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MICRO- AND NANOTECHNOLOGIES TO ENGINEER BONE REGENERATION

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10.1 INTRODUCTION

The prevalence of bone fractures in the United States is a major health care concern with more than 1 million new incidences every year.[1] Non-union fractures occur when the broken bone loses the ability to self-heal and can be classified as atrophic, lacking healthy cells or vasculature; hypertrophic, containing healthy cells and vasculature and able to heal when stable; or oligotrophic, a transition stage between the previous two fracture types.[2] Bone loss in non-union fractures requires specialized treatment strategies such as the use of a set and cast or in some cases surgery in which the fracture is stabilized by a pin or plate.[3,4] These methods are accompanied by medications to alleviate pain and delay healing times depending on the site of fracture.[4] In more severe cases, when damaged bone is either removed or lost, bone implants play a vital role in tissue regeneration and healing.

Bone implants can be autografts or allografts. Autograft bone implants are patients' own bone used for grafting procedures to replace damaged bone tissue. Although autografts are highly successful because of low risk of immunological rejections, they require extraction of bone from a healthy part of the individual, leading to deterioration of the donor site, pain, and risk of infection. Allografts, bone implants harvested from other individuals, can be rejected by the host immune system. Apart from immune rejection, they have a high risk of infection with the

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additional possibility of acquiring fatal diseases from the donor.[5] Recently, artificial bone grafts have been developed that eliminate the risks associated with autografts and allografts. They can be formulated specifically for every patient depending on the fracture site and host immune response. Although artificial bone grafts have improved the quality of bone implants, concerns of biocompatibility, biodegradability, and reduced mechanical properties are associated upon implantation in humans.[5] Bone tissue engineering, incorporating the knowledge of biological systems, and engineering, has the potential to address the aforementioned concerns associated with the use of articifial bone implants in humans. One of the most widely used strategies in bone tissue engineering is the use of scaffolds for temporary structural support. Scaffolds are porous biomaterials and play a central role in tissue engineering approaches by guiding cell proliferation and assisting the exchange of nutrients and waste.

The goal of contemporary bone tissue engineering research is to formulate a scaffold that mimics the mechanical properties of native tissue. The mechanical properties of human bones have been extensively studied and characterized.[6] The compression modulus and compression strength of human cortical bone have been reported as 17–20 GPa and 106–133 MPa, respectively.[7] The flexural modulus of bone, as reported, is 15.5 GPa, and the flexural strength is 180 MPa.[8]

Nanoparticles have been incorporated into tissue engineering scaffolds to increase their mechanical properties.[6,9–15] A widely accepted definition of a nanoparticle/nanofiber is a material having size less than 100 nm in one dimension. Nanomaterials interact with the scaffold matrix by weak Van der Waals interactions or hydrogen bonds or may covalently bind to the polymer. The nanoparticle-incorporated scaffolds provide mechanical support and the microenvironment necessary for cells to differentiate and mature. The surface properties of nanoparticle-incorporated scaffolds allow better interaction of cells with proteins, creating an extracellular matrix (ECM), which in turn facilitates cell growth and tissue regeneration.[1] To increase the mechanical properties of nanomaterial-incorporated scaffolds, covalent bonds between nanoparticles or nanofibers and polymer chains are highly desired, permitting efficient mechanical load transfer and formulation of tougher nanocomposites.[16]

In this chapter, selected published articles pertaining to micro- and nanotechnologies for bone tissue engineering are reviewed with a focus on development of scaffolds.

10.2 NANO-HYDROXYAPATITE REINFORCED SCAFFOLDS

Hydroxyapatite (HAp or $Ca_{10}(PO_4)_6OH_2$),[17] a ceramic widely used in bone tissue engineering applications, reduces stress shielding and increases the biocompatibility of the implant.[18,19] HAp is responsible for the release of calcium (Ca) and phosphorus (P) ions, which are used as substrates during bone remodeling.[18] Recent studies have shown that nanohydroxyapatite (nHAp) has improved protein adsorption capabilities compared with macro- and micro-HAp.[19–21]

In bone, HAp exists as needle-shaped crystals with a size distribution of 20–60 nm, whereas nHAp can be found in rods, fiber, or particulate form.[18,22]

The morphology of nHAp is governed by the mode of synthesis, which may be mechanochemical based, in which the material is created by a heterogeneous reaction between solids dependent on the perturbation of surface-bonded species;[23] may be combustion based, in which calcium carbonate is subjected to a temperature of 900 °C, resulting in CO_2 release and the formation of CaO, which yields HAp in a phosphate solution;[24] or may use wet chemistry techniques involving direct precipitation via sol-gel synthesis.[25] Precipitation synthesis of HAp crystals involves the use of modifiers, chemical compounds that may influence the morphology of HAp crystals. Some of these modifiers include citric acid, amino acids, and ethylenediamine-tetra-acetic acid (EDTA).[26] The morphology of HAp crystals can also be governed by changes in pH with a pH greater or equal to 7 resulting in the formation of nHAp crystals and a pH below 7 leading to the formation of microcrystals.[26]

HAp crystals are brittle, and scaffolds formulated with HAp in matrix have significantly low mechanical properties, making them unsuitable for load-bearing applications.[27] HAp is widely used as a bone void filler.[28] Owing to its morphology, nHAp possesses greater surface area compared to micro-HAp, which can be exploited to yield a dense packaging of nHAp in the scaffold matrix.[26] A dense packaging leads to significant enhancements in the mechanical properties, which in conjunction with the similarity of nHAp to native tissues (with respect to size and chemistry) make nHAp a favorable material for bone tissue engineering.[18] However, one of the prime reasons for the use of nHAp is to increase the mechanical properties of the polymer matrix.[9,17–19,27,29–32] This is analogous to natural bone, a composite of apatite crystals within a collagen matrix.[13]

nHAp and chitosan nanocomposites have been studied as scaffolds for bone tissue engineering applications.[13,33] Polymers such as poly(L-lactic acid) (PLLA),[34]poly (ester urethane) (PU),[19] poly(vinyl alcohol) (PVA),[17] and poly(D,L-lactic-*co*-glycolic acid) (PLGA) have been widely investigated as a scaffolding matrices, containing HAp crystals.[27] Uniform dispersion of nHAp within the polymer matrix is of utmost importance because unequal distribution can lead to voids after bone formation (Fig. 10.1).[9]

HAp-incorporated scaffolds can be formed by various techniques such as electrospinning,[13,15,31] gas foaming and particulate leaching,[27] salt leaching–phase inversion[19] followed by mixing in an acidic environment, and lyophilizing.[33] Electrospinning is an extensively used technique for the production of polymer fibers. A well-dispersed nanomaterial–polymer composite is an essential prerequisite before electrospinning scaffolds to prevent the agglomeration of nanomaterials.[13] Electrospinning involves the exposure of nanomaterial–polymer solution to an electric field within a capillary tube. When the electric field overcomes the surface tension of the material, a jet of polymer solution is released from the capillary. The polymer solution undergoes stretching as the collector is grounded, resulting in the formation of fine electrospun fibers.[35] Gas foaming and particulate leaching involve exposure of salt containing polymer matrix to a high-pressure gas before the salt leaching step.[27] The salt leaching–phase inversion technique involves the mixing of nHAp–polymer solution with a porogen, followed by exposure to air to evaporate solvents, and washing steps to remove the porogen (Fig. 10.2).[19]

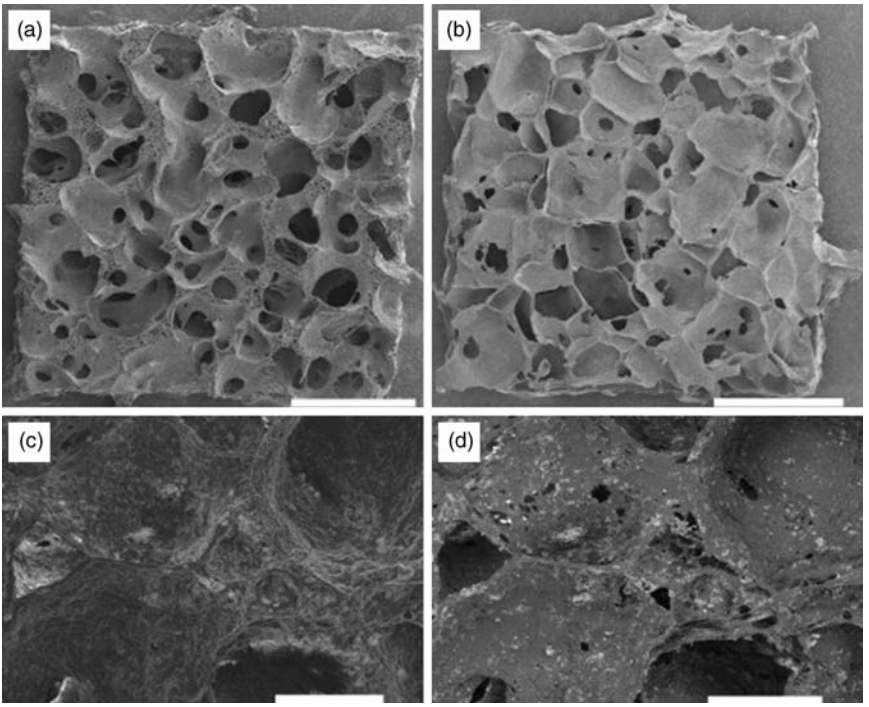


FIGURE 10.1 Scanning electron microscope micrographs of (a) polyurethane (PU) and (b) nHAp/PU scaffold. Note that nHAp scaffolds exhibit a decreased microporosity compared with the control. Scale bars: 1 mm. (c and d) Higher magnification images of (a) and (b). Scale bars: 100 μm. Adapted from Ref. [29] © Elsevier 2010.

The toxicity of nHAp sponges, assessed by a trypan blue viability assay, confirmed the absence of necrosis of human bone marrow stromal cells (HBM) after 14 days of exposure.[36] Another study reported the cytotoxicity of varying amounts of HAp (0, 10, 20, 30, 40 or 50 wt%) added to PLLA on rat mesenchymal stem cells. Cellular viability assessed by alamarBlue assay after 1 and 2 weeks of exposure indicated the nontoxic nature of materials.[18] Two studies on the toxicity of nHAp composites, one with chitosan and nanosilver (nano-Ag) containing 1:1 ratio of nHAP:chitosan, and the other with copper (Cu) and polyethylene glycol 400 (PEG 400), reported the absence of toxicity of the composites on rat osteoprogenitor cells, assessed by MTT assays after 24 h incubation.[33,37]

In addition to nHAp, nano-Ag and Cu have also been dispersed in the polymer matrix. The addition of nano-Ag or Cu imparts antibacterial properties to the polymer material. Specifically, nano-Ag imparts antibacterial properties against both gram-positive and -negative bacteria, but the addition of Cu preferentially inhibits the growth of gram-positive bacteria.[33,37] nHAp–multi-walled carbon nanotube (MWCNT) composites have also been formulated for bone tissue engineering applications. Addition of 7 vol.% of MWCNTs increased the biaxial strength and

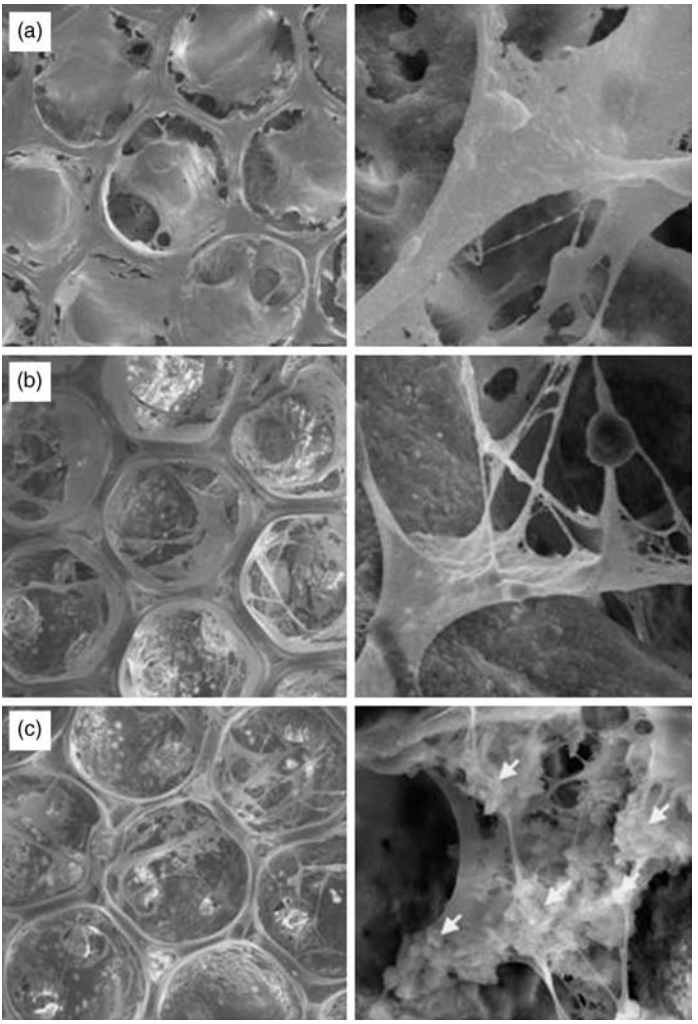


FIGURE 10.2 Scanning electron microscope micrographs of cells cultured on (a) PLGA scaffolds, (b) PLGA-HAp, and (c) Ap-coated PLGA-HAp scaffolds for 28 days. Large numbers of nodules such as minerals (indicated as *arrows*) were observed on the surface of Ap-coated PLGA-HAp scaffolds. Adapted from Ref. [9] © ACS 2010.

toughness of the composites by 28 and 50%, respectively. Further increase in MWCNT loading concentration decreased the strength and toughness of the composites due to formation of MWCNT aggregates resulting in weakness at the interface between nHAp and MWCNTs (Fig. 10.3).[38]

nHAp–polymeric scaffolds seeded with marrow-derived (40 and 200 μg/10 mesenchymal stromal cells (MSCs) have been reported to stimulate the growth of MSCs at concentrations less than 20 μg/10^4 cells. However, at higher

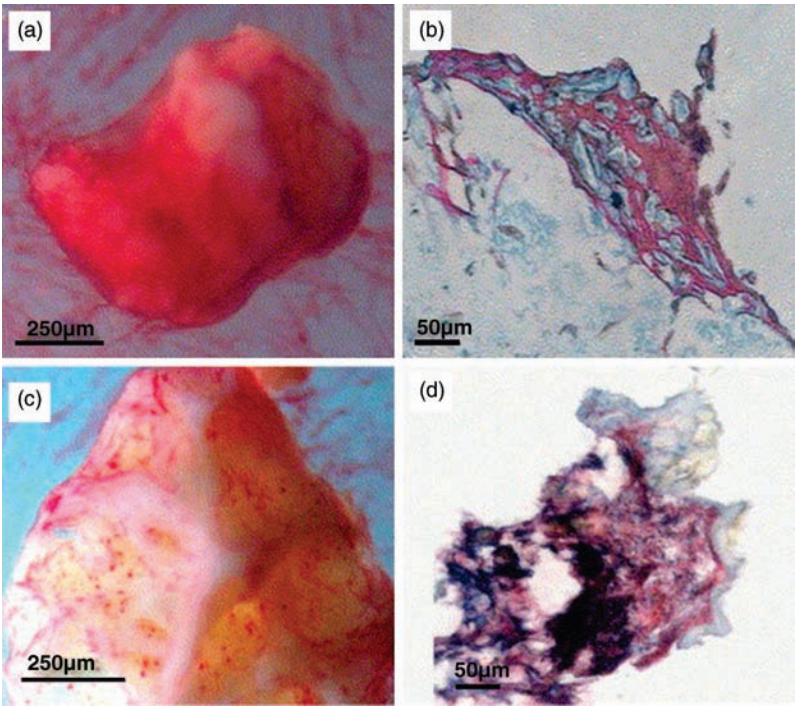


FIGURE 10.3 Photomicrographs of HBM (human bone marrow) cells grown on HAp-alanine and HAp-dextran spongelike scaffolds. (a and c) Expression of alkaline phosphatase (red staining) and (b and d) collagen production (Sirius red and Alcian blue staining). Adapted from Ref. [36] © Elsevier 2005.

concentrations 40 and 200 μg/104 cells, nHAp inhibited cell growth. Moreover, differentiation of cells occurred when the cells and nHAp were in osteogenic media coupled with an inhibitor of mineralization of cells.[20] In another study, nHAp–PLGA scaffolds have been reported to stimulate the osteogenic differentiation of preosteoblast cells after 6 weeks in culture. Micro computed tomography analysis revealed the even distribution of secreted minerals throughout the scaffold.[9] When included in cyclic acetal hydrogels, nHAp particles enhanced the differentiation of MSCs into osteoblasts, observed by an increased osteogenic gene expression (bone morphogenic protein -2, alkaline phosphatase, and osteocalcin).[21]

10.3 BIODEGRADABLE POLYMERIC SCAFFOLDS AND NANOCOMPOSITES

Synthetic polymers such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) and their copolymer PLGA have been investigated for bone tissue engineering applications. PLGA is a biocompatible, biodegradable polymer with enhanced

mechanical properties compared to PLA and PGA. The Food and Drug Administration (FDA) has approved PLGA for clinical and basic research in drug delivery, vaccination, cardiovascular diseases, and tissue engineering applications.

PLGA synthesis involving ring-opening co-polymerization of PLA and PGA uses tin (II) 2-ethylhexanoate, tin (II) alkoxides, or aluminum isopropoxide as catalysts.[11] During the reaction, the monomers PLA and PGA are linked together by ester linkages. Depending on the ratio of monomers present at the onset of the reaction, various forms of PLGA can be synthesized. PLGA 75:25 contains 75% PLA and 25% PGA. Similarly, PLGA 65:35, PLGA 50:50, and PLGA 85:15 are also commercially available. PLGA is hydrolyzed to its monomers (PLA and PGA) in the presence of water. Because these monomers are the byproducts of various metabolic pathways in the body, they can easily be metabolized and degraded without any complications.[39]

A challenge in bone tissue engineering is to design a scaffold that mimics the mechanical properties of natural bone ECM. Polymers by themselves do not have the mechanical properties comparable to native bone tissue. Therefore, nanomaterials have been used as reinforcing agents to improve the mechanical properties of polymeric composites. Some of the nanoparticles that have been incorporated into PLGA scaffolds are HAp nanoparticles, single-walled carbon nanotubes (SWCNTs), and titanium oxide microsphere.[9,11,12,40] HAp incorporated in electrospun PLGA at concentrations of 1 and 5% improved the mechanical properties of PLGA fibers. However, an increase in the concentration of HAp particles to 10 and 20% resulted in defects in the fiber, thereby decreasing the mechanical properties. In another study, HAp–PLGA nanocomposites exhibited a decreased biodegradability compared with neat PLGA, desirable for long-term stability of the scaffold.[41] Incorporating 1% SWCNT in PLGA scaffolds prepared by solvent casting increases the Young's modulus from 5.0 to 7.8 MPa.[12] Carboxylated SWCNTs in the same concentration further increased the Young's modulus, 8.3 MPa. In addition to an increase in the Young's modulus, carboxylated SWCNTs nanocomposites, also accelerated hydrolytic degradation and weight loss. Addition of pristine SWCNTs showed no significant effect on the degradation or the weight loss of the scaffolds. Nanomaterials incorporated into PLGA scaffolds can thus be used to tailor the properties of the scaffolds depending on the desired application.

Poly(propylene fumarate) (PPF), a polyester of propylene glycol and fumaric acid, is a biocompatible, biodegradable, and osteoconductive polymer widely studied for bone tissue engineering applications.[42] PPF is highly viscous and can be cross-linked with methyl methacrylate, *N*-vinyl pyrrolidinone (NVP), PPF–diacrylate, poly(ethylene glycol)–diacrylate, or itself.[42,43]

In biological systems, PPF is hydrolyzed into biocompatible fumaric acid and propylene glycol with traces of acrylic acid and poly(acrylic acid-*co*-fumaric acid). PPF and its degradation products possess low *in vitro* cytotoxicity and minimal inflammatory responses.[44,45] PPF scaffolds lack suitable mechanical properties required for bone tissue engineering applications. Nanoparticles incorporated in PPF scaffolds enhance the mechanical properties.

Recent study shows that addition of two-dimensional carbon and inorganic nanostructures such as single- and multi- walled graphene oxide nanoribbons,

graphene oxide nanoplatelets and molybdenum disulfide nanoplatelets at low loading concentrations (0.01–0.2 wt%) increase the mechanical properties (i.e. Young's modulus, compressive yield strength, flexural modulus and flexural yield strength) of PPF nanocomposites.[46]

Ultra-short single-wall carbon nanotubes (USCNTs) have been incorporated into PPF scaffolds for bone tissue engineering applications. USCNTs, homogeneously incorporated into PPF scaffolds at 0.5wt%, improved the mechanical properties of the PPF scaffold by up to 200% for the flexural and compressive properties compared with PPF alone.[47] *In vitro* cytotoxicity studies showed 100% cell viability and excellent cytocompatibility, although some adverse effects on cells were observed during degradation of the scaffold.[48] To study *in vivo* cytotoxicity, USCNT scaffolds were implanted in rabbit femoral condyles and subcutaneous pockets. Histology and histomorphometric analysis of soft and hard tissue exhibited good biocompatibility over a period of 12 weeks. Scaffolds containing USCNTs resulted in an enhanced bone regeneration compared with PPF scaffolds (Fig. 10.4).[49]

Alumoxane nanoparticles have been investigated for the fabrication of alumoxane–PPF bone tissue engineering scaffolds. Mechanical properties of alumoxane–PPF composites were characterized by compressive and flexural testing. Composites containing 1 wt% alumoxane nanoparticles exhibited more than threefold increase in flexural modulus compared with PPF controls. The enhancement of mechanical properties was attributed to the fine dispersion of alumoxane nanoparticles, and covalent bonding between nanoparticles and PPF.[16]

Degradation and biocompatibility of alumoxane–PPF nanocomposite scaffolds have been studied *in vitro* and *in vivo*. Nanocomposite scaffolds degrade significantly faster compared to PPF controls and exhibit negligible *in vitro* cytotoxicity in fibroblasts. Minimal adverse effects such as inflammation of the surrounding tissue was observed *in vivo*. It was also observed that predegraded particles increase cytotoxicity and inflammation because of their increased surface area and roughness.[50]

10.4 SILK FIBERS AND SCAFFOLDS

Silk, originating from the silkworm (*Bombyx mori*), is widely used in biomedical applications such as tissue engineering. Spider silk, not widely commercialized, is also of interest as it possesses better mechanical properties. Spider silk produced by various species of spiders vary in their amino acid content.[51] Silk has been conventionally used as a biomaterial for sutures and recently been investigated for applications in bone tissue engineering.[52]

Silk from *B. mori* is composed of two types of proteins, sercin and fibroin. Sercin forms a coating on the inner core protein, allowing self-adhesion between silk fibers; fibroin forms the core of the fiber. Fibroin, composed of nonpolar amino acids such as glycine, alanine, and serine, is made up of heavy (325 kDa) and light chains (25 kDa).[53,54] β-Pleated sheets on heavy chains form crystals in an amorphous matrix.[53,55] Silk from *Nephila clavipes*, one of the most comprehensively studied

spider silk, is composed of a single chain of fibroin (275 kDa).[51] Black braided silk in which the protein sercin has been stripped from the fiber is also used as a biomaterial for nonallergic sutures.[52]

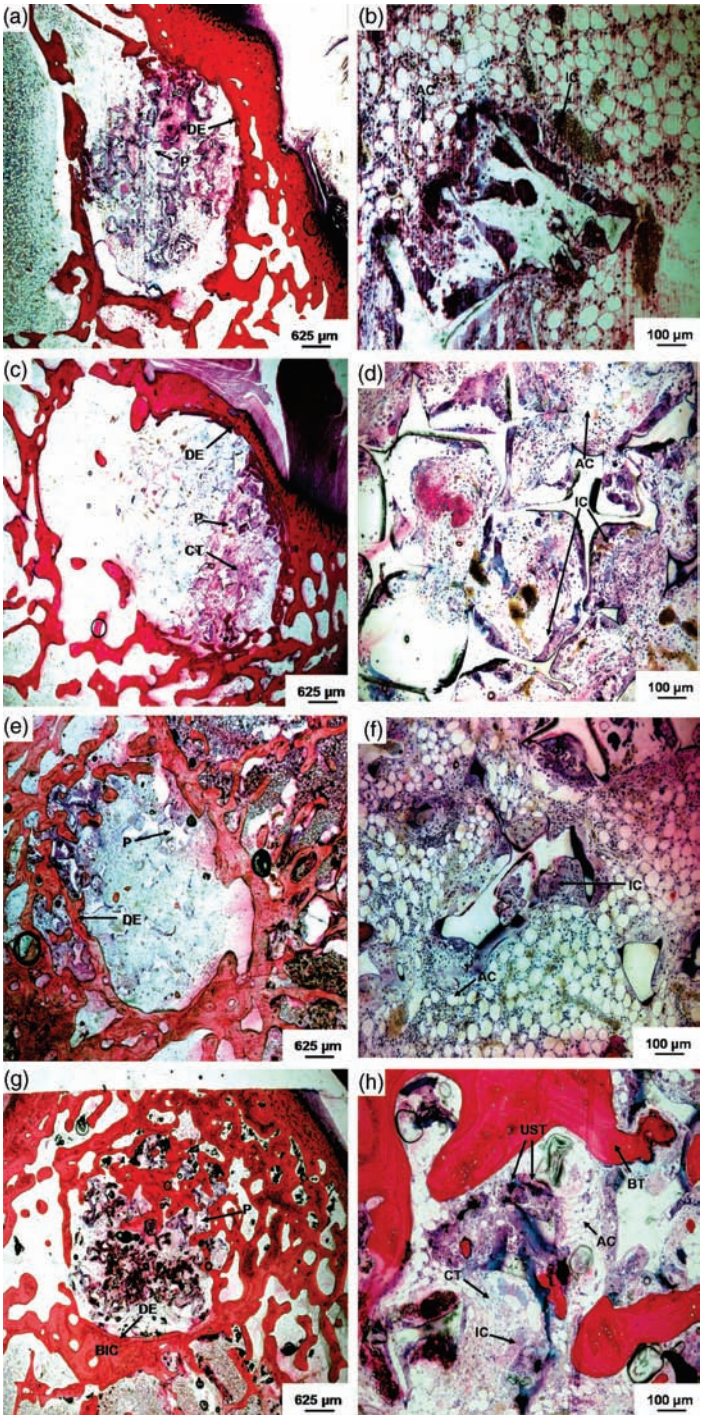
Silk line of the spider *N. clavipes* is the strongest natural fiber known.[56] Silk possesses high tensile strength and elongation capabilities. The mechanical properties of silk shows negligible changes in response to variations in strain rate. This is attributed to the decrease in viscous and elastic behavior, and an increase in the plasticity of silk fibers. However, viscosity of silk is directly proportional to strain rate, resisting elastic and plastic behavior.[57] The viscoelastic behavior of silk is a result of the stretch of amorphous regions along with the elastic deformation of β-pleated sheet crystals under stress.[55] Silk scaffolds lack sufficient mechanical properties and cannot be used to provide mechanical support to the bone structure. Silk particles have been incorporated to reinforce polymeric scaffolds, thereby increasing the mechanical properties. Compressive modulus of silk particle reinforced scaffolds is significantly silk controls.[58]

Silk scaffolds can be fabricated using electrospinning which produces nanoscale diameter silk fibers by using the same method as described in the HAp section earlier. Additionally silk scaffolds in the form of films can be developed[46] by dissolving fibrin protein in LiBr, dialyzing in water before freeze drying, and redissolving in hexafluoro-2-propanol.[14] Another method to incorporate silk into a scaffold is by creating hydrogels. To formulate hydrogels containing silk, a silk solution (created similarly to the film method by dissolving in LiBr and dialyzing in water) is mixed with ethanol in various ratios (silk solution/ethanol: 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, and 9/1).[59]

Virgin silk fibers, originally used as suture materials, can induce hypersensitive responses in patients, characterized by an increase in IgE levels, severe allergic response, and asthma.[52] Allergic responses are attributable to the protein sercin coated on the silk fibers.[60] To reduce these adverse effects, sercin is stripped from the fibroin, creating black braided silk fibers.[52] Silk, manufactured in a twisted and braided type, uses virgin silk and is not used as a suture material. Black braided silk, which does not induce allergic responses, can lead to hypersensitivity after multiple exposures. Although not considered an allergen, black braided silk is capable of inducing a foreign body response stimulating eosinophils, macrophages, and giant cells to attack foreign material, which may become chronic due to the formation of granular tissue around the suture.[60]

Cytotoxicity of silk films, assessed by MTT assay on bone marrow stromal cells, showed that the number of cells increased significantly after 14 days.[14] Correspondingly, the cytotoxicity of silk hydrogels tested by MTS assay showed increased

FIGURE 10.4 Histologic sections of PPF scaffolds implanted in femoral condyle defects. (a and b) PPF scaffold after 4 weeks of implantation. (c and d) a US tube–PPF scaffold after 4 weeks, (e and f) a PPF scaffold after 12 weeks of implantation, (g and h) a US tube–PF scaffold after 12 weeks of implantation. The PPF scaffold appears as white areas in the image, and bonelike tissue (BT) appears red. US tubes (USTs), connective tissue (CT), adipose cells (ACs), and inflammatory cells (ICs) are also shown. Adapted from Ref. [49] © Elsevier 2008.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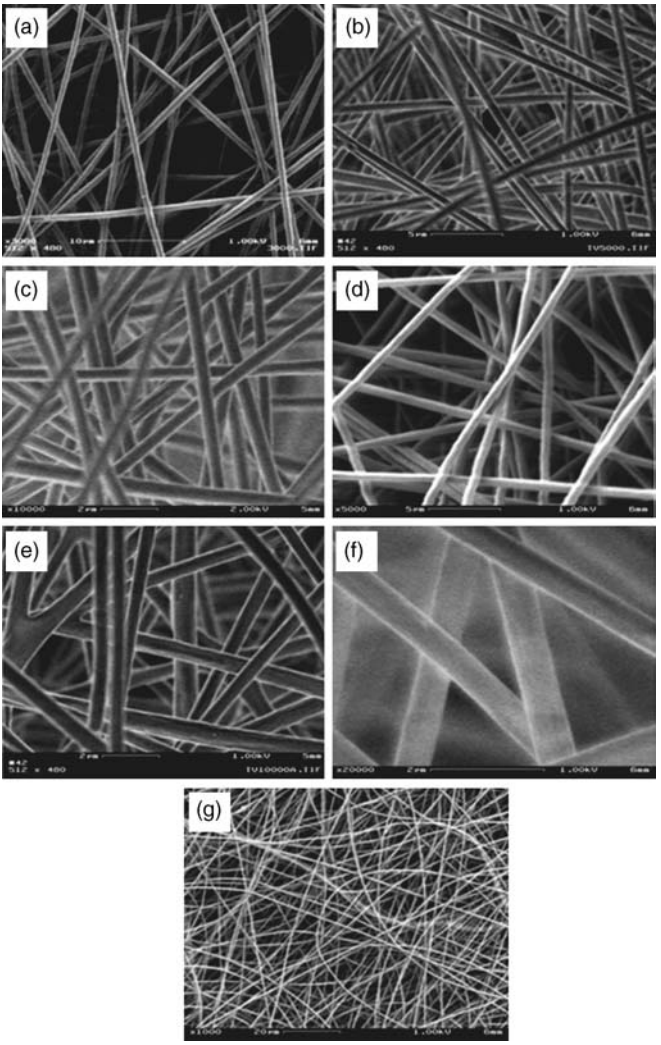

FIGURE 10.5 Scanning electron microscope micrographs of electrospun silk fibers with different diameters. (a) Fiber diameter = 840 ± 80 nm, (b) fiber diameter = 740 ± 150 nm, (c) fiber diameter = 700 ± 100 nm, (d) fiber diameter = 730 ± 50 nm, (e) fiber diameter = 720 ± 100 nm, (f) fiber diameter = 850 ± 60 nm, and (g) fiber diameter = 880 ± 50 nm. Adapted from Ref. [54] © ACS 2002.

viability of human MSCs with increasing concentration of silk after 48 h of exposure. This increase in viability is believed to be caused by an increase in β-pleated sheet crystals, elastic modulus, network size, and bound water.[59]

In nature, silk lacks cell-binding domains; however, these domains can be added to the fiber. The addition of domains renders silk fibers susceptible to macrophages,

which degrade the silk over a period of time. The addition of cell-binding domains allows native tissue growth in the matrix, allowing tissue to attain normal physiological function.[53] Addition of the peptide arginine-glycine-aspartic acid (RGD) to the surface of silk films increases cell density. In a study, films with RGD peptide on the surface had higher cell counts after 24 h, and cells continued to increase after 14 days (Fig. 10.5).[14]

In vitro studies on electrospun silk scaffolds reported cell growth and ECM formation after 14 days of incubation. Various combinations of silk, polyethylene oxide (PEO), bone morphogenic protein 2 (BMP-2), and nHAp, were used to fabricate scaffolds. BMP-2 and nHAp integrated scaffolds exhibited significant increase in calcium deposition and BMP-2 transcription levels.[35] Enhanced attachment and spreading of human MSCs and anterior cruciate ligament (ACL) fibroblasts was observed on RGD-modified silk scaffolds.[14] RGD functionalization also increases cellular mineralization; osteoblast-like cells (Saos-2) mineralized significantly on substrates containing parathyroid hormone.[55]

10.5 SUMMARY

The research to date suggests that nano- and microparticles or fibers have immense potential for applications in bone tissue engineering. Nanoparticles and nanofibers have shown to improve the mechanical properties of biodegradable polymeric implants. A few studies show that nano- and microparticle incorporated composite and scaffold implants are cytocompatible (*in vitro*) and biocompatible (*in vivo*). Some of the nanoparticles such as carbon nanotubes can also be functionalized for targeting, drug delivery, and bioimaging. Furthermore, their intrinsic physical properties can be harnessed for therapeutic and imaging applications. Although these nano- and microparticles improve the mechanical properties of bone tissue engineering scaffolds, little is known about their long-term biocompatibility and biodistribution upon their release from the scaffolds *in vivo*.[61,62] Although silk scaffolds produced by electrospinning are biocompatible, their mechanical properties can be improved by the dispersion of micro- and nanoparticles as reinforcing agents. Furthermore cell-binding domains can be modified to limit their susceptibility toward macrophage degradation. The future direction of tissue engineering field will see attempts to overcome these challenges and continue to create more biomimetic scaffolds because these nano- and microtechnologies show great promise with multifunctional capabilities for bone tissue engineering.

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