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REVIEW

Gradient Nanofiber Scaffolds for Tissue Engineering

Azadeh Seidi[1,*], Kaarunya Sampathkumar[2], Alok Srivastava[2], Seeram Ramakrishna[3,4], and Murugan Ramalingam[2,5,6,*]

[1] *Technology Center, Okinawa Institute of Science and Technology (OIST), Onna-son, Okinawa 904-0412, Japan*
[2] *Centre for Stem Cell Research (CSCR), A Unit of Institute for Stem Cell Biology and Regenerative Medicine, Bengaluru, Christian Medical College Campus, Vellore 632002, India*
[3] *Faculty of Engineering, Center for Nanofibers and Nanotechnology, National University of Singapore (NUS), 2 Engineering Drive 3, Singapore 117576, Singapore*
[4] *Department of Biomedical Engineering, Jinan University, Guangzhou 510632, China*
[5] *Faculté de Chirurgie Dentaire, Institut National de la Santé Et de la Recherche MédicaleUMR977, Université de Strasbourg, Strasbourg 67085, France*
[6] *WPI-Advanced Institute for Materials Research (WPI-AIMR), Tohoku University, Sendai 980-8577, Japan*

Scaffolds are one of the key factors for the success of tissue engineering, in particular when dealing with anchorage-dependent cells. The concept of using scaffolds in tissue engineering lies in mimicking the physical, chemical and biological features of native extracellular matrix (ECM) in order to support cell function, which in turn regulates cellular microenvironment that directs cell growth and subsequent tissue formation. Nanofibers fabricated from both synthetic and natural polymers are being used as scaffolds in many tissue engineering applications. At the molecular level, native ECM is made up of a gradient of fibrous proteins and polysaccharides that are nanoscale structures. The gradient cues of ECM, directs critical cell behaviors such as alignment, motility and differentiation, particularly in the region between soft and hard tissues called interfacial tissue. Therefore, it is essential to develop gradient nanofiber scaffolds particularly for interfacial tissue engineering applications. Keeping these points in view, in this article, we review the recent developments of gradient nanofiber scaffolds, their design strategies, and their applications in tissue engineering.

Keywords: Gradient Nanofibers, Scaffold, Electrospinning, Interfacial Tissue Engineering.

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1. INTRODUCTION

The interdisciplinary field of tissue engineering applies the principles and methods from engineering, physical, chemical and biological sciences with the aim of designing and fabricating biological tissue alternatives to repair or regenerate defective tissues or organs that fail to heal spontaneously.[1–5] In general, the concept of scaffold-based tissue engineering involves the *in vitro* culture of specific cells, obtained from a patient or donor, onto a scaffold that mimics the microenvironment of tissue to be engineered in order to grow a physiologically functional tissue, which is to be transplanted back to the patient's body where the tissue gets repaired or regenerated. The concept is schematically represented in Figure 1. In the context of tissue engineering, scaffolds play a major role by providing structural and temporal support for cell culture, and facilitating delivery of bioactive molecules such as growth factors to cells, which in turn enable adequate environment suitable for cell attachment, proliferation, differentiation and subsequent tissue organization. Therefore, scaffolds are considered as a key element for success of engineering functional tissues.

*Authors to whom correspondence should be addressed.

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Azadeh Seidi is a Biochemist at Okinawa Institute of Science and Technology, Japan. Since earning her Ph.D. from Tokyo Institute of Technology, she has focused her activities on biomedical researches, both on biochemical and engineering levels.

Ms. Kaarunya Sampathkumar is a Junior Research Fellow at the Centre for Stem Cell Research, Christian Medical College Campus, India. She received her M.Tech. degree in Medical Nanotechnology from SASTRA University. Her research interests include the development and characterization of nanobiomaterials for stem cell engineering, tissue regeneration, and smart drug delivery.

Alok Srivastava, MD, FRACP, FRCPA, FRCP is Professor of Medicine at the Christian Medical College, Vellore, India. He is the head of the department of Haematology and the Center for Stem Cell Research which is unit of in Stem, Bengaluru. He is among the pioneers who have developed stem cell transplantation in India and is responsible for initiating the Indian Stem Cell Transplant registry. He has been the co-chair of the Task Force for Stem Cell Research and Regenerative Medicine of the Department of Biotechnology, Ministry of Science and Technology, Government of India and is also the chair of the National Apex Committee for Stem Cell Research and Therapy of the Department of Health Research of the Ministry of Health, Government of India. He also serves as the Vice-chair for Asia, Africa and Australia on the Center for International Blood and Marrow Transplantation Research advisory committee. His research involves both basic and clinical aspects of hematopietic stem cells. He has also been involved with research in hemostasis, both clinical and genetic aspects and is currently coordinating the development of gene therapy for hemophilia in India.

Seeram Ramakrishna, FREng, FNAE, FAIMBE is the Professor and Director of Center for Nanofibers and Nanotechnology at the National University of Singapore. He specialises in design, processing and validation of biomimetic scaffolds for regeneration of various tissues. He pioneered translucent biomaterials and devices, which are now manufactured and marketed globally by Biomers International. He is acknowledged as number one in the field of materials by electrospinning (http://researchanalytics.thomsonreuters.com/m/pdfs/grr-materialscience.pdf). He authored five books and ~ 500 international journal papers, which attracted $\sim 20{,}000$ citations with H-index of 71. He delivered more than one hundred plenary and key note lectures worldwide including Kavli Prize Nanoscience Symposia Lecture. He is placed 25th in the world in biocompatable materials experts by Elsevier Science. He mentors research teams in China, India, Malaysia, Japan, South Korea, Taiwan, Germany, and Italy. Various global databases including Thomson Reuters, Scopus, Google Scholar and Microsoft Academic place him among the top one percent of materials scientists worldwide (ISI Web of Knowledge Essential Science Indicators, ESI rank is 27). He is an elected fellow of major engineering academies and professional societies in Singapore, ASEAN, India, UK and USA.

Murugan Ramalingam is an Associate Professor and Scientist G at the Centre for Stem Cell Research, Christian Medical College and Hospital Campus, India. Concurrently he is an Adjunct Associate Professor at the Tohoku University, Japan. Prior to joining the CSCR, he was an Associate Professor of Biomaterials and Tissue Engineering at the Institut National de la Santé et de la Recherche Médicale, Faculté de Chirurgie Dentaire, Université de Strasbourg, France. He has worked at the WPI Advanced Institute for Materials Research, Japan, as an Assistant Professor. He has also worked at the National Institute of Standards and Technology, USA and the National Institutes of Health, USA, under the U.S. National Academies Associateship program. He received his Ph.D. in Biomaterials from the University of Madras. He has also undergone training in Ethical and Policy issues on Stem Cells from Harvard University, USA, and in Operations Management from the University of Illinois-Chicago. His current research interests are focused on the development of multiphase biomaterials, through conventional to nanotechnology to biomimetic approaches, cell patterning, stem cell differentiation, and tissue engineering. He is the author of over 150 publications, including peer-reviewed journal papers, conference proceedings, book chapters, authored books, edited books, and patents relevant to biomaterials, stem cells, and tissue engineering. He has organized several international conferences and chaired Biomaterials, Nanotechnology and Tissue Engineering sessions. He also serves as a board member of several international scientific and research committees in various public and private bodies and grant reviewer of international funding agencies. He serves on the editorial boards of multiple biomaterials and tissue engineering-related journals, including as the Editor-in-Chief of the *Journal of Biomaterials and Tissue Engineering*, and the *Journal of Bionanoscience*. He is a recipient of several prestigious fellowships and awards, including CSIR Fellowship (India), SMF Fellowship (Singapore), NRC National Academies Fellowship (USA), Nationale Professeur des Universités (France) and Fellow of Royal Society of Chemistry (UK).

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Native extracellular matrix (ECM) consists of structural proteins, specialized soluble and insoluble factors, and proteoglycans, which together constitute a highly hydrated and viscoelastic network, to support and regulate cellular functions and subsequent tissue organization. The structural proteins of ECM exhibit nanoscale features in the form of fibrils and pillars, which control the cell-matrix interactions.[2,6] This nanoscale structure of native ECM components is essential to guiding specific tissue formation through a phenomenon known as contact guidance. In this regard, nanofiber-based scaffolding systems have shown unique potential for tissue engineering by offering amazing functional properties such as high surface area to scaffold's volume ratio, adequate porous structure and nanotopographical features, which promote cell adhesion, migration, orientation, proliferation, infiltration and differentiation.[7–9]

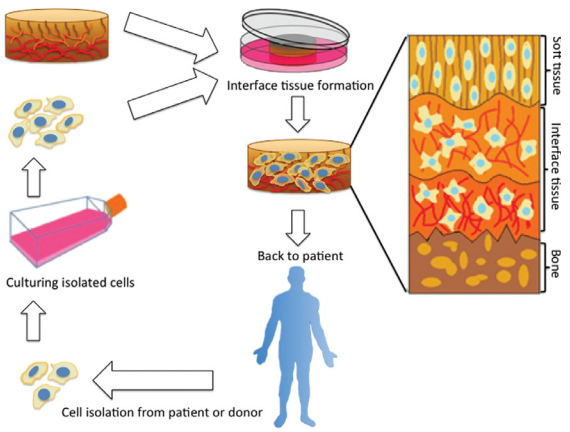

Fig. 1. Schematic of the concept of interface tissue engineering, using scaffolds.

In addition to nanoscale structure, surface functionality of ECM with appropriate biomolecules also plays critical role in regulating cell behavior and subsequent tissue organization. The surface functionality often depends on biophysical and biochemical cues, which are provided by ECM fibrous proteins, soluble and insoluble macromolecules. They often exist in non-homogeneous fashion and particularly appear in the form of gradients to guide cell behavior within a native tissue. For example, during a phenomenon known as chemotaxis, chemical gradients in the body cause cell migration along the concentration gradient of a chemo-attractant. Chemotaxis is responsible for important biological events such as the migration of leukocytes toward sites of inflammation and wound healing.[2] Therefore, in order to provide cells with biomimetic gradient features of their native microenvironments, gradient nanofibers have recently been explored to take advantage of the gradient nature of structure and function, which support the growth of heterotypic cell lineages in a gradient manner specifically suitable for interface tissue engineering applications such as cartilage-to-bone, tendon-to-bone or ligament-to-bone in addition to high-throughput drug or biomolecule screening, as shown in Figure 2.[3,10] Therefore, tissue scaffolds that best mimic the native ECM of the body is essential to support cell functions and to develop tissue grafts with the ability to integrate into a patient's body and restore the function of the lost or damaged tissue.

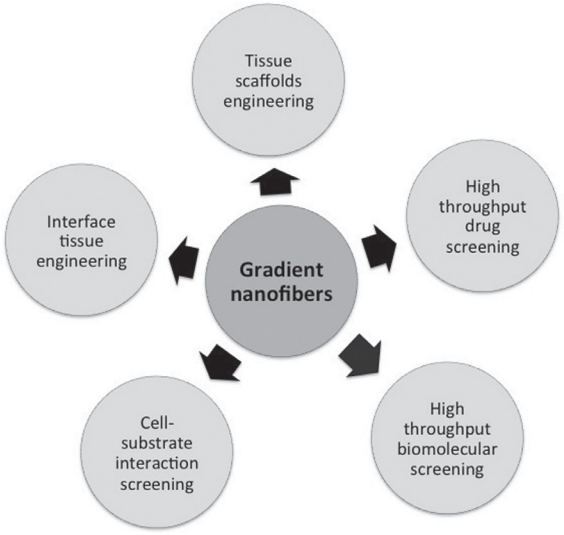

Fig. 2. The widespread applications of gradient nanofibers.

In view of the above mentioned points, this article reviews the impact of nanofiber scaffolds with special emphasis on the emerging role of gradient nanofibers, their fabrication methods and their applications in tissue engineering.

2. METHODS OF FABRICATING NANOFIBER SCAFFOLDS

Engineering of nanofiber scaffolds with structural and functional features similar to native ECM is a pre-requisite for engineering of physiologically functional tissues. It is necessary to ensure a large surface area, optimum aspect ratio, high porosity and adequate pore size of the scaffold to facilitate better cell adhesion, infiltration and continued function. Cell adhesion is the very initial step during the process of cell response to scaffolds and is considered as one of the prime factors that influence other cellular behaviors such as cell migration, proliferation, and differentiation. Cell infiltration is another important feature, which is essential for three-dimensional (3D) cell growth, transport of nutrients and oxygen supplies, and tissue remodeling. Tissue scaffolds can be manufactured by different methods. However, only a very few methods can have the ability to fabricate nanofiber scaffolds. The most commonly used methods are electrospinning, self-assembly, and phase separation. In the following sections, how these methods can be utilized to fabricate the scaffolds and how it can be used in tissue engineering are briefly discussed.

2.1. Electrospinning

Electrospinning is almost a century-old simple and versatile technique that essentially employs electrostatic forces

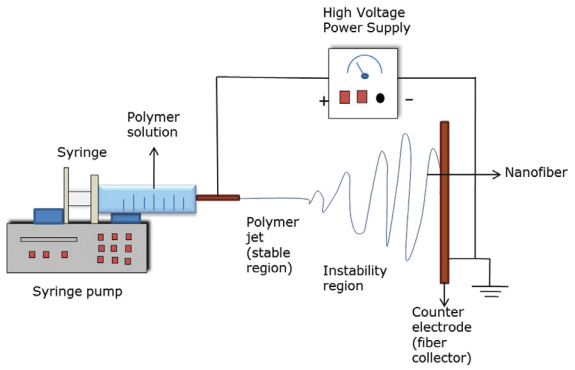

Fig. 3. Schematic representation of electrospinning set-up.

to produce polymer fibers and their composites, ranging in size from a few microns down to tens of nanometers. This is one of the widely exploited techniques for the manufacture of scaffolds suitable for basic cell studies and tissue engineering applications owing to its ability to fabricate scaffolds with functional properties favorable for the culture of most of the cells and subsequent tissue organization.[4]

Schematic representation of electrospinning set-up is shown in Figure 3, which consists of a syringe pump, fiber collector, and a high-voltage power system. As illustrated in the figure, the polymer solution is taken in the syringe and can be delivered at a controllable feed rate with the help of an automated syringe pump. The fiber-collecting device is positioned at an appropriate distance from the needle. A high-voltage/low-current power system is required for the conversion of polymer solution into a charged polymer jet. The electric power (usually up to 30 kV) is applied across the needle and the metallic counter electrode (fiber collector), which is grounded, to facilitate ejection of the charged polymer jet from the needle tip towards the fiber collector, which leads to fiber formation.

The size and shape of the electrospun fibers can be controlled by varying the solution and system parameters such as the molecular weight and viscosity of the polymer, the size of the spinneret, the flow rate of the polymer, the distance between the needle tip and the fiber collector, and the intensity of the applied electric potential. Moreover, depending on the collector's static or dynamic motion, it is also possible to fabricate nanofiber mats with random or aligned fibers. The mentioned parameters impart versatility to the electrospinning technique to produce nanofibers with suitable specification and function, which can be tailored for various applications.[11–13] A wide range of natural polymers, synthetic polymers and polymer blends[14] have been utilized for producing nanofibers by electrospinning. Examples include natural polymers such as collagen,[15–18] gelatin,[19,20] chitosan,[20] hyaluronic acid,[21] and silk fibrion.[22] Examples of synthetic polymers include poly(lactic

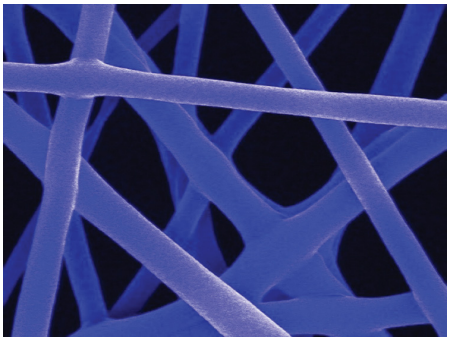

Fig. 4. A representative SEM micrograph of polycaprolactone nanofibers fabricated by electrospinning.

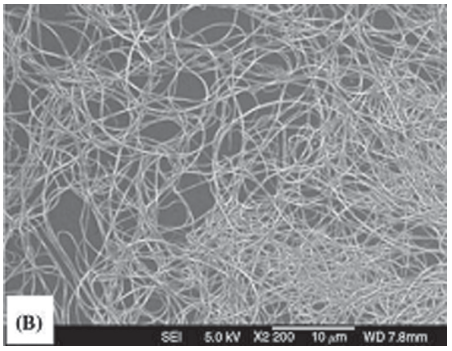

Fig. 5. Scanning electron micrograph image of self-assembled peptide amphiphiles nanofibers with incorporated RGD molecules. Reprinted with permission from [36], H. Hosseinkhani et al., *Biomaterials* 27, 4079 (**2006**). © 2006, Elsevier.

acid) (PLA),[23] polyurethane (PU),[24] poly(ε-caprolactone) (PCL),[25] poly(lactic-co-glycolic acid),[26] poly(ethylene-co-vinylacetate),[27] and poly(l-lactide-co-ε-caprolactone),[28,29] poly(vinyl alcohol),[30] polyacrilonitrile,[31] polyaniline.[32] Figure 4 shows polycaprolactone nanofibers fabricated by electrospinning. However, fiber meshes produced by this method have limited mechanical properties unless otherwise they are further processed.

2.2. Self-Assembly

Self-assembly is a process in which individual, pre-existing components spontaneously organize themselves into an ordered structure by means of weak interactions. The process of self-assembly takes place by non-covalent bonding, which typically includes hydrogen bonding, ionic bonding, hydrophilic, hydrophobic and Van der Waals force interactions. Although each of these forces is rather weak, their collective interactions could produce a very stable structure that could match the structural features of biological systems. Peptide amphiphiles (PAs) are notable examples of such molecules, which consist of a hydrophobic tail component (such as dialkyl chain moiety) attached to a hydrophilic head component (such as *N*-alpha amino group of a peptide chain), forming a "peptide amphiphile," which readily self-assemble into a stabilized helical conformation in an aqueous solvent.[33,34] Furthermore, bioactive sequences have been added to the PA assembling components to improve the bioactivity of the resulting nanofibers to guide cell response to the bioactive molecule patterns on the exterior of the assembled helix, hence giving these PA structures the potential to be used as surface coatings for biomaterials to improve biocompatibility.[35] One such PA self assembled nanofiber, with RGD functional motifs on the surface as in Figure 5, prepared by Hosseinkhani et al. has been used by the authors to demonstrate the successful attachment of mesenchymal stem cells and their differentiation to osteogeniclineage.[36] Engineering of the assembling components could alter the characteristics of the nanofibers fabricated by self-assembly technique. For instance, it has been demonstrated that due to the hydrophobic interaction between alkyl chains, increasing the chain length of the hydrophobic component, increases the thermal stability of the PAs.[37] In another example, Hartgerink et al. developed a pH-sensitive assembly of di- and tri-block PAs into a rod-like architecture with 5–8 nm in diameter and several microns in length by engineering the peptide head group of the PA.[38,39] Further, self-assembly of oppositely charged PAs by electrostatic interaction at neutral pH has been developed by Niece et al.[40] Thus, self-assembly can be utilized for the fabrication of nanofiber scaffolds with desired chemical and physical properties. However, engineering suitable molecular building blocks that can undergo spontaneous organization into a well-defined pattern that mimics complex biological systems is necessary and the research should be progressed on this direction.

2.3. Phase Separation

Phase separation is a simple technique for manufacture of nanofiber scaffolds, which involves various key processing steps such as dissolution of raw materials, gelation, solvent extraction, freezing, and drying.[7] Yang et al. have fabricated a nanofiber poly(l-lactic acid) (PLLA) scaffold, using the above processing steps. The authors have used the scaffold to successfully culture nerve stem cells (NSC) resulting in its differentiation and neurite outgrowth.[41] He et al. have fabricated a nanofiber scaffold by liquid–liquid phase separation of a ternary system of PLLA/water/dioxane and used the same to study the attachment and proliferation of mesenchymal stem cells.[42]

Of the three methods described above, electrospinning has been the most widely used technique for the fabrication of scaffolds suitable for cell culture and tissue engineering purpose. In the following section, we will further focus on how the electrospinning technique can be extended for the fabrication of gradient nanofiber-based scaffolds suitable for tissue engineering.

3. GRADIENT NANOFIBERS

Nanofibers as scaffolding material find applications in engineering homotypic tissues such as bone,[43,44] meniscus,[45] cartilage,[46] and ligament[25] owing to their structural and other functional properties quite similar to native ECM.[5,8,9,47–52] Gradient nanofibers have been increasingly gaining attention as scaffolding material for tissue engineering applications mainly due to their ability to accommodate multi cell types that support the organization of complex heterotypic tissues in particular interfacial tissues (soft-to-hard, for example), which can not be expected from the scaffolds made of conventional homologous nanofibers. Nanofiber scaffolds with gradient of chemical or structural functionalities also have ability for use in high-throughput cell and drug screening. This can also be extended to high-throughput screening of biomolecules. The gradient nanofiber scaffolds retain other functional properties that favor cell growth and function similar to homologous nanofiber scaffolds.[53] The field of gradient nanofibers is still in its infancy and technologies are being developed to design such gradient nanofiber scaffolds suitable for tissue engineering applications. In the following sub-sections, we discuss currently available techniques for fabricating gradient nanofiber scaffolds.

3.1. Fabrication of Gradient Nanofibers During Electrospinning

In an approach to produce gradient nanofibers directly using electrospinning, Ramalingam et al. developed a technique using two-spinnerets system that were placed in close proximity to dispense simultaneously two different types of polymeric solutions in order to make gradient of nanofibers, which were collected in an overlapping pattern, resulting in a gradient in nanofiber composition.[54] The formation of such a gradient nanofibers, as illustrated in Figure 6, was obtained using the two-spinnerette assembly. One spinnerette spun clear PCL fibers while the second spinnerette spun PCL fibers with Sudan IV yielding overlapping mats of nanofibers with different compositions that can be easily visualized by the naked eyes. This clearly indicates the formation of gradients during the electrospinning process.

Zhang et al. have prepared gradient nanofibers using a microfluidic device to mix the polymer solution and an electrospinning nozzle to deposit the fiber on a dynamic 3D platform. The authors also used the system to successfully create a gradient of Teflon nanoparticles, drug and biomolecules.[55] Electrospinning technique can also be used in combination with extrusion to impart various ingredients into electrospun nanofiber mat in a time-dependent way during the spinning process. This hybrid technique is based on integrating a twin-screw extruder with fully intermeshing and co-rotating screws,to the electrospinning process.[56] The time-dependent way of extruding various ingredients into the electrospinning polymeric

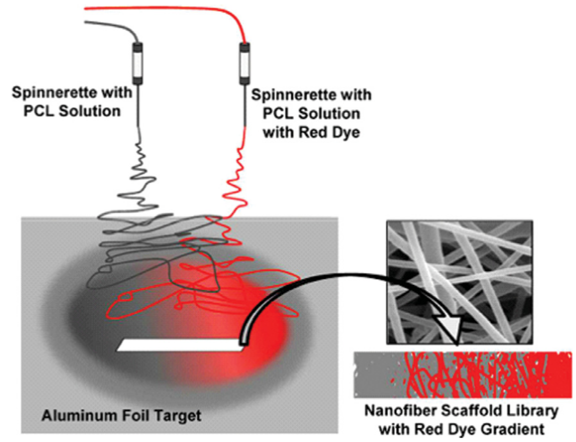

Fig. 6. A two-spinnerette electrospinning device for fabricating gradient nanofibers. Two spinnerettes are placed side-by-side and used to electrospin two different polymer solutions to yield overlappingmats of nanofibers with different compositions. Reprinted from with permission from [54], M. Ramalingam et al., *J. Biomater. Appl.* (**2012**). © 2012, SAGE Publications.

solution results in the formation of nanofiber mats with spatially graded properties. Erisken et al. introduced this method to fabricate a gradient composite of PCL and ß-tricalcium phosphate (ß-TCP) nanofibers.[57] The authors controlled the feed rates of ß-TCP nanoparticles extrusion into PCL during electrospinning process, which generated a nanofiber material with a linear variation in ß-TCP concentrations, ranging from 0–15%wt (Fig. 7).

3.2. Fabrication of Gradient Nanofibers After Electrospinning

Gradient features can also be imparted to nanofibers even after electrospinning, which is in contrast to the above one step direct spinning process. For example, Shi et al. generated a fibronectin gradient in a polymethylglutarimide nanofiber matrix by placing it in a chamber, which was then filled in a controlled manner from the bottom with the fibronectin solution.[58] Li et al. utilized a similar technique to fabricate a nanofiber matrix with a gradient in mineral composition (see Fig. 8).[59] The authors further developed their method to fabricate gradient mineralized electrospun nanofibers with change in fiber orientation from aligned to random, attempting to mimic the change in collagen fiber orientation at the tendon-to-bone interface.[60] In another study Valmikinathan et al. have developed a polycaprolactone-poly(ethyleneglycol) (PCL-PEG) nanofiber scaffold with a laminin gradient. Laminin was cross-linked to ferritin and the protein deposition was controlled by magnetic field.[61] Du et al. have fabricated a chitosan-polycaprolactone (CS-PCL) gradient nanofiber matrix by co-electrospinning of two polymer solutions wherein when the flow rate of one polymer was increased sequentially, the flow rate of the other was decreased.

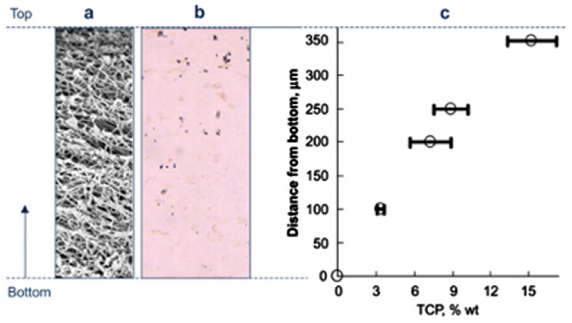

Fig. 7. Scanning electron micrograph of cross-section of the functionally graded non-woven mesh (a), von Kossa staining for distribution of β-TCP nanoparticles along distance (b), and the quantification of β-TCP concentration with thermo-gravimetric analysis apparatus (c). Reprinted with permission from [57], C. Erisken et al., *Biomaterials* 29, 4065 (**2008**). © 2008, Elsevier.

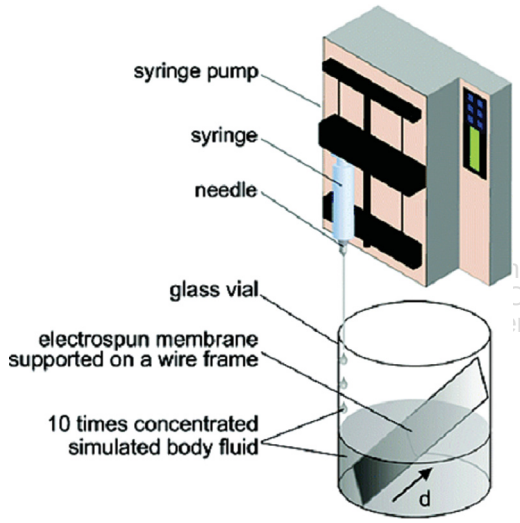

Fig. 8. Fabrication of electrospun nanofibers with a gradient in mineral composition by graded exposure of the scaffold to mineral solution. Reprinted with permission from [59], X. Li et al., *Nano Lett.* 9, 2763 (**2009**). © 2009, American Chemical Society.

They have also functionalized the scaffold with heparin gradients by covalent binding to chitosan backbone.[62] This system has been utilized for vascular tissue engineering application, which is also briefly described in Section 4. In the next section, we discuss applications of gradient nanofiber-based scaffolds in basic cell studies and in interface tissue engineering.

4. APPLICATIONS OF GRADIENT NANOFIBERS IN TISSUE ENGINEERING

The complex interface between damaged soft-to-hard tissues (see Fig. 9) cannot be restored using homogeneous biomaterials, which fail to integrate into the host tissues due to the lack of ability to create an interface tissue.[63]

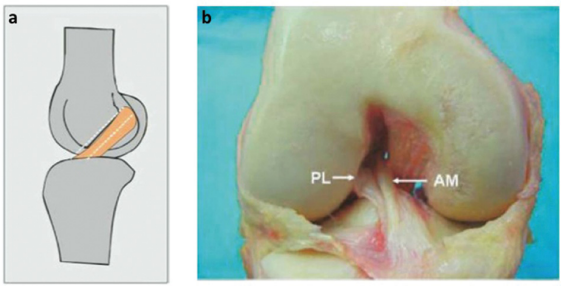

Fig. 9. Anatomical shape of the insertion areas at the femoral and tibial insertion site. Common reconstruction techniques do not replicate the foot type insertion at the tibia. A straight graft (dotted lines) such as a bone–patellar tendon–bone or hamstring graft may impinge at the notch in knee positions close to extension (a). Human right knee joint from anterior. The patellar tendon and the surrounding soft tissue have been removed to inspect the anterior cruciate ligament (ACL). Note the 2 distinct bundles, the Anteromedial (AM) and Posterolateral (PL) bundles of ACL (b). Reprinted with permission from [63], T. Zantop et al., *Operative Techniques in Orthopaedics* 15, 20 (**2005**). © 2005, Elsevier.

Gradient nanofiber-based scaffolds have been particularly investigated for engineering of interface tissues in the body. Such gradient nanofiber scaffolds have been evaluated for their potential use in various tissue engineering applications. In a notable study, Li et al. generated a non-woven mat of electrospun nanofibers with a linear gradient of calcium phosphate minerals that would match tendon-to-bone insertion site.[59] The gradient in mineral content created a gradient in the stiffness of the scaffold, where regions with 23% higher mineral content exhibited two-fold higher Young's modulus. This gradation in material composition and mechanical properties imparted 'functional gradation' to the material for a smooth stress transfer at the interface. Moreover, the authors observed that MC3T3-E1 cells, cultured on this gradient scaffold, exhibited a graded level of attachment, with the highest cell density at the region with highest calcium phosphate content. The same group further altered the fiber orientation to fabricate nanofiber scaffolds with aligned orientation, which mimicked the structure of tendon-to-bone insertion sites.[60] The authors observed that the cells cultured on the aligned scaffold, showed better alignment and expression of collagen type I. Valmikinathan et al. have used the laminin coated PCL-PEG scaffold to culture Schwann cells. Their results indicate that the scaffold could have potential use in nerve tissue engineering to bridge gaps more than 10 mm for injuries in the peripheral nervous system.[61]

In another example, Ramalingam et al. fabricated PCL nanofibers with gradients in amorphous calcium phosphate nanoparticles (*n*ACP), and demonstrated that osteogenic cells cultured on this scaffold responded to *n*ACP gradient by exhibiting a graded pattern of adhesion and proliferation, which is a similar phenomenon observed in the native interface tissues.[54] In an attempt to mimic ECM structure of a typical cartilage-bone interface, Erisken et al. developed a hybrid twin-screw extrusion/electrospinning system

to generate PCL nanofibers with a gradient incorporation of tricalcium phosphate.[57] The authors observed that culturing MC3T3-E1 cells on these nanofibers resulted in the synthesis of ECM proteins such as collagen, as well as scaffold mineralization in a graded fashion, as seen in a typical cartilage-bone interface. Du et al. have shown that sandwich co-culture of human umbilical vein endothelial cells and smooth muscle cells on a CS-PCL nanofiber scaffold with heparin and vascular endothelial growth factor gradient, results in rapid endothelialization mimicking the lumen and adventitia of natural blood vessels. Such heparinised gradient nanofibers have potential applications in vascular tissue engineering.[62] These experimental examples discussed in this section, in addition to others, demonstrate the emerging of gradient nanofibers as scaffolds for use in tissue engineering, in particular when engineering interfacial tissues or graded tissues.

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5. CONCLUSION

Biomaterials in the form of nanofiber scaffolds are widely used in tissue engineering. Cells grow in a distinct pattern to organize specialized tissue formation, function and homeostasis. Heterotypic tissues in body possess a complex structure and properties replete with graded physical and biochemical cues to fulfill their intended functions. This natural complexity demands more sophisticated design of tissue scaffolding system to aid in tissue repair and regeneration. Therefore, it is essential to develop techniques for engineering biomaterials with biomimetic gradients in composition, structure, and functional properties as tools for engineering interface tissues or graded tissue patterns, and studying cellular response.

In this article, the three major techniques for fabricating nanofibers were described. Of these, electrospinning is the most extensively used technique to fabricate nanofiber scaffolds for tissue engineering applications. It's also being used in the development of gradient nanofiber scaffolds that mimics the native ECM at the interface tissues. The experimental examples discussed in this article, and other literatures, clearly show the impact of gradient nanobiomaterials in engineering interface tissues and other complex graded tissues. However, the methods for fabricating such scaffolds are limited. Therefore, future research is tasked with developing new methods that impart gradient functionality within the nanofiber scaffolds to recreate the physical and chemical complexity of native tissues. Smart nanofiber scaffolds incorporating multiple gradient cues could then be fabricated for mimicking the cellular and structural characteristics of *in vivo* cellular microenvironments. In addition, there should be further attempts to impart better mechanical properties to nanofiber scaffolds by improving material synthesis techniques. This is an exciting time to be involved in gradient nanobiomaterials in order to formulate them as a clinically ideal scaffolding system for high-throughput cell screening and tissue engineering, with great challenges and also great expectations ahead.

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