Synthetic Multi-level Matrices for Bone Regeneration

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Chapter 6
Synthetic Multi-level Matrices for Bone Regeneration

Nicholas R. Boyd, Richard L. Boyd, George P. Simon, and David R. Nisbet

Abstract Current bone replacement strategies are clinically inadequate, yet there is great promise in the use of synthetic adjuvant matrices. Electrospinning provides a three-dimensional platform in which matrices can be designed to mimic features of the extracellular matrix and improve bone regeneration. Composite nanofibers can be functionalised with therapeutic molecules, and/or may permit the delivery of growth factor combinations as required to stimulate bone healing. Collectively, these should more precisely direct repair by exogenous and endogenous stem and progenitor cells. The real novelty will be in combining multiple levels of scaffold-based tissue engineering developments in an "off the shelf" clinic-ready product. Until then, application of bioactive nanofiber analogues, with dual-scale three-dimensional porosity that can be co-interfaced within effective stem cell treatment regimes, will be crucial in developing smart matrices for skeletal repair. This review presents holistic concepts for more effective bone regeneration and the methods in which they can be incorporated into nanotechnology-based scaffolds from a materials engineering perspective.

Abbreviations

ALP	Alkaline phosphate
BMP-2	Bone morphogenetic protein-2
BMSCs	Bone-marrow-derived stromal cells
BSP	Bone sialoprotein-2
ECM	Extracellular matrix
FAp	Fluoroapatite

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FDA	Food and Drug Administration
FGF-2	Fibroblast growth factor-2
HAp	Hydroxyapatite
Hep	Heparin
Hep-S	Heparan sulphate
LbL	Layer-by-layer
MMT	Montmorillonite
MSCs	Mesenchymal stem cells
MWCNT	Multi-walled carbon nanotube
MWNT	Multi-walled nanotube
OC	Osteocalcin
OP	Osteopontin
OPG	Osteoprotegerin
PCL	Poly(ε-caprolactone)
PDGF	Platelet-derived growth factor
PEO	Polyethylene oxide
$P_{DL}LA$	Poly($_{DL}$lactide)
PGA	Polyglycolic acid
PLA	Polylactic acid
P_LLA	Poly-L-lactide acid
RANKL	Receptor activator of NF-κB ligand
RGD	Arg-Gly-Asp
rhBMP-2	Recombinant human BMP-2
SBF	Simulated body fluid
SEM	Scanning electron microscopy
SIS	Small intestinal submucosa
SPARC	Osteonectin
TCP	Tricalcium phosphate
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor β
TSP	Thrombospondin
VEGF	Vascular endothelial growth factor

6.1 Introduction

One of the ironies of improvements to general health and living conditions that lead to an increase in average life span is a higher incidence of skeletal disease. While some treatments do exist, they are usually problematic due to a lack of long-term success and insufficient clinical efficacy. More sophisticated approaches, such as artificial bone substitutes, are thus required on a global scale. The rapidly evolving interface between nanotechnology and biological sciences provides an optimistic outlook.

Bone-related diseases, skeletal abnormalities and physical trauma can often lead to defective skeletal support, such that bone may no longer be able to regenerate and

repair naturally [1]. Osteoporosis, osteonecrosis, bone cancer, osteoarthritis, rickets, Paget's disease and osteogenesis imperfecta [2–8] cause an enormous strain economically and on the patients quality of life. In 1997, it was estimated that osteoporosis affected 75 million people in Europe, USA and Japan alone [9], with alarming projections estimating that the incidence in hip fracture as a result of osteoporosis will double by 2025 [10]. Throughout orthopaedics, neurosurgery and dentistry, some 2.2 million bone-graft surgeries are already performed annually [11]. Two major issues are rapidly becoming clear: (1) the need for bone grafts is increasing and (2) the longevity of existing bone-graft therapies needs to improve in order to avoid revision surgery and to accommodate the expected increase in patient lifespan post-surgery [12, 13]. Consequently, new strategies and more sophisticated materials are required for restoration of bone/joint functionality.

Current bone grafts are burdened by limited availability, infection and morbidity, poor mechanical properties and over-elevated bone resorption [1, 14]. While the traditional "gold standard" autograft has shown a degree of clinical success, only small skeletal defects can be treated and they are very invasive [14]. Alternatively, problems with allografts are compounded by immune rejection, and immunosuppressive drugs carry the added risk of susceptibility to infection [1, 15]. Synthetic biomaterials have provided an alternative solution with advantages in availability, versatility, precision, reduced immune rejection and the potential for smarter, biologically instructive and active bone-graft templates. Most common hard orthopaedic biomaterials, e.g., titanium hip implants, are largely bio-inert which leads to limited integration with endogenous tissue and poorly sustained functional restoration [16]. Numerous attempts to improve the integration of the implant via surface treatment of the titanium [17] have included mechanical roughening, chemical treatment, sol-gel coating, ion implantation and thermal spraying of HAp. However, major limitations still persist and the ability to achieve appropriate mechanical and biological properties has yet to be achieved simultaneously.

Potentially, the most promising fabrication technique for the next generation of synthetic bone grafts is electrospinning. This provides a simple, yet highly versatile bottom-up approach [18], in which a vast array of porous nanofiber matrices can be manufactured by accelerating a jet of charged polymeric liquid under the presence of an electric field [19–21]. A wide range of polymeric, ceramic and composite nanofibers can be produced via electrospinning [18]. In turn, the diversity of electrospun membrane properties and functionality can be manipulated relatively easily. Electrospun fibers are amenable to post-processing surface treatments including deposition of bioactive nanoparticles, attachment of growth factors and bioactive proteins. However, while the approach is simple, the physical science behind electrospinning is quite complex [20, 21] and difficult to control. The challenge is clear: can innovative new strategies be developed to optimise the utility and function of three-dimensional tissue analogues?

Currently, the mechanical properties of electrospun scaffolding for bone tissue engineering are generally inadequate for therapeutic applications as load bearing bone-graft substitutes. While their in vivo implementation is in its infancy, direct avenues for their clinical application exist and have advanced as guided-bone-regeneration membranes in areas such as dental restoration. Moreover, augmentation in non-union/delayed fracture healing and in replacement of segmental gap defects

may support revolutionary therapeutic advances if improvements can be made to tailor their mechanical properties.

This review firstly introduces basic bone biology and healing redevelopment processes as a platform for scaffold design. A hierarchy of physical and biochemical inputs have been utilised for stimulating bone regeneration, yet have not been successfully co-incorporated to date. Hence, the focus of this review discusses the most recent, novel and promising advances in scaffold engineering designed primarily for bone tissue reconstruction. Effective three-dimensional synthetic biomatrices for bone regeneration are thought to require surface and structural biomimicry, in conjunction with effective stem cell treatment strategies.

6.2 The Platform for Design: Biology

The successful growth of cells on synthetic matrices is dependent on synergies between topographical, biochemical and mechanical stimuli that best reflect the in vivo microenvironment. Understanding these biological functions, structures and healing processes is therefore crucial for creating relevant inputs into the adjuvant bone graft. Material engineering strategies alone have not yet progressed to the point where they can recreate bone analogues accurately, due to the dynamic nature and intricate hierarchy of bone.

6.2.1 Bone Structure

Bone is a highly organised connective tissue that principally provides structural support for skeletal motion, protection of internal organs and a mineral reservoir for homeostasis of chiefly calcium and phosphate [22–24]. Furthermore, bone supports the primary production of blood cells via haematopoiesis [16], has unique self-regenerative capabilities [25] and an exceptional light-weight combination of strength, stiffness, fatigue resistance, fracture toughness and porosity [26, 27]. The specialised physical and biological capabilities of bone are founded on the complex ECM hierarchy (reviewed in refs. [26, 28]) and subsequent dynamic relationship with bone-associated cells.

At the nanoscale, intricately organised mineralised collagen fibrils (individual fibril (1.5–3.5 nm), fibers (50–70 nm), and bundles of fibers (150–250 nm) [26] form the building blocks of the ECM. A complex, interpenetrative and cooperative network of organic and inorganic components underpins the highly advanced mechanical properties of bone at all scale lengths [26, 29–31]. Bone-apatite nanoplatelets based around the chemical structure of HAp, $Ca_5(PO_4)_3OH$, occupy approximately 70 wt% of bone [32] and are dispersed throughout a highly organised and cross-linked [33, 34] periodic stacking [26, 30, 34] of triple helical collagen type I molecules [31, 35], referred to as tropocollagen. HAp platelets act

as stiffening components and mineral reservoirs, and have been reported to be located on the fiber surface, as well as within the fiber [36, 37]. The organic component makes up about 25 wt% of bone [32], with more than 90% being collagen type I [28]. A small fraction of type V collagen coexists, which facilitates the assembly of fibrillar structures [38, 39], as do several non-collagenous proteins [40]. While only accounting for a minor contribution (approximately 10% [28]) [41], OC [42], SPARC [43, 44], fibronectin [44], BSP [45] and TSP [46–48] play crucial biochemical roles in the ECM.

6.2.2 Bone Maintenance and Remodelling

Bone, like most tissues, undergoes continual self-renewal and requires repair and remodelling following damage. Homeostatic maintenance of bone structure essentially involves a continuing flux between bone resorption by osteoclasts and reformation by osteoblasts, which is initiated through mechanical stimuli and ECM-cellular communication. Osteoclasts are bone-resorbing macrophage-cells, which locally secrete hydrochloric acid that facilitates localised mineral dissolution, followed by up-regulation of cysteine-proteinases (importantly cathepsin K) that degrade the organic matrix [49, 50]. Osteoblasts are bone-forming cells derived from MSCs which are present predominantly in the endosteal niche and surrounding marrow vasculature [51]. New bone is developed through osteoblastic synthesis, deposition, mineralisation and organisation of the ECM. This includes production and secretion of type I collagen, bone apatite and a vast variety of non-collagenous proteins [40], cytokines and growth factors [24, 52].

Virtually all tissues derive growth and differentiation occurs through dynamic interaction with the surrounding ECM. Bone, however, displays uniquely adaptive properties, as the cells and bone remodelling is distinctly sensitive to mechanical stress [53]. Physical forces imposed on bone are translated and interpreted via three-dimensional osteocyte networks through mechanotransduction (ECM-cell cross-talk) [54], which correspondingly feeds instructive signals to regulate osteoblastic and osteoclastic behaviour [53]. On a molecular basis, the structure of bone converges around two key molecules: OPG and OPG-ligand, also known as RANKL [55]. With structural damage to the ECM, including tropocollagen uncoiling, fibrillar sliding and tearing, generation of microcracks, lamellar sliding [34] and macroscopic fracture, new remodelled bone is required.

6.2.3 Bone Healing

Bone healing is complex. A highly regulated sequence of multi-tissue activated events, referred to as the regeneration cascade, proceeds at the initial point of healing. Overlapping of each developmental stage occurs, with multiple cell types and signal

cascades acting in harmony [56, 57]. Unlike soft-tissue healing, bone healing does not leave an obvious scar, the process being regenerative, rather than solely reparative [32, 58, 59]. There are direct links between the mechanisms involved in initial skeletal tissue formation and the fracture healing cascade [60]. Fracture size and mechanical stability are crucial in defining the success of the healing mechanism [61].

The intricacies and multiple-tissue development involved in secondary bone healing have yet to be effectively incorporated in synthetic bone tissue engineering scaffolds. It is thought that the endogenous healing mechanisms activated in response to bone-graft implantation follow similar progressive regeneration as are involved in fracture repair. Indeed understanding the involvements that the various components play in fracture healing is a key feature for designing relevant scaffolds for bone grafts. Bone fracture is followed by an inflammatory response that involves haematoma formation, recruitment of MSCs and activation of inflammatory signals and osteogenic stimulators. A temporary cartilaginous matrix replaces the initial haematoma in order to stabilise the fracture site, which is crucial for facilitating subsequent angiogenesis and primary bone formation. Finally, the woven primary bone fracture callus is reorganised into functional bone. The biological processes, cellular involvement and predominating growth factors involved in each stage of bone regeneration have been previously reviewed in detail [56, 57, 62–64]. Many of these growth factors have successfully been incorporated into synthetic adjuvant scaffolding with therapeutic stimulation of bone repair.

6.3 Levels of Matrix Sophistication

The evolving forefront of nanotechnologies, cellular and molecular microbiology and biomaterial science provides locus for developing highly effective tissue engineering materials on a range of size scales. Each level of sophistication provides an essential biomimetic design input to assist in instructing and stimulating the in vivo cellular niche.

6.3.1 Electrospun Fibers

6.3.1.1 Natural and Synthetic-Based Polymeric Nanofibers

Electrospun fibers alone are by no means ideal for biomatrices, despite some preliminary success in bone tissue engineering [65]. They are, however, of crucial importance as they form the base-framework in the design of advanced bone tissue constructs since they are morphologically and dimensionally similar to collagen in the endogenous ECM. Initial studies were based on this premise and made important contributions to the mimetic prototypes.

Investigation of cellular response on different fiber diameters have lead to a variety of results [66–70], few of which have been well substantiated [71].

Cellular preference to fiber size is intrinsically difficult to isolate, as with changes in electrospun fiber size, there is subsequent change in the inter-fiber spacing [72, 73], mechanical properties [74] and biodegradability; all of which have been shown to mediate cellular behaviour and underpin the entire performance of the synthetic bone graft. Furthermore, depending on the cell phenotype, preferential fiber size and porosity for maximum cell adhesion may peak at multiple points [71]. It has been well shown that osteoblastic cells respond more favourably to nano-topographic cues demonstrating superior cellular adhesion, growth, proliferation and differentiation [75], similarly seen with co-incorporating nanofibers in microporous scaffolds [73, 76]. Interestingly, cellular behaviour on nanofibers was improved with enhanced levels of bio-stimulative factors in the culture medium [68, 69], thus suggesting synergistic interactions and the necessity for multiple microenvironmental inputs to satisfy the cellular niche.

More recent developments have focussed on advanced electrospun systems and the incorporation of bone-specific bioactivity. As bone has the inherent capacity to regenerate, biodegradable materials have been predominately used. Synthetic polymers, most commonly polyesters (PLA, PGA, PCL, etc.) and their various copolymers, offer the advantage of substantial flexibility in electrospinning processability, mechanical modification, biodegradability and are of relatively low expense. Furthermore, of clinical translational importance, many have FDA-approved biocompatibility and have been widely investigated in other areas of tissue engineering and medical applications. Polymers, such as PCL, have the capacity to be synchronised with the redevelopment rate of endogenous bone in vivo, and the degradation by-products do not overly increase the cellular microenvironment acidity [77], making it a more viable solution for sustained cellular mobilisation and angiogenesis [77]. However, most synthetic polymers are hydrophobic (hence showing poor cell attachment) with limited bioactivity and functional groups for post-tethering of growth factors and proteins [78]. Electrospun natural polymers, which include collagen [79], gelatin [80], silk fibrin [81, 82] and chitosan [83], have the key advantages of exhibiting enhanced biofunctional motifs, are much more hydrophilic and in turn support superior cell adhesion [78]. Initial cell adhesion and survival is eminent in the consequential success and effectiveness of the bone graft. However, natural polymers have greater structural, chemical and molecular weight variability and a higher possibility of immune rejection. Furthermore, there is much less versatility in regards to electrospinning control and ease, in that only few solvents do not denature natural polymers, narrowing the window of solution and operating conditions to permit electrospinnability.

There has been substantial investigation into combining the properties of both natural and synthetic polymers via electrospinning various polymer blends such as PCL with Hep-S [84], gelatin [85] and silk fibrin [78, 86]. The benefits of co-incorporating natural and synthetic polymers have been demonstrated in a comparative study by Lee et al. [87], highlighting that the addition of collagen into PCL significantly increases hydrophilicity, degradation rate, mechanical elongation, cell adhesion and growth in vitro, cellular infiltration in vivo, and up-regulation of OP, ALP activity and collagen type I in osteoprogenitor cells. Furthermore, the blending of SIS with PCL in an layered micro/nano dual fiber size construct provided

approximately a fourfold increase in proliferation of BMSCs, possibly linked to an increase in hydrophilicity and thus cell adhesion [86]. Importantly, incorporation of layers of electrospun fibers without SIS had no significant impact on the cellular response. Blending PCL with Hep-S [84], a carrier for human MSCs, improved the in vitro growth of these osteoprogenitor cells. Heparin alone showed some improvement, but only half that of Hep-S. Indeed Hep-S has the intrinsic ability to regulate numerous functional growth factors involved in post-fracture bone [88]. When implanted, there was no protective carrier for the cells, so any impact of the Hep-S on bone formation could not be accurately evaluate given the likely intervention of host inflammatory responses [84].

6.3.1.2 Inorganic Nanofibers

A novel approach to overcoming the polymer nanofibers shortcomings (which include limited integration with the host tissue and insufficient mechanical properties) is to electrospin inorganic (ceramic) nanofibers, shown in Fig. 6.1. While the material processing of such nanofibers has been well reported [89], their involvement in the bio-industry has been relatively untapped, in particular their effects on cellular responses in vitro and in vivo. Inorganic electrospun fibers have predominately been obtained via electrospinning sol-gels of polymer and ceramic components, followed by heat treatment up to 700 °C [90] to burn the binding polymer off, thus leaving ceramic fibers. Nanofibrous matrices of silicate [91], bioactive glass [90, 92, 93], FAp [94] and HAp [95] are potential integrative materials for bone tissue regeneration. Bioactive glass has intrinsic anti-inflammatory and anti-microbial capabilities [96], the capacity to up-regulate the secretion of growth factors [97], and can stimulate mineralisation [92]. Cell viability and ALP activity of bone-marrow-derived human MSCs on bioactive glass nanofibers significantly superseded that of bioactive glass discs and PCL nanofiber controls [90]. Despite superior bioactivity and dramatic increases in modulus and mechanical strength, inorganic nanofibers have the limitations of being too brittle as bone-graft candidates [95, 98].

6.3.1.3 Composite Nanofibers

The hybrid material properties obtained via biodegradable polymers and bioactive particles within electrospun nanocomposites are intuitively more suitable for functional bone substitutes. Pre-blending HAp nanoplates [99, 100], carbon nanotubes [101], bioactive glass [102] and various calcium phosphates within electrospun biodegradable polymers has been a logical, exciting and successful approach for promoting cell anchorage and bone-specific bioactivity with improvements in fiber mechanical properties, nano-roughness and hydrophilicity. Advances in osteoconductivity (the recruitment and stimulated in-growth of osteoprogenitor cells and neo-blood vessels from the host tissue into the scaffold [59, 103]) and osteoinductivity (the capacity to induce bone formation directly [59, 103]) have been achieved with the integrated complexity of such a composite system.

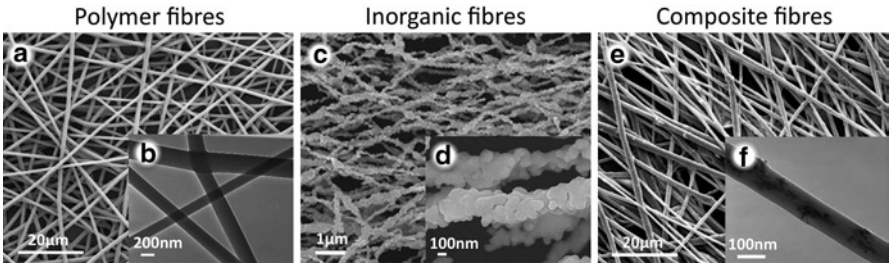

Fig. 6.1 Electrospun polymer, inorganic and composite fibers. (**a**) SEM image of electrospun P_LLA (860 ± 110 nm) (adapted with permission from [100]). (**b**) TEM image of electrospun PVA/collagen nanofibers (~245 nm) (adapted with permission from [104]). (**c**) SEM image of aligned HAp nanofibers post-removal of poly(vinyl pyrolidone) via heat treatment at 600 °C for 6 h. (**d**) High magnification SEM image of HAp nanofibers displaying fusing of adjacent HAp nanorods (diameter of ~7 nm and length of ~27 nm); (Both (**c**) and (**d**) adapted with permission from [95]). (**e**) SEM image of blended P_LLA/HAp (wt ratio 4:1) electrospun composite fibers: 845 ± 140 nm (adapted with permission from [100]) (**f**) TEM image of composite PVA/collagen/HAp nanorod blended nanofibers (~320 nm) (adapted with permission from [104])

Apatite/Polymer Composite Fibers

Biomimetic electrospun structures consisting of nano-HAp with various biodegradable polymers have been well explored [99, 104] (refer to Fig. 6.1). The effectiveness of nanoparticulate, reinforced polymeric matrices is critically dependent on the dispersion uniformity and binding affinity at the polymer–particle interface. Surfactant adsorption of hydroxysteric acid [105] and Hep [106], grafting of PCL [107], electrostatic dispersion via pH changes [108] and HAp silica coating [109] feature as some of the possible techniques to reduce the inherent tendency for nano-HAp particles to aggregate, thereby improving their interaction with the matrix.

Recently, more sophisticated additions including incorporation of chemical variations of apatite have shown potent effects in stimulating the cellular response. TCP [110] and carbonated-apatite [111] dissolve faster than nano-HAp and are more readily taken up by osteoclasts, whereby an initially rich calcium and phosphorous microenvironment facilitates more rapid bone redevelopment. Stable bone-specific inorganics, such as FAp [94], sustain slower calcium dissolution rates, where combining it with HAp (40% FAp:60% HAp) enhanced osteoblastic cell survival and expression of OPG, OC and BSP-1 in vitro on flame-sprayed orthopaedic coatings [112]. Similarly, Arinzeh et al. reported a combination of HAp with β-TCP (20:80) promoted far higher MSC bone deposition than each individual component in vitro and in vivo [113]. Intuitively, a combination of inorganic crystals that can lead to rapid osteoconductivity and osteoinductivity for primary fixation stability and integration with the host tissue, while maintaining osteogenesis until the defect has healed, are best suited. However, dissolution is only one parameter that determines the choice of inorganic bioactive ceramics; structural integrity during degradation, biocompatibility, particle size, shape, crystallinity, surface chemistry, and binding and dispersive interaction with the polymer co-component are all vital for controlling the endogenous response.

The necessity to provide a combination of bio-stimulative phases has been supported by statistically higher cell number and ALP activity of adult human BMSCs on combinations of HAp (50:50) with two different bioactive glasses than on each bioactive glass and HAp nano-rough ceramic surface alone [114]. The combinatorial benefits of HAp with bioactive glass has also recently been applied to Ti-orthopaedic implants and shown to supersede the initial cell adhesion, proliferation and sustained osteogenic differentiation of commercially available HAp coatings [96].

Carbon Nanotubes in Electrospun Fibers

As a result of their highly specialised combination of aspect ratio (surface area), strength, stiffness, light weight and versatile surface chemical functionalisation [115], the incorporation of carbon nanotubes within electrospun polymers has provided a unique platform for bone tissue engineering. Dramatic increases in stiffness, strength and elongation have also been reported [116]. The addition of 0.02 wt% MWCNTs into silk fibrin nanofibers increased the modulus from 231.2 ± 13.7 MPa to 342.7 ± 16.2 MPa; tensile strength 6.7 ± 0.7 MPa to 10.0 ± 1.0 MPa, but elongation decreased from 16.8% to 11.7% [117]. However, the properties were dependent on dispersion, functionalisation and nanotube concentrations. Reduction in mechanical properties, slower biodegradation rate and increases in fiber diameter were observed with increases in non-functionalised MWCNT concentration beyond 0.5 wt% [116]. Similar to blending apatite nano-crystals, surface functionalisation has been critical for dispersion to enable the significant addition of nanotubes without aggregation and decline in material properties. These properties will also improve adhesion with the polymeric matrix [118] and biocompatibility [119]. PCL has been grafted onto MWNTs to create a hard-core/soft-shell structure, again targeted for "artificial bones" [120]. However, the addition of carbon nanotubes into electrospun fibers has had varied effects on scaffold mechanical properties. While covalently-functionalised carbon nanotubes may provide better dispersion throughout the fiber, their effect can be mechanically detrimental. Functional groups in the graphene lattice also act as defect points that can weaken the nanotube and corresponding electrospun fiber [121].

Biologically, the presence of nanotubes has also enhanced bone-specific bioactivity: fibrous blends of biocompatible MWCNTs and PLGA (2.15 μm), stimulate apatite mineralisation from SBF without the presence of any cells [101]. Furthermore, co-blending of both HAp nanoparticles and MWNTs within PLGA [122] has been proposed as attractive nanocomposites for bone tissue engineering, exhibiting both attachment and proliferation of bone-marrow-delivered MSCs.

Core-Shell Electrospinning

Despite the advantages in pre-blending particles with biodegradable polymers to yield electrospun nanocomposite fibers, the spatial organisation within the fiber is

still random. As a result, it is not yet possible to mimic the physical and chemical organisation of mineralised fibrils in native bone. However, improvements may be realised through core-shell electrospinning (also referred to as co-axial electrospinning). This is conceptually similar to single jet electrospinning, however, it involves a dual-orifice spinneret whereby two separate solutions are simultaneously fed though an outer and inner needle to create dual-layered nanofibers [123, 124].

One of the crucial advantages in core-shell electrospinning is that there are fewer restrictions on the solution properties, with the shell acting as a guide to form appropriately layered structures [124]. While not yet extensively explored, core solutions including low-molecular weight polymers, molecules with limited solubility, metallic and ceramic particles [124] and even cells (which have been demonstrated to maintain full functionality post-electrospinning) [125] are potential candidates for unique advances in bone tissue engineering.

6.3.2 Dual Porosity, Cellular Infiltration and Three-Dimensional Structures

Scaffold three-dimensionality with interconnected microporosity for cellular infiltration, vascularisation and integration with the patient's tissue is paramount for the clinical viability of the adjuvant synthetic matrix. Host tissue in-growth and consequential mechanical interlocking is of key importance for effective osseointegration and implant stability. Not only does the stability of the implant influence healing time, pain and physical support, the micromechanics imposed in the endogenous niche have a critical influence over the formation of neo-blood vessels, osteoprogenitor differentiation, proliferation, migration, inflammatory response [59, 126], and ultimately the quantity and quality of newly formed bone. This is one of the major inherent limitations with electrospun matrices: the pores are too small.

The increasingly prevalent need to provide micropores while maintaining nanomimetic features and porosity for which cell nutrients and metabolic waste are transported [127] has lead to combining a range of fabrication technologies with electrospinning. Dual-scale porosity in electrospun nanofiber matrices has been improved through co-incorporating electrospun water-soluble sacrificial fibers [128–130], ice crystals [131–133], micron-sized fibers produced by heated melt deposition [76, 86, 134] or co-electrospinning [135], salt leaching [136, 137], salt leaching/gas foaming techniques [127, 138], drawing a metallic comb through the fibers to expand the mesh mechanically [139], using a metallic spherical dish as a collector of the electrospun fibers [140] and cutting pores out using laser ablation [141], some of which are shown in Fig. 6.2.

While many of these approaches increase cellular infiltration and/or survival, a major pitfall that accompanies increased porosity is the subsequent reduction in mechanical strength. In an attempt to overcome this issue, Lee et al. [127] co-blended MMT nano-sized platelets as reinforcements in electrospun P_LLA and used salt leaching/gas foaming to create a reinforced three-dimensional nanofibrous mesh

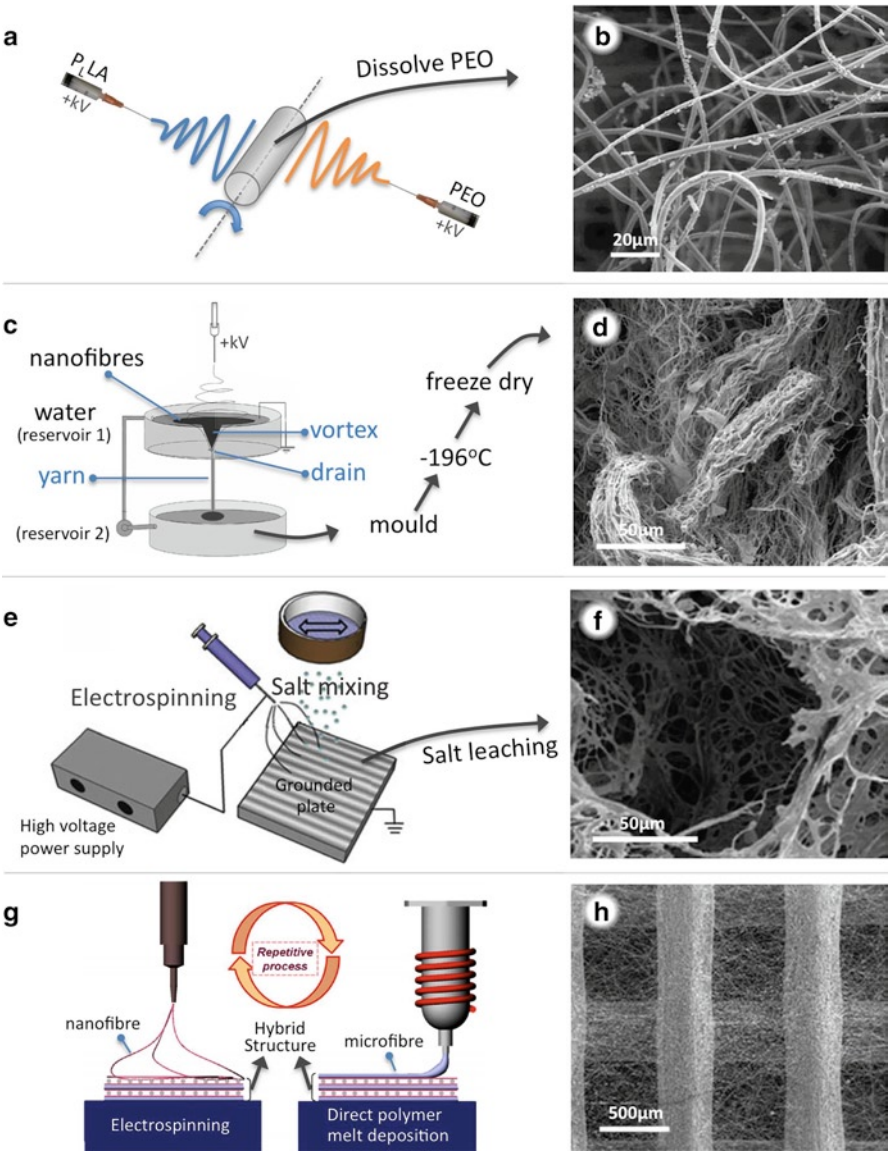

Fig. 6.2 Methods for generating microporosity within electrospun nanofibrous scaffolds. (**a**) Schematic simultaneous electrospinning of P_LLA with PEO onto a common rotating drum (concepts derived from [130]). (**b**) SEM image of electrospun P_LLA/PEO (1:3), post-removal of PEO and fiber mineralisation in SBF (adapted with permission from [130]). (**c**) Electrospun PCL collected in an electrically grounded reservoir of water. Nanofibers drained into a lower reservoir where the vortex fluid motion confined PCL fibers into yarned bundles that were collected in reservoir 2. Slurry of PCL fibrous yarns and water from reservoir 2 was moulded into cylindrical three-dimensional structures, immersed in liquid nitrogen (−196 °C) for 15 min and freeze dried; (**d**) corresponding SEM image; ((**c**) and (**d**) adapted with permission from [133]). (**e**) Simultaneous deposition of salt particles (100–200 µm) during electrospinning of collagen/sodium hyaluronate

with microsized pores. Opportunistic bone-specific strengthening systems as discussed in sect. 6.3.1.3 may be important in combination with dual-scale porous scaffolds to create fully functional three-dimensional bone-graft implants.

Open microporosity may also be crucial in effective tissue regeneration, yet without active surface chemistry as discussed below, host infiltration is likely to be ineffective. It has been suggested that modified surface chemistry on its own can encourage tissue infiltration, and is likely to be a prerequisite. This has been supported through in vitro and in vivo studies of Hep-coated P_LLA nanofibers, where in-growth of bovine aortic endothelial cells into three-dimensional scaffolds was improved with Hep coating, and interestingly through using aligned fibers [142]. More potently, as neural cell migration can be driven by neurotrophin-2 and nerve growth factor gradients [143], there may be potential for encouraging bone-specific infiltration via bone-specific gradients.

6.3.3 Surface Functionalisation

The ability to modify the surface chemistry on the nanofibers facilitates manipulation and tuning of biological responses for bone regeneration. Greater surface exposure of bone-specific inorganics, cell adhesive molecules and growth factors attachment has been achieved through various novel surface modification techniques discussed below. The ability to expose multiple drugs and growth factors from electrospun systems is an exciting prospect for sophisticated bio-regenerative signalling, yet currently in its developmental infancy.

6.3.3.1 Surface Activation Techniques and Hydrophilicity

An alternative technique for increasing cell affinity and biocompatibility of synthetic electrospun polymers involves modification of the surface of electrospun fibers via the attachment of hydrophilic chemical groups such as amides, carboxyl and hydroxyl groups [144]. In situ grafting of hydrophilic acrylic acid on to PGA, P_LLA and PLGA electrospun fibers for increased presentation of carboxyl groups facilitated significant improvements in fibroblast attachment, spreading and proliferation in vitro [145]. Importantly, these reactive groups are prerequisites for providing docking sites for adsorption and/or the immobilisation of an extensive range of growth factors, proteins and peptides. Often spacer molecules are also attached onto the fiber via polymerisation grafting, to change the surface biochemistry and provide docking sites for immobilisation of more powerful biological molecules and to maintain bioactivity [144].

Fig. 6.2 (continued) (80/20); (**f**) High magnification SEM image of dried scaffold post-collagen/sodium hyaluronate cross-linking and salt leaching; ((**e**) and (**f**) adapted with permission from [136]). (**g**) Schematic stacking of PCL/collagen type I blended electrospun nanofibers with microfibers via direct polymer melt deposition; (**h**) SEM image of electrospun nanofibers on top of microfibers; ((**g**) and (**h**) adapted with permission from [76])

6.3.3.2 Macromolecules, Natural Polymers and Signalling Factors Involved in Bone Healing

Following surface pre-activation, collagen [146], gelatin [147] and fibronectin [29] have been tethered onto electrospun synthetic polymers to increase biofunctionality. Cell adhesive ligands, including RGD-containing peptides, have also been attached to electrospun synthetic biodegradable polymers, leading to enhanced control over attachment, spreading [148], proliferation and differentiation of a variety of osteoprogenitor stem cells [149, 150]. Owing to its high negative charge, Hep attachment onto electrospun fibers has been important for electrostatic adsorption of a variety of growth factors involved in bone morphogenesis, such as FGF-2 [151], VEGF, TGF-β [152] and PDGF [153]. Furthermore, dual-immobilisation of laminin and FGF onto Hep functionalised P_LLA nanofibers showed enhanced bioactivity [154]. Conversely, Hep has been shown to suppress BMP-2 osteogenic bioactivity [155] and thus control over Hep location, concentration and timed exposure should therefore be taken into careful consideration in designing multiple growth factor delivery systems for bone tissue engineering.

Not only does the selection of bio-signalling molecules have a great influence over regulating cellular response, the immobilisation mechanism is crucial. The two major surface attachment techniques include covalent conjugation and adsorption via secondary forces. Typically, conjugation has been linked with sustained signal exposure yet greater chances of protein bio-deactivation, while adsorption only supports burst delivery. Importantly, a comparison between these two techniques has been shown for attachment of BMP-2 [156]. BMPs can stimulate osteoblastic differentiation of MSCs in vitro [157], initiate bone osteoinduction and osteoconduction in endochondral and intramembranous regenerative cascades [158], and even play a concurrent role in mediating angiogenesis [159]. Conjugation of BMP-2 onto cast PCL films showed greater concentrations of BMP attached with a slower accumulated release, in contrast to adsorption of BMP [156]. Greater ALP and OC expression from BMSCs (15 days in culture) was observed with covalent attachment of BMP-2, suggesting that this conjugation maybe a more favourable attachment mechanism [156].

6.3.3.3 Calcium Phosphate Coatings

One convenient technique utilised in developing bone-specificity on electrospun fibers has been coating biodegradable fibers with HAp and various calcium phosphates via immersion in SBF [160]. Surface presentation of selective chemical groups on biodegradable fibers is a prerequisite for stimulating surface nucleation and growth of HAp when incubated in calcium- and phosphate-rich SBF solutions [161]. Various techniques have been used to create surface active groups which can support the nucleation of calcium phosphate phase, some of which include plasma treatment [145, 162], alkaline erosion [163] and LbL coating [164]. An elegant study by Cui

et al. utilised covalent grafting of chitosan on amino-functionalised $P_{DL}LA$ fibers to facilitate controlled HAp deposition with uniform surface coverage. The presence of chitosan and mineralisation showed improved proliferation and differentiation of osteoprogenitor cells in comparison to controls [165]. However, a distinct disadvantage in surface deposition of HAp, rather than blending within the fiber, is that there is virtually no improvement in mechanical properties.

6.3.3.4 Electrospin/Electrospray Systems

Utilisation of electrospraying has been recently proposed as an alternative technique to enable strategic engineering control over the spatial organisation of each constituent phase in electrospun nanocomposites [166, 167]. Francis et al. [168] recently utilised simultaneously electrospraying of HAp and electrospinning of gelatin fibers onto a common rotating drum collector. Gelatin fibers were cross-linked, which increased the mechanical strength and elongation. As HAp particles were electrosprayed onto the surface of the gelatin fibers, this resulted in greater HAp exposure and enhanced surface "nano-roughness," alleviating problems such as the loss of HAp platelet bioactivity that may result if blended within the fiber. This spin-spray system provided appreciable enhancement in human foetal osteoblast proliferation, mineralisation and ALP after 15 days of culture, in comparison to blended gelatin/HAp electrospun fibers [168].

Spraying technologies also offer potentially more effective incorporation of mechanically mismatched materials, which may otherwise be detrimental if physically mixed together. However, despite various improvements in mechanical properties, material technologies are yet to be able to produce electrospun bioactive composites that can be utilised as stand-alone, temporary bone substitutes.

6.3.3.5 Layer-by-Layer Surface Modification of Nanofibers

Alternate electrostatic assembly of anionic and cationic electrolyte layers is a highly attractive means of altering nanofiber surface chemistry and coating a nano-thin biological reservoir of growth factors, drugs and proteins onto the electrospun fibers. Within the cellular environment, growth factors are released by degradation of inter-layer bonding as a result of aqueous, enzymatic or cellular activity. A wide variety of lipid vesicles, nucleic acids, DNA, proteins and more recently growth factors have been immobilised in LbL coatings with broad biological benefits [169–171]. Higher concentrations of molecules can be accumulated in a robust and controlled manner by simply increasing the number of assembled layers [144, 170]. Loading efficiency currently limits the clinical application of many growth factors such as rhBMP-2 [172]. Encapsulation of rhBMP-2 in cross-linked poly-L-lysine/hyaluronan LbL films, which could be coated onto nickel-titanium implant surfaces, maintained near zero-order release kinetics and protein bioactivity over 10 days, and promoted in vitro differentiation of myoblasts into osteoblasts [173]. A variety of bonding

forces, beyond the most common electrostatic interactions, including hydrogen bonding, hydrophobic interaction and covalent bonding, can also be used to control the biomolecular retention and release kinetics [174].

6.3.4 Cellular-Based Matrices

The design of artificial microenvironments for repair needs to accommodate the specific cell types and their interface with region-specific physical and pharmacological parameters. With bone, these should be specific to those activated during endogenous regeneration. As cells ultimately define the repair process, their involvement with the scaffold is an eminent step in therapeutic tissue engineering.

Various cell-encapsulating devices have been implemented to increase delivery efficiency, longer-term viability and function as opposed to direct cellular re-injection. MSCs can proliferate and differentiate on two-dimensional culture surfaces with the addition of growth factors, proteins, cytokines and hormones active in the bone healing cascade along with various anti-inflammatories and antimicrobials added into the culture medium [175]. Once dense differentiated populations of MSCs are achieved, they can be seeded on three-dimensional electrospun scaffolds as discussed throughout this review and correspondingly implanted. Alternatively, proliferation could be encouraged directly on the scaffolds in vitro. There have been few innovative approaches to cell-encapsulation within electrospun scaffolds. Recently, this has began to change with research involving electrospraying of cells [176], encapsulating cells inside individual electrospun fibers [125, 177] and three-dimensional stacking of cells between electrospun layers [178].

6.4 Summary and Future Perspectives

There is an ever-increasing need to improve the clinical management of skeletal disorders. While not necessarily life threatening, these conditions severely impact quality of life and present a major economic burden on society. Recently, the process of bone repair has received great impetus from progress made in engineering (nanotechnology) and biomedicine (bone stem and progenitor cells). Innovative strategies to combine each field have provided a rational and strategic framework to transform the clinical management of skeletal disease repair.

Establishing a nano-framework using natural and synthetic polymers, in combination with apatite crystals, functionalised nanotubes and/or bioactive glass underpins the mechanical properties of scaffolds that are critical for functional bone development. It is envisaged that combining techniques will yield more biomimetic and structurally sound synthetic ECM. Importantly, improved design of smart biomatrices that target bone regeneration is of great importance. Surface engineering via adsorption immobilisation, covalent conjugation and LbL coatings provide unique platforms

for releasing/presenting growth factors that stimulate bone regeneration from electrospun scaffolds. It is visioned that cooperative interaction of tissue engineering at multiple levels of matrix sophistication with stem cell-based therapies will ultimately expedite bone healing. The challenge is how they can be strategically combined.

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