds since 1972.[133–135] Fibrous keratins consist of two major morphological parts: (1) the cuticle layer composed of overlapping cells that surround the cortex and (2) the inner part of the fiber (the cortex). The cortex comprises spindle-shaped cortical cells that are separated from each other by a cell membrane complex, made of nonkeratinous proteins and lipids.[136–139] The cuticle cells constitute 10% of total weight and are laminar with a rectangular shape.[137,140,141]

Some studies classify keratin proteins into two groups: intermediate filament proteins (IFPs) and matrix proteins.[136–139] The IFPs are also known as α-keratin that reside in the fiber cortex. They have an α-helical secondary structure with low sulfur content. They have an average molecular mass of 40–60 kDa. The matrix proteins (γ-keratin) are low-weight globular molecules. These are mostly distinguished using their high content in cysteine, glycine, and tyrosine residues. The high-sulfur matrix proteins are divided in high-sulfur proteins (HSPs) or ultra-high-sulfur proteins (UHSPs) depending on their cysteine content and molecular weight (in the range of 11–26 kDa). The high-glycine/tyrosine proteins (HGTPs) have a molecular mass of 6 and 9 kDa.[142–144] The matrix protein functions surround the IFPs that interact through intermolecular disulfide bonds.[139] The formation of the cross-linked IFP composite matrices plays a critical role in the rigidity of α-keratin.[160] The combination of IFPs and matrix proteins forms macrofibrils within the cortex, which makes another group of keratin proteins called β-keratin.[145,146] This kind of protein forms the primary content of the cuticle whose function is protecting keratin fibers from physical and chemical damages. It is difficult to extract β-keratins

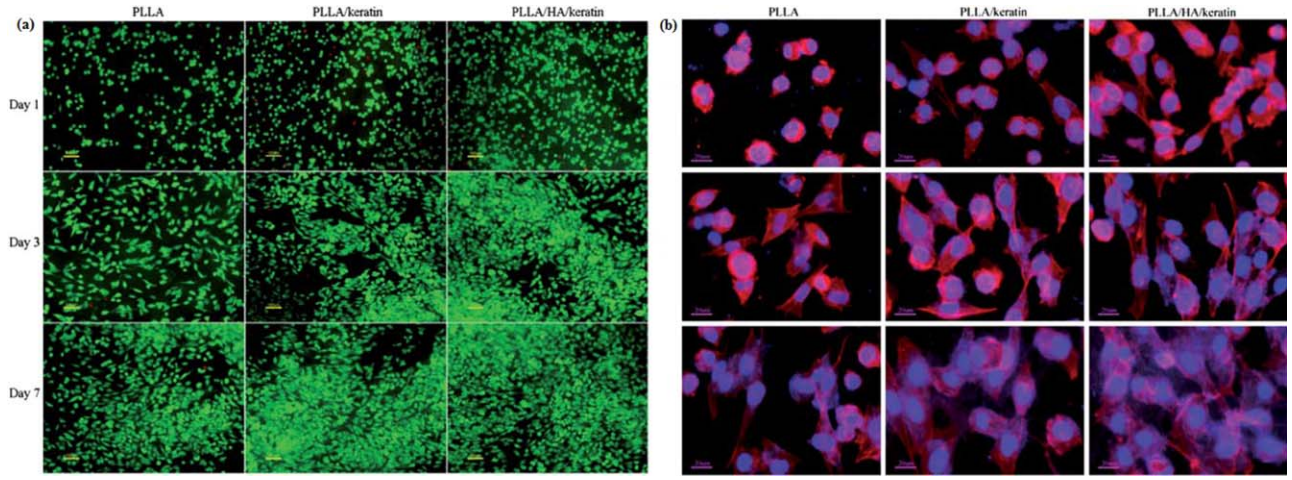

Figure 4. SEM of MC3T3 osteoblast on (A) electrospun PLLA, (B) PLLA/keratin after 7 days culturing, and (C) degradation of electrospun PLLA/keratin fibrous membrane after 3 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and, moreover, they do not form useful reconstituted structures.[146,147]

Similar to many naturally derived biomolecules, keratins have intrinsic biological activity and biocompatibility.[137] They have shown potential for wide use in wound healing,[138,139] drug delivery,[140–142] tissue engineering, trauma, and medical devices.[142–145] These qualities encourage many researchers to extract and purify keratin proteins from cheap keratin wastes such as hair, wool, feathers, nail, and other similar sources. The extracted keratin contains arginine–glycine–aspartic acid (RGD) and leucine–aspartic acid–valine (LDV). These are the same as amino acid sequences found in ECM proteins[148,149] and make favorable biomaterials for cellular attachment and growth.[150,151] Purified keratin can be polymerized into fibrous and porous films, microcapsules,[142,143] and sponge structures both at the microscale[152–154] and macroscale levels.[155]

The first study on the use of keratin biomaterials for coating on vascular grafts is presented in Ref. 156. In this study, the coated graft was successfully implanted into a dog for more than 200 days, without thrombosis. Since then, keratin has been

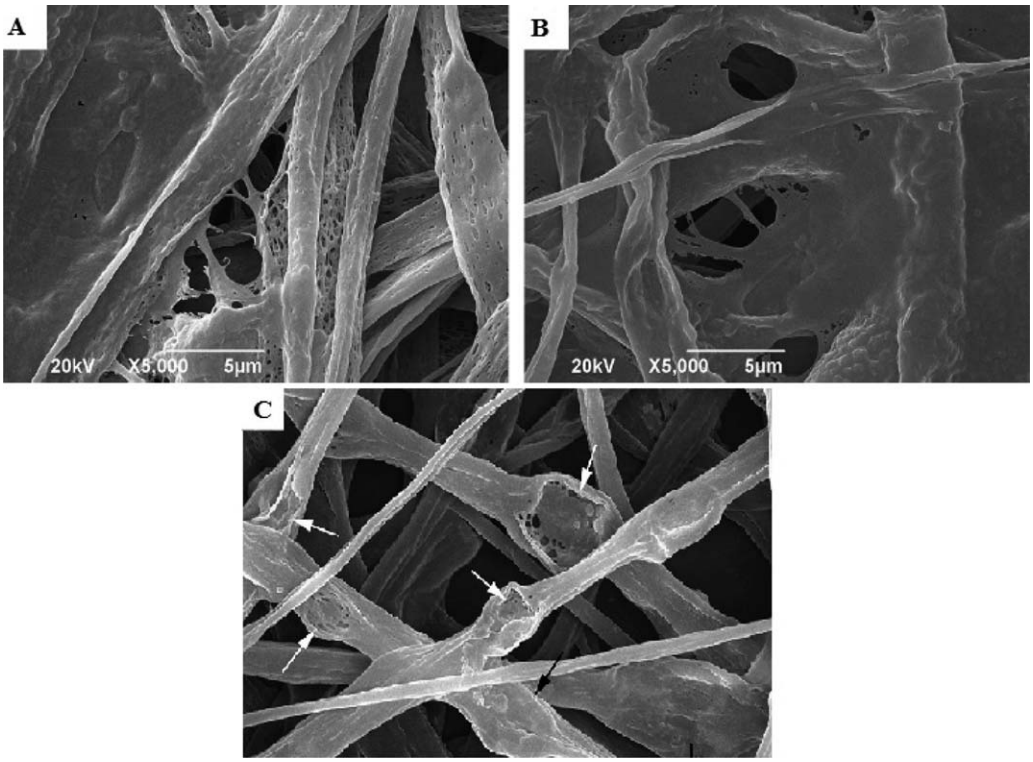

Figure 5. (a) Live (green)/dead (red) assay of Saos-2 and (b) cytoskeletal and adhesion structures of Saos-2 cells after 1, 3, and 7 days of *in vitro* culture cultured on PLLA, PLLA-keratin, and PLLA-HA-keratin membranes.[165] (Copyright 2013, reproduced with permission from Royal Society Ltd.)

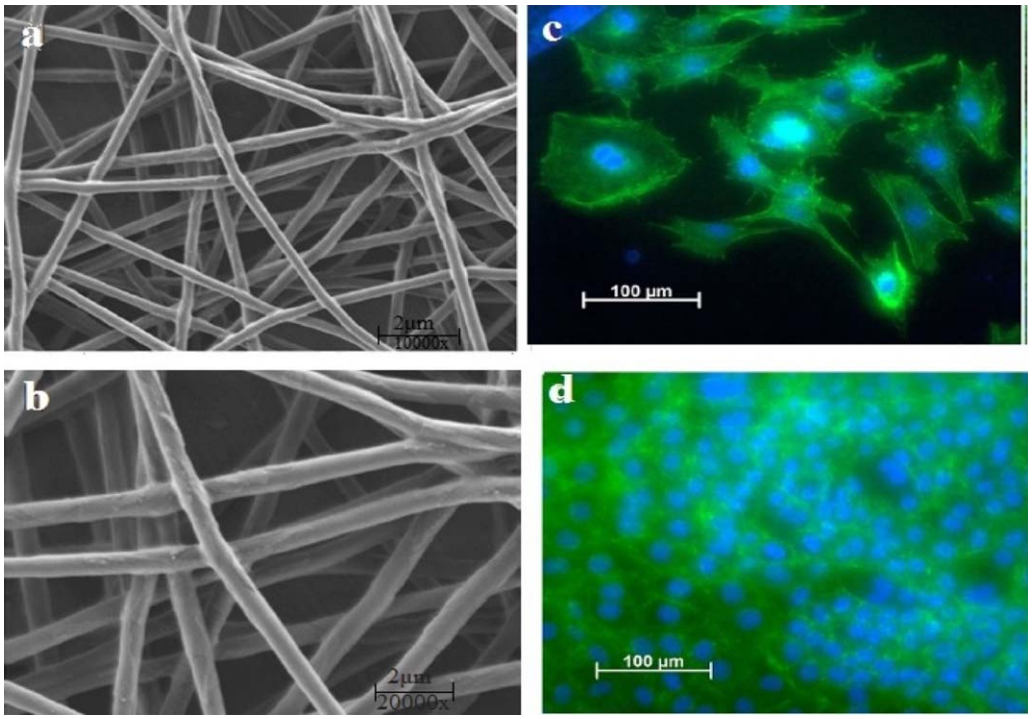

Figure 6. Electrospun chitosan (PEO)/SiO_2 (a) magnification 10,000×, (b) magnification 20,000×, and fluorescence images of bone cells on electrospun nanofibers (c) after 1 day and (d) 6 days.[192] (Copyright 2013, reproduced with permission from Elsevier Ltd.) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

evaluated for its usage in biomedicine such as wound healing,[157–161] bone regeneration,[162] hemostasis,[163] and recently, in the peripheral nerve repair.[164,165]

Keratin, as a natural protein, can be used instead of collagen and gelatin. Like collagen and gelatin, however, because of their low molecular weights (9–60 × 10^3), keratin or keratin-based materials have relatively poor mechanical properties, and pure keratin membranes are brittle. Therefore, appropriate polymer blends are provided to improve keratin properties. Li *et al.* (2009, 2013) tested wool keratin/HA particles to improve the cell affinity of poly-L-lactic acid (PLLA).[166,167] The PLLA/keratin/HA nonwoven fibrous membrane was fabricated via electrospinning the blended solutions. Besides, osteoblast cells were used to evaluate the cellular behavior of the composite membrane. The presence of keratin was found to enhance the interactions between osteoblast cells and the polymeric membranes [Figures 4 and 5(A,B)]. Figure 5(C) shows the two factors contributing to the fast release of keratin within a few hours of degradation. These keratin particles were expected to be exposed gradually with the degradation of PLLA. The polylactic-co-glycolic acid/wool keratin fibrous composite was electrospun for bone regeneration. Further, the results indicated that the presence of keratin enhances cell adhesion and proliferation, and makes nanofibers thinner and more homogeneous.[168]

Gelatin. It is a natural protein obtained from hydrolyzed as a collagen and it is derived usually from animal skin and bone. Gelatin fibers can simulate ECM structure of human tissues and organs. They can be used widely in the tissue engineering field because of their excellent biocompatibility, biodegradability, and commercial availability.[169] For electrospinning, gelatin needs to be blended with other polymers. Gao *et al.* (2013) fabricated electrospun gelatin/bioactive glass nanofibers and evaluated them *in vitro*.[170] The authors found the formation of HA material after 12 h which covered the whole surfaces of the fibers after 5 days. Zhang *et al.* (2009) worked on cross-linking the electrospun gelatin fibers by 1-ethyl-3-dimethyl-aminopropyl carbodiimide (methanediimine) hydrochloride and *N*-hydroxyl-succinimide to improve their stability and mechanical properties.[171] Kim *et al.* (2005) produced the HA/gelatin nanofibers using the electrospinning technique.[172] These nanofibers showed a significant improvement in bone-derived cellular activity in comparison with pure gelatins.[173] The same results were obtained from gelatin/PCL[174,175] and gelatin/calcium phosphate particles.[176]

Chitosan (CS). As a natural biodegradable, biocompatible, and nontoxic polymer polysaccharide, it is produced by deacetylation of chitin. Recent studies have demonstrated the potential of electrospun chitosan nanofibers for[177] bone tissue engineering and the high affinity of bone cells to their nanofiber scaffolds.[178–182] In Ref. 183, the electrospun chitosan was found to enhance cell growth with spherical morphology and mineral-rich matrix deposition by osteoblasts in culture. This study noticed that it has a positive influence on osteogenesis *in vitro* and *in vivo*. Chitosan membranes can promote the differentiation of osteoprogenitor cells and bone formation.[184,185]

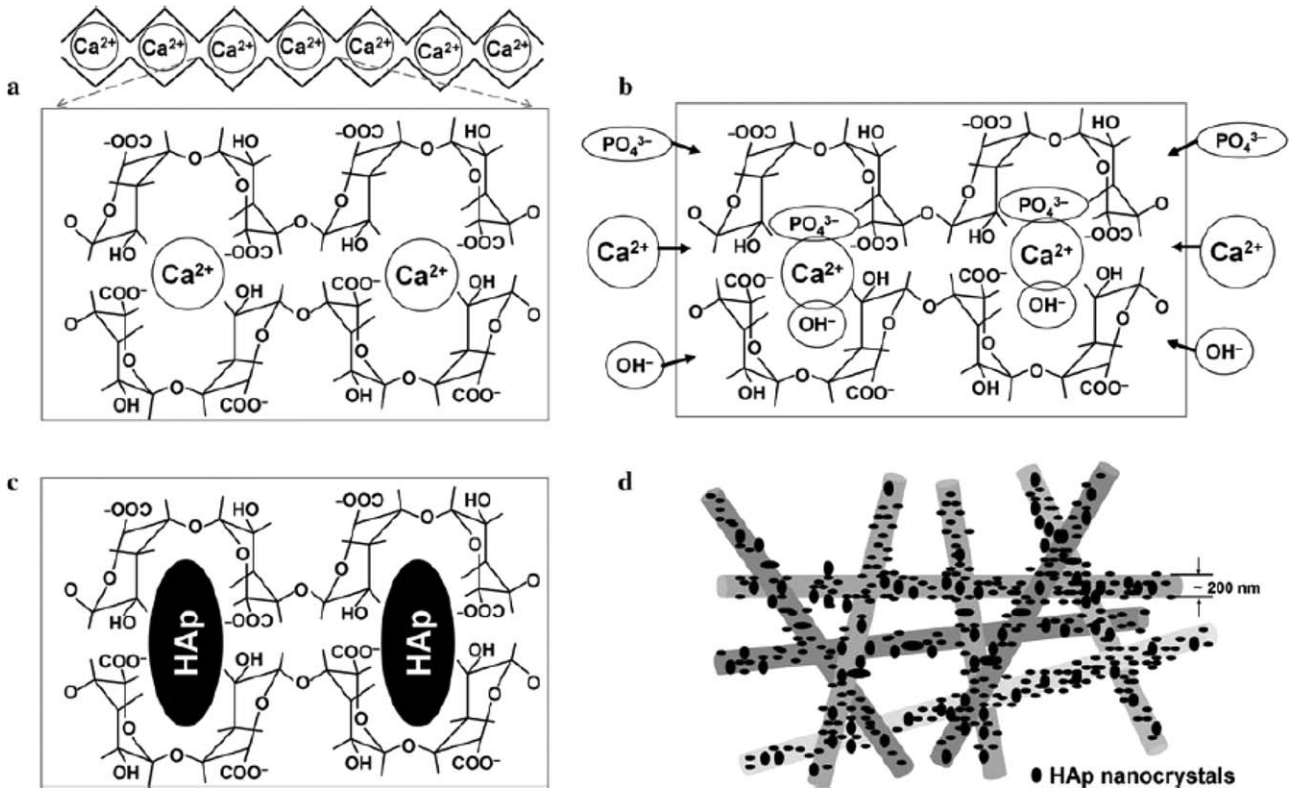

Figure 7. Chemical structure of (a) "egg box" model of calcium alginate, (b) "egg box" model of calcium alginate with precursor ions for HA nucleation, and (c) mineralized "egg-box" structure with HA and (d) illustration of cross-linked/in situ synthesized HA/alginate nanocomposite fibrous scaffold.[199] (Copyright 2013, reproduced with permission from Springer Ltd.)

Their low mechanical properties and high swelling ability lead to easy deformation of chitosan. Therefore, chitosan may be blended with other biopolymers such as hyaluronic acid,[186] alginate,[16] gelatin,[187] collagen,[188] and silk fibroin.[99] Adding other biopolymers, CS still preserves its osteogenesis characteristics, while improving its mechanical properties.[189] A blend of CS/biopolymer would be a feasible agent to develop a composite scaffold for bone tissue engineering to obtain better biomechanical features and tissue regeneration capacity.[190] For example, Bhattarai *et al.* (2005), reported the ability of electrospun chitosan/poly ethylene oxide nanofibers to promote human osteoblast cell attachment and viability.[191] Toskas *et al.* (2013) prepared hybrid nanofibers with chitosan, containing polyethylene oxide and silica.[192] Silica was synthesized from tetraethoxysilane and 3-glycidyloxypropyltriethoxysilane precursors through the sol–gel process. Figure 6(a,b) shows the SEM photographs of electrospun chitosan/SiO_2 with different magnifications. The adhesion and formation of bone cells on nanofiber mats were excellent after 1 and 6 days [Figure 6(c,d)]. Further, several mechanical properties of the resultant samples, such as elastic modulus and loss modulus, were investigated as well. Due to entangled polymer chains, the loss modulus was higher than the elastic modulus ($G'' > G'$).

Hydroxyapatite (HA) (Ca_{10} $(PO_4)_6$ $(OH)_2$) has been used to improve the osteoconductivity of chitosan scaffolds.[193–200] The composites of polyvinyl alcohol (PVA)/chitosan/HA,[201] collagen/chitosan/HA,[202] chitosan/HA,[203,204] chitosan/BMP-2,[205] and carboxymethyl chitin/PVA/HA[206] might be mentioned as examples applied to seed osteoblast cells. Thein *et al.* (2013) fabricated electrospun nanofibers in an HA solution.[204] The chitosan/HA nanofibers had higher cell proliferation and alkaline phosphate (ALP), than that of electrospun nanofibers. In another study, the effects of nano- and micro-HA composites were compared. The results showed that the nano-HA/chitosan nanofibrous scaffolds had better human bone marrow mesenchymal stem cell (MSC) attachment and proliferation than micro HA/chitosan nanofibers, without osteogenic supplementation. The osteogenic gens including smad1, BMP-2/4, Runx 2, ALP, and collagen I were upregulated in MSC culture on HA/chitosan nanofiber scaffolds.[207] Some organic solvents, such as 1,1,1,3,3,3-hexafluoroacetic acid, are employed in the fabrication of chitosan electrospun solutions.[206] The organic solvents or acids are harmful when electrospun scaffolds are used in human tissues. Shalumon *et al.* (2009) developed water soluble

Table I. Cytotoxicity of Different Scaffold After 7 days

Type of scaffold	The method of measurement	Cytotoxicity	Reference
Collagen/PCL	MTT assay	0.2	84
Chitosan	MTT assay	0.2	204
Alginate	MTS assay	87.5	216
PCL	MTT assay	0.4	84

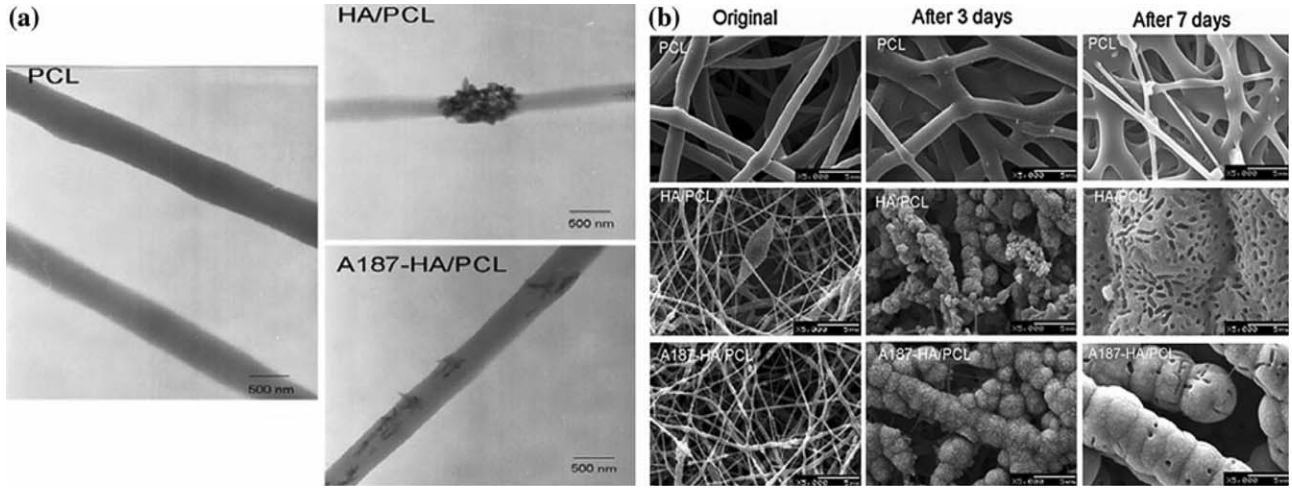

Figure 8. (a) TEM images of PCL, HAp/PCL, and A187-HAp/PCL and (b) SEM images of fiber before and after immersion in 1.5SBF.[3] (Copyright 2013, reproduced with permission from Springer Ltd.)

carboxymethyl chitin through electrospinning an aqueous solution of PVA.[206]

Alginate. It is a linear natural polysaccharide with negative charge (—COO^-), obtained from brown seaweed and bacteria.[208] Alginate contains α-L-guluronic acid and β-D mannuronic acid, which forms a hydrogel by means of ionic cross-linking with divalent cations such as calcium.[209] Alginate has been used for a variety of tissue engineering applications such as skin,[210,211] cartilage,[70,212] bone,[71,212,213] and nerve.[213,214] Chae *et al.* (2013) fabricated HA/alginate nanofibrous composites for engineering the bone tissue.[215] The HA was synthesized in situ to generate a uniform deposition of HA nanocrystals on alginate mats. Figure 7 illustrates the in situ synthesis of HA on alginate nanofibers. The HA deposition on nanofibers increased rat osteoblast cell attachment compared to the pure alginate nanofibers. Jeong *et al.* (2011) fabricated electrospun alginate/chitosan nanofibrous mats and investigated their osteogenic capabilities.[216] According to this study, alginate is naturally non-adhesive to cells. In an alternative research, Yu *et al.* (2013) electrospun scaffolds composing of alginate, chitosan, collagen, and HA for the purpose of bone tissue engineering with scaffolds.[217] Table I presents the cytotoxicity of different scaffolds such as collagen/PCL, chitosan/HA, alginate, and PCL.

Synthetic Polymers

Polycaprolactone (PCL). It is a kind of biodegradable polyester, synthesized via ring-opening polymerization of ε-caprolactone using catalysts. PCL is degraded by means of hydrolysis of its ester linkages in physiological conditions (such as in the human body) and has been, therefore, used as a potent substance for tissue engineering.

PCL can be blended with other materials such as β-TCP,[218,219] nHA,[220–222] gelatin,[223–225] collagen,[226] chitosan,[227] and calcium phosphate[228] in order to improve the differentiation and proliferation capacity. Blended with collagen, gelatin, and silk, PCL shows better mechanical properties. Costa *et al.* (2014)

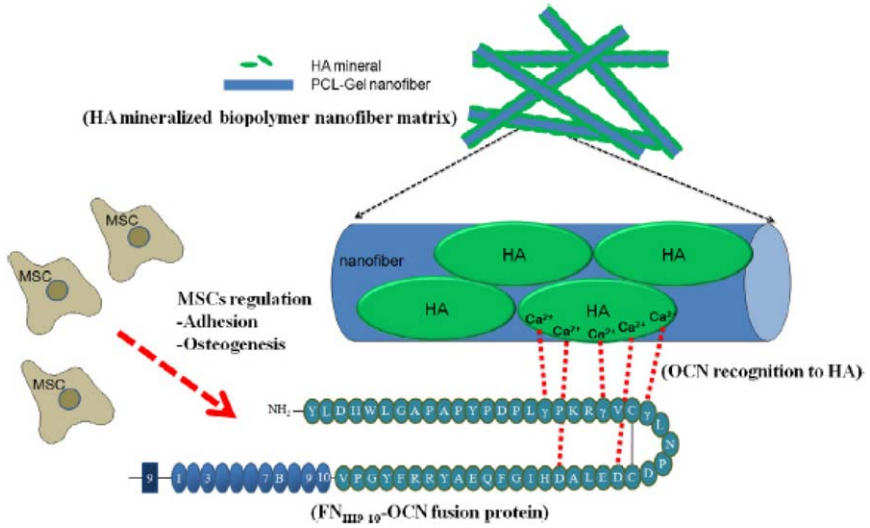

Figure 9. Schematic illustration of MSC adhesion of PCL–gel fiber matrix containing HA and FN-OCN.[213] (Copyright 2013, reproduced with permission from Elsevier Ltd.) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

fabricated a PCL scaffold compromising about 20% of β-TCP using the melt electrospinning technique, and then coated it with calcium phosphate.[229] This scaffold exhibited the potential for establishing better interconnectivity between the bone and periodontal ligament compartments. Figure 8(a) shows the TEM images of HA/PCL scaffolds. It is seen that nHA agglomerated in HA/PCL scaffolds; whereas, HA treated with A-187 was well dispersed in PCL matrix. *In vitro*, the activity of fibers was assessed in 1.5 simulated body fluid (1.5SBF), where no apatite formation was observed for PCL fibers after 7 days. However, apatite deposited on A187-HA/PCL and HA/PCL fibers after 3 days of immersion (Figure 8), confirming that polymeric fibers containing HA represent promising candidates for guided bone regeneration. In their study, Eap *et al.* (2014) functionalized PCL with neural growth factor (dental germ) for bone and tooth regeneration.[230]

Lee *et al.* (2014) investigated the function of mesenchymal stem cell functions in PCL-gel/HA scaffolds containing fibronectin 9–10 domain ($FN_{III9–10}$) and osteocalcin (OCN).[231] The OCN attached to the interface of the HA-mineralized fiber surfaces through binding γ-carboxylglutamic acid (γ) and aspartic acid (D) sequences to five calcium ions in the HA crystal lattice (Figure 9). Binulal *et al.* (2014) produced electrospun composite nanofibrous scaffolds with various PCL/gelatin ratios in a diluted acetic and ethyl acetate mixture. It was found that viscosity and solution conductivity improved due to the addition of gelatin.[223]

Poly (L-lactide) (PLLA). It has been widely used as scaffolding materials for bone tissue engineering because of its superior mechanical properties, biodegradability, and biocompatibility.[232–234] With high spatial interconnectivity, high porosity, and controlled alignment, electrospun 3D nanofibrous matrices provide cell migration ability.[235] For instance, Cai *et al.* (2012) demonstrated a practical 3D macroporous nanofibrous (MNF) scaffold obtained from aligned electrospun nanofibrous yarns for bone tissue engineering.[236] Human embryonic stem cell-derived mesenchymal stem cells (hESC-MSCs) were well attached on the 3D MNF scaffolds and the cells changed their original rounded shape to elongated and spindle-like shapes. *In vivo*, radiography and histology results showed that the MNF scaffold caused 3D bony tissue to form at 6 weeks. This study demonstrated that the 3D MNF scaffold could provide a structural support for hESC-MSC growth and guide bone formation, which may help promote the clinical translation of electrospun nanofibers for regenerative medicine in the future.[236]

Torricelli *et al.* (2014) fabricated PLLA/gelatin scaffolds by co-electrospinning.[237] He *et al.* (2014) produced mineralized PLLA nanofibrous scaffolds using the electrodeposition method. Afterward, they compared it with an extensively explored simulated body fluid (SBF) incubation method in terms of the deposition rate, chemical composition, and morphology of calcium phosphate formed on electrospun fibrous thin matrices.[238] It was found that electrodeposition was two to three orders of magnitude faster than the SBF method in mineralizing the fibrous matrices. As a result, it is able to reduce the mineralization time from about 2 weeks to 1 h while the same amount of mineralization is achieved. Nano-hydroxyapatite,[238–242] type I collagen,[242] and calcium phosphate[243] were appeared to be efficient in inducing mineralization. When subcutaneously implanted into nude mice, these scaffolds were able to form a new bone matrix only within 2 weeks. Furthermore, the development of bone matrix can be accelerated within 1 week, when the nanofibrous scaffold is enriched with human mesenchymal stem cells before implantation. Akkouch *et al.* (2014) designed a collagen/HA/poly(L-lactic-co-ε-caprolactone) (PLCL) scaffold for bone regeneration.[244] This collagen/HA/PLCL nanofiber was combined with hMSC cells for dental pulp stem cell differentiation in order to regenerate bone tissue *in vitro*. It was found that the 3D Coll/HA/PLCL composite enabled the osteoblast-like cell adhesion and growth owing to its highly porous structure. The Coll/HA/PLCL scaffold showed the ability to produce alkaline phosphatase and form nodules more than did PLCL alone.

The blend of polylactic and polyglycolic scaffold matrix (PLGA) has also shown proper biomimetic structure, good mechanical strength, and desirable bio-activity. Moreover, it could be further integrated with inorganic minerals to facilitate bone regeneration.[245–250] The PLLA and poly-D-lactide (PDLA) require at least 24 months and 12–18 months to be degraded, respectively; while the PGA completely degrades only after 6–12 months.[21,250] The copolymer of polylactide-co-glycolide also shows proper mechanical properties and its degradation time can be manipulated by controlling the ratio of lactide to glycolide (GA); thereby, it seems more flexible for clinical applications.[250] Haider *et al.* (2014) chemically grafted insulin on the surface of hydroxyapatite nanorods (nHA) to prepare electrospun PLGA/nHA-I composite nanofiber scaffolds.[251]

Electrospun PLGA/nHA-I nanofiber scaffolds led to enhanced osteoblastic cell growth. Samavedi *et al.* (2014) fabricated 2D and 3D meshes consisting of an aligned PCL fibers region and randomly oriented PLGA fibers.[252] Cell culture on 2D meshes showed that bone marrow stromal cells (BMSCs) were highly aligned and possessed high aspect ratios when cultured on the aligned PCL fiber region; however, they were polygonal shaped and randomly oriented when grown on a random PLGA fiber region. Lee *et al.* (2013) developed bioactive electrospun fibers based on PLGA through immobilizing bone-forming peptide 1 (BFP1) derived from the immature region of bone morphogenetic protein 7 (BMP7).[253] However, the poor bioactivity and serious local inflammation induced by the acidic degradation products of PLGA nanofibers may limit their applications.[245,254] Therefore, some studies focused on electrospinning nanofibrous by the introduction of gelatin[255–257] and collagen[258] biomimetics as to improve the biocompatibility of PLGA. Recently, Adegani *et al.* (2014) investigated PLGA coated with bioceramic (Zn_2SiO_4) on bone reconstruction.[259]

Other Electrospun Polymeric Scaffolds

Polyhydroxyalkanoates are considered as biodegradable and noncytotoxic thermoplastic polyesters.[260] Among various polyhydroxyalkanoates, poly3-hydroxybutyrate has been shown to preserve the chondrocyte[261] and osteoblast phenotypes,[262] promote chondrocyte regeneration,[263,264] and initiate chondrogenesis of MSCs.[265] Ramier *et al.* (2014) designed poly-3-hydroxybutyrate (PHB)-based scaffolds. The authors fabricated

Table II. Proliferation of Bone Cell on Different Electrospun Scaffold

Type of scaffold	Unit of cell proliferation	Cell proliferation (day)								Mineralization (day)			Ref.
		3	4	5	7	10	14	15	21	5	10	15	
Collagen	RFU (MTT assay)	-	8125	-	13125	-	25000	-	31250				90
Collagen/nHA(3.5%)	RFU (MTT assay)	-	13750	-	19375	-	29375	-	35000				90
Collagen/PCL	OD_{570} (MTT assay)	0.08	-	-	0.129	-	-	-	-				80
Collagen/gelatin/genepin/10%HA	%	100	-	-	162	-	-	444	300				92
Silk	OD (MTT assay)	0.19	-	0.4	0.6	-	-	-	-				118
Silk/collagen (85 : 15)	Number of cell (MTT assay)	-	-	-	-	-	128571	-	-				275
Silk/HA	OD	0.35	-	0.53	0.48	-	-	-	-				118
Silk/Ti	OD_{570} (MTT assay)	0.1	-	-	0.14	-	-	-	0.29				132
Silk/anodization Ti	OD_{570} (MTT assay)	0.16	-	-	0.22	-	-	-	0.33				132
Chitosan	OD_{492} (MTS assay)	0.9	-	-	1.3	-	1.9	-	1.8				109
Chitosan	OD_{570} (MTT assay)	0.1	-	0.4	-	-	-	-	-				204
Chitosan/silk	OD_{492} (MTS assay)	0.9	-	-	1.8	-	2.8	-	2.8				109
Chitosan/HA	OD_{570} (MTT assay)	0.4	-	1.5	-	-	-	-	-				204
Chitosan/HA	Absorbance, 490 nm (MTS assay)	-	-	656	-	1218	-	1437	-				202
Chitosan/collagen/HA	Absorbance, 490 nm (MTS assay)	-	-	875	-	1656	-	1937	-	19.2%	22.3%	21.5%	202
Chitosan/BMP-2	Cells/cm^2	-	-	-	70000	-	150000	-	170000				206
PCL	OD_{570}	0.03	-	-	0.04	-	-	-	-				80
PCL/βTCP	OD_{570}	0.05	-	-	0.08	-	-	-	-				80
PCL/collagen/βTCP	OD_{570}	0.09	-	-	0.18	-	-	-	-				80
PCL/chitosan/BMP-2	%	10.45	-	-	16.36	-	16.36	-	24.54				226
PCL/gelatin (50 : 50)	Cell number (10^4) (Picogreen assay)	0.6	-	1.3	1.6	-	-	-	-				223
PLLA	WST1 450/625 nm	-	-	1.05	-	1.16	-	-	-				237
PLLA/keratin	Absorbancy (MTS assay)	1.67	-	2.67	-	-	-	-	-				166
PLLA/keratin/HA	Absorbancy (MTS assay)	2.07	-	-	2.6	-	-	-	-				166
PLGA	OD_{570}	0.83	-	-	1.285	-	-	-	-				168
PLGA/keratin	OD_{570}	1.34	-	-	1.74	-	-	-	-				168
PLCL	Absorbance, 550 nm (WST-I assay)	-	1.6	-	2.2	-	3	-	2.3				244
PLCL/collagen/HA	Absorbance, 550 nm (WST-I assay)	-	1.36	-	1.64	-	2.85	-	4				244

RFU, relative fluorescence unit; OD, optical density.

Table III. ALP Activity of Different Electrospun Scaffolds

Type of scaffold	Unit of ALP	ALP activity								Ref.
		3 days	4 days	5 days	7 days	10 days	14 days	15 days	21 days	
Collagen/gelatin/genepin/10%HA	Absorbance/min	0.011	–	–	0.150	–	–	0.020	0.026	92
Silk	OD_{405}	0.008	–	–	0.020	–	0.060	–	0.520	109,118
Silk/Ti	$mu/mg_{protein}/min$	4.57	–	–	7.43	–	12	–	–	132
Silk/anodization Ti	$mu/mg_{protein}/min$	5.42	–	–	10.28	–	16	–	–	132
Chitosan	OD_{405}	0.280	–	–	0.060	–	0.12	–	0.1	204
Chitosan	mgALP/mg protein	0.100	0.160	0.150	–	–	–	–	–	204
Chitosan/silk	OD_{405}	0.012	–	–	0.044	–	0.1	–	0.1	109,118
Chitosan/HA	Absorbency (405 nm)	–	–	400	–	777	–	977	–	202
Chitosan/HA	mgALP/mg protein	0.12	0.19	0.16	–	–	–	–	–	204
Chitosan//collagen/HA	Absorbency (405 nm)	–	–	533	–	1000	–	1311	–	202
Chitosan/BMP-2	$Units/min/mg_{protein}$	–	–	–	15.5	–	24.5	–	25	205
PCL	ng/mg	–	–	–	416	–	466	–	315	223
PCL/gelatin (50:50)	ng/mg	–	–	–	583	–	683	–	416	223
PLLA	Mg/mg	–	–	–	0.003	–	–	–	0.003	237
PLLA/keratin	mmol/L	90	–	–	140	–	–	–	–	166
PLLA/keratin/HA	mmol/L	110	–	–	150	–	–	–	–	166
PLCL	ALP activity/10^5 viable cell	–	0.64		0.7	–	1.36	–	2.4	244
PLCL/collagen/HA	ALP activity/10^5 viable cell	–	1.36	–	1.64	–	2.85	–	4	244

three types of poly-3-hydroxybutyrate (PHB)-based scaffolds using different approaches, namely, electrospinning of a PHB solution, electrospinning of a mixed solution with PHB and nHAs, and simultaneous electrospinning of a PHB solution and electrospraying of an nHA dispersion.[266] As a consequence of incorporating nHAs within the fibers, the PHB/nHA-based nanofibrous scaffolds showed better mechanical properties than neat PHB mats. Nanofibers fabricated using the electrospinning/electrospraying approach had higher porosity which led to less mechanical properties, but they demonstrated better biological properties. Ito *et al.* (2005) used poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHHx), a copolymer of microbial polyester, as a scaffold.[267] Wang *et al.* (2012) investigated the differential effect of electrospun poly-3-hydroxybutyrate-co-3-hydroxyhexanoate fibers on the adipogenic and osteogenic potential of MSCs.[268]

To produce bioactive 3D scaffolds with a cotton-wool-like structure, Poologasundarampillai *et al.* (2014) electrospun the calcium-containing SiO_2 fibers using sol–gel solutions for bone regeneration.[269] Tetraethylorthosilicate (TEOS) was hydrolyzed and condensed under acid-catalyzed conditions to obtain Si—O—Si linear chains.

Mi *et al.* (2014) produced electrospun thermoplastic polyurethane (TPU)/hydroxyapatite scaffolds for bone tissue engineering.[270] The authors evaluated the effect of properties and particle size of (micro- and nano) HA polymer on scaffold physical properties and osteoblast-like cell performance. The addition of micro-HA led to decreased diameter in the electrospun fiber. On the contrary, nano-HA increased the fiber diameter in both soft and hard TPUs. The soft TPU had significantly lower Young's modulus and higher strain-at-break than the hard TPU. The addition of both TPUs decreased tensile properties; in other words, tensile properties were decreased as a result of adding both mHA and nHA. However, decrease rate resulted from mHA was more considerable. The cells on hard scaffolds actively proliferated and migrated compared to those on soft scaffolds. On the other hand, soft scaffolds had effective osteoblast attachment than hard scaffolds. Moreover, the soft scaffolds with nHA more effectively improved osteogenesis of hMSCs than those without nHA. Therefore, it was suggested that the soft TPU scaffolds containing nHA had the potential for being employed in bone tissue engineering applications.

Leszczak *et al.* (2014) electrospun a natural demineralized bone matrix (DBM) without the use of a carrier polymer.[193] DBM is an allograft bone with its inorganic material removed, and it contains the protein components of bone. These components consist of important growth agents such as adhesive ligands and osteoinductive signals. In another study, 3D conductive scaffolds were prepared through applying a biocompatible conductive polymer poly(3,4-ethylenedioxythiophene)-poly(4-styrene sulfonate), gelatin, and bioactive glass by sol–gel method.[271] Abdalhay *et al.* (2014) produced nylon 6/nHA nanofibers with HA nanoplates deposited on electrospun nylon 6. The surface property (wettability) of the fabricated scaffolds can influence on the behavior of osteoblasts.[200] An HA coated onto nylon 6 fibers leads to increased surface roughness which, in turn, improves surface wettability and some biological properties of nanofibers.[272] Furthermore, surface hydrophilicity regulates the adsorption of ECM proteins.[273] Gentilini *et al.* (2012) fabricated a poly-γ-glutamic acid scaffold. γ-PGA involves free carboxyl side groups that make it a versatile candidate for producing scaffolds. Modified γ-PGA with benzyl was found to have higher viability and adhesion in comparison with PLLA scaffolds.[274]

Table II summarizes the bone cellular activity of different electrospun polymers and Table III indicates the ALP activity of different electrospun scaffolds.

CONCLUSION

Bone defects and damages are a major clinical problem and nanofibrous scaffolds can be considered as a promising solution this problem and facilitate bone regeneration. Development of more effective scaffolds with the aim of bone regeneration is a challenging topic nowadays. Electrospinning technique is being constantly developed to produce nanofibers with similar architecture of natural fibrillar ECM. Highly porous 3D structure of scaffolds not only implies the better support for cell adhesion but also provides an ideal environment for the migration and proliferation of cells. Moreover, using some additives (such as hydroxyapatite, bone morphogenic protein (BMP-2), tricalcium phosphate (β-TCP), and type I collagen) on nanofibrous scaffold enhance cell adhesion, stem cell differentiation, and tissue formation. Nevertheless, developments in this area are being more challenging and many practical and clinical problems have to be solved. For example, nanofibrous scaffolds require *in vitro* investigation for practical use.

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