Acta Biomaterialia 27 (2015) 88–100

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

3D imaging of cell interactions with electrospun PLGA nanofiber membranes for bone regeneration

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ARTICLE INFO

Article history:
Received 27 May 2015
Received in revised form 2 August 2015
Accepted 4 September 2015
Available online 5 September 2015

Keywords:
3D tomography
FIB–SEM
3D imaging
Electrospinning
Nanofibers
PLGA
Osteoblast
Tissue engineering

ABSTRACT

The interaction between resident cells and electrospun nanofibers is critical in determining resultant osteoblast proliferation and activity in orthopedic tissue scaffolds. The use of techniques to evaluate cell–nanofiber interactions is critical in understanding scaffold function, with visualization promising unparalleled access to spatial information on such interactions. 3D tomography exploiting focused ion beam (FIB)–scanning electron microscopy (SEM) was used to examine electrospun nanofiber scaffolds to understand the features responsible for (osteoblast-like MC3T3-E1 and UMR106) cell behavior and resultant scaffold function. 3D imaging of cell–nanofiber interactions within a range of electrospun poly(D,L-lactide-co-glycolide acid) (PLGA) nanofiber scaffold architectures indicated a coherent interface between osteoblasts and nanofiber surfaces, promoting osteoblast filopodia formation for successful cell growth. Coherent cell–nanofiber interfaces were demonstrated throughout a randomly organized and aligned nanofiber network. Gene expression of UMR106 cells grown on PLGA fibers did not deviate significantly from those grown on plastic, suggesting maintenance of phenotype. However, considerably lower expression of *Ibsp* and *Alpl* on PLGA fibers might indicate that these cells are still in the proliferative phase compared with a more differentiated cell on plastic. This work demonstrates the synergy between designing electrospun tissue scaffolds and providing comprehensive evaluation through high resolution imaging of resultant 3-dimensional cell growth within the scaffold.

Statement of Significance

Membranes made from electrospun nanofibers are potentially excellent for promoting bone growth for next-generation tissue scaffolds. The effectiveness of an electrospun membrane is shown here using high resolution 3D imaging to visualize the interaction between cells and the nanofibers within the membrane. Nanofibers that are aligned in one direction control cell growth at the surface of the membrane whereas random nanofibers cause cell growth into the membrane. Such observations are important and indicate that lateral cell growth at the membrane surface using aligned nanofibers could be used for rapid tissue repair whereas slower but more extensive tissue production is promoted by membranes containing random nanofibers.

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1. Introduction

The production of nano- and micro-structured scaffolds for tissue engineering has required concurrent development in imaging techniques to evaluate cell interaction and growth on biomaterials.

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http://dx.doi.org/10.1016/j.actbio.2015.09.003
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While the effectiveness of tissue-engineered scaffolds has been reported as being dependent on their mechanical stability [1], chemical composition [2], and biological compatibility [3], the interaction between the scaffold and cells [4] is critical for resultant viability, cell activation [5] and focal adhesion formation [6]. Thus, imaging techniques are often employed to quantify these scaffold–cell interactions through direct visualization. Orthopedics is an important area where tissue engineering exploits biomaterials to promote cell adhesion, but understanding osteoblast behavior and adhesion is required to effectively optimize bone–biomaterial interfaces [7]. Electrospun scaffolds are used widely in regenerative medicine for orthopedic applications due to the high porosity of the 3D spun network that has been shown to promote cell proliferation [8,9] and invasion of host tissue. Thus, the architecture of electrospun scaffolds and their surfaces is advantageous in tissue engineering for shaping and directing cell growth [10]. The fibrous architecture is easily controllable in electrospinning and has been notably varied to increase the pore size and spacing between fibers using low-temperature electrospinning [11] or controlling fiber organization by employing patterned and rotating collectors [12]. Such control of electrospun fiber network architecture allows engineering of cell migration through the scaffolds [13]. Prevalent examples of polymers electrospun into effective tissue scaffolds include polydioxanone [14], poly(ε-caprolactone) [15], polyglycolic acid (PGA) [16], polylactic acid (PLA) [17], poly(L-lactide) [18] and their copolymers poly(D,L-lactide-co-glycolide) (PLGA) [19–23] that are often exploited as high surface area fibrous membranes [24,25]. Electrospinning is particularly notable as the predominant method used to produce synthetic fibers in the nanometer range to mimic the collagen matrix and is therefore most promising in bone regeneration and cartilage regeneration [17,26]. Electrospun materials including PLGA have the potential to biomimic the structure of natural bone [19].

Production of electrospun fibers for tissue scaffolds is currently popular due to the ease of selecting processing parameters, particularly to control fiber diameters ranging from 10 nm up to a few microns [27,28]. Further control of the fiber organization into an aligned network has been achieved by deposition of nanofibers onto a rotating drum collector [29–31]. Such a method reduces spacing between fibers and decreases the resultant pore size in the spun membrane comparing to a randomly deposited system. The resultant increase in surface area to volume of electrospun nanofibers in both random and aligned arrangements has a considerable geometric advantage over larger fiber diameters [30]. However, optimization of the electrospinning process and understanding cell growth from their interaction with nanofiber surfaces is yet to be fully determined. Previous work has used PLGA fibers to rebuild the natural 3D environment for enhanced skin cell and tissue growth by imitating the fibrillar structure and ECM, as well as providing the necessary direction for cell function, organization and survival [21]. Many studies have also shown the biocompatibility of PLGA with osteoblasts, highlighting PLGA as a preferred material to promote bone regeneration [19–24]. Bone regeneration is complex [32] and therefore any orthopedic application utilizing PLGA electrospun tissue scaffolds must be evaluated to ensure that normal osteoblast behavior is maintained in the new environment.

Adhesion between osteoblasts and the biomaterial substrate surface is critical in guiding growth and is characterized by focal adhesion contacts and F-actin supported finger-like protrusions of the plasma membrane known as lamellipodia and filopodia [7]. The elongation of these structures facilitates osteoblast migration and adhesion to the extracellular matrix (ECM) in wound healing [33]. Filopodia regulate cell motility and therefore require understanding of their growth in 3D. Moreover, quantifying filopodia in terms of size, numbers and growth direction are relevant for cell motility studies [34] and assessing the suitability of the manufactured substrate. Evaluation of membranous outgrowths is typically achieved using fluorescence microscopy but is relatively ineffective as some filopodia diameters are of the order of 200 nm, which is below the resolution of many optical techniques. Despite confocal microscopy commonly being employed to view cells in 3D structures, the penetration of light in dense scaffolds is limited to 200 microns [35]. X-ray computed tomography (CT) can be used to image through dense scaffolds to characterize the electrospun fibers but the resolution is limited to microns [11]. Thus, scanning electron microscopy (SEM) has been shown to be highly effective in evaluating sub-micron filopodia development at surfaces in 2D [36]. While SEM is recognized as possessing sufficient (nanometer) resolution to image a range of biomaterial surfaces, recent work has extended SEM by combining focus ion beam (FIB) microscopy to study cell–substrate interfaces at patterned surfaces, cells and ECM [37]. FIB–SEM uses both FIB to section through a material and SEM to image exposed surfaces following the FIB sectioning, which is typically referred to as 'slice-and-view' [38,39]. Collection of 2D SEM images during this sectioning is subsequently reconstructed to provide a 3D image of the interrogated sample. FIB–SEM tomography is widely used in studying microstructure changes in superalloys [40] as well as biological and geological materials [41]. Recently, FIB–SEM has been used to investigate the cell–substrate interaction between microneedle arrays [42] and complex structures including interconnections of dentine tubules [39]. Despite the introduction of FIB–SEM as a high-resolution imaging tool, evaluation of the cellular interactions with porous tissue scaffolds is lacking. Indeed, FIB–SEM is particularly suited to the study of tissue scaffolds with structural features below the resolution of optical microscopy. Such imaging is applied to PLGA, as a medically approved material, organized into random and aligned nanofiber architectures to investigate 3D cell proliferation across the relatively large scaffold volume as well as at the smaller cell–nanofiber interfaces. This paper therefore attempts to quantify fiber stability and visualize the interaction of osteoblasts with electrospun PLGA fiber networks intended for guided bone regeneration for bone scaffold applications. The interaction between filopodia and the nanofiber membrane is visualized using 3D imaging based on FIB–SEM 'slice-and-view' methods and correlated with confocal microscopy and gene expression. This 3D analysis allows direct investigation of cell proliferation depth into the electrospun membrane and considers the influence of fiber spacing on cell proliferation using aligned and random fibrous organizations.

2. Materials and methods

2.1. Electrospinning PLGA scaffold

Polymer solutions for electrospinning were prepared using poly (D,L-lactide-co-glycolide acid) (PLGA – lactide:glycolide (75:25), molecular weight: 66,000–107,000, Sigma Aldrich, U.K.) dissolved in a mixture of chloroform (analytical reagent grade, Fischer Scientific, U.K.) and *N,N*-dimethylformamide (DMF, 99.8%, Sigma Aldrich, U.K.) (85/15 mass ratio) within a glass vessel to produce a resultant polymer concentration of 15 wt.% in solution. Electrospinning of PLGA was achieved using a single nozzle setup and a voltage of 14–15 kV applied between the nozzle and a ground electrode positioned 20 cm below the spinning nozzle. Polymer solution was supplied with a syringe pump at a flow rate of 0.5–1 μl h^{-1}. The applied voltage at the metal needle caused charge build-up at the polymer solution meniscus hanging at the end of the nozzle until cone-jet formation and stretching jets towards

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