Review

Title: Electrospun fibers for recruitment and differentiation of stem cells in regenerative medicine†

Sharanya Sankar¹, Chandra S. Sharma², Subha N. Rath¹*, Seeram Ramakrishna³*

¹Department of Biomedical Engineering, Indian Institute of Technology, Telangana-502285, Hyderabad, India.

²Department of Chemical Engineering, Indian Institute of Technology, Telangana-502285, Hyderabad, India.

³Center for Nanofibers & Nanotechnology, National University of Singapore, 110077, Singapore

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/biot.201700263].

This article is protected by copyright. All rights reserved
Received: June 24, 2017 / Revised: September 12, 2017 / Accepted: September 29, 2017
Abstract
Electrospinning is a popular technique used to mimic the natural sub-micron features of the native tissue. The ultra-fine fibers provide a favorable extracellular matrix-like environment for regulation of cellular functions. This article summarizes and reviews the current advances in electrospun fiber application and focuses on the novel strategies applied for tissue regeneration and repair. It explores the different factors affecting the attachment and proliferation of mesenchymal stem cells (MSCs) on the electrospun substrates. The influence of different features of electrospun fibers in the differentiation of MSCs into specific lineages (bone, cartilage, tendon/ligament and nerves) has been elaborated. In addition, the different techniques to mimic the hierarchical features of tissues and its effect on cellular functions are reviewed. Additionally, the new developments like three-dimensional (3D) electrospinning, 3D spheroid double strategy and the comparative analysis of dynamic and static culture on electrospun scaffolds are discussed. With the intricate understanding of the interaction between the cells and the electrospun fiber matrix we can aim to combine the newer strategies to overcome the existing challenges and improve the potential application of electrospun fibers in the field of tissue regeneration and repair.

Keywords: Electrospinning, mimic, differentiation, 3D electrospinning, 3D spheroid.

1. Introduction:

The simple and versatile method of electrospinning has received a lot of attention in the past few years. There has been considerable progress in the process due to the changing demands and application requirements. Due to its similarity with the extracellular matrix (ECM) of the tissue the process of electrospinning has gained great interest in the last decade for tissue regeneration and repair. Plethora of synthetic polymers like poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(caprolactone) (PCL) and poly(ethylene oxide) (PEO); natural polymers like collagen, gelatin, silk fibroin; and polysaccharides like chitosan and alginate have been used for tissue regeneration and repair [1,2]. In addition, the substantial attention towards multipotent differentiation capacities of MSCs into different lineages in the recent past has also been combined with the ECM like electropun nanofibers for tissue engineering applications [1]. Elaborate research over the last few years has been done to study the effect of the cells on electrospun fibers. However, a critical factor to be considered is that in a native environment the cells are contained in a three-dimensional (3D) niche, and this complex environment controls the
cellular functions like proliferation, migration and differentiation. Originally electrospun fiber mats have relatively flat (two dimensional) surface, low thickness, and size limitations [2]. Owing to these problems a number of techniques have been devised to make the process suitable for three dimensional applications. Here arises the need for 3D electrospinning which is the key to mimic the natural tissue hierarchy [5]. Hence, electrospinning has evolved to fabricate scaffolds with higher dimensions. But problems like cellular infiltration, nutrient transport and mechanical stability demands further improvement in scaffold design [6]. Recent advancement includes patterning of electrospun fibers to achieve structural similarity with the native tissue hierarchy. Topography and patterns have found to influence cell alignment, orientation and differentiation of MSCs into specific lineages [7]. A bottom-up approach to mimic the nano- as well as micro-structure of tissues using electrospinning assists in controlling the cellular functions and fulfills the growing need for biomimetic scaffolds [8]. Considering the clinical application, the action of biomechanical dynamic forces comes into play. Hence studying the effect of dynamic culture on the scaffolds is a primary requirement for relevant clinical translation. Studies demonstrate the enhanced rate of proliferation and differentiation of MSCs into specific cells under dynamic conditions as compared to static culture [7–10]. Additionally, recent advances include 3D cell culture on 3D electrospun fiber mats. It consists of 3D cell aggregates (also called spheroids/micro-tissue) where cells adhere to each other and sustain tissue function better compared to normal two dimensional (2D) cultures. These spheroids cultured in electrospun fiber meshes act as building blocks for tissue regeneration. The nanofibers act as a supporting template for developing thick tissue constructs [11].

This current review highlights the importance of electrospinning for tissue regeneration with specific emphasis on MSC growth, proliferation and differentiation on electrospun
substrates. The effect of surface functionalization, coating, fiber roughness on cell proliferation and differentiation of MSCs into different lineages (bone, cartilage, tendon/ligament and nerves) have been discussed. The effect of patterned electrospun fibers on MSC alignment and differentiation have also been discussed briefly. A comparison between static and dynamic culture conditions for electrospun scaffolds and their effect on cell functions are summarized. In addition, the recent advances in the field of 3D electrospinning and 3D spheroid/micro tissue double strategy have been addressed.

2. Factors effecting cell adhesion and proliferation:

2.1 Surface roughness

The interaction between cells and their environment plays a vital role in determining cell function. Hence, the surface property of the scaffold is an important element to be considered in the fabrication process for proper adhesion and proliferation of cells. The roughness of the surface influences the initial attachment and proliferation of cells. It also increases the attachment of proteins to the surface which further enhances cell attachment. “Plasma treatment on poly (ethylene oxide terephthalate)/poly (butylene terephthalate) electrospun scaffolds showed an increase in surface roughness value. With the increase in the time of plasma treatment, an increase in roughness value ($R_a$)was noted (~3nm-27nm for 0-30 minutes) [12]. The surface roughness is proportional to the increase in surface area available for protein adsorption and hence assists in cell adhesion and proliferation. The surface treated fibers for bone regeneration application showed an increased expression of bone sialoprotein and osteonectin expression compared to untreated samples. Another study demonstrated that the treatment with oxygen and argon plasma on polycaprolactone electrospun fibers increased the $R_a$ values (2.51 and 4.72 µm for 5 minutes) compared to control (2.01 µm) [13]. Polycaprolactone fibers coated with polymethyl methacrylate showed a $R_a$ ~2.75 µm compared to same fibers without coating with $R_a$ value of ~0.26 µm, which showed improved cytocompatibility[14]. Nanofibers with micro- and nano-scale roughness have found to provide a
higher surface area for cell adhesion [15]. Studies show that the fiber diameter is directly proportional to roughness. Hence, larger the fiber diameter more is the affinity of the cells towards the surface [16]. Smaller diameter fibers support the initial adhesion of cells, but over a period, larger diameter fibers in culture seem to produce more ECM gene expression compared to the former [17]. A combination of micro- and nanofibers in electrospinning produces greater pore interconnectivity and larger pore size which leads to better cell penetration and infiltration. Other techniques like salt leaching, cryogenic electrospinning and sacrificial fiber induced pore generation allow fabrication of fibers with controllable pore size which leads to enhanced nutrient transport, better cell proliferation, increased cell migration and strong integration with the host tissue [6,18].

2.2 Surface coating/Functionalization:

The surface property of the nanofiber matrix is very crucial for cell interaction and function. For rapid tissue regeneration, the interaction of proteins, biomolecules and cells with the surface is of primary importance. Common methods include surface coating with natural molecules like fibronectin, vitronectin, and collagen to enhance the initial cell adhesion to the substrate [19]. For example, a recent study on multifunctional mesoporous silica-shelled PCL hybrid nanofiber scaffolds combines the mechanical function of constructs with cellular functions. The mesoporous silica functionalized fibers allowed loading of drugs and biomolecules which alter the surface charge of the substrate. Furthermore, differentiation of MSCs to osteogenic lineage and enhanced mineralization was observed [20]. A recent study on nanoencapsulation of functional biopeptides in electrospun poly(lactic-co-glycolic acid) and nanohydroxyapatite fibers using layer-by-layer approach showed increased matrix mineralization for bone tissue regeneration[21].The functional biopeptides are grafted into discrete nanolayers, which include KRSR (lysine-arginine-serine-arginine) sequence to enhance cell adhesion and proliferation, NSPVNSKIPKACCVPTELSAI to guide bone marrow mesenchymal stem cells differentiation, and FHRRIKA (phenylalanine-histidine-arginine-arginine-isoleucine-lysine-alanine) to improve mineralization matrix formation.. Studies show that pDA coating stabilizes MSC adhesion and proliferation and maintains the self-renewal property of pluripotent stem cells for a long time [24]. The hydrophilicity of pDA coating increases the adhesion of cells to the substrate and also enhances the growth of the attached cells as compared to pristine scaffolds. “In-situ pDA
functionalized electrospun PCL fibers showed high cell seeding efficiency and cell proliferation and regulates stem cell differentiation. pDA coated fibers were found to enhance osteogenesis of bone marrow stem cells, suggesting its role in commitment of MSCs towards osteogenic lineage. Another study showed the multi-walled carbon nanotube coating on polycaprolactone fibers enhanced cell adhesion and proliferation for nerve tissue regeneration [25]. Hence, surface functionalization of electrospun fibers provides an environment which is more favorable for cells to interact with the substrate. Identification of the required functional groups and surface coating for the electrospun fibers is a crucial parameter to make the substrate more biocompatible.

2.3 Coaxial-electrospinning

Co-electrospinning, a method producing nanofibers which encases material within a polymer sheath, is an effective strategy to stimulate the growth and proliferation of cells in addition to specific tissue differentiation by providing all the necessary factors in an optimum controlled manner. A study using Poly(ε-caprolactone)-co-poly(ethylene glycol) (PCE) copolymer co-electrospun nanofibers embedded with BMP-2 and dexamethasone loaded BSA nanoparticles stabilized with chitosan demonstrated the differentiation of MSCs to osteoblasts (Figure 1). A time-programmed release of both the drugs promoted the repair of critical-sized bone defect. The dual effect of the drug played an important role. The Initial release of dexamethasone leads to osteogenesis of MSCs followed by the simultaneous release of BMP-2 after a couple of weeks which led to bone formation [26]. Another example demonstrates the therapeutic bone scaffolds with core-shell nanofiber delivering two growth factors, FGF-2 and FGF-18 in a sequential manner for bone regeneration. The initial release of FGF-2 initiates angiogenesis followed by the release of FGF-18 which induces osteogenesis at a later stage. For the sequential release of factors, a novel nano-carrier was preloaded with FGF-18 and mixed with the FGF-2 into a core shell-hollow structured fiber. The combined effect of the growth factors plays an important role in osteogenesis, and such therapeutic scaffolds have good
potential for regeneration of tissue [27]. Recombinant human transforming growth factor-b1 (rhTGF-b1), which induces the differentiation of bone marrow derived MSCs (B-MSC) into chondrocytes, was co-axially spun as core part and PCL with B-MSC-affinity peptide (E7) as the outer sheath. The fibers supported cell growth; the E7 peptide allowed adhesion of B-MSCs; and the bioactive factor rhTGF-b1 induced differentiation of stem cells to chondrocytes [28].

3. Effect of patterning on stem cell alignment and differentiation

Patterned surface guides the cell movement during morphogenesis. A close relation exists between the substrate and the cell integrins [29]. The topography of the surface plays a crucial role in controlling the cell behavior. Studies show that the micro- and nano-topographies enhance specific cell function. Patterned electrospun fibers by photolithography have been used to produce microstructures on different substrates for modifying the surface topography of implants [30]. These geometric cues have a significant effect on cell adhesion, migration and differentiation [31]. Gap junctions are crucial for communication between adjacent cells, and some studies show that they help in osteoblastic differentiation MSCs. Aligned electrospun fibers showed an increased differentiation of MSCs as compared to cross-aligned fibers. The gap junctions are said to align along the pattern, and the polarization effect of gap junctions has an effect on the differentiation of cells [32]. A novel multilayered aligned Poly(ε-caprolactone) (PCL) electrospun scaffolds showed an increased cellular infiltration, development of tendon ECM and differentiation of MSCs into tendon fibroblasts as compared to non-aligned fibers (Figure 2A) [33]. The alignment of fibers provides a mechanical anisotropy closely mimicking the native tendon tissue and results in the organization of cells and matrix which further stimulates the differentiation of MSCs. The role of focal adhesion kinase (FAK), one of the cellular mechanical structures, in orientation and
spreading of MSCs on aligned electrospun scaffolds show that beyond the morphological changes in cells these patterned nanofibers also control other cellular functions like cell migration, growth and differentiation [34]. Oriented poly(L-lactic acid)-co-poly(ε-caprolactone) P(LLA-CL)/collagen type I (Col-I) nanofiber yarn mesh fabricated using liquid electrospinning method demonstrated an increase in the migration and chondrogenic differentiation of rabbit MSCs as compared to non-aligned sponge substrate (Figure 2B) [35]. The oriented fibers create a more realistic micro-environment and provide both physical and biological cues for chondrogenic differentiation. An interesting study on novel electrospinning system to manufacture continuous uniaxially aligned, nanofiber yarns (UANY) evaluated the differentiation capacity of human adipose derived stem cells (HADSC) on the fiber yarns (Figure 2C). Different techniques used in textile processing like braiding, weaving, and knitting were used to fabricate the 3D fibrous scaffolds to mimic the tissue anisotropy. The scaffolds supported cell growth and differentiation into osteogenic cells and smooth muscle cells [36]. Human-induced pluripotent stem cells (hiPSCs) seeded on well-aligned chitosan-based fibers underwent stepwise differentiation into tendon-like cells. This combination of physical cue and novel method of obtaining patient-specific stem cell for tissue regeneration can be used for clinical applications [37]. Three-dimensional (3D) aligned silk fibroin (SF) hybrid scaffolds showed the differentiation of MSCs into tendon fibroblast lineage under static conditions. But an enhanced level of tenogenic marker expression was observed under dynamic conditions [38]. The synergistic effect of mechanical stimulation and physical cues intensified the differentiation of MSCs. Hence a combination of different factors is very important to mimic the conditions in-vivo.
4. Differentiation of MSCs on electrospun scaffolds

4.1 Osteogenic differentiation

Composite fibers with different materials provide specific functionalities which initiate the process of cell differentiation into the desired lineage. For example, Graphene oxide (GO) doped PLGA electrospun scaffolds impart hydrophilicity to the fibers as compared to plain PLGA nanofibers and increase protein adsorption capacity of the fibers. It was found to enhance the adhesion and proliferation of cells on the surface. Interestingly, the GO doped fibers induced differentiation of MSCs to osteoblasts. An increased expression of ALP, collagen I and osteocalcin gene expression was observed [39]. iPSC - MSC on nanofibers of hydroxyapatite/collagen/chitosan (HAp/Col/CTS) showed good proliferation and enhanced biomineralization of osteoblasts. Cell migration was found over a large area which was attributed to the nanofiber composition. Differentiation of iPSC-MSC into osteogenic lineage was observed even in the absence of differentiation medium. The osteoconductive and osteoinductive capabilities of HAp and collagen seem to play an important role in the differentiation of iPSC-MSCs [40].

4.2 Chondrogenic differentiation

Due to the poor regenerative capacity of the cartilage tissue, treatment for cartilage repair or regeneration is a great challenge [42]. A recent study demonstrated the effect of 3D ultrafine keratin electrospun fibers derived from feathers in the differentiation of adipose derived mesenchymal stem cells (AD-MSCs) into chondrocytes. The 3D keratin scaffolds showed better growth, proliferation and differentiation of AD-MSCs to chondrocytes compared to 2D Poly lactic acid electrospun scaffolds [3]. Poly (L-lactic acid) scaffolds with hydrogen treated multi-walled carbon nanotubes (MWCNTs) and poly-L-lysine coating was used to control
differentiation of MSCs to chondrocytes for cartilage regeneration. The MWCNTs increased the mechanical strength of the scaffolds and poly-L-lysine assisted in chondrogenic differentiation of MSCs [43]. Co-culture of B-MSCs with chondrocytes has found to enhance the differentiation of B-MSCs into chondrocytes and initiates a stable cartilage tissue regeneration as compared to culturing only B-MSCs on electrospun gelatin/polycaprolactone nanofibers [44]. In a co-culture environment, the MSC population is more prone to chondrogenic differentiation, and this reduces the total number of chondrocytes needed for regeneration of tissue [45].

4.3 Neural differentiation

Neurodegenerative disorders are treated with stem cell therapy, but the percentage of cell viability and the differentiation of stem cells into neuronal lineage are very low. Hence, a matrix or support for the stem cells can initiate cell viability and differentiation leading to successful nerve regeneration. The nanofibrous scaffold of PCL/collagen was found to initiate differentiation of mesenchymal stem cells derived from Wharton’s jelly (WJ-MSCs) into motor-neuron like cells in the presence of induction factors [46]. A study shows that the chorion-derived mesenchymal stem cells (C-MSCs) with induction factors like retinoic acid and sonic hedgehog differentiated into motor nerve cells on 3D gelatin electrospun scaffolds [47]. MSCs cultured on oxygen plasma treated PCL electrospun fibers showed differentiation into neuron cells. The upregulated expression of Map-2 proteins, indicative of the nanofiber morphology initiated differentiation of MSCs [48]. The topography of the fibers along with the induction factors is said to upregulate the expression of markers specific to nerve cells. This explores the potential of electrospun scaffolds in the treatment of neuronal disorders.
4.4 Tenogenic differentiation

Owing to the poor self-regenerative capacity of tendon and ligament tissues the application of MSCs is a good therapeutic alternative. Studies show that the differentiation of MSCs into the desired lineage requires a microenvironment which can provide the necessary growth factors in a controlled way similar to the native ECM [49]. 3D fiber yarns with aligned twisted fibers provide better topography for the differentiation of MSCs to tendon-like cells as compared to 2D sheets. The yarns provide support for cell adhesion and proliferation and guide the cells parallel to the fiber alignment [50]. Another study demonstrated an increase in tendon-like tissue formation on electrospun PLGA scaffolds. The newly formed tissue was thinner and mechanically weak as compared to the native tissue. However, mechanical stimulation of the scaffolds, was found to stimulate the production of aligned collagen fibers which increased the strength of the scaffolds [51]. Hence the current therapies require different strategies to repair and regenerate functional tendon tissue with mechanical integrity similar to the native tissue.

Table 1: Summary of electrospun fibers for differentiation of MSCs.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cells cultured</th>
<th>Differentiation inducer</th>
<th>Tissue type</th>
<th>Stage of development</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graphene oxide (GO) doped PLGA</td>
<td>MSC</td>
<td>Dexamethasone</td>
<td>Bone</td>
<td>Lab studies</td>
<td>GO increased cell adhesion, proliferation and differentiation.</td>
<td>[39]</td>
</tr>
<tr>
<td>hydroxyapatite/collagen/chitosan</td>
<td>iPSC - MSC</td>
<td>No</td>
<td>Bone</td>
<td>Lab studies</td>
<td>Hydroxyapatite induced differentiation of MSCs</td>
<td>[40]</td>
</tr>
<tr>
<td>Collagen</td>
<td>MSC</td>
<td>Cyclic tension</td>
<td>Tendon/ligament</td>
<td>Lab studies</td>
<td>collagen III and tenascin-C upregulation</td>
<td>[41]</td>
</tr>
<tr>
<td>Keratin from chicken feather</td>
<td>AD-MSC</td>
<td>Chondrogenic medium</td>
<td>Cartilage</td>
<td>Lab studies</td>
<td>3D fibrous structure provides guidance for cell proliferation and differentiation</td>
<td>[3]</td>
</tr>
<tr>
<td>Multi-walled carbon nanotubes (MWCNTs) and poly(L-lactic acid) (PLLA) with poly-L-lysine coating</td>
<td>B-MSC</td>
<td>Chondrogenic medium</td>
<td>Cartilage</td>
<td>Lab studies</td>
<td>poly-L-lysine enhanced differentiation. MWCNTs increased mechanical strength of scaffolds</td>
<td>[43]</td>
</tr>
<tr>
<td>gelatin/polycaprolactone</td>
<td>B-MSC/chondrocyte co-</td>
<td>No</td>
<td>Cartilage</td>
<td>Lab studies</td>
<td>Co-culture strategy with the gelatin/PCL fibers induced differentiation of MSCs.</td>
<td>[44]</td>
</tr>
</tbody>
</table>
5. Electrospinning to mimic hierarchical structure of tissues

Biomimicking, the 3D nanoscale network of the native tissue, is a huge challenge. The design of the fabricated scaffold should mimic the features of the native tissue at all hierarchical levels to ensure proper cellular function. An interesting work demonstrates the fabrication of bi-layered laser micropatterned hierarchical scaffolds. The electrospun fibers mimic the nanostructures, on which the microstructures are created using laser cutting [53]. The hybrid surface features assisted in MSC differentiation into cardiac myogenic lineage even in the absence of external chemical factors for induction. This highlights the importance of biomimicking tissue micro- and nano-topography in deciding stem cell fate. Further, mimicking the anisotropy of tissue is very critical for cell attachment, migration, infiltration, differentiation and to maintain mechanical stability. PCL/collagen electrospun fibers were fabricated by modified rotary jet spinning to produce 3D aligned fibers. Cells cultured on these scaffolds showed enhanced cell proliferation and were aligned along the fiber direction [54]. Thermally induces self-aggregated PCL nanofiber scaffolds showed hierarchically interconnected porous structures similar to native ECM. In-vitro studies showed good cell viability, osteogenic and chondrogenic differentiation induced by bone morphogenetic protein-2 (BMP-2) [55]. In-vivo studies performed by subcutaneous transplantation in mice showed bone regeneration through endochondral ossification. Hence, 3D nanofibrous scaffolds with hierarchical pore structure could act as a suitable biomimetic and functional implant for bone regeneration. Another recent study mimics the unique criss-cross fiber structure of tendon by using “chinese finger-trap design”. The mechanical response exhibited by the fabricated 3D structure was similar to native tendon tissue. Also, MSCs cultured on these structures showed contact guidance and growth along the topography of the pattern.
Cell differentiation towards tenogenic lineage was also observed with increase in expression of genes related to tenogenesis [56]. Another study shows a robust method for continuous collection of polydioxanone fibers on a thin conductive wire collector. The fibers can then be stretched, twisted or annealed like common textile processes to achieve the desired hierarchical structures. The yarns found to increase cell attachment and proliferation compared to monofilaments [57]. Hence, such simple and robust techniques are required to scale up the yield to develop potential therapeutic implants for clinical applications.

6. Guided tissue regeneration

Guided tissue regeneration (GTR) membranes prevent the infiltration of fibroblasts but allow the proliferation and adhesion of specific cells for tissue regeneration. Many natural and synthetic polymers have been used to fabricate GTR membranes using electrospinning technique. Metronidazole loaded poly(e-caprolactone) and gelatin fibers was successfully developed as an anti-infective GTR membrane which could be used in drug delivery at the site of application owing to the controlled drug release from the nanofibers [58]. Chitosan grafted Poly (L-lactide) nanofibers was used as a novel composite GTR membrane for periodontal tissue regeneration. The biodegradability, bioactivity and the enhanced cell proliferation on the modified membrane makes it a potential candidate for guided regeneration of periodontal tissue [59]. Poly (lactide-co-glycolic acid) immobilized with bone-forming peptide 1 (BFP1) was used as a guidance membrane for regeneration of tissue in critical size bone defects. An enhanced osteogenic differentiation of MSCs was observed. In-vivo implantation of the membrane in a calvarial defect in mice model for eight weeks showed an integration of the electrospun membrane with the tissue and improved bone tissue regeneration. Hence these membranes can be used for guided bone regeneration especially in cases of critical size defects [60]. The synergistic effect of growth factor encapsulated aligned electrospun fibers have shown to facilitate peripheral nerve regeneration for peripheral nerve injury in rats. Thus the combination of growth factors and topographical cues has shown to enhance regeneration of tissue for critical size defects [61]. Another study showed that the combination of electrospun PCL and polymethylmethacrylate (PMMA) could be a biocompatible and stable implant as compared to the conventional extrudable polymers. These implants were tested in-vivo by creating skull defects in rats. Two months post implantation the implant site showed superior bone regeneration and enhanced
healing. This suggests that PCL/PMMA electrospun scaffold initiated both *in-vitro cell growth* and *in-vivo* bone regeneration [62]. The effect of aligned poly(l-lactic acid) (PLLA) electrospun fibers coated with pDA in guided regeneration of bone tissue both in-vitro and in-vivo was demonstrated by a study. Scaffolds implanted in critical sized calvarial defects in mouse showed that aligned nanofibers induced the formation of collagen matrix in an aligned manner and guided cell migration along the matrix direction. Hence nanofibers provide a spatial guidance both for in-vitro cell growth and in-vitro tissue formation and regeneration [63].

7. Recent advances:

7.1 3D electrospun scaffolds:

A critical point to be considered is that a 2D sheet of electrospun mat restricts cellular penetration. When implanted in the body the cells would be exposed to nutritional supply and stresses in a 3D way [64]. Currently, researchers intend to replace the 2D scaffolds with 3D scaffolds which could truly mimic the *in-vivo* conditions. 2D electrospun mats are generally few millimeters thick which cannot be used for filling large sized critical defects of tissues and are currently used as models for mimicking ECM for *in-vitro* cultures. For the clinical advancement of electrospun fibers, a different approach is required which would emphasize on micro-structure as well as macro-structure of the scaffold which would ultimately mimic the native tissue hierarchy. Recent reports have shown a comparative study between 2D random electrospun sheets; 2D aligned electrospun fibers and aligned fibers twisted into bundles to make a 3D construct. Although, 2D aligned fibers show guided regeneration of tissue, the scaffolds with 3D structure mimics the native tissue more closely and would be a better alternative to replace the natural tissue owing to its greater mechanical properties and closeness to the actual tissue hierarchy. However, cell penetration in the 3D scaffolds is still a challenge [64]. 3D scaffolds with modulated surface patterns (knitted-type mesh) showed better cell adhesion, proliferation,
and osteogenic differentiation as compared to the 2D counterpart. The large surface to volume ratio provided by the modulated 3D electrospun scaffolds allowed cellular migration and penetration within the nanofiber layers [65]. Rod-like 3D aligned electrospun poly (3-hydroxybutyrate-co-3-hydroxyvalerate)/Hydroxyapatite scaffolds have been reported to show a significant effect on in-vivo rabbit models for critical sized defects (Figure 3A). Interestingly the mechanical properties of the aligned 3D electrospun scaffolds were found to be higher compared to random-oriented fibers [66]. Layer-by-layer paper stacking of electrospun nanofibers has been studied extensively by research groups to obtain 3D structures similar to living tissues. It has an advantage of having control over cell seeding density in each layer, layer thickness and efficient drug/growth factor loading (Figure 3D) [67]. Chemical gradients can also be made by using different polymers in different layers. This facilitates the modification of scaffold property which is vital for tissue functioning and mechanical stability [68]. Controllable 3D micro- and nano-porous structures with a thickness greater than 3mm were made using a modified electrospinning method. Ethanol solvent bath was used as a collector for the electrospun fibers. To increase the porosity of the 3D fibrous structures for cell infiltration and proliferation femtosecond laser pulse was provided. This resulted in a pore size of around 200-400 μm, thereby providing a suitable niche for cell migration and free transport of nutrients through the pores (Figure 3B) [69]. Methanol solvent has also been used as a collector followed by salt leaching and scaffold molding in a cylindrical glass vessel to obtain silk gland nanofiber scaffolds with micro- and nano-porous structure [70]. An interesting study mimics the hierarchical structure of the articular cartilage by developing a five layered electrospun collagen scaffold. Each layer had a specific alignment of the electrospun fibers and material specific to the individual layer. This method combines chemical gradient (collagen I and collagen II) as well as structural gradient (random
and aligned) thus closely mimicking the cartilage tissue hierarchy [71]. By replacing the traditional flat collector design with modified 3D printed collector uncompressed low-density fiber scaffolds was fabricated. A complicated arrangement of needle-like structures on the collector allows the fibers to deposit loosely without compression. A functional 3D neuronal network mimicking the philological environment was successfully developed (Figure 3C) [72]. A study shows the fabrication of 3D electrospun scaffolds using the principle of electrostatic repulsion between the fibers. The scaffold allowed cells to proliferate in a 3D manner without loss of its native morphology. Such stereoscopic structures can closely mimic the native ECM due to the spatial arrangement of the fibers [73]. Table 2 summarizes the different methods to fabricate 3D electrospun scaffolds.

Table 2: Different methods to fabricate 3D electrospun fibers and effect on cell functions.

<table>
<thead>
<tr>
<th>Material</th>
<th>3D shape/design</th>
<th>Method</th>
<th>Cells cultured</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
</table>
| poly(e-caprolactone) (PCL) | 3D fiber bundles | Twisting 2D aligned fiber sheets | Tendon fibroblasts | • Guided parallel growth of tendon fibroblasts  
• Mechanically stable  
• Mimics natural tendon tissue | [64] |
| poly (e-caprolactone) (PCL) | Knitted-mesh | Modified chain-like electrospinning collector (rotating shaft) | hBMSC | • Provides larger surface area for cell functions  
• Knitted structure supported better cell infiltration  
• 3D structures supported better differentiation of MSCs | [65] |
| poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and Hydroxyapatite (HaP) | Rod-like | Fiber sheets cut into trapezoids and rolled into rod shaped structures | Rat B-MSC | • HaP induces osteogenic differentiation  
• Ideal for critical size defect repair  
• Degradation rate still needs to be optimized | [66] |
| Polycaprolactone/gelatin | Stack of fiber | Layer by layer | AD-MSC | • Enhanced | [67] |
sheet | stacking of 2D fiber mats | osteogenic capability
- Problem of cell infiltration is solved as cells can be seeded in each layer
- Permeable to nutrients and oxygen

| Poly(3-caprolactone) (PCL) | Cylindrical micro/nano fibrous scaffolds (> 3mm thickness) | Ethanol bath used as collector and femtosecond laser to make micropores of 189 ±28 µm to 380 ± 21 µm | MG-63 | Micro and nano pores together allow better cell adhesion, proliferation and infiltration
- Controllable pore size | [69] |

| Poly(3-caprolactone) (PCL) | 3D uncompressed porous fibers | Modified 3D printed spherical collector | Human neural progenitor cell line | Uncompressed scaffolds led to increase in differentiation into nerve cells
- Topography played an important role in differentiation of neural progenitor cells | [72] |

### 7.2 Spheroids/Micro-tissue on electrospun scaffolds: 3D double strategy

Studies show that aggregated cells respond in a different way as compared to cells in 2D condition. The conventional 2D environment does not mimic the natural microenvironment as it provides a homogenous and controlled environment as opposed to the actual 3D microenvironment found *in-vivo* [74]. Hence, current research aims at a double 3D strategy where a combination of nano-structured electrospun scaffolds along with 3D spheroids/micro-tissue assists in superior tissue regeneration both *in-vitro* as well as *in-vivo* conditions. 3D spheroids can be fabricated using some techniques like hanging drop method, liquid overlay technique, centrifugation, and in round bottom ultra-low attachment plates [75]. Electrospun scaffolds with hierarchical structures (micro-wells) facilitated the aggregation of seeded MSCs
into spheroids/micro-tissue. The nanofibrous material allows proper diffusion of nutrients across it and assists in cell adhesion. The combination of 3D spheroids with the electrospun fibers showed improved cell functions in rodent models [76]. A recent study has demonstrated that the sophisticated double 3D strategy could increase the efficiency of implants even in the absence of bioactive molecules. Moreover, the regeneration of bone tissue was found to be enhanced with 3D spheroids as compared to single cells. As opposed to the classical tissue engineering technique, which uses single cells on biomaterials the 3D strategy initiates robust bone regeneration and accelerates faster mineralization (Figure 4) [77]. An interesting study shows the trans-differentiation of MSCs on Poly(L-lactic acid)-co-poly-(ε-caprolactone)/collagen electrospun scaffolds to generate 3D hepatic constructs. The nanostructure modulates the MSCs to form functional hepatospheres. Such a construct may serve as a model to study chronic liver diseases [78]. Modulating the fiber porosity and structure plays a vital role in the aggregation of seeded cells on the electrospun scaffolds. Higher porosity increases cell infiltration and cell aggregation. An optimal balance between cell-cell and cell-matrix interaction is the key for self-aggregation of cells to form spheroids on scaffolds. Further modification of nanofibers with RGD groups maintains cell function and initiates cell migration and adhesion by providing optimal chemical and mechanical microenvironment to promote the retention of cells within the fibers [79]. This new concept is promising to be clinically relevant and can evolve as a major therapeutic implant in the near future.

7.3 Static Vs Dynamic culture of electrospun scaffolds

Besides chemical cues, biomechanical stimulation has an important role to play in the successful differentiation of MSCs into specific lineages. It has been observed that the flow-induced stress causes differentiation of cells. Differentiation of MSCs into endothelial cells has
been demonstrated in tubular poly(L-lactide-co-e-caprolactone) electrospun scaffolds subjected to a shear stress of 2.5 dyne/cm² and circumferential stretching in the presence of chemical factors [80]. This suggests that the combination of mechanical and chemical cues is very critical in the differentiation process. The different mechanical parameters in addition to the chemical cues should be tested to study the differentiation of MSCs into different lineages. Electrospun fibers in combination with bioreactor culture have shown improved cell proliferation and infiltration as compared to static cultures. Poly (ethylene oxide terephthalate)-co-poly (butylene terephthalate) (PEOT/PBT) electrospun fibers cultured with MSCs under dynamic condition was used to mimic the fibrous tympanic membrane layer of the eardrum [62]. The idea was to initiate MSC differentiation later inside the physiological environment by exploiting the native chemical factors. These biocompatible biohybrid constructs showed great potential as a replacement for the tympanic membrane in otosurgery. In addition, dynamic forces are said to enhance MSC differentiation on electrospun fibers into bone, cartilage and tendon like tissues as compared to static culture [38,41].

8. Conclusion

This paper highlights the recent advances in fabricating electrospun scaffolds for regeneration of tissue. The proliferation, migration and differentiation of MSCs in different fiber morphologies, compositions, and substrate topography and culture conditions have also been discussed. Novel techniques of electrospinning developed to mimic the natural tissues in combination with the advanced cell culture methods like 3D spheroid culture is the key to meet the current challenges of cell infiltration and differentiation. The challenge of developing a 3D functional scaffold which is clinically applicable can be achieved with the help of inter-
disciplinary research which integrates the new approaches of fabrication and 3D cell growth to achieve the goal of successful tissue regeneration.

References

List of figures:

Figure 1: Schematic showing (A) BMP-2-loaded BSA nanoparticles stabilized with chitosan, (B) FGF18-preloaded mesoporous bioactive glass nanospheres incorporated within the FGF2-loaded core–shell electrospun polymeric fiber and (C) coaxial electrospun fiber scaffold containing rhTGF-β1 in the core and E7 in the PCL shell of the fibers. Reproduced with permission from [26], Copyright 2015, Elsevier, and [27], Copyright 2015, Elsevier, and [28] Copyright 2014, Elsevier.
Figure 2: Image showing (A) total collagen content in aligned scaffolds to be more as compared to non-aligned scaffolds, (B) yarn-CH group with more ECM, homogenous structure and chondrogenic differentiation as compared to sponge scaffolds and (C) braided architecture supporting differentiation of AD-MSC to smooth muscle and osteogenic lineage. Reproduced with permission from [30], Copyright 2015, Elsevier, and [32], Copyright 2015, Elsevier, and [33] Copyright 2016, American Chemical Society.
Figure 3: Schematic of (A) preparation of 3D electrospun PHBV/HA scaffolds, (B) the 3D electrospinning process with an EtOH bath, (C) modified electrospinning collector for uncompressed low-density electrospun fibers for neural applications and (D) layer by layer paper-stacking of electrospun membranes to form 3D structures. Reproduced with permission from [66], Copyright 2013, Elsevier, and [69], Copyright 2013, Elsevier, [72] Copyright 2017, Elsevier, and [67] Copyright 2015, Dove medical press Limited (http://creativecommons.org/licenses/by-nc/3.0/).
Figure 4: 3D double strategy for tissue regeneration.