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journal homepage: www.elsevier.com/locate/addrElectrospun materials as potential platforms for bone tissue engineering[☆]Jun-Hyeong Jang^c, Oscar Castano^{d,e}, Hae-Won Kim^{a,b,e,*}^a Department of Biomaterials Science, School of Dentistry, Dankook University, South Korea^b Biomaterials and Tissue Engineering Lab, Department of Nanobiomedical Science & WCU Research Center, Dankook University, South Korea^c Department of Biochemistry, Inha University College of Medicine, South Korea^d Department of Material Sciences and Metallurgical Engineering, Institute of Bioengineering of Catalonia (IBEC), Universitat Politècnica de Catalunya, Barcelona, Spain^e Institute of Tissue Regeneration Engineering (ITREN), Dankook University, South Korea

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ABSTRACT

Nanofibrous materials produced by electrospinning processes have attracted considerable interest in tissue regeneration, including bone reconstruction. A range of novel materials and processing tools have been developed to mimic the native bone extracellular matrix for potential applications as tissue engineering scaffolds and ultimately to restore the degenerated functions of the bone. Degradable polymers, bioactive inorganics and their nanocomposites/hybrids nanofibers with suitable mechanical properties and bone bioactivity for osteoblasts and progenitor/stem cells have been produced. The surface functionalization with apatite minerals and proteins/peptides as well as drug encapsulation within the nanofibers is a promising strategy for achieving therapeutic functions with nanofibrous materials. Recent attempts to endow a 3D scaffolding technique to the electrospinning regime have shown some promise for engineering 3D tissue constructs. With the improvement in knowledge and techniques of bone-targeted nanofibrous matrices, bone tissue engineering is expected to be realized in the near future.

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1. Introduction

The treatment of bone defect sites with medical-grade materials is widely performed with some degree of clinical success. The manipulation of biomaterials in concert with tissue cells is considered a promising and alternative therapy to the autologous surgery [1]. This tissue engineering approach to bone reconstruction, having gained significant interest and research input over the last decade, requires a suitable cell supporting matrix, namely a scaffold, to provide a 3-dimensional substrate for cells to populate on and function appropriately during the formation of bone analog tissue [2,3].

There have been significant advances in the development of bone scaffolds with various compositions and 3-dimensional configurations using a variety of techniques [4,5]. Recently, the electrospinning process and the nanofibrous matrices thus fabricated have gained tremendous interest, mainly due to the structural similarity to the tissue extracellular matrix (ECM), the processing availability to a wide range of materials, as well as simple set-up and operation at low cost [6–10]. Several studies have reported the performance of nanofibrous materials in guiding cells to initially adhere to and spread over the material, as well as further triggering them to secrete the appropriate ECM molecules targeted to the skin, blood vessel, cartilage, muscle, adipose, nerve and bone. The intriguing features of a fibrous morphology with diameters ranging from tens of nanometers to a few micrometers have attracted considerable attention focused on exploiting the properties as well as structural tuning to the tissue of concern for the applications as a tissue engineering scaffold.

In the bone reconstruction area, the electrospun nanofibers have also attracted considerable attention from scientists aimed at identifying suitable material compositions and exploiting them into electrospinning [11,12]. As the bone-associated cells and their progenitor/stem cells show initial responses in a similar manner to those in other tissue cells, which are anchorage-dependent, the nanofibrous substratum may provide favorable conditions for cell anchorage and growth. In tandem with the initial cell responses, further osteoblastic differentiation and mineralization have also been reported to be regulated in a positive manner on nanofibrous surfaces compared to a dense substrate of polymers [13].

Although studies on the *in vivo* feasibility of electrospun nanofibers in bone reconstruction and tissue engineering progress are currently in the early stages, recent reports of electrospun nanofibers with new compositions targeted for bone as well as some processing tools to design 3-dimensional scaffolding and tissue engineering have highlighted the potential use of electrospun materials in bone tissue engineering.

This review consists of three parts: a brief introduction of the bone structure, which is to be mimicked by electrospun nanofibrous matrices, and the bone tissue engineering concept; a research summary of electrospun materials targeted for bone regeneration, including polymers, inorganics and their composites/hybridized compositions; and a description of on-going efforts aimed at employing nanofibrous

matrices for drug delivery and tissue engineering, which was facilitated by surface functionalization, drug encapsulation and 3D scaffolding technique.

2. Bone and tissue engineering

2.1. Bone structure and ECM mimics

2.1.1. Bone structure: bone cells, ECMs and organization

It is important to understand the biomechanical and biological properties of bone in order to gain insight into choosing the type of materials that can best be used to reconstruct the degenerative functions of bone. Bone is a complex, highly organized and specialized connective tissue. Compared to soft tissues, bone is physically hard, rigid and strong, and microscopically contains relatively few cells with abundant intercellular matrix in the form of collagenous fibers and stiffening inorganic substances. There are three types of cells comprising bone as illustrated in Fig. 1.

Osteoblasts located on the surfaces of bone are responsible for the formation and organization of the extracellular matrix of bone and its subsequent mineralization. These cells are responsible for the synthesis of organic components of the bone ECM. They are derived from mesenchymal precursor cells in the marrow, which also has the potential to differentiate into fat cells, chondrocytes or muscle cells [14]. The principal products of mature osteoblast are type I collagen (90% of the protein in bone), bone specific vitamin-K dependent proteins, osteocalcin and matrix Gla protein, phosphorylated glycoproteins including bone sialoproteins I and II, osteopontin and osteonectin, proteoglycans and alkaline phosphatase.

A proportion of osteoblasts become trapped as osteocytes in the lacunae within the bone matrix. These cells may be responsible for intercellular communication. They possess long thin cytoplasmic processes called filopodia located in thin cylindrical spaces or canals in the bone matrix. Nutrients and oxygen pass between the blood vessels and distant osteocytes via the arrangement of the canaliculi. Osteocytes also break down the bone matrix through osteocytic osteolysis to release calcium for calcium homeostasis [15].

Osteoclasts are polarized cells with a ruffled border region of the cell membrane that is surrounded by an organelle-free region, or 'clear zone'. They adhere to the bone surface via integrins, which are specialized cell surface receptors [16]. Osteoclastic bone resorption initially involves mineral dissolution, followed by degradation of the organic phase. These processes take place beneath the ruffled border and depend on lysosomal enzyme secretion and an acid microenvironment [17]. Osteoclasts actively synthesize lysosomal enzymes, particularly the tartrate-resistant isoenzyme of acid phosphatase (TRAP) (used as a marker of the osteoclast phenotype), and cysteine-proteinases, such as cathepsins, which are capable of degrading collagen. Lysosomal enzymes are released only at the ruffled border region of the osteoclast cell membrane [18].

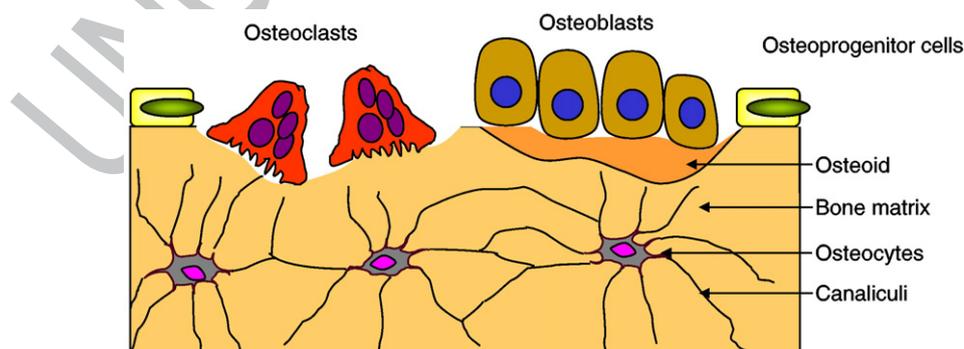


Fig. 1. Schematic diagram of bone structure at cellular level.

2.1.2. Bone ECM components

Type I collagen is a major organic component of mineralized ECM, comprising 90–95% of the organic material and serves as a template upon which mineral is deposited. Type V collagen is also present in small quantity, as are a number of non-collagenous proteins, some of which are relatively specific to bone [19].

In addition to the major collagen matrix, bone contains several other non-collagenous proteins. Osteocalcin is a 6-kDa noncollagenous protein and comprises up to 15% of the noncollagenous protein of the mature bone [20]. The osteocalcin expression is largely restricted to the osteoblasts of bone as well as the odontoblasts and cementoblasts of teeth [21]. The structure of osteocalcin is characterized by three glutamic acid residues that undergo vitamin K-dependent carboxylation. The γ -carboxyglutamic acid residues (Gla) provide osteocalcin with the ability to bind bone mineral hydroxyapatite with high affinity [22]. Osteocalcin is the second most abundant protein in the bone matrix, and it is highly conserved in all vertebrate species [23]. The biological function of osteocalcin is probably related to the regulation of bone turnover and/or mineralization [24].

Osteopontin is a secreted, glycosylated phosphoprotein that is found normally in mineralized tissues, such as bones and teeth, in addition to the kidneys, urine and epithelial lining cells in numerous organs [25]. Osteopontin supports cell adhesion through its Arg-Gly-Asp (RGD) integrin recognition motif. Osteopontin is also rich in aspartic acid residues and can be heavily glycosylated. The acidic nature of osteopontin probably accounts for its ability to modulate the growth of calcium crystals in both bone [26] and urine [27]. Osteopontin is a multifunctional protein that promotes cell adhesion and migration, inhibits bone mineral formation, and binds Ca^{2+} [28,29]. Osteopontin can exist in a variety of forms depending on the extent of post-translational modification. A highly phosphorylated form of osteopontin can be isolated from the mineralized extracellular matrix of bone tissue, and is synthesized by osteoblasts [30].

The ECM plays an important role in the function of growth factors [31]. This cooperative/synergistic process may involve the convergence of intracellular signaling pathways triggered by the ECM proteins and growth factors, and becomes important in the tissue regeneration process. In addition to its serving as a scaffolding for mineralization, the ECM proteins function as a substratum for bone cell adhesion and differentiation. Once engaged with the matrix, the bone cells sense deformation and other changes within the bone (matrix–cell crosstalk) [32]. On the other hand, they may interact with their surroundings by anchoring and pulling on the matrix, as has been shown for other cell types (cell–matrix crosstalk) [33]. The summary of the bone ECM proteins is shown in Table 1.

2.1.3. ECM mimicking approach

Given that defective bone can recover with the use of artificial materials, bone-associated cells should be directed to recognize and respond appropriately to form bone ECM that is analogous to the native bone matrix. Therefore, it is favored to design and engineer materials with structure, composition and properties similar to the

bone ECM [34]. Bone mimicking materials should play active roles in assisting cells to follow processes that are effective in bone formation. The major organic bone matrix consists of collagenous fibrils interwoven within hydrated polysaccharide chains, acting efficiently in response to external stress, and transmitting signals to the cell membrane receptors that reach the nucleus via intracellular signaling cascades. More importantly, within the organic network, inorganic nanocrystallites (mostly hydroxyapatite phase) are mutually incorporated. Therefore, the bone ECM is a type of organic–inorganic nanocomposite, organized on the nanoscale, in which bone is allowed to perform good biomechanical functions and biological roles [35]. Besides collagenous fibers and inorganic mineral nanocomponents, a variety of key proteins and growth factors are present in the bone matrix and are involved in bone formation, and should also be considered in the design of ECM mimicking materials. Overall, a nanofibrous matrix that can be produced by electrospinning is believed to be able to retain bone ECM components and be engineered to modulate the microenvironments further to form tissue mimics in the course of ex vivo tissue engineering or under *in vivo* situations [36]. This drives us to focus on a tissue engineering approach where the native bone structure can be better mimicked because bone actually contains both ECM and cell components.

2.2. Bone tissue engineering

2.2.1. Progenitor/stem cells

The recent emerging strategy in bone tissue engineering is to use stem cells. Many adult tissues contain populations of stem cells that have the capacity for renewal. These cells may be found within the tissue or in other tissues that serve as stem cell reservoirs. For example, although bone marrow is a major source of adult hematopoietic stem cells (HSCs) that renew circulating blood elements, these cells can also be found in other tissues [37]. Adult bone marrow also contains mesenchymal stem cells (MSCs), which contribute to the regeneration of mesenchymal tissues, such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma [38]. Therefore, they are an attractive cellular source for bone tissue engineering applications. Under permissive stimulation, MSCs undergo osteogenic differentiation through a well-defined pathway, acquiring osteoblastic markers and secreting extracellular matrix and calcium crystals [39]. *In vitro* and animal implantation studies have suggested that the population is either multipotent MSCs or mixtures of committed progenitor cells, each with a restricted potential [40]. However, clinical translation is impeded by the low population of MSCs in bone marrow, particularly in older age groups in whom fractures and non-union are common.

Blood mesenchymal precursor cells (BMPCs) have been a central focus in regenerative medicine for bone regeneration ever since these cells were first found to exist in the circulation of healthy patients. BMPCs were discovered by Zvaifler et al., who reported that these cells adhere to plastic and glass and proliferate logarithmically in DMEM-20% fetal calf serum without growth factors, which suggests that these cells are relatively easy to expand *in vitro* [41]. After adding osteogenic supplements (e.g., dexamethasone, ascorbic acid, and beta-glycerophosphate) into the culture, fibroblast formation is inhibited, and the BMPCs then assume the more cuboidal shape of osteoblasts, as confirmed by alkaline phosphatase (ALP) and osteocalcin staining. This group further demonstrated that circulating osteocalcin positive cells also deposit minerals *in vitro* and bone *in vivo* in immunodeficient mice [42]. They also reported that circulating osteocalcin positive cells are predominantly small, round cells that are phenotypically similar to the cells originally isolated from the nonadherent bone marrow population by Long et al. [43]. Given the osteogenic potential of circulating blood mesenchymal cells, exposing these cells to osteogenic factors is a potent stimulus for bone formation. Otsuru et al. recently reported that osteoblast progenitor cells in the circulation that originate from the blood mesenchyme form ectopic

Table 1

The ECM proteins found in bone.

ECM proteins	Function	Comments
Collagens	Collagen type I Collagen type V	Tensile strength Tensile strength
Noncollagen proteins	Osteocalcin (bone Gla protein)	Mineralization
	Osteopontin	Cell adhesion, Mineralization
	Bone sialoprotein-2	Mineralization
	Osteonectin (SPARC)	Cell adhesion
	Fibronectin	Cell adhesion
	Thrombospondin	Cell adhesion

bone after being implanted with a bone morphogenetic protein (BMP)-2-containing collagen pellet into skeletal muscle beds of mice [44]. When these pellets were implanted into GFP transgenic mice, there was a significant number of GFP-positive osteoblastic cells engrafting into the ectopic bone after circulatory migration to the osteogenic site.

More recently, MSCs with osteogenic potential have been isolated from a wide variety of tissue types, including adipose tissue, umbilical cord blood, amniotic fluid and fetal blood [45,46]. However, it is unclear how these novel fetal perinatal and adult MSC sources compare with their standard adult blood MSC counterparts for osteogenic differentiation and potential for bone tissue engineering.

2.2.2. Osteogenesis and angiogenesis

The development of osteogenesis occurs through two distinct processes: intramembranous and endochondral ossification. In intramembranous ossification, bone is formed by the differentiation of mesenchymal cells into osteoblasts in the absence of a cartilaginous model. The flat bones of the skull, sternum, and scapula are examples of bones that develop through intramembranous ossification. The term endochondral refers to the close association of the developing bone with the pre-existing hyaline cartilage model of that bone. The long bones of the limbs (including the phalanges) and ribs develop through endochondral ossification.

Recently, studies using *in vitro* and *in vivo* models of osteogenesis highlighted the importance of blood vessels in the formation of the

skeleton and bone repair [47]. The vasculature transports oxygen, nutrients, soluble factors and numerous cell types to the bone tissues. There are a number of factors involved in angiogenesis, and the main factors are Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor-2 (FGF-2), and various members of the Transforming Growth Factor beta (TGF- β) family [48]. Recent studies have shown that a combination of angiogenic and osteogenic factors can stimulate bone repair and regeneration [49]. Therefore, the delivery a combined system of growth factors at different rates locally from an engineered biodegradable nanofibrous scaffold might enhance the reparative mechanism of critical sized bone defects, thereby mimicking the *in vivo* bone repair conditions. The multiple release of growth factors, such as VEGF and BMP, may mimic the conditions in bone fracture repair. Hence, scaffolds capable of releasing an active angiogenic factor will promote early vascularization and attract osteogenic precursor cells. Huang et al. reported that PLGA scaffolds containing a combination of plasmids encoding DNA for BMP-4, VEGF and human bone marrow stromal cells promoted greater bone formation when implanted into the subcutaneous tissue of SCID mice than those containing a single factor or a combination of two factors [50].

2.2.3. Bone tissue engineering

Bone tissue engineering has become a rapidly expanding research area because it offers a new and promising approach for bone repair and regeneration [51]. Typically, bone tissue engineering approaches involve the use of scaffolding materials in combination with tissue

Table 2
Summary of electrospun nanofiber systems produced for the bone reconstruction.

Composition	Fiber diameter	Assays	Remarks	Ref.	
Synthetic polymers	PLA (<i>L</i> - and <i>DL</i> -type)	141–2140 nm	MC3T3-E1	Effect of osteogenic factors and fiber size	[55]
	PCL	20–5000 nm	BMSC, <i>in vivo</i> (rat)	Tissue engineering	[11,54]
Natural polymers	PHB, PHBV, blend	2000–4300 nm	SaOS-2 & L929		[56]
	Collagen I	50–1000 nm	hMSC		[63]
	Chitosan	200 nm	MG63, <i>in vivo</i> (rabbit)	Bone formation at 4 weeks	[69]
Polymer blends	Silk fibroin	217–610/183–810 nm	MC3T3-E1		[66]
	Silk fibroin	700 nm	BMSC	Poly(ethylene oxide) (PEO) addition	[65]
	PCL–gelatin	tens of nm–1000 nm	BMSC	Cell penetration with gelatine addition	[57]
	PLLA–gelatin	190–390 nm	MC3T3-E1	Enhanced cell responses on blends	[58]
Inorganics	PCL–heparan sulfate	–	BMSC	Osteogenic differentiation	[59]
	Bioactive glass	84–630 nm	Production, bone bioactivity, rBMSC	Excellent bone bioactivity and BMSC responses	[70]
	Bioactive glass	320 nm	Production, osteoblast adhesion	FN-introduction, enhanced cell adhesion	[75]
	Hydroxyapatite and fluoro-hydroxyapatite	240–1550 nm	Production, dissolution	Reduced dissolution by fluorine addition	[71]
	Hydroxyapatite	10–30 nm	Production	Microfibers	[72]
	Hydroxyapatite	200–500 nm	Processing		[73]
Composites/hybrids	Silicate	–	<i>In vitro</i> (MG63)	Apatite forming ability	[74]
	Gelatin–hydroxyapatite	200–400 nm	Production, osteoblasts	Enhanced osteoblastic differentiation	[79]
	Collagen–hydroxyapatite	75–160 nm	Production, osteoblasts		[80]
	Chitosan–hydroxyapatite	~214 nm	hFOB	PEO addition	[81]
	PCL–CaCO ₃	~760 nm	Mechanical test, <i>in vitro</i> (hFOB)	GBR membrane application	[82]
	PLLA–hydroxyapatite	~1000–2000 nm	Production, MG63	Surfactant introduction	[83]
	Siloxane–gelatin	40 to 670 nm	Production, MC3T3-E1	Hybridized structure, Ca requirement	[84]
	PCL–HA–collagen	~370 nm	hFOB		[85]
Surface functionalized	PCL– β TCP	200–2000 nm	Osteoblast responses	Better cell adhesion due to β TCP	[86]
	PCL	~250 nm	Production, osteoblasts, PDL fibroblasts	Apatite mineralized, higher osteogenic responses	[87]
	PLLA	200–2200 nm	Production	NaOH-treatment	[89]
	PDLLA	–	Production	Ca(NO ₃) ₂ addition	[90]
Drug/gene delivery	PLLA, PLLA–collagen	287–364 nm	hFOB	Mineralization with collagen	[91]
	PLGA, PLGA–PEG	–	Fibroblast adhesion	Amination, RGD-immobilization	[94]
	PLA, PCL	–	Antibacterial effects	Antibiotic delivery	[96]
	Silk, Silk-PEO (+ hydroxyapatite)	510–590 nm	hMSC responses	BMP2 efficacy on osteogenesis	[97]
	PLGA–HA	250–875 nm	<i>In vitro</i> gene transfection	BMP encapsulation in chitosan nanoparticles	[101]

Abbreviations; PLA: poly(lactic acid), PCL: poly(ϵ -caprolactone), PHB: poly(hydroxybutyric acid), PHBV: poly(hydroxybutyric-co-valeric acid), PLGA: poly(lactic-co-glycolic acid), PEO: poly(ethylene oxide), PEG: poly(ethylene glycol), β -TCP: β -tricalcium phosphate, HA: hydroxyapatite, hMSC: human mesenchymal stem cell, BMSC: bone marrow stem cell, hFOB: human fetal osteoblast.

cells and biological cues. An advanced scaffolding material for tissue engineering must exhibit high quality, reliability, sustainability and cost-effectiveness throughout the individual's life and provide new advanced levels of medical assistance in therapy and surgery. One particular requirement of bone tissue engineering is that the scaffold be porous because large numbers of cells can be incorporated in that form. The three dimensional scaffolds provide the necessary support for cells to attach, grow and differentiate, and define the overall shape of a bone tissue engineered transplant [1]. A range of biomaterials have been investigated for use in bone tissue engineering scaffolds, which can be classified mainly into three categories according to the composition: bioactive inorganics, degradable polymers and their composites/hybridized forms [52]. Gigante et al. evaluated the behavior of human MSCs cultured on various scaffolds to determine if their differentiation can be induced by cell–matrix interactions [53]. They reported that MSCs grown on type I + II collagen differentiated to cells expressing chondrocyte markers, while those grown on type I collagen + hydroxyapatite differentiated into osteoblast-like cells. Their study highlighted that human MSCs grown on different scaffold matrices can display different behaviors in terms of cell proliferation and phenotype expression [53].

Recent technological advances has facilitated the generation of a variety of scaffolds with a modulated pore configuration and nanostructure. Electrospun nanofibers are one of these recently highlighted systems that may find applications as a scaffolding material in bone tissue-engineered constructs.

3. Electrospun bone regenerative materials

Designing matrices suitable for the recruitment of osteoprogenitor/stem cells has been promoted by the approach of mimicking the composition, morphological traits and mechanical function of the native bone ECM. The beneficial features of a nanofibrous structure by electrospinning were first realized with degradable polymers, which stimulate cells into osteogenic pathway assisted via well-controlled differentiation cues.

However, a major part of the bone ECM also contains calcium phosphates mineral phases, which requires a mineralization step that is essential in the bone regeneration process. The existence of bone-bioactive inorganic components within biomaterials generally favors calcium phosphate mineralization followed by an osteogenic differentiation process. Therefore, recent studies have focused on introducing a range of inorganic phases within the polymeric nanofibers with the ultimate aim of achieving both bone-specific bioactivity and mechanical properties.

A new strategy to designing nanofibers involves endowing biofunctionality onto the surface of nanofibers because the cells first recognize the surface of the material, which mostly regulates their responses. Modulation of a polymeric surface with materials that are more friendly and active to bone cells, such as a bone mineral-like phase, is one example of surface tailoring methods targeted for bone regeneration. Moreover, nanofibers that are surface-conjugated or incorporated internally with proteins and genes are an elegant way of utilizing nanofibrous matrices in drug delivery systems. *In vitro* data have demonstrated the potential of introducing cell adhesive proteins or peptides as well as osteogenic stimulatory signals including growth factors and genes. In Table 2, the electrospun nanofiber systems produced for the reconstruction of bone tissue are summarized.

Because of the inherent processing nature of electrospinning, which contains pores with sizes at best a few micrometers, the introduction of larger sized pores within the nanofibrous network are needed in order to identify extended and potential uses of bone tissue engineering 3D scaffolds. A few recent trials carried out to generate macro-sized pores and engineer 3D tissue constructs provided some insights into future work on bone tissue engineering using electrospun nanofibers as a scaffold.

3.1. Polymeric nanofibers

The electrospinning of degradable polymers, either with a synthetic or natural origin, was first reported to generate suitable bone cell matrices largely due to their ease of processing including solution preparation. Furthermore, the flexibility and shape-availability of polymeric materials gives them great potential in the bone regeneration area.

Among all polymeric materials, a group of poly(α -hydroxyl acid), such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ϵ -caprolactone) (PCL) and their copolymers, has been the most extensively studied nanofiber system for the regeneration of tissues, including bone [3]. PCL was first suggested to be a degradable nanofiber matrix for the bone regeneration [11], which demonstrated good support of the rat bone marrow stromal cells (rBMSCs) and *in vitro* matrix formation at 4 weeks, such as collagen I and calcium phosphate mineral. Moreover, a cell–nanofiber construct implanted in

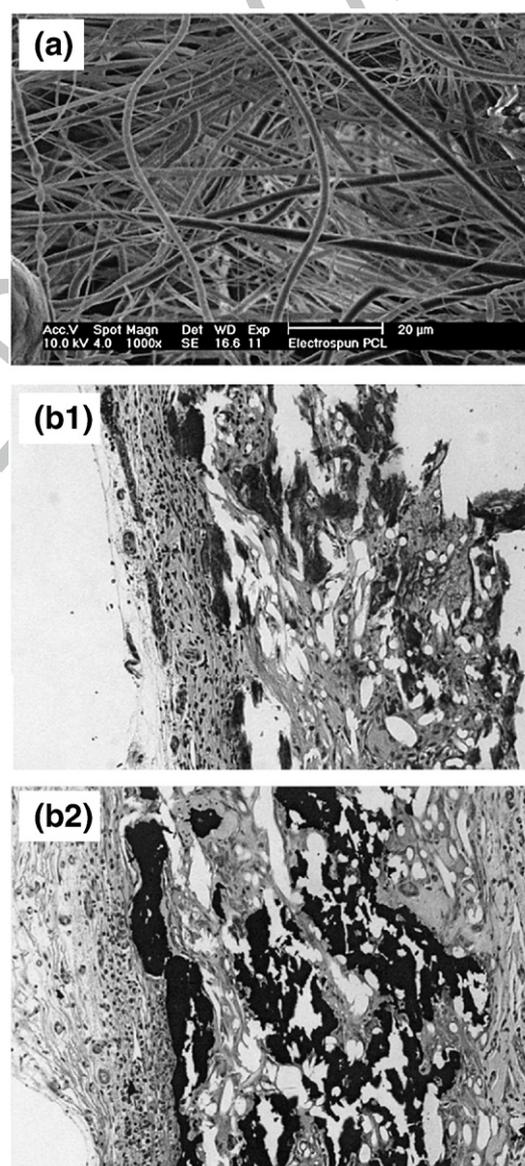


Fig. 2. (a) PCL electrospun nanofiber. (b1,b2) Histology cross-section of the explanted specimens after 4 weeks of *in vitro* culture and 4 weeks of implantation in the omentum of rat. (b1) Osteocyte-like cells embedded in bone matrix are present (H&E; original magnification, $\times 3100$). (b2) Mineralization has occurred throughout the specimen (von Kossa; original magnification, $\times 3100$). Adapted with permission from [54] copyright 2004 Mary Ann Liebert.

rat omenta for 4 weeks revealed the formation of collagen I and mineralization similar to bone-like ECM, highlighting its usefulness in bone tissue engineering (Fig. 2) [54]. The PLA electrospun nanofibers with variable sizes were observed to affect the MC3T3-E1 cell responses [55]. Interestingly, when an osteogenic medium was used, a higher cell density was observed on the PLA nanofibers than on flat PLA. On the other hand, there was little difference observed when no osteogenic medium was used, suggesting the possible influence of osteogenic factors on the osteoblastic responses to the nanofibrous topology. Poly(hydroxyalkanoate)s, another class of degradable polyester polymer, was also developed into electrospun nanofibers for bone regeneration [56]. Poly(hydroxybutyrate) (PHB) and poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) nanofibers with relatively large diameters (approximately 2 to 4 μm on average) exhibited better cell growth behavior (SaOS-2 cell line) than their equivalent flat film counterparts, and maintained the osteoblastic phenotype [56].

However, due to the innate hydrophobic nature, the initial cell adhesion behavior to the synthetic polymers is limited. Given that nanofibers are to be used as cell matrices for tissue engineering, it is essential to confirm the initial cell adhesion and high population. Blending with natural polymers is another way of improving the cell compatibility [57,58]. When PCL was mixed with gelatin at a 1:1 ratio, the blending nanofiber exhibited good penetration of BMSCs within the nanofiber matrix. On the other hand, there was little growth observed within the pure PCL nanofiber [57]. Our recent study on the blending nanofibers of PLA with gelatin at various ratios (1:3, 1:1 and 3:1) showed that the osteoblastic cells (MC3T3-E1) were more viable than those on pure PLA nanofiber [58]. Moreover, a range of bone-related genes were expressed at significantly higher levels on a blended hydrophilic nanofiber substrate. Another report developed heparan sulfate-containing PCL nanofibers, where human MSCs pre-committed to an osteogenic lineage were observed to secrete bone matrix and bone formation under a subcutaneous model in nude mice [59]. Together with the blending approach, the surface of the synthetic nanofibers was coated with natural polymers, such as collagen and gelatin, which showed good initial adhesion and growth of cells including osteoblasts [60,61].

As natural polymer sources, collagen has long been studied for the electrospinning into nanofibers [62–64]. Type I collagen is the major organic component of bone ECM, and has attracted considerable attention for use as a bone cell supporting matrix. Nanofibers of collagen type I can be electrospun to various diameters and provide good substrate conditions for BMSCs to adhere and grow [63].

Although electrospun collagen mimics the nanofibrous morphology of native ECM, there is some debate as to whether the native structure and biological characteristics are preserved [64]. Whilst one report showed native periodic bands in electrospun collagen [62], Jeugolis et al. insisted the electrospun collagen was only a denatured form of gelatin, when electrospun out of fluoroalcohols which limit the typical biological properties of collagen derived from the triple helical structure, and suggested the method of collagen coating of the electrospun nanofibers [64]. Nevertheless, cross-linked electrospun collagen is believed to have strong potential as a nanofibrous substrate for cells to anchor and populate as well as in osteogenic development and mineral deposition provided appropriate differentiation cues are present.

Silk fibroin has also been explored as a potential electrospun substrate because of its useful properties for tissue engineering, such as cell compatibility, biodegradability and minimal inflammatory reaction [65]. Electrospun nanofibers of silk with sizes ranging from 500 nm to 1 μm were observed to support the initial adhesion and growth of BMSCs [65] and osteoblastic cells [66]. One merit of silk fibroin in bone regeneration is its ability to promote the deposition of calcium phosphate minerals thus to form an apatite–silk nanocomposite [67].

Compared to other natural polymers, chitosan is considered relatively difficult to electrospin mainly due to the limited solvents and high viscosity at low concentrations [68]. A recent study developed an electrospun chitosan nanofibrous mesh for use as a dental barrier membrane to selectively guide hard tissues within the periodontal pocket. The *in vivo* result at 4 weeks of implantation using the membrane within a critical-sized defect of a rabbit calvarium demonstrated almost full coverage of the defect and bone formation, which highlights its potential use in bone regeneration (Fig. 3) [69].

3.2. Inorganic nanofibers

Although the degradable polymeric nanofibers with a synthetic or natural origin have been shown to support the growth of osteoblasts and their progenitor/stem cells as well as to recruit their phenotypic expression and differentiation under the appropriate microenvironment, bone-bioactive inorganics, including calcium phosphates and bioactive glasses/glass ceramics have been a fascinating choice of materials for the reconstruction of hard tissues. In practice, the electrospinning of inorganic materials into a nanofibrous structure is well documented, even though they were mainly not for biomedical purposes. It was not until a few years ago that some studies exploiting

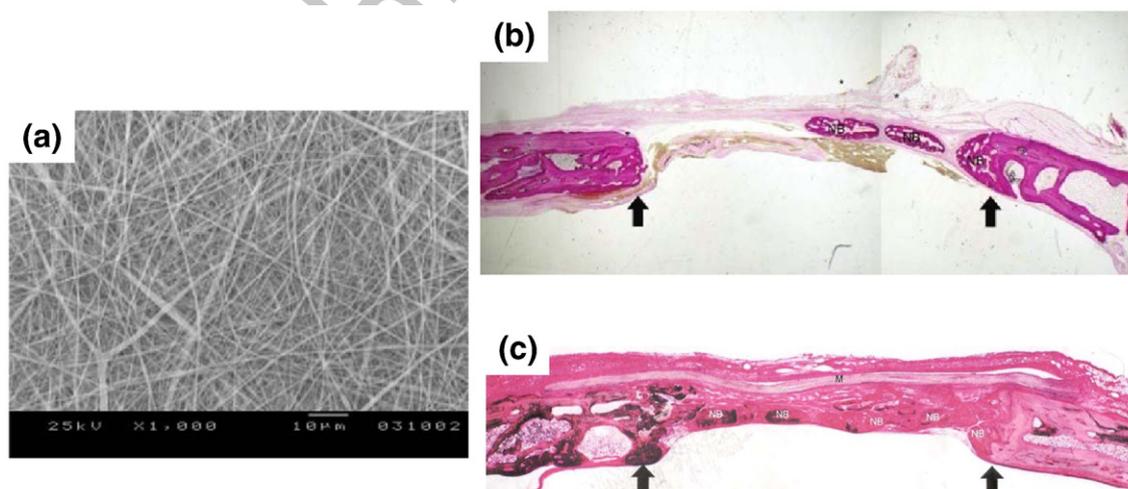


Fig. 3. (a) chitosan nanofibrous membrane. (b,c) histological view of implantation without the membrane (b) and (c) with the chitosan membrane within a rabbit calvarium defect at 4 weeks (arrows: defect margin, NB: new bone, M: membrane). H & E staining in (b) and Masson-Trichrome–Goldner staining in (c). Adapted with permission from [69] copyright 2007 American Academy of Periodontology.

490 the bone-bioactive inorganic composition into electrospun nanofibers
491 were reported.

492 A recent study reported the generation of bioactive glass nanofibers
493 by electrospinning [70]. Silica-based sol-gel glass ($70\text{SiO}_2 \cdot 25\text{CaP} \cdot 5\text{P}_2\text{O}_5$)
494 mixed with a polymer binder was electrospun into a nanofibrous mesh
495 and heat-treated to produce fibers with sizes ranging 84 nm to 640 nm
496 by varying the sol concentration. The glass nanofiber induced the
497 formation of a bone mineral-like apatite phase on the surface in a
498 simulated body fluid, which was attributed to the extremely large surface
499 area of the nanofiber and the consequent ionic reaction with the
500 surrounding medium (Fig. 4). Moreover, the nanofibrous substrate

501 actively supported a population of rat BMSCs and osteogenic differentia-
502 tion to a level significantly higher than that on dense sintered bioactive
503 glass or PCL polymer nanofiber, highlighting the potential of bioactive
504 glass nanofibers in terms of both morphological and compositional
505 benefits. A parallel approach has also been realized on the production of a
506 range of inorganic nanofibers including hydroxyapatite [71–73], fluoro-
507 hydroxyapatite [71], and silica nanofibers [74], by using the sol-gel
508 solution which was mixed with a polymeric binder either with poly
509 (vinyl pyrrolidone) and poly(vinyl butyral) and subsequent heat
510 treatment. One elegant study applied the in-situ mineralization behavior
511 of the bioactive glass to the introduction of biomolecules on the

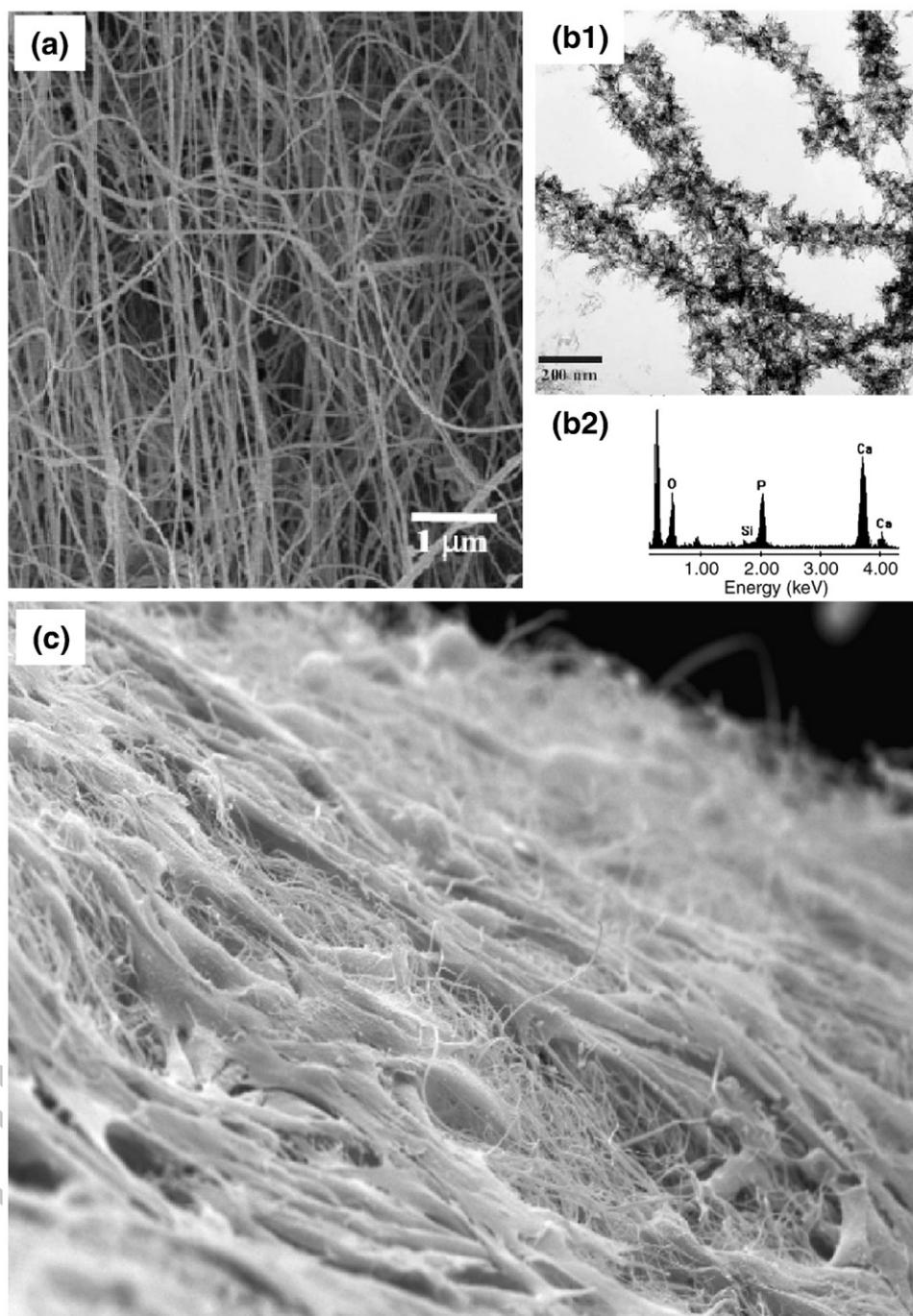


Fig. 4. (a) Inorganic nanofiber with a bone-bioactive composition (sol-gel glass $70\text{SiO}_2 \cdot 25\text{CaP} \cdot 5\text{P}_2\text{O}_5$) obtained by electrospinning and heat-treatment at 700°C . (b1,b2) Acellular bone-bioactivity of the nanofiber showing the formation of bone mineral-like apatite on the nanofibrous surface after soaking in simulated body fluid for 3 days, as observed by TEM (b1) and composition analysis by EDS (b2). Adapted with permission from [70] copyright 2006 Wiley-VCH Verlag GmbH & Co. (c) Rat BMSCs grown on bioactive glass nanofibers for 7 days exhibiting good cell population and active cytoplasmic extension in concert with the underlying nanofibrous substrate.

nanofiber surface [75]. Cell-adhesive fibronectin was effectively coupled with apatite mineral onto the surface of bioactive glass nanofiber which demonstrated significant enhancement in the initial osteoblast adhesion and spreading.

However, regardless of their attractive bone-bioactivity, electrospun nanofibers of inorganic materials, including calcium phosphates and bioactive glasses, may have limited use as tissue regeneration matrices on account of their brittleness. Moreover, post heat-treatment can limit their drug delivery potential. In this respect, future knowledge and advanced technology need to be developed in order to overcome the disadvantages of bone-bioactive inorganic nanofibers as well as to identify appropriate uses as bone tissue engineering matrices. At the moment, nanofibrous inorganic materials are being studied as nanofillers for the production of nanocomposite scaffolds with degradable polymers [76,77]. In particular, electrospun nanofibrous bioactive glass, being used as a novel inorganic nanocomponent, is well homogenized with collagen or a PLLA solution to produce uniform scaffolds and membranes, ultimately improving the bone-bioactivity of the organic phase and osteogenic differentiation and cellular mineralization (Fig. 5). The approach, which aimed to combine the bone-bioactivity of the inorganic component with shape-formability of the organic phase, highlights the useful application of the electrospun inorganic nanofibers as a bone-bioactive nanocomponent.

3.3. Polymer-inorganic composite/hybridized nanofibers

Combining degradable polymers with bioactive inorganic materials during the course of electrospinning is considered a fascinating

and reasonable way of generating nanofibers with the appropriate properties targeted for bone regeneration. The inorganic phase may act to improve the biological properties of polymeric nanofibers, such as cell compatibility and bone forming process, involving the osteogenic differentiation and calcification of bone matrix. Moreover, given that the brittleness of inorganic materials is a major limitation to their use as suitable cell substrates, the introduction of a polymeric phase should provide some degree of mechanical flexibility. In addition, the fact that there is no need for thermal treatment because of the binding polymer matrix is another attractive point for its use in drug delivery systems. Basically, the bone ECM is a type of composite constituted mainly of collagenous fibers embedded with hydroxyapatite nanocrystallites, which highlights the need for the development of nanocomposites mimicking bone structure [35].

In practice, the combinatorial/synergistic mechanical and biological properties of polymers and inorganics have been well documented in cases of porous scaffolds and membranes [78]. The ideas beyond those nanocomposites might well be applied to nanofibrous systems. However, it should also be noted that the electrospinning of organic-inorganic compounds requires special consideration in the preparation of solutions. Some elegant methods have been used to produce organic-inorganic composite nanofibers by electrospinning. One example is the gelatin-hydroxyapatite nanofiber, which was designed to mimic the bone ECM, wherein gelatin and hydroxyapatite precipitates were dissolved in an organic solvent and subsequently electrospun to produce nanofibers with hundreds of nanometers in diameter (Fig. 6) [79]. Hydroxyapatite nanocrystallites were evenly distributed in the gelatin matrix within the nanofibrous morphology, which was

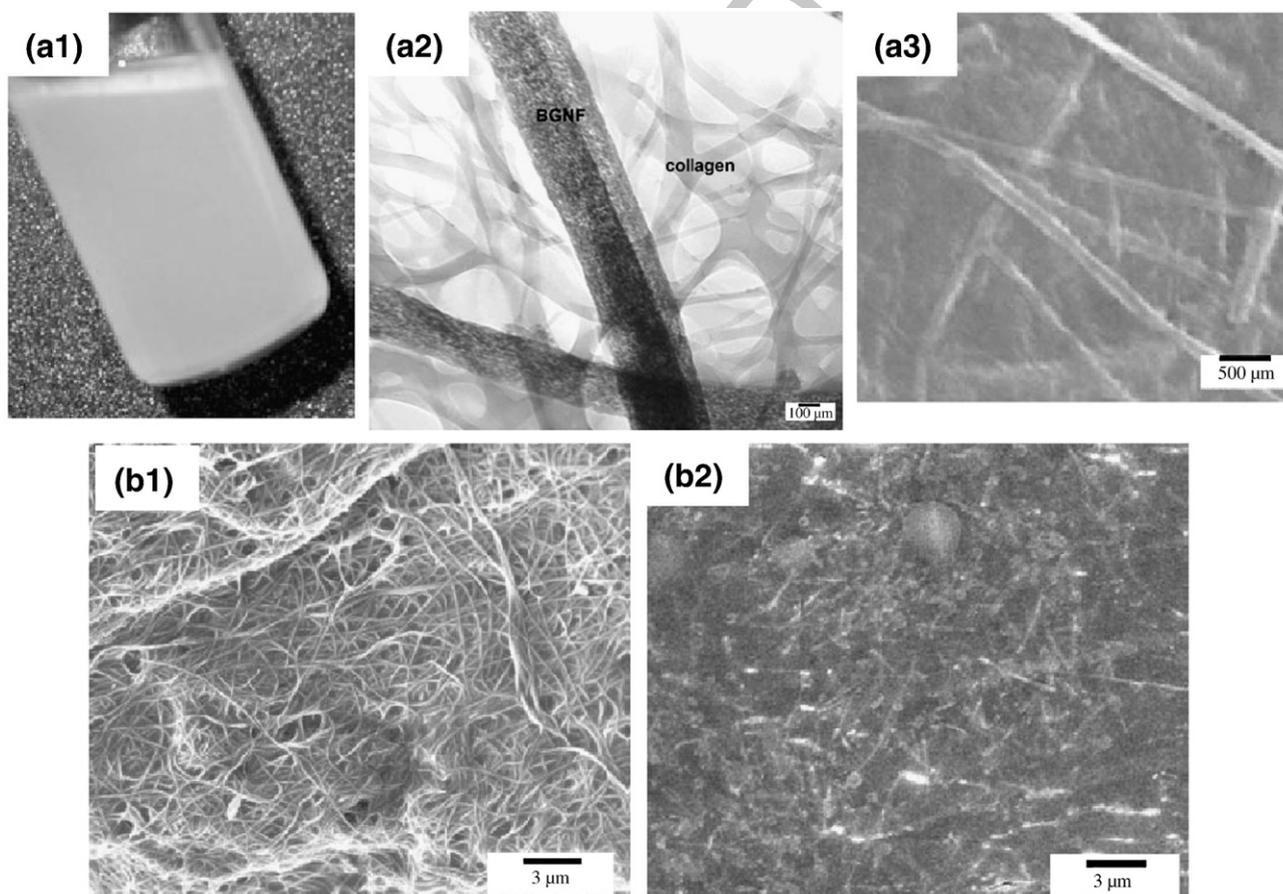


Fig. 5. Use of electrospun bioactive glass as an inorganic nanofiller for the production of nanocomposites with collagen (a1–a3) and PLLA (b1,b2). The nanofibrous component was homogenized with a collagen solution (a1) and further dried into a nanocomposite (a2,a3), showing an inter-organized glass nanofiber (BGNF) and collagen fibers (a2,a3). Nanocomposite with PLLA showed the permeation of a PLLA solution well into the interspacings of the nanofibrous network (b1), which was pressed to produce a dense nanocomposite (b2). The bone-bioactivity of the nanocomposites with collagen and PLLA was significantly enhanced showing the induction of a bone mineral-like phase when immersed in SBF. Adapted with permission from [76,77] copyright 2007 Wiley Interscience Co.

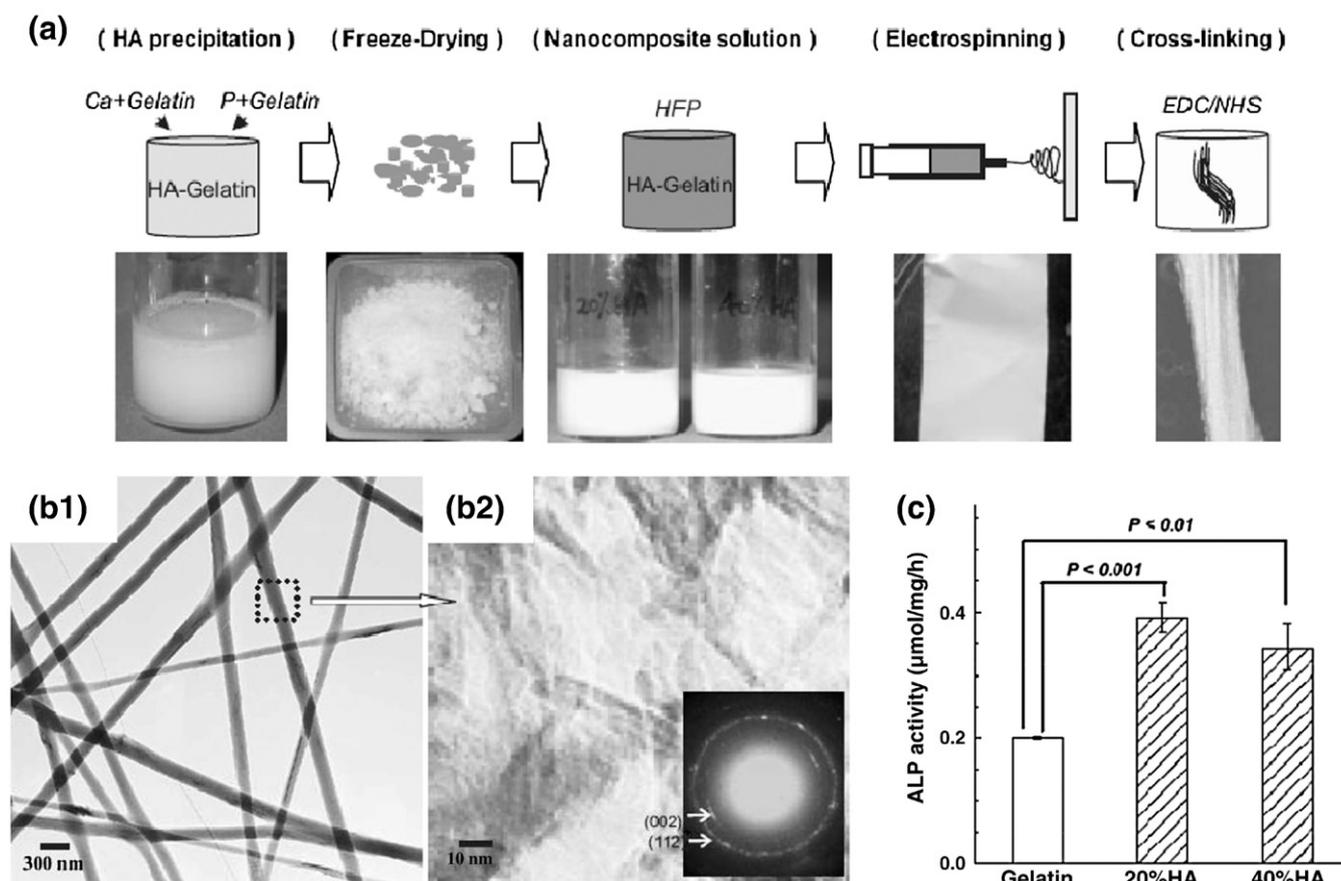


Fig. 6. (a) Experimental procedure to generate gelatin-apatite bone-mimicking nanofibers and (b1,b2) TEM image of a gelatin-20%apatite nanofiber and the organization of apatite within the gelatin matrix (apatite crystalline pattern revealed in the inset). (c) Osteoblastic cells exhibiting higher levels of alkaline phosphatase phenotypic expression on the nanocomposite nanofibers after 7 days of culturing. Adapted with permission from [79] 2005 Wiley-VCH Verlag GmbH & Co.

566 attributed to the role of the gelatin amino acid sequences modulating
 567 the precipitation of hydroxyapatite crystals. On the other hand, when
 568 hydroxyapatite nanopowders were mixed directly with a gelatin
 569 solution, electrospinning into nanofibers was impeded significantly
 570 resulting in a number of beads. The organized hybrid matrix showed
 571 significant enhancement in osteoblastic differentiation, and was
 572 proposed for use as a guided tissue regeneration membrane in dentistry.
 573 This approach was also realized in the collagen-hydroxyapatite system
 574 to generate a nanofibrous matrix to better mimic the bone ECM [80] as
 575 well as applied to other composite nanofiber of chitosan-hydroxyapatite
 576 [81].

577 Apart from natural polymers, synthetic degradable polymers have
 578 also been used in the electrospinning of composite fibers with bioactive
 579 inorganic materials. However, unlike hydrophilic natural polymers,
 580 which are easier to homogenize and be organized with inorganic
 581 crystallites, degradable synthetic polymers, such as PLA, PCL and PHBV,
 582 present a significant challenge in their combination with the inorganic
 583 phases on account of their hydrophobic nature. A recent work by
 584 Fujihara et al. developed PCL-CaCO₃ composite fibers with submicrometers
 585 in size, by introducing ultrafine CaCO₃ particles (~40 nm in
 586 size) [82]. Composite fibers containing CaCO₃ nanoparticles at 25 and
 587 75 wt.% showed good water affinity and mechanical tensile properties,
 588 as well as directed favorable osteoblastic adhesion and growth, thus
 589 being suggested for use as a guided bone regeneration membrane
 590 (Fig. 7).

591 However, inorganic nanoparticles generally agglomerate easily and
 592 cannot be intermixed well or homogenized with synthetic polymer
 593 solutions, resulting in bead formation during electrospinning. In an
 594 attempt to overcome this, we recently exploited PLA composite fibers
 595 containing ultrafine hydroxyapatite nanocrystallites obtained by a sol-

596 gel process (~35 nm in size) and by introducing a surfactant, 12-
 597 hydroxyteristic acid (Fig. 8) [83]. The amphiphilic nature of the surfactant
 598 was suggested to act as a stabilizing mediator at the interface of the
 599 hydroxyapatite nanocrystallites and PLA-organic solvent. Bead-free
 600 electrospun fibers were obtained with fiber sizes of a few micrometers
 601 wherein the hydroxyapatite nanocrystallites well distributed within the
 602 PLA matrix. The composite fiber was shown to promote the growth of
 603 osteoblastic cells and their phenotype expression to a significantly
 604 higher level than on pure PLA fiber. Overall, the current electrospinning
 605 of composite fibers has focused mainly on incorporating bioactive
 606 inorganic nanoparticles evenly within a polymeric matrix without
 607 breaking down the fibrous morphology. This has been possible to a large
 608 extent through the introduction of ultrafine particles or control of the
 609 level of homogenization.

610 Instead of introducing particulate forms of the bioactive inorganic
 611 phases within a polymeric solution, degradable and bioactive hybrid
 612 nanofibers were recently produced through the hybridization
 613 approach of using inorganic and organic phases in solution, such as
 614 the sol-gel process [84]. An aqueous solution of gelatin was mixed
 615 with polysilane (3-(glycidopropyl) trimethoxysilane) at various ratios
 616 (siloxane/gelatin = 0.5, 1 and 2) containing a small concentration
 617 (2.5 wt.%) of CaCl₂, which was hydrolyzed, condensed and then
 618 electrospun into nanofibers. In particular, the involvement of siloxane
 619 groups within the gelatin significantly improved the chemical stability
 620 of gelatin by forming linkages with amide groups of gelatin to produce
 621 a hybridized network. Moreover, the hybridized nanofibers signifi-
 622 cantly enhanced osteoblastic differentiation, suggesting their poten-
 623 tial use as a bone regeneration matrix (Fig. 9).

624 The approach of using bioactive inorganic phases in concert with
 625 degradable polymers is continuing to attract attention in finding

PCL Nano Fibers

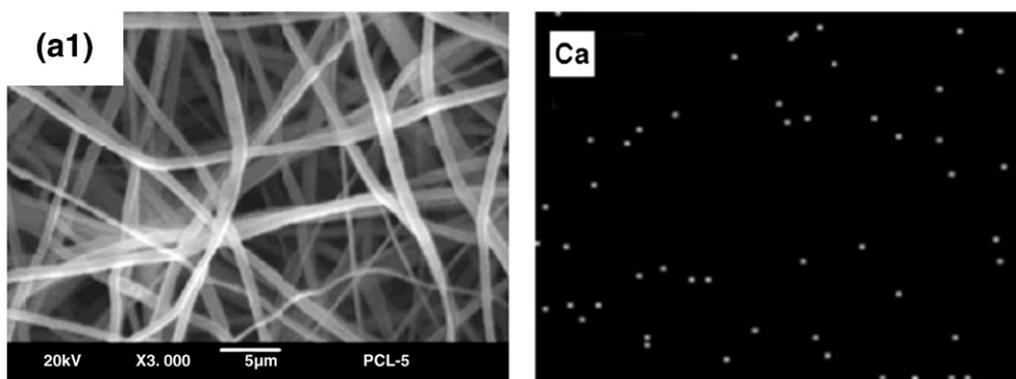
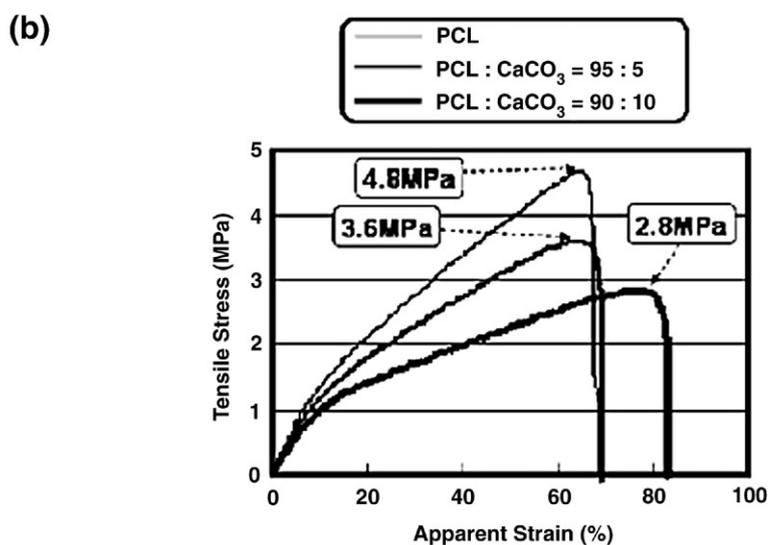
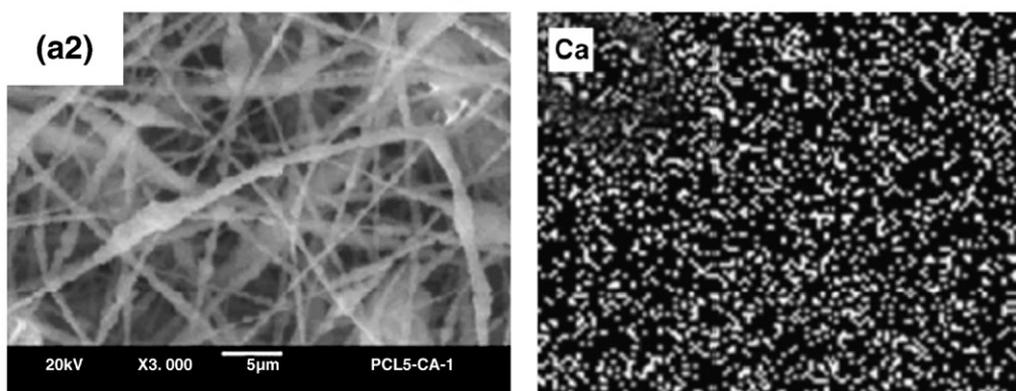
PCL/CaCO₃ Composite Nano Fibers
(PCL : CaCO₃ = 25:75)

Fig. 7. (a1,a2) Image of electrospun nanofibers of PCL and PCL/CaCO₃ composite and EDS mapping. (b) Tensile stress-strain curves of the nanofibers. Reprint with permission from [82] 2005 Elsevier.

626 suitable matrices for the regeneration of bone and its interfaced zone
 627 with cartilage [85,86]. Therefore, many more studies are expected to
 628 focus on developing composite nanofibers with new compositions
 629 with suitable mechanical properties and biological functions in bone
 630 regeneration. Although some challenges still remain, such as
 631 morphological and compositional control, including a reduction of
 632 fiber size, level of homogenization, and securing mechanical stability,
 633 more promising results are expected to come out from the composite
 634 nanofibers with respect to the polymeric single component.

4. Bio-functionalization and scaffolding for tissue engineering

635

636 Given that nanofibrous matrices have an extremely large surface
 637 area relative to volume, the surface-related properties of nanofibrous
 638 materials, such as materials release, protein adsorption and cell
 639 adhesion, are very important. Therefore, it is essential to tailor the
 640 surface properties of nanofibers to induce the appropriate biological
 641 reactions. The surface-functionalization of the nanofibers, as a post-
 642 treatment following the electrospinning process is another important

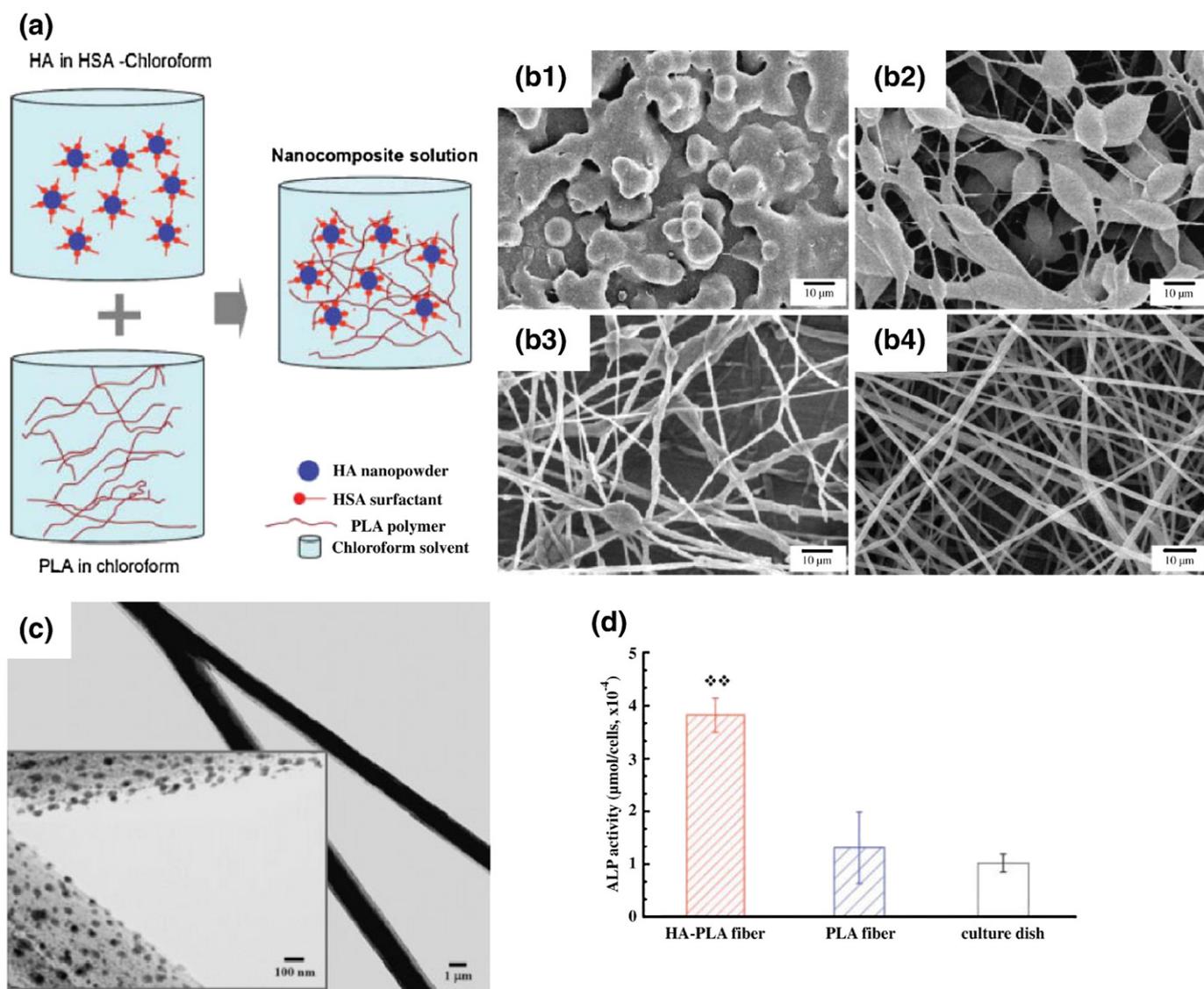


Fig. 8. (a) Schematic diagram showing the experimental design of the HA-PLA biomedical nanocomposite fiber mediated with HSA surfactant through the electrospinning process. (b1–b4) Electron morphology of the HA-PLA nanocomposite fiber electrospun under different conditions: HA commercial powder without HSA (b1), HA sol-gel powder without HSA (b2), HA sol-gel powder with 0.1% HSA (b3), and concentration of (b4) 12.5% was thickened to 25% by evaporation of the solvent (b5). (c) TEM morphology of the nanocomposite electrospun fiber consisting of HA sol-gel powder and PLA obtained with the mediation of 0.1% HSA. Fiber image in the inset prepared as an ultrathin film (<100 nm) using a microtome reveals the dispersion of HA fine particles within the PLA matrix. (d) ALP activity expressed by the cells after culturing for 7 days. A glass coverslip was used as a fiber-supporting substrate. Data on tissue culture dish was included as a control. The cell seeding density was 1×10^4 /ml. The data is reported as the mean \pm std., for $n = 6$, and a statistical comparison by ANOVA one-way analysis showed significant differences between the HA-PLA fiber and PLA fiber at $p < 0.01$ (*) and 0.001 (**). Reprint with permission from [83] 2006 Wiley Interscience Co.

643 area for regulating and improving the potential of nanofibers as a cell
 644 matrix. The initial cell adhesion and growth, osteogenic differentiation
 645 and matrix synthesis, and therapeutic stimulations can be tuned
 646 by bio-functionalization of the surface, which include the surface
 647 coverage with bone-reactive materials and spatially distributed
 648 conjugation with macromolecules, such as proteins, peptides and
 649 antibiotics. In the latter case, surface-tailored nanofibers will have
 650 therapeutic impact as an implantable drug delivery system [6].
 651 However, in order to gain intended biological performance, the
 652 surface conjugated molecules should maintain their biological activity
 653 and exhibit therapeutic functioning in a timely and proper manner.
 654 However, in such systems for long term delivery, drugs sometimes
 655 need to be encapsulated within the nanofiber to elicit therapeutic
 656 effect in a sustained manner.

657 The improvement in 3D scaffolding techniques is another
 658 challenge in electrospun nanofibers if they are to find potential use
 659 in bone tissue engineering. Electrospun nanofibrous meshes contain

660 small sized channels, at best a few micrometers in size, which can
 661 restrict cell migration and angiogenesis to form neo-blood vessels.
 662 Many recent attempts have been made to produce macropores within
 663 or to construct 3D tissue analogs with electrospun nanofibers, which
 664 may extend their potential use in bone tissue engineering.

665 4.1. Surface functionalization

666 Specific focus has recently been made on utilizing bone mineral
 667 phase in surface-tailoring of polymeric nanofibrous matrices which
 668 targeted for bone regeneration. As the bone mineral-like calcium
 669 phosphates, mainly hydroxyapatite phase, have good biocompatibility
 670 related to cell affinity and osteogenic regulation, a surface treatment of
 671 degradable polymeric nanofibers with a mineral phase is a promising
 672 route for up-regulating the bone cell functions [87–89]. A recent study
 673 mineralized a PCL nanofibrous surface with hydroxyapatite using a
 674 series of surface-modification steps involving the activation of

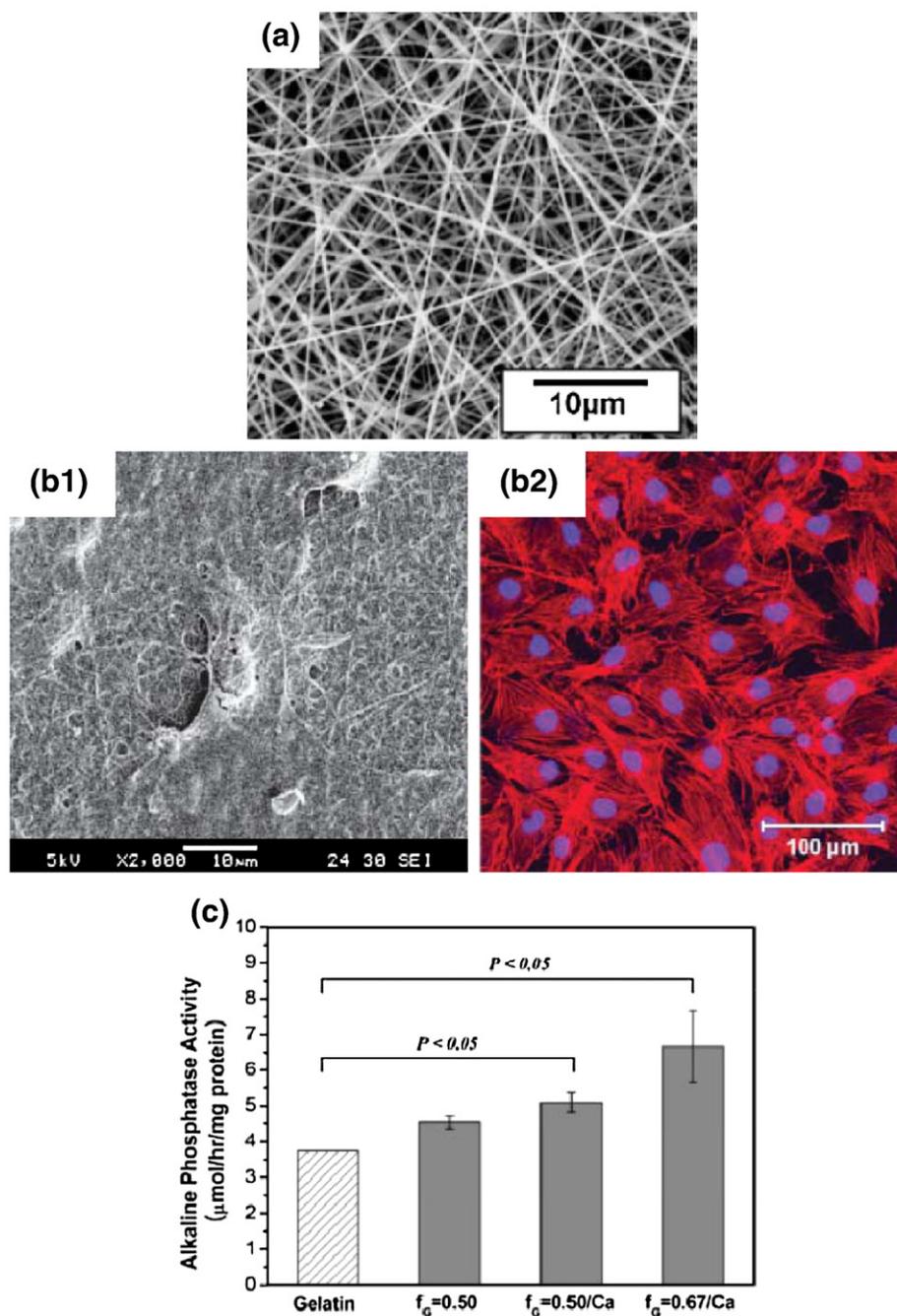


Fig. 9. (a) Morphology of gelatin–siloxane hybridized nanofibers (gelatin:polysilane = 1:1, CaCl₂ addition = 2.5 wt.%). (b1,b2) MC3T3-E1 growth on the hybridized nanofiber after 3 days (scanning electron microscopy (b1) and confocal laser scanning microscopy (b2)). (c) Alkaline phosphatase activity of osteoblastic cells on the nanofibers showing a significantly higher level on the hybridized ones (ANOVA). Reprint with permission from [84] 2008 Wiley Interscience Co.

nanofibers in an alkaline solution (2 N NaOH) to generate carboxylic groups, followed by alternate dipping in Ca and P-rich solutions (150 mM of Ca²⁺ and HPO₄⁻) to allow mineral nucleation followed by further soaking in a Ca–P pseudo saturated solution (simulated body fluid) [87]. The mineralized PCL nanofiber showed active osteoblastic responses, such as cell adhesion and growth, and significantly higher expression levels of the genes related to bone ECM than those on pure PCL nanofiber [87] (Fig. 10). Through surface mineralization, significant osteogenic induction was also observed on the periodontal ligament fibroblasts, highlighting the mineralized polymeric nanofiber for use as a guided bone regeneration membrane [88].

A similar approach has also been found in other degradable polymers, including PLA (*L*- and *DL*-type), wherein the mineral induction was facilitated more easily by treatment in an alkaline

solution [89] or by incorporating calcium [90]. When collagen was added to the PLA nanofiber, hydroxyapatite induction was possible without treatment with an alkaline solution, where collagen plays a key role in mineralization [91]. The hydroxyapatite mineral phase obtained by the solution-mediated process is generally poorly crystallized and carbonated, being similar in composition and structure to the native bone mineral, which is thus believed to regulate a series of biological reactions in a favorable manner, including the selective adsorption of bone-associated proteins, osteogenic stimulation of progenitor/stem cells, and the acceleration of subsequent bone formation. Moreover, modification of mineralized nanofibers with bio-functional molecules is expected to be a promising area of future research because the apatite mineral has strong affinity to certain bone-specific proteins which contribute enhanced bonding to bone tissue [92,93].

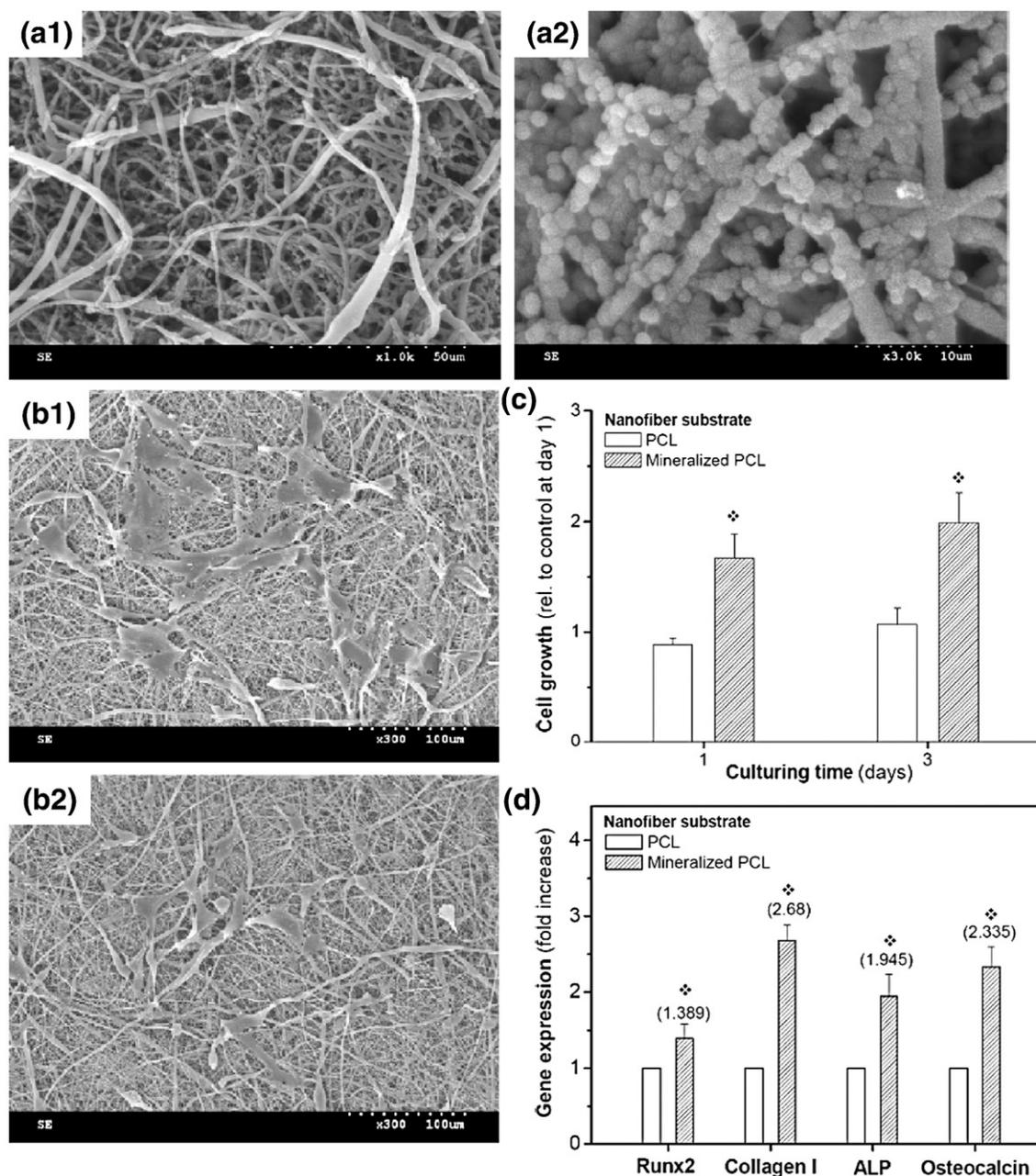


Fig. 10. (a1,a2) Morphology of the surface-mineralized PCL nanofibrous matrix obtained by a series of solution-mediated mineralization steps involving activation in an alkaline solution, alternate soaking in Ca- and P-solutions, and immersion in SBF. (b1,b2) Initial osteoblastic cell response showing better spreading behavior on the mineralized PCL nanofiber (b1) than on pure PCL nanofiber (b2) at 8 h of culturing. (c) Cell viability and (d) expression of bone-associated genes were significantly enhanced by surface mineralization. Adapted with permission from [87] 2007 Wiley Interscience Co.

703 More general than the mineral phase is the bioactive macromole-
 704 cules that have been introduced on the surface of polymeric nanofibers,
 705 including proteins, peptides and drugs, to regulate and improve specific
 706 biological functions. Nonetheless, few studies have examined the
 707 applicability of macromolecules in bone regeneration area. A few
 708 studies used RGD (Arg-Gly-Asp) peptides to adhere onto polymeric
 709 surfaces and reported the biological effects of the cell adhesive ligands,
 710 such as adhesion, spreading and growth, using a range of cell types
 711 [94,95]. Fibroblast adhesion, spreading and growth were enhanced
 712 when the GRGDY peptide was immobilized on PLGA and its copolymer
 713 with PLGA-b-poly(ethylene glycol) (PEG)-NH₂ nanofiber [94]. Given
 714 that the major weakness of the synthetic polymeric surface is the poor
 715 cell affinity, the use of adhesive proteins or peptides is believed to be an
 716 appropriate way of improving the initial bone cell responses and
 717 possibly further biological steps. Our recent study also developed the

718 surface of poly(lactic-co-caprolactone) (PLCL) nanofibers by covalently
 719 linking with a fibronectin peptide containing a central cell binding
 720 domain to improve the initial cell adhesion and spreading behavior of
 721 osteoblastic cells. Parallel applications were also suggested using bone
 722 target proteins and peptides, such as growth factors and bone
 723 morphogenetic protein family. Together with the types of macromole-
 724 cules, the selection of a coupling method and the maintenance of their
 725 biological activity should be fully considered to gain the optimal
 726 performance of biomolecules on a nanofiber surface.

4.2. Drug encapsulation within nanofibers

727
 728 When macromolecules are coupled onto the surface of nanofibers,
 729 maintenance of their chemical stability and biological activity for
 730 prolonged time course is of special importance. Therefore, the

encapsulation of drugs within the nanofibers might be favored, which will be particularly useful for controlled release systems. In this case, the encapsulation method and drug efficiency as well as the eluting profiles need to be designed and investigated carefully. In particular, the drugs chemically bound to the matrix with respect to those physically mixed/adsorbed may sustain their elution for longer period, and depending on the encapsulated status, drug release kinetics is greatly affected. A range of drugs have been encapsulated within the nanofibers of polymers, including antibiotics, bone morphogenetic protein, and even genes [96–101]. Although not all were targeted for bone tissue, the method is believed to be suitable for bone reconstruction. Poly(lactic-co-glycolic acid) (PLGA) nanofibers mixed with a hydrophilic block copolymer

were incorporated with antibiotics (Mefoxin®, cefoxitin sodium), and the nanofiber mesh showed potential to entrap drugs and then release them in a sustained manner, ultimately inhibiting bacterial activity [96,97]. For the specific delivery of osteogenic signals, BMP-2 was encapsulated directly within the blending polymer of silk and polyethylene oxide to show enhanced mesenchymal stem cell differentiation into the osteogenic lineage and calcification [98]. For gene delivery within the nanofibrous matrix, DNA was first encapsulated within a block copolymer poly(lactide-co-glycolic acid)–poly(ethylene glycol), which was further electrospun in concert with the PLGA solution [100]. The results showed that the nanofibrous matrix delivered DNA that was capable of cellular transfection and encoding protein β -galactosidase [100]. Recent

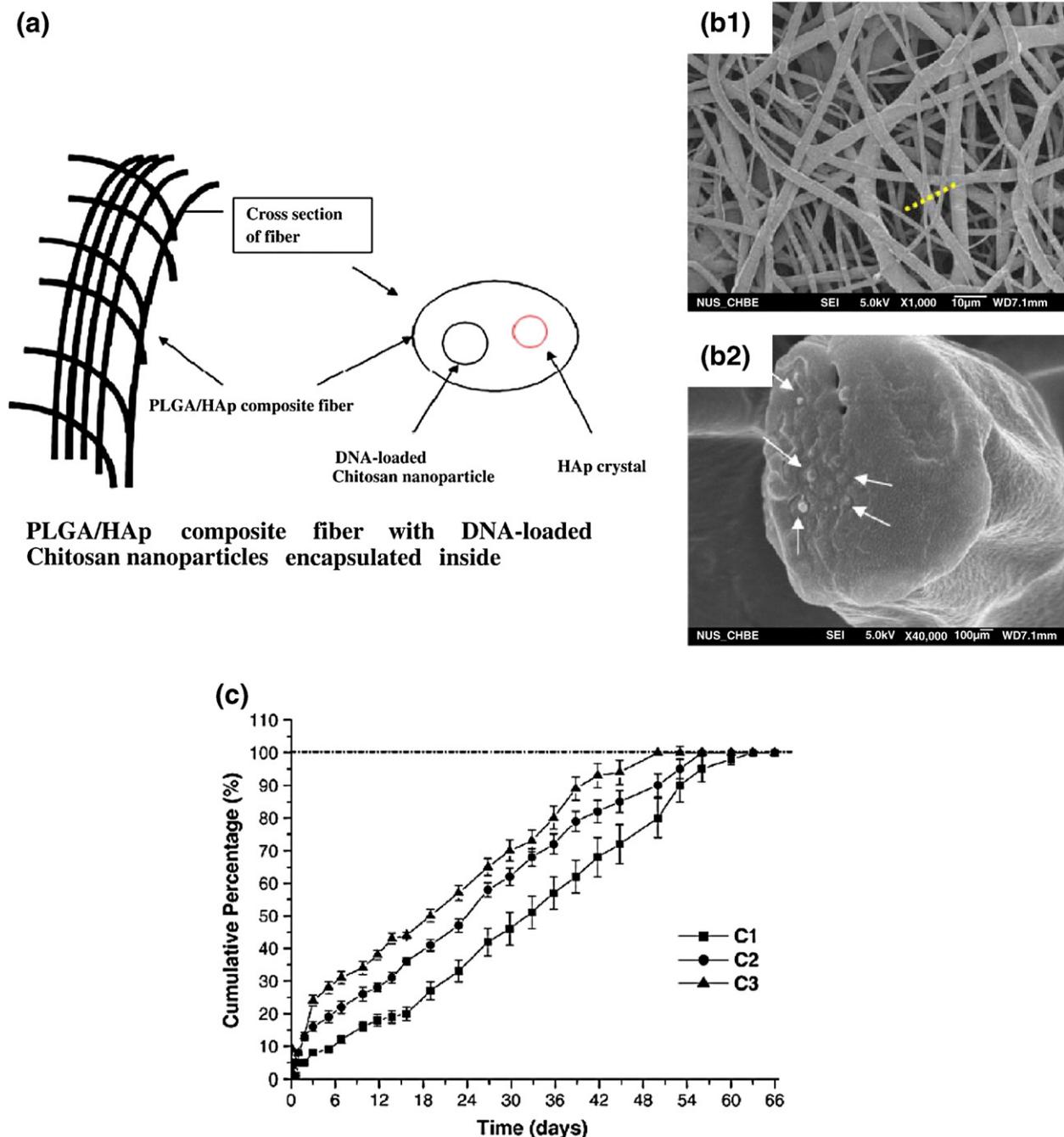


Fig. 11. (a) Illustration of the gene-delivering nanofiber scaffold showing the DNA first secured within the chitosan nanoparticles and then electrospun with a PLGA solution to generate a DNA-incorporated nanofiber. (b1) SEM image of the nanofiber incorporating DNA/chitosan. (b2) Enlarged image of the fiber cross-section revealing the presence of DNA/chitosan nanoparticles. (c) *In vitro* release profile of DNA from nanofibers incorporated with DNA/chitosan nanoparticles, showing continuous release for up to ~60 days. Adapted with permission from [101] copyright 2007 Elsevier.

study on specific targeting for bone tissue has been reported by Nie et al., where they used the PLGA/hydroxyapatite composite nanofibers to deliver BMP-2 plasmid DNA [101]. In particular, the DNA was pre-loaded within chitosan before electrospinning the PLGA/hydroxyapatite solution. The results demonstrated that the nanofiber encapsulated with DNA/chitosan had higher cell attachment and viability as well as more desirable transfection efficiency than the nanofiber surface-adsorbed with naked DNA or surface-adsorbed with DNA/chitosan (Fig. 11) [101]. Pre-encapsulating genes within nano-vehicles before electrospinning is thus considered an appropriate way of securing the biological stability of genes and improving the transfection efficiency. Although studies on gene delivery with nanofibrous matrices are still in the early stages, this area may be a future direction in the bone regenerative medicine using the nanofibers [102].

Novel designing of the electrospinning apparatus permits advances in the drug delivery technology. A dual tip (syringe) apparatus, so-called

co-axial electrospinning, which was designed to produce a core-shell structure of the nanofiber, was reported to contain and release drugs more efficiently [103,104]. Drug-containing solution to be placed in the core part was electrospun simultaneously with the material solution to be allocated at the outer layer. In this case, while the inner solution affects the drug loading efficiency and stability, the properties of the shell layer can control the drug release profile. Furthermore, depending on the drug properties, suitable materials and solutions should be selected for the core-shell nanofiber structure. Modulation of the morphological and chemical properties of nanofiber materials is the key to controlling the drug delivering ability [9]. This drug delivering potential greatly strengthens the ability of artificial scaffolds to guide osteogenic differentiation of stem cells and to generate bone analogs in bone tissue engineering approach. As new knowledge on novel materials becomes available, more extensive works are expected in tissue engineering nanofibrous scaffolds with therapeutic targeted for bone.

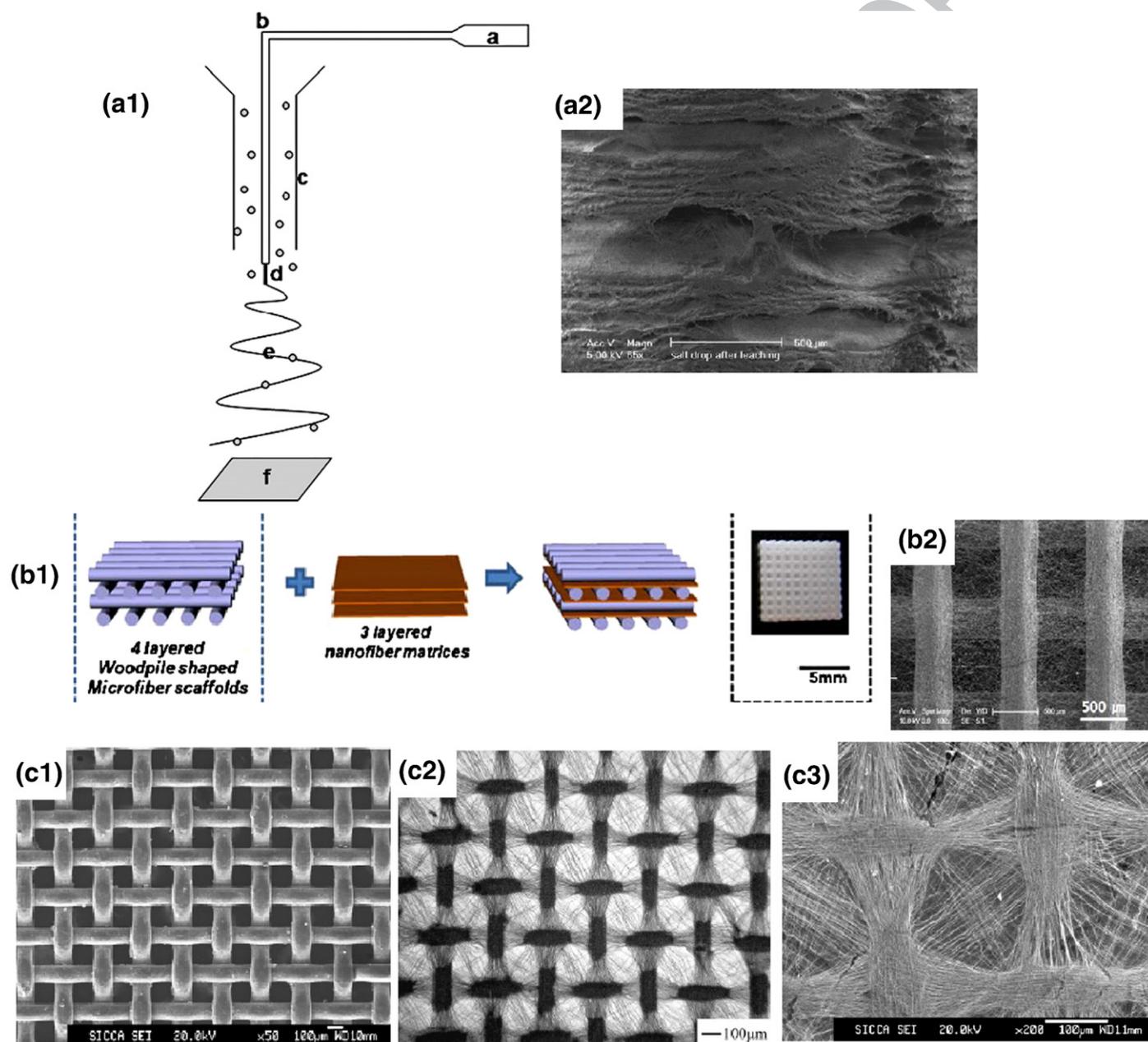


Fig. 12. Scaffolding techniques of the electrospun nanofibers: (a1,a2) Salt particle incorporation and leaching method; (a1) schematic diagram and (a2) generated macropores ~100 μm in size. Adapted with permission from [109] 2008 copyright Mary Ann Liebert. (b1,b2) Electrospinning combined with a direct deposition method; (b1) schematic diagram of the process and (b2) generated micro-nanoscaffold. Adapted with permission from [112] 2008 copyright Acta Materialia. (c1–c3) Patterned conducting polymer method; (c1) knitted conducting polymer collector and (c2,c3) produced patterned scaffolds. Adapted with permission from [113] 2007 copyright Wiley-VCH Verlag GmbH & Co.

787 4.3. Scaffolding for cell growth and tissue engineering

788 More widespread use of electrospun nanofibers for tissue engineering
 789 applications has been a challenge due to their difficulty in 3-
 790 dimensional shaping and macroporous scaffolding. Processed by a type
 791 of line-of-sight approach, electrospun fibers are first gathered in the
 792 form of a 2-dimensional sheet and then piled up 3-dimensionally with
 793 increasing spinning time. Although some collector designs help shape
 794 nanofibers into simple forms, such as tubular forms, much more
 795 complex shapes are still on demand [105]. Above all, interconnected
 796 macro-pores are essential for vascularization in order to supply oxygen
 797 and nutrients, provide sufficient space for cell ingrowth and drain the
 798 consumed metabolites [106,107]. Although the electrospun nanofibrous
 799 structure generates a network of open-pores, the pore sizes are about
 800 the same order of the fiber sizes, i.e., at best a few micrometers. Some
 801 studies provided evidence of *in vitro* cell penetration and *in vivo* tissue
 802 formation within the nanofibrous network, where thin membranous

803 substrates were used [57,108]. In particular, the ex-vivo culturing of
 804 tissue cells within nanofibers to construct uniform cell-material
 805 constructs is a significant challenge. Moreover, the reconstruction of
 806 larger and complex-shaped bone defects requires 3-dimensional
 807 shaping of the nanofibrous scaffolds with interconnected macropores.
 808 Otherwise, new technological tools to develop 3-dimensional tissue
 809 mimicking cell-nanofiber constructs should be explored.

810 Some studies have reported a level of success on the scaffolding of
 811 electrospun nanofibers [109–113] (Fig. 12). Salt particles were
 812 incorporated within the polymer nanofibrous matrix, which then
 813 leached out to generate some macropores [109]. Furthermore, salt
 814 leaching and gas foaming techniques have been combined to produce
 815 some macropores within a clay-reinforced PLA nanofibrous structure
 816 [110]. One approach used the microfibrinous mesh as a rigid
 817 supporting structure upon which the nanofibrous network was covered
 818 by electrospinning to produce a micro-nano fibrous scaffold [111].
 819 However, the process can only produce a scaffold with a limited

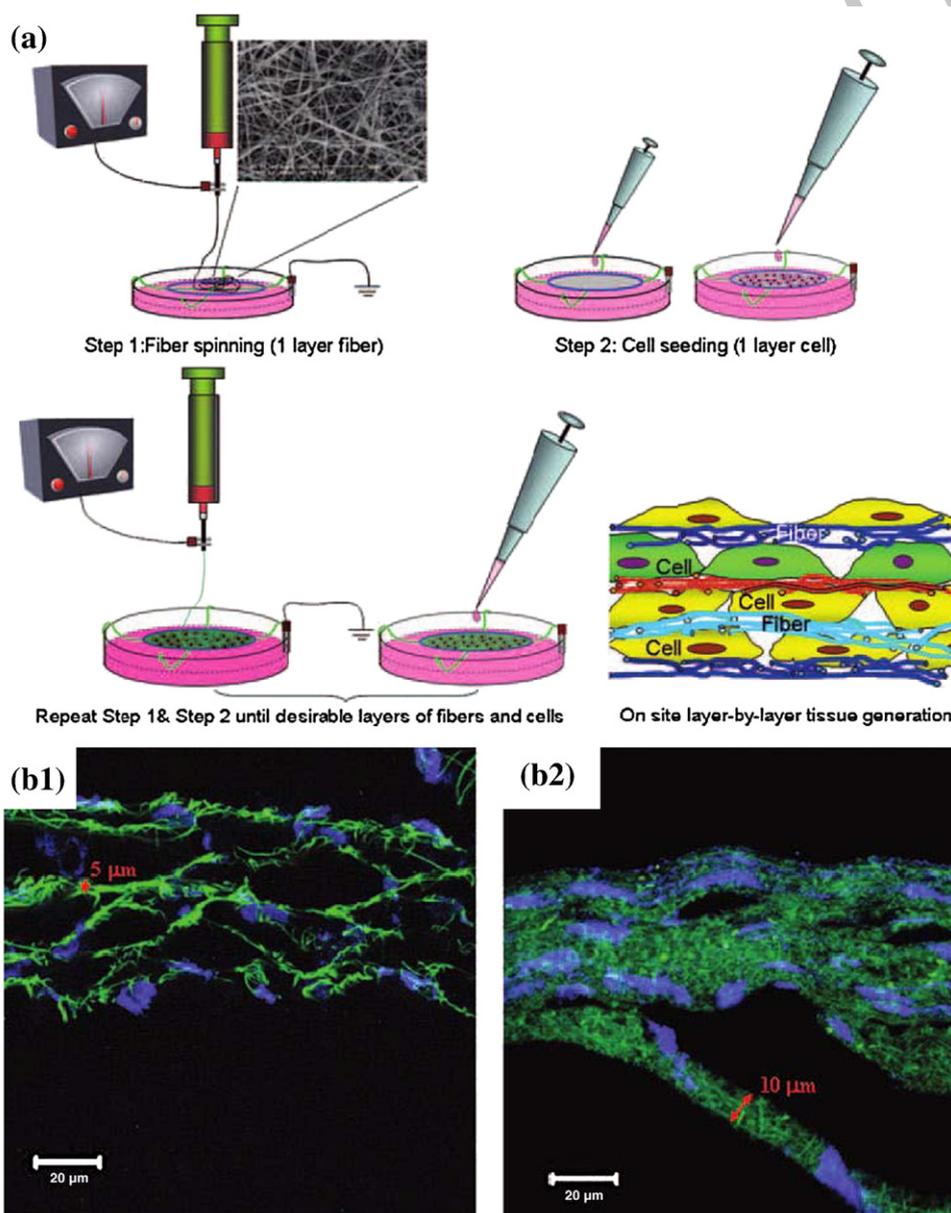


Fig. 13. Layer-by-layer approach for tissue engineering: (a) Schematic diagram of the on-site layer-by-layer cell assembly on the electrospun fibers. Both fiber and cell layers can be varied during cell assembly to create a customized final 3D construct. (b1,b2) Multi-layered cell–fiber constructs with two different fiber layer thicknesses. Fluorescent micrograph of DAPI-stained cross sections of fiber–cell constructs cultured for 2 days. Nuclei, blue. The fibers were labeled with FITC (green), and the cells were stained blue by DAPI. Adapted with permission from [114] 2008 copyright Mary Ann Liebert.

thickness. A similar approach aimed at producing a thicker nanofibrous network on a microfibrous structure using alternate processes of electrospinning and direct deposition of polymer melt [112]. Ultimately, those approaches attempted to combine the 3D scaffolding merit of a microfibrous support with a nanofibrous network. The cell responses were significantly enhanced when the electrospun nanofibrous network was present on the microfibrous scaffold. Modifying a collector part with conducting patterned polymers made it possible to pattern a nanofibrous network [113].

One recent report showed the engineering of 3D tissue constructs using a thin nanofibrous substrate [114]. A tissue-mimicking 3D construct was developed by the alternate stacking of cells and thin nanofiber substrate (Fig. 13). The idea was to culture the cells on the thin 2D nanofibrous substrate and then build 3D cell-nanofiber constructs using a layer-by-layer approach. As the cells can easily penetrate a thin layer of nanofibers, the method was proposed to mimic the native 3D tissue structure. Although suggested particularly effective for engineering layered tissues, such as skin, the approach can also be applied to the elaboration of 3D bone structure.

As described above, some technological advances are in progress to fully utilize the electrospun nanofibers in tissue engineering applications, including bone regenerative area. Given that the scaffolds for bone tissue engineering need to be qualified for specific mechanical properties as well as for biological compatibility, the 3D structural design of nanofibers and their scaffolding with tissue cells should be considered carefully in order to achieve properties analogous to the native bone ECM. Although few studies have been carried out using a nanofibrous matrix in bone tissue engineering, promising outcomes may be reported in the near future.

5. Concluding remarks

A significant amount of research has been directed to electrospinning nanofibrous materials targeted for bone regeneration. The selection of materials with the appropriate composition is of utmost importance in the successful generation of bone ECM mimicking matrices suitable for neo-bone formation. As described in this review, a range of degradable polymeric materials have demonstrated utility for bone regeneration. In particular, recent efforts have been focused on the incorporation of bioactive inorganic nanoparticles within the polymeric phase reaping up the combinatory roles of bone-bioactivity and rigidity of inorganic phase and degradability and shape-formability of polymers. Moreover, there is increasing research on the surface functionalization of nanofibers, such as mineralization of the polymeric surface and coupling with proteins/peptides, to regulate cell functions from the initial cell adhesion to osteogenic stimulation of progenitor/stem cells. Materials that can elicit therapeutic effects by incorporating bio-signaling molecules within the nanofibers, such as antibiotics and proteins and genes pre-loaded in nanocapsules, hold great promise as scaffolds with drug delivery potential. To make full use of 3D cell culturing and tissue engineering, there has been considerable research aimed at developing macroporous morphology as well as shaping the nanofibrous structure by apparatus design and engineering cell-material constructs.

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