

Multiscale Fibrous Scaffolds in Regenerative Medicine

Sowmya Srinivasan, R. Jayakumar, K.P. Chennazhi, Erica J. Levorson, Antonios G. Mikos, and Shantikumar V. Nair

Abstract In recent years, multiscale fibrous scaffolds containing a combination of micro- and nanoscale fibers have attracted a lot of attention in the tissue engineering field. The multiscale concept is inspired by the hierarchical structure of many tissues, such as bone. Fibrous scaffolds have been traditionally microscale; however, it has been determined that many physicochemical and biological properties are influenced by fiber scale. For this reason, in an effort to optimize tissue regeneration the use of multiple scales has been investigated for obtaining innovative property combinations not otherwise attainable with a single fiber scale. Multiscale architectures have been found to be favorable not only in bone regeneration but also in the regeneration of soft tissues including cardiovascular tissue, neural tissue, cartilage, and skin. The unique properties of multiscale scaffolds have been pivotal in better mimicking the extracellular matrix and promoting vascularization, a key step towards the development of engineered tissue. In this review, we present current designs of multiscale scaffolds and discuss their physicochemical characteristics, as well as their potential applications in regenerative medicine.

S. Srinivasan, R. Jayakumar (✉), K.P. Chennazhi, and S.V. Nair (✉)
Amrita Center for Nanosciences and Molecular Medicine, Amrita Institute of Medical Sciences
and Research Centre, Cochin 682 041, India
e-mail: rjayakumar@aims.amrita.edu; shantinair@aims.amrita.edu

E.J. Levorson
Department of Bioengineering, Rice University, MS-142, P.O. Box 1892, Houston, TX
77251-1892, USA

A.G. Mikos
Department of Bioengineering, Rice University, MS-142, P.O. Box 1892, Houston, TX
77251-1892, USA

Department of Chemical and Biomolecular Engineering, Rice University, MS-362,
P.O. Box 1892, Houston, TX 77251-1892, USA

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1 Introduction

Replacement of damaged tissues or organs by natural tissue instead of using synthetic implants is now seen as a realistic goal [1]. The emerging field of tissue engineering essentially involves engineering a suitably shaped biocompatible scaffold that has the potential to regenerate the host tissue when infused with the right mix of cells and other growth factors [2–7]. The regeneration can be conducted in vitro giving the opportunity to partially develop the host tissue extracorporeally, followed by implantation. An ideal scaffold must exhibit a porous, interconnected, and permeable structure to permit the infiltration of cells and nutrients and also should exhibit the appropriate surface structure and chemistry for cell adhesion and proliferation [8–12]. The ideal scaffold must also have mechanical properties comparable with the tissue being engineered and have just the right degree of biodegradability, such that it degrades to form soluble nontoxic products when the tissue is fully formed [13, 14].

The question of scale in this review is uniquely concerned with the scaffold structure. Early studies focused on scaffolds containing micrometer-sized interconnected pores to allow for infiltration of cells into the scaffold [15–19]. Since 2000, with advances in new nanomaterials and processing techniques a large number of studies [20–24] have focused on the role of the nanostructure of scaffolds in tissue regeneration. This was also prompted by the recognition that the environment of the cells in actual tissues (the extracellular matrix; ECM) is fundamentally nanostructured. On the other hand, microscale fibers allow the fabrication of scaffolds with controlled cell–cell interactions. Microfibers have the potential to be utilized as tissue engineering scaffolds in different forms for numerous applications such as wound dressings and bone regeneration [25, 26]. The appeal of nanofibers in tissue engineering is their structural similarity to native ECM;

however, ECM not only has a structural role but also a functional one. This network creates a dynamic, three-dimensional (3D) microenvironment in which cells are maintained. Signals are transmitted from cell surfaces in contact with ECM to the cell nucleus, enabling communication influencing cell adhesion, migration, growth, differentiation, programmed cell death, modulation of cytokine and growth factor activity, and activation of intracellular signaling [27]. Spun nanofibers also offer several advantages such as an extremely high surface-to-volume ratio, tunable porosity, flexibility with respect to scaffold size and shape, as well as the ability to control the nanofiber composition to achieve the desired results from its properties and functionality [27]. Though there are many studies that have proposed nanofibrous polymeric mats for tissue engineering, they have a limitation for 3D applications due to their pore size, which is smaller than a cellular diameter and restricts cell migration within the structure [28–30].

The use of toxic organic solvents for the fabrication of micro- and nanofibers is a major limitation of fibers obtained from synthetic polymers, which would require thorough washing or solvent evaporation treatment prior to use with cells. Natural polymers act as alternatives to synthetic polymers and offer a few advantages such as solubility in aqueous media due to their hydrophilic nature in addition to limited or nonexistent immunogenicity and cytotoxicity [31]. A variety of natural and synthetic polymers have been used to develop scaffolds; however, these scaffolds do not interact with cells in the desired manner. The growth of these cells may be affected by their interaction with the substrate [32–34]. In vivo, the complex structure of vasculature ensures a maximum nutrient diffusion distance of nearly 200 μm for the supply of nutrients to tissue. However, this distance exceeds 200 μm within in vitro constructs, resulting in reduced nutrient supply that compromises cell growth and differentiation [35–37]. Very few studies have looked at the vascularization of scaffolds, which is also of major concern [38]. Most of the current scaffolds lack an interconnected microcapillary network that would help cells to survive within these scaffolds. Also, the cells seeded onto larger and thicker scaffolds have limited success as the cells in the deeper layers are devoid of nutrients and oxygen. Hence, there are more cells at the surfaces of thick 3D scaffolds than in the deeper layers [39]. Therefore these scaffolds should be provided with an inbuilt nutrient distribution network to support the uniform growth of cells. Some fabrication techniques fail to form interconnected pores, which are an important requirement of tissue engineering scaffolds [40–43]. In addition, fabrication techniques in general fail to develop 3D constructs that mimic the complex natural tissues, specifically with regard to mechanical properties [38, 44, 45].

To overcome the current limitations, one approach developed mainly in the last 5 years has been to study multiscale scaffolds in an attempt to mimic hierarchical tissue structures. This review essentially focuses on studies involving multiscale fibrous scaffolds, their development, use of different fabrication methods, their properties, and specific applications in tissue engineering.

2 Multiscale Scaffolds: Principles and Fundamentals

ECM is a hierarchical structure comprised of both micro- and nanoscale structures. The micrometer scale offers a porous structure large enough for the migration of cells through the construct, whereas the nanoscale allows for enhanced protein adsorption, thus improving protein-mediated interactions with the cell surface and so favoring cell adhesion and improving cell viability [46]. The nanoscale fiber meshes exhibit high porosity and reduced pore sizes. Fiber diameter plays an important role in adhesion of cells to the fibers. Reports have suggested that nanofibers may serve as suitable biomaterials for tissue engineering since they can be used to enhance cell differentiation, adhesion, and proliferation [47]. Cells attach at multiple focal points, extend filopodia along the length of the fibers, and spread throughout the nanofiber matrix [48]. However, the effect of fiber diameter on tissue regeneration still remains unknown. Scaffold architecture and design are key factors in determining vascularization and nutrient supply, which are crucial for the development of regenerating tissue [49]. Studies have shown that pores of 100–300 μm favor vascularization and nutrient supply [50]. However, the relatively small pore size of nanoscale fibrous scaffolds compared to cell diameter (5–20 μm) limits cell migration and infiltration within the scaffold, leading to the formation of a monolayer of cells on the scaffold surface rather than a 3D cellular construct. For this reason, nanoscale fibrous scaffolds cannot mimic the natural thickness of certain tissues such as human articular cartilage, which ranges from 0.5 to 7.1 mm thick [51].

Microfiber scaffolds could be potentially advantageous because they are comprised of larger pores than nanofiber scaffolds. These larger pores could facilitate cellular infiltration and/or diffusion of nutrients during *in vitro* culture. To study the mechanism of interaction between cells and nanofibers, it is important to control the architecture of nanofibers to minimize the interaction of a single cell with neighboring fibers [47]. Controlling the nanofiber to microfiber ratio can easily vary the porosity of multiscale scaffolds. Pham et al., who developed a bimodal scaffold consisting of a top nanofiber layer and a bottom microfiber layer, demonstrated this [52]. These scaffolds were developed by a sequential electrospinning technique wherein the multiscale layers were electrospun using the same polymer with different morphologies. These scaffolds were characterized to evaluate the extent of nanofiber deposition as a function of duration of electrospinning time. Electrospinning for longer periods increased the amount and thickness of the nanofibrous layer. Hence, thickness and coverage of nanofibrous layer could be controlled by modulating the electrospinning time. Cross-sectional images helped in distinguishing the alternating layers, even in the presence of a thin nanofiber layer. Also, scaffolds with different densities of nanofiber layers were considered to investigate the effect on cellular infiltration into these bimodal scaffolds. Cell attachment at different intervals of time was found to be similar; however, cell spreading was affected by the presence of nanofibers. The cells initially appearing to exhibit rounded morphologies became

more spread with time, with the greatest amount of spreading being observed on scaffolds with nanofibers. Complete infiltration of cells was only observed in the presence of a flow perfusion bioreactor. Thus the presence of nanofibers was found to hinder cell migration due to the presence of smaller pores, but at the same time influenced cell spreading, proliferation, and differentiation [52]. However, these 3D scaffolds combining nano- and microscale fibers (nano/microfiber scaffolds) have a layered nanofibrous structure instead of a randomly mixed structure composed of nanofibers and microfibers. Therefore, the nanofibrous layers, which were formed in sheet-like structures over microfibers, can prevent the cells from infiltrating adequately into the scaffolds.

3 Mechanical, Physicochemical, and Biological Properties of Multiscale Scaffolds

The success of a tissue engineering scaffold ultimately depends on the interplay of various biological, mechanical and physicochemical properties. For every successful application, these tissue engineered constructs must satisfy the essential requirements of biological, mechanical, and physical properties. The scaffold architecture should support and withstand compressive forces and contraction in vivo. The scaffold should provide adequate support for cell attachment and growth in vitro and facilitate mass transfer of nutrients and oxygen when implanted in vivo. For biodegradable scaffolds, the rate of degradation should balance the rate of neo-tissue formation and remodeling, wherein the degree of remodeling depends on the type of tissue. Shalumon et al. prepared multiscale poly(ϵ -caprolactone) (PCL) fibrous scaffolds [46]. Various processing parameters such as viscosity, applied voltage, and flow rate influence the fiber diameter and result in the formation of a simultaneous micro/nanofiber matrix. The presence of nanofibers embedded in the microfiber matrix was found to enhance the mechanical strength in comparison to microfibers alone. Also, the strain values were increased, which may be attributed to the cumulative elongation effect in the presence of the nano/microfiber combination. The incorporation of nanocrystalline hydroxyapatite (nHA) into these multiscale fibers did not alter the fiber morphology; however, the fiber diameter was slightly larger. This was probably due to the increase in polymer viscosity. The cellular activity on multiscale scaffolds, regardless of the incorporation of nHA, was higher than on microfibers or nanofibers alone. Cells attached to the multiscale fibers were flattened, laterally stretched, and extended (Fig. 1) [46].

Similarly Shalumon et al. also fabricated electrospun porous poly(lactic acid) (PLA) multiscale scaffolds and evaluated their physicochemical and biological properties in detail [53]. These multiscale scaffolds were developed through a bimodal fiber fabrication system. The three main solution properties, solution viscosity, conductivity, and surface tension, were determined to be the governing

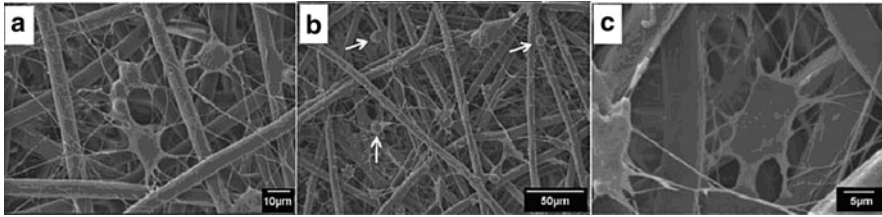


Fig. 1 SEM images of cell attachment after 12 h of incubation. (a) Cell attachment on multiscale scaffold. (b) Cell growth into the multiscale scaffold; *arrows* indicate spherical morphology of cells attached to microfibers. (c) Cell access to the interior of the multiscale scaffold through the pores in the microfibers (Adapted from [46])

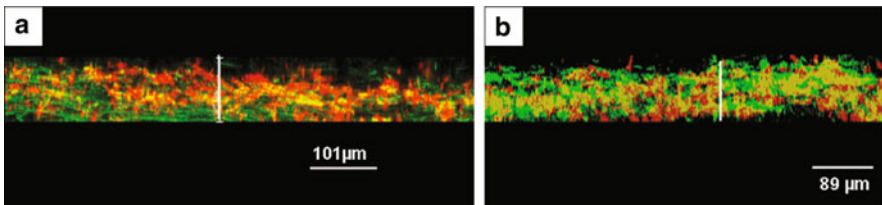


Fig. 2 3D projection images of cell infiltration behavior into (a) micro/nanofibrous scaffolds and (b) micro/nano/nHAp fibrous scaffolds (Adapted from [53])

factors in the formation of multiscale fibers. Cells seeded on the multiscale scaffold displayed a well-flattened and laterally stretched morphology over the scaffold surface. Cell penetration and proliferation on multiscale scaffolds was enhanced in comparison to nano- or microscale scaffolds (Fig. 2). This was due to the combined effect of porosity and low contact angle offered by the microfibers and the capacity for spreading offered by nanofibers. Increased cell penetration also resulted in homogenous distribution of the cells throughout the multiscale scaffold [53].

Santos et al. elucidated the importance of endothelial cell (EC) colonization and angiogenic potential of nano/microfibrous combined scaffolds [54]. The ECs spanned between microfibers with the support of nanobridges formed by the nanofibers, yielding a higher density of adherent ECs. This spanning of cells between the fibers was absent in the microfiber control. This could be due to the presence of a nanonetwork that induced a different cytoskeletal arrangement, which was reflected in the stretched shape with numerous cellular extensions. Besides improving the interconnectivity, these nano/micro scaffolds provided a means of physical support for the enhanced growth of ECs, thus showing close resemblance to morphology of a capillary-like network [54]. Edwards et al. also described similar work wherein a multiwalled carbon nanotube–PLGA nanofiber composite presented ideal pore spanning, allowing uniform distribution of fibroblast-like cells throughout the scaffold surface [55]. With an increase in cell spanning and stretching, the receptors also get stretched and activated. Tuzlakoglu et al.

developed polymeric micro/nanofiber scaffolds from a blend of starch and PCL. The results indicated changes in cell morphology and cytoskeletal rearrangement. The cell receptors were stretched and activated, leading to the expression of different genes in comparison to the unstretched cells [48]. Mota et al. developed dual-scale scaffolds consisting of aligned PCL microfilaments and PLGA nanofibers [32]. They observed that in the dual-scale scaffolds, cells were able to colonize the interfibrillar gap caused by the microfilaments. This was due to the effect of the guidance in cellular adhesion exerted by the electrospun nanofibers, which were also responsible for the morphological changes between cells adhered on fibers with different orientations. These dual-scale scaffolds are also characterized by fiber alignment and high spatial connectivity capable of directing cell orientation and migration [32]. The interaction of cells with ECM is not only an anisotropic behavior, but is also dependent on the chemical changes experienced from the surrounding environment. The cells endure chemical stimulation by direct contact with the surrounding ECM or the neighbors, which greatly depends on the spatial concentration distribution of the fibers. By altering the composition of nanofibers while electrospinning, multilayered scaffolds with specified chemistry, thickness, and morphology can be obtained [56].

Porosity and pore size are among the key factors responsible for the success of tissue engineered scaffolds. Optimum porosity enhances cell spreading and migration while supporting the exchange of nutrients between the scaffold and the surrounding environment. Porosity and pore size in electrospun scaffolds are mainly dependent on the fiber diameter and their packing density. Soliman et al. developed a bio-inspired multiscale 3D scaffold for soft tissue engineering wherein the fiber distributions were intermixed into a single, multimodal layer [57]. They also stated the importance of combining nano- and microfibers in a single scaffold wherein cell motility and adhesion was improved by the network of nanofibers. The web of nanofibers helped in bridging the cells across the microfibers, thus colonizing the entire scaffold, while the microfibers produced larger pores to accommodate the cells. This is a valuable characteristic because in a single-scale scaffold the cells are frequently confined to a single fiber due to the absence of bridging across microfibers that are far apart. The multimodal scaffold also exhibited superior strength and stiffness in comparison with the controls [57].

Multiscale scaffolds composed of nano- and microscale fiber meshes using two different electrospinning techniques, multilayer electrospinning and mixing electrospinning, were reported by Kidoaki et al. [58]. Initially, four different polymers were individually electrospun: type I collagen, styrenated gelatin (ST-gelatin), segmented polyurethane (SPU), and poly(ethylene oxide). Then, these individual fiber meshes were deposited layer-by-layer to form a trilayered electrospun mesh and mixed electrospun fiber mesh. A bilayered tubular construct composed of a thick SPU microfiber mesh as an outer layer and a thin type I collagen nanofiber mesh as an inner layer was fabricated as a prototype scaffold of artificial grafts and may provide compliance matching with native arteries and tissues. Further insights into detailed in vitro and in vivo studies are essential to visualize the functioning of the fiber mesh after implantation [58]. Thus, the

synergistic combination of micro- and nanofiber hierarchical scaffolds developed using combinations of a wide variety of biopolymers showed unique mechanical, biological, and physicochemical properties that are suitable for diverse tissue engineering applications and are absent in single-scale scaffolds.

4 Applications of Multiscale Scaffolds in Tissue Engineering

4.1 Bone

Bone tissue engineering is a branch of tissue engineering that aims to repair and/or regenerate bone using scaffolds with cell-based therapies and growth supplements. It may be used to restore skeleton function in the field of orthopedic and oral-maxillofacial surgery [59–61]. Scaffolds for bone tissue engineering are developed with the primary aim of closely mimicking the biophysical structure of natural ECM. Tuzlakoglu et al. developed nano- and microfiber combined scaffolds from starch/PCL-based biomaterials to support bone cells [48]. The nanofibers were electrospun in a random manner between the microfibers, thereby providing nanobridges similar to those found in ECM. In the presence of nanofibers, a well-spread cellular morphology and cytoskeletal organization of the SaOs-2 human osteoblast-like cell line and rat bone marrow stromal cells was observed. Osteoblasts were directed to bridge between microfibers and this resulted in scaffolds completely filled with cells after 2 weeks of culture. With cell stretching, the receptors also got stretched and activated, resulting in the expression of various genes in comparison to the unstretched cells. Cell viability and alkaline phosphatase (ALP) activity for both of the cell types was found to be enhanced in nano/microfiber combined scaffolds as compared to control scaffolds based on fiber meshes without nanofibers. Consequently, the developed structures are believed to have a great potential for the 3D organization and guidance of cells that are important for engineering 3D bone tissues. Their unique architecture, which supports and guides the cells, makes them suitable candidates for bone tissue engineering applications [48]. Martins et al. also developed bone ECM-inspired structures by conjugating electrospun chitosan (Cht) nanofibers within biodegradable polymeric microfibers [poly(butylene succinate) (PBS) and PBS/Cht], assembled in a fiber mesh structure [62]. The physical properties of these scaffolds could be enhanced by developing a highly connected porous framework. The osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) produced an increased amount of calcium phosphates on these nanofiber-reinforced composite scaffolds, confirming ECM deposition and mineralization, mainly in the PBS/Cht-based fiber meshes. The osteogenic genotype of the cultured hBMSCs was confirmed by the expression of osteoblastic genes, namely those coding for ALP, osteopontin, bone sialoprotein and osteocalcin, and the transcription factors Runx2 and Osterix, all involved in different stages of osteogenesis [62]. Osathanon et al.

fabricated micro- and nanoporous fibrin/calcium phosphate composite scaffolds with tightly controllable pore size and interconnected pores using sphere-templating methods [63]. Murine calvarial cells attached, spread and showed a polygonal morphology on the surface of the scaffold. Multiple cell layers and fibrillar matrix deposition were observed. Cells seeded on mineralized fibrin scaffolds exhibited significantly higher ALP activity as well as osteoblast marker gene expression compared to fibrin scaffolds and nHA-incorporated fibrin scaffolds. The scaffolds also promoted bone formation in a mouse calvarial defect model, and the bone formation was enhanced by addition of recombinant human bone morphogenetic protein-2 (rhBMP-2) [63]. These works have demonstrated the combination of micro- and nanofiber scaffolds to be a feasible option for the design and development of hierarchical, fully functional, synthetic ECM substitutes.

Vascularization following implantation is a major concern in bone regeneration as well as tissue engineering as a whole because diffusion limitations are a major barrier to the production of 3D engineered tissues. Strategies that include seeding ECs on biomaterials and promoting their adhesion, migration, and functionality might be a solution for the formation of vascularized bone. Nano/microfiber combined scaffolds have a unique characteristic inspired by ECM, the nano-dimensions, which promote cell adhesion, together with a microfiber mesh that provides the mechanical support. Santos et al. described a work showing the influence of a nanonetwork on growth pattern, morphology, inflammatory expression profile, expression of structural proteins, homotypic interactions, and angiogenic potential of human ECs cultured on a scaffold made of a blend of starch and PCL [54]. These nanonetworks on microfiber meshes not only increased the adhesion surface area and interconnectivity in the constructs but also provided structural and organizational stability for ECs. As mentioned earlier, the human dermal microvascular ECs (HDMECs) spanned between the microfibers using the nanobridges formed by the nanofibers, thus favoring high levels of cell adhesion. On the microfibers, the cells exhibited a flattened morphology characteristic of their location inside larger blood vessels and an extremely stretched shape reminiscent of the angiogenic phenotype, with multiple cellular protrusions anchoring them to several nanofibers (Fig. 3). The cells also covered the entire surface of the scaffold without hampering the scaffold porosity. Furthermore, on nanofibers as well as on microfibers, ECs maintained the physiological expression pattern of the structural protein vimentin and platelet EC adhesion molecule (PECAM-1) between adjacent cells. In addition, ECs growing on the nano/microfiber combined scaffold were sensitive to pro-inflammatory stimulus. Under pro-angiogenic conditions in vitro, the ECM-like nanonetwork provided the structural and organizational stability for migration and organization of ECs into capillary-like structures with branching. The architecture of nano/microfiber combined scaffolds, thus, elicited and guided the 3D distribution of ECs without compromising the structural requirements for bone regeneration [54].

Multiscale scaffolds thus satisfy the required criteria of an ideal bone regenerating scaffold, namely biocompatibility. Likewise, they promote cellular adhesion, growth, and migration. The bone-specific cells align themselves along the

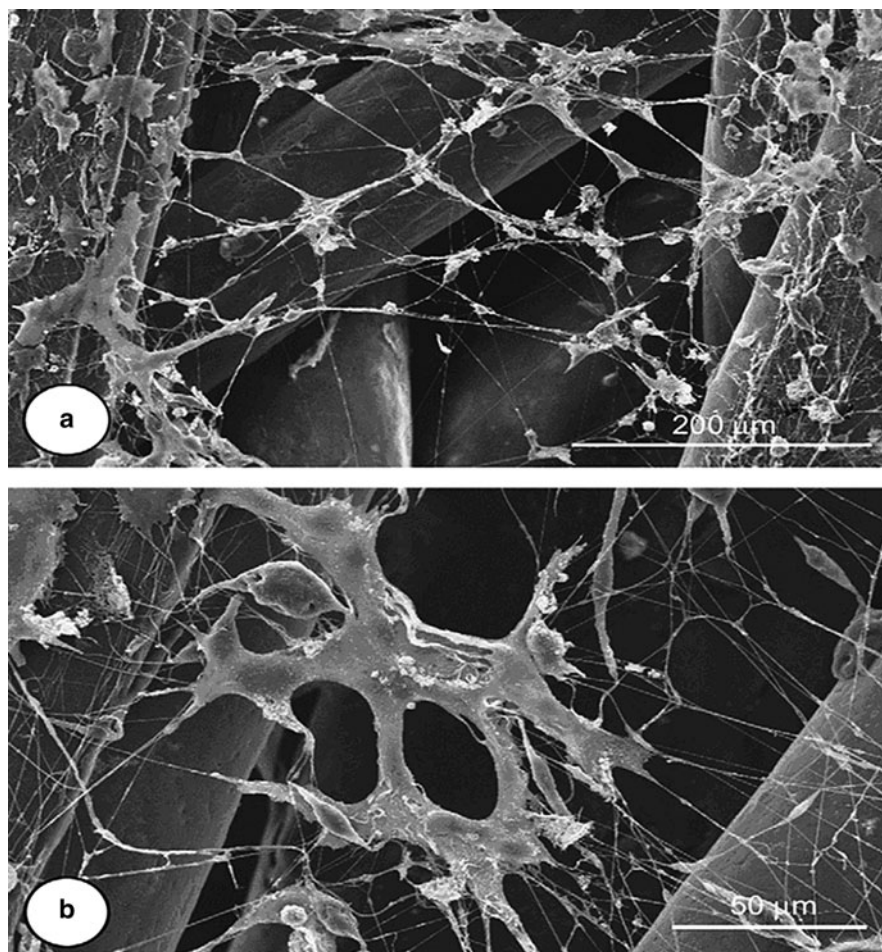


Fig. 3 (a, b) SEM micrographs of HUVEC cells on fibronectin-coated starch/PCL scaffolds, showing the nano/microfiber combined scaffold after 3 days of culture. Note the ability of the ECs to use the nanofibers to span across the microfiber structure (Adapted from [54])

long axis of the micro- and nanofibers, bond to the existing bone surface facilitated by the presence of nanoscale structures, and stimulate osteogenesis. The presence of microfibers in the construct provides the necessary porous structure for the cells to migrate easily, enable the supply of nutrients and oxygen, and enhance vascularization. These constructs are biodegradable and have a controlled degradation rate. They also exhibit mechanical and physicochemical properties that match the native bone. The architecture, properties, and biological requirements can be tuned during fabrication to obtain a construct of desired morphology and characteristics. All these factors should be further confirmed with *in vivo* studies to ensure that multiscale scaffolds are a suitable option for bone tissue engineering.

4.2 Cartilage

Cartilage is a tissue in which the ECM plays a very important role in both the biological and mechanical function of the tissue. For this reason, it follows that a well-designed scaffold that mimics the structure of native ECM could potentially lead to more successful development of engineered cartilage. Park et al. fabricated highly functionalized polymeric 3D scaffolds by a combination of direct polymer melt deposition (DMPD) and electrospinning [64]. Between the microfiber layers formed by DMPD, PCL/collagen nanofiber matrices were deposited via electrospinning. The hybrid scaffold showed a 3D woodpile structure composed of layers of PCL microfibers with a PCL/collagen nanofiber matrix (Fig. 4). This stacked structure was thought to be beneficial for the penetration of cells, also leading to increased cell migration through the side channels. Results confirmed enhanced chondrocyte attachment and proliferation on the hybrid scaffold. An increase in cell number was noted with an increase in culture duration. This could be due to direct reaction of cells to the nanofibrous topography such as groove, ridge, pore, step, and node topographies. Hence, the nanofibrous topography could be easily sensed by the seeded chondrocytes thereby influencing cellular interactions. The nanofibrous structure can also enhance serum protein adsorption

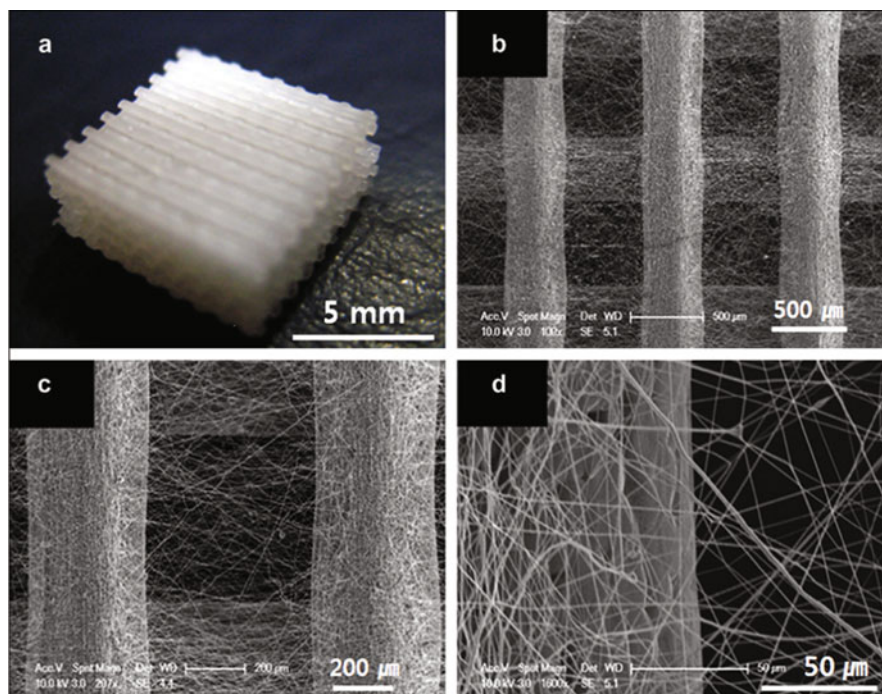


Fig. 4 (a) Photograph of the overall 3D woodpile structure with dimensions of $9 \times 9 \times 3.5$ mm. (b) Hybrid basic unit layer composed of microfibers and the electrospun nanofiber matrix. (c, d) Magnified images of (b) (Adapted from [64])

on the surface of the hybrid scaffold. An increase in serum protein adsorption further enhances cell adhesion. With the exposure of collagen on the surface of the hybrid scaffold, cells were provided with a suitable environment for growth and differentiation. Hence, the surface nanotopography enhanced the cytocompatibility of the scaffold to make it more suitable for cartilage tissue regeneration [64].

4.3 Skin

Kim et al. reported the development of 3D scaffolds with randomly mixed interconnected network of micro- and nanofibers [65]. The hybrid process was designed by combining melt electrospinning and solution electrospinning. This hybrid structure with a randomly mixed network of micro- and nanofibers also had similar pore dimensions to microfiber scaffolds, and the scaffold thickness could also be varied to the centimeter scale making it adaptable for 3D culture of various tissues. The mechanical properties of the poly(D,L-lactic-co-glycolic acid) (PLGA) nano/microfiber composite scaffold were assessed using tensile testing. The PLGA nano/microfiber composite scaffold had higher tensile strength and elongation at break than the PLGA microfiber scaffolds, which may be due to the entangled structure of nanofibers with the microfibers. The nanofibers in the nano/microfiber scaffolds can provide more contacts and/or physical junctions with the microfibers or nanofibers, and thus act as physical crosslinks. The response of normal human epidermal keratinocytes (NHEKs) and primary normal human epidermal fibroblasts (NHEFs) forming this tissue was analyzed in vitro. The attachment of NHEF cells was significantly higher on PLGA micro/nanofiber scaffolds in comparison to NHEK cells. Regardless of culture duration, NHEKs did not spread well on the PLGA microfiber scaffolds. Conversely, NHEFs spread and spanned across the pores between fibers in the PLGA microfiber scaffolds within a single day of culture, yet cell contacts appeared broken in these scaffolds after 3 days, which could negatively influence further construct development. Nano/microfiber scaffolds seeded with either NHEK or NHEF cells displayed spreading and cellular infiltration after only 1 day of culture. Since the PLGA scaffolds possess similar tensile properties to human skin, and due to the increased cell spreading and infiltration of nanofiber containing constructs, it was anticipated that multiscale PLGA scaffolds would be a suitable tissue engineering scaffold for skin regeneration [65].

4.4 Neural

Neural tissue repair in order to treat nerve damage or neuropathy arising from a spinal cord or cerebral injury essentially requires the intervention of tissue engineering because axons are unable to regenerate on their own. Hence, recent advances in neural tissue engineering provide suitable and promising substitutes

for nerve regeneration [66, 67]. Wang et al. developed chitosan bilayered tubes and micro/nanofiber mesh tubes [68]. These tubes were prepared using the electrospinning technique. Bilayered tubes were formed by combining an inner nano/microfibrous tube with a chitosan film outer coating. These nano/microfiber mesh tubes, bilayered tubes, and film tubes were tested as bridge grafts in injured rat sciatic nerves. Although the mechanical properties of micro/nanofiber mesh tubes were inferior in comparison to the bilayered tubes, these properties were sufficient to serve as a nerve conduit preserving permeation of the mesh tube. Both function recovery and nerve regeneration were enhanced as a result of good permeation ability, thereby enabling the exchange of nutrients and metabolic wastes. The micro/nanofiber mesh also had the ability to interact with the ECM on both sides of the amputated nerve and to promote Schwann cell migration into the lesion area to provide a suitable environment for the growth of the nerve into the lesion site. The graft may also approximate the two sides of the lesion [68]. Panseri et al. used tubes made of biodegradable polymers (a blend of PLGA/PCL) fabricated by electrospinning to regenerate a 10-mm nerve gap in a rat sciatic nerve in vivo [69]. The flexible fibrous structure was easily sutured to the proximal and distal ends of the nerve stump. The graft also showed a porous structure allowing the passage of nutrients, and a necessary barrier to prevent the infiltration of unwanted tissue into the conduit. Four months after surgery, the electrospun tubes were found to induce neural tissue regeneration and functional reconnection of the two severed sciatic nerve tracts in comparison to the untreated control. Myelination and collagen deposition were detected in accordance with the newly regenerated nerve fibers. Thin fibrous tissue capsule formation around the surface of the conduit and negligible inflammatory response proved the biocompatible nature of the conduit. These results effectively showed that the prosthesis did not produce any mechanical stress-related nervous degeneration and favored the reestablishment of functional neuronal connections evoking re-innervation of the target muscles in the majority of treated animals [69]. The results from these studies confirmed the efficacy of multiscale scaffolds as suitable constructs for neural tissue engineering. Some of the remarkable features such as the migration of Schwann cells into the injury site favoring the growth of nerve, functional innervation, nerve regeneration as a result of good permeation ability with exchange of nutrients, and graft approximation clearly satisfy the needs of neural tissue engineering.

4.5 Cardiovascular

Regenerative medicine is of paramount importance for treating patients with severe cardiac diseases. Various strategies for regenerating the damaged myocardium are under investigation with varying degrees of success. These strategies include cell therapies, gene or protein therapy, and the application of passive or bioactive materials or a combination of cells, growth factors, and scaffolds to repair or regenerate cardiac tissues [4, 7, 70]. Kwon et al. fabricated nano/microstructured

biodegradable poly(L-lactic acid-co- ϵ -caprolactone) (PLCL) fabrics of different compositions by electrospinning [71]. Different architectures of the elastomeric copolyester, the equimolar PLCL copolymer, were prepared using different solvents. The decrease in the fiber diameter of the fabric resulted in a decrease in porosity and pore size, as well as an increase in fiber density and mechanical strength. Human umbilical vein endothelial cells (HUVECs) were well attached and proliferated on the small fiber diameter fabrics. Results suggest that these electrospun elastomeric fibers could be combined into multiscale scaffolds to yield more advantageous responses in cardiovascular and muscular tissue engineering endeavors by harnessing the positive characteristics of the differently scaled fibers in a single scaffold [71]. Further *in vivo* studies are essential to confirm its application for cardiac tissue engineering.

5 Logistics and Limitations

Often when nanofibers are incorporated into a microscale scaffold you see increased cellular attachment and spreading because nanofibers more closely resemble the scale of ECM than do microfibers and in turn act as cellular bridges across larger pores. While this is often a positive result for a multiscale scaffold, when the density of nanofibers is too great cellular infiltration of the scaffold is also limited, which is a detriment to the development of larger 3D tissues. Previous studies have shown that dense layers of nanofibers formed by as little as 300 s of electrospinning can significantly hinder the infiltration of cells into a scaffold [52]. For this reason, it is important to optimize the proportion of nanofibers to microfibers in multiscale scaffolds in order to maximize the beneficial effects of nanofibers while still harnessing the positive attribute of larger pore sizes usually associated with microscale scaffolds.

Due to the nature of the electrospinning process it is very difficult to independently vary the proportions of two differently scaled fiber diameters while maintaining consistent fiber characteristics. This is due to the fact that one of the primary ways to regulate fiber proportions is by adjusting the polymer extrusion flow rate. Consequently, flow rate also has a major influence on fiber diameter. Another strategy for controlling the relative proportion of different fiber scales is by interrupting the flow of one polymer stream to reduce the relative amount of that particular fiber. However, in order to interrupt the flow of a polymer stream while electrospinning, one must: (1) interrupt the voltage source, thus stopping the propulsion of the polymer solution; or (2) pause the extrusion of the polymer solution, this halts polymer flow yet a small amount of polymer may still be drawn out of the spinnerette due to the active electrical field; or (3) physically block the fibers from reaching the target, which may be difficult to do if other scaled fibers are being electrospun in close proximity to the fibers being manipulated. Furthermore, all of these methods of controlling fiber proportions generate issues regarding reproducibility. A summary of the tissue engineering applications of the multiscale fibrous scaffolds discussed in this review is given in Table 1.

Table 1 Applications of multiscale fibrous scaffolds for tissue engineering

Tissue	Diameter	Fiber fabrication	Application	Key results	Ref.
General tissue engineering	10 μm , 3–5 μm , and 500 nm	Aligned electrospun PGA/collagen fibers	In vitro culture of NIH-3T3 fibroblast-like cells	Increased cellular adhesion and spreading on nanofibers as compared to microfibers, regardless of fiber composition	[47]
	8 μm , 9–9.5 μm , 700–750 nm, 850–980 nm	Electrospun PLA micro/nanofibers without and with nHA	In vitro culture of MG-63 osteoblast-like cells	Cell penetration and proliferation on multiscale scaffolds was higher than for the control. Cells were dispersed throughout the scaffold. The addition of nHAp further improved the bioactivity	[53]
	34.1 $\pm$ 2.9 μm yarn and nanoscale fibers	Knitted tube of carbon nanotube yarn coated with electrospun PLGA nanofibers	In vitro culture of NR6 fibroblast-like cells	Electrospun nanofibers supported cell spanning across large pores while limiting infiltration of the tube	[55]
	3.3 μm and 600 nm	Electrospun PCL micro/nanofibers	In vitro culture of murine MSCs	Compared to monoscale micro- and nanocontrols, multiscale scaffolds led to increased adhesion, viability, and scaffold infiltration	[57]
Bone	365.4 $\pm$ 18.3 μm and 1 μm	Aligned melt-extruded PCL microfibers and electrospun PLGA fibers	In vitro culture of MC3T3 preosteoblast-like cells	Compared to extruded microfiber controls, dual-scale scaffolds led to increased cell viability and adhesion	[32]
	6.8 $\pm$ 0.4 μm with 244.7 $\pm$ 18.8 nm or 11.3 $\pm$ 1.9 μm with 269.5 $\pm$ 42.2 nm	Electrospun PCL micro-, nano-, and multiscale fibrous scaffolds with and without nHA	In vitro culture of MG-63 osteoblast-like cells	Cell activity, proliferation, and total protein adsorption were higher on scaffolds containing nanofibers than on those containing microfibers	[46]

(continued)

Table 1 (continued)

Tissue	Diameter	Fiber fabrication	Application	Key results	Ref.
	160 µm and 400 nm	Starch/PCL fiber bonded microscaffolds with electrospun nanofibers	In vitro culture of SaOs-2 osteosarcoma-like cells and rat MSCs	Compared to microfiber scaffolds, multiscale scaffolds displayed higher cell viability, ALP activity, and stretched cell morphology	[48]
	Bilayered scaffolds of 5 µm and 600 nm	Electrospun PCL micro/nanofibers	In vitro culture of rat MSCs	Nanofibers increased cell spreading and decreased infiltration while having little to no effect on attachment	[52]
	160 µm and 400 nm	Starch/PCL fiber bonded microscaffolds with electrospun nanofibers	In vitro culture of HUVECs and HDMECs	Cells on nanofibers spanned adjacent microfibers and adopted capillary-like morphologies while remaining sensitive to pro-inflammatory stimuli	[54]
	460 µm or 500 µm and nanoscale fibers	Melt-extruded PBS or PBS/Chit microfiber structures with electrospun Chit	In vitro culture of human bone marrow MSCs	Nanofibers led to increased deposition of calcium phosphate, especially in combination with PBS/Chit microfibers	[62]
Cartilage	200–250 µm pore size and 40–80 nm	Fibrin, mineralized fibrin, or fibrin/nHA microporous, nanofibrous scaffolds	In vitro culture of murine calvarial cells and in vivo implantation of acellular scaffolds	Mineralized fibrin scaffolds showed higher ALP activity than fibrin and fibrin/nHA scaffolds. Scaffolds promoted bone formation in a mouse defect model	[63]
	405 ± 15 µm and 431 ± 148 nm or 325 ± 128 nm	DPM deposited PCL microfiber structures with electrospun PCL or PCL/collagen nanofibers	In vitro culture of bovine chondrocytes	Cell attachment and proliferation was improved by the addition of electrospun nanofibers, with the largest increase due to PCL/collagen nanofibers	[64]

Skin	28 μm and 530 nm	Hybrid electrospun PLGA micro/nanofibers	In vitro culture of human epidermal keratinocytes and fibroblasts	Compared to microfiber controls, the cell attachment, spreading, and infiltration were higher in micro/nanofiber composite scaffolds [65]
Nerve	Microscale fibers and 200–600 nm	Electrospun Cht nano/microfibrous tubes with or without Cht film outer coat	In vivo implantation in rat sciatic nerve	The Cht nano/microfiber mesh tubes effectively maintained defect space and enabled superior cell migration, permeation, and functional recovery [68]
	Bilayered tubes of 7.5 $\pm$ 2.0 μm and 279 $\pm$ 87 nm	Electrospun PCL microfibers with PCL/PLGA nanofibers	In vivo implantation in rat sciatic nerve	Implants led to nerve regeneration, muscle re-innervation, as well as some evidence of myelination [69]
Cardiac	7.0 $\pm$ 1.0 μm , 1.2 $\pm$ 0.2 μm , and 320 $\pm$ 80 nm	Electrospun PLCL nano-to microfiber fabrics	In vitro culture of HUVECs	Cell attachment and proliferation was higher on the highly packed nano- and 1- μm -scaled-fiber fabrics in comparison to the control [71]

ALP alkaline phosphatase, *Cht* chitosan, *DPM* direct polymer melt, *HDMECs* human dermal microvascular endothelial cells, *HUVECs* human umbilical vein endothelial cells, *MSCs* mesenchymal stem cells, *nHA* nanocrystalline hydroxyapatite, *PBS* poly(butylene succinate), *PCL* poly(ϵ -caprolactone), *PGA* poly (glycolic acid), *PLA* Poly(lactic acid), *PLGA* poly(D,L-lactic-co-glycolic acid), *PLCL* poly(L-lactide-co- ϵ -caprolactone)

Conversely, it is also challenging to alter fiber diameters while keeping the relative fiber scale proportions constant, for the same list of reasons. This makes it challenging to design effective studies to examine the effect of fiber size or relative fiber type proportions on cellular responses within multiscale scaffolds. It would be easier to alter the fiber diameter or relative proportions of micro- to nanoscale features if electrospun nanofibers were coupled to a different fabrication technique such as polymer melt deposition. However, it can be argued that this might not produce a reasonably homogenous scaffold with an even mixing of nano- and microfeatures throughout the entire scaffold, which could in turn influence results.

6 Conclusions and Future Directions

Techniques to produce multiscale biomaterial scaffolds with designer geometries are ‘the need of the hour’ to provide improved biomimetic properties for functional tissue replacements. While micrometer fibers generate an open pore structure, nanofibers support cell adhesion and facilitate cell–cell interactions. This was further proven by cell penetration studies, which showed superior ingrowth of cells into hierarchical structures. Mixed bimodal scaffolds of two different polymers are another promising approach, because they exhibit hierarchical pore/surface systems and combine the beneficial properties of both polymers at two different scales. Various 3D micro- and nanoscale multiscale scaffolds have been fabricated through various techniques and were found to have the potential to essentially recreate natural bone, cardiac, neural, and vascular tissues.

The multiscale system also appears to be capable of providing more enhanced biological functionality, particularly for vascularization, which is favored by the interaction of ECs with the nanofibrous networks that allow suitable cell architecture and orientation for microtubule formation. Thus, the synergistic effect of micro- and nanoscales could successfully regenerate natural tissues *in vivo* in the near future. Future work should focus on optimizing this process to better recapitulate key features of the native ECM, including its mechanical and biochemical properties, which would enhance the functionality of these 3D multiscale scaffolds in order to fabricate functional tissue engineered constructs.

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