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The engineering of patient-specific, anatomically shaped, digits

Peng Wang [a], Jiang Hu [a], Peter X. Ma [a,b,c,*]

[a] Department of Biologic and Materials Sciences, University of Michigan, Ann Arbor, MI 48109, USA
[b] Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA
[c] Macromolecular Science and Engineering Center, University of Michigan, Ann Arbor, MI 48109, USA

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ABSTRACT

It is now recognized that geometric structures of scaffolds at several size levels have profound influences on cell adhesion, viability, proliferation and differentiation. This study aims to develop an integrated process to fabricate scaffolds with controllable geometric structures at nano-, micro- and macro-scales. A phase-separation method is used to prepare interconnected poly(L-lactide) (PLLA) nanofibrous (NF) scaffolds. The pore size of the NF scaffold at the scale of several hundred micrometers is controlled by the size of porogen, paraffin spheres. At millimeter scale and above, the overall shape of the scaffold is defined by a wax mold produced using a three-dimensional printer. The printer utilizes a stereo lithographic file generated from computed tomographic files retrieved from the National Library of Medicine's Visual Human Project. NF PLLA scaffolds with a human digit shape are successfully prepared using this process. Osteoblast cell line MC3T3-E1 cells are then seeded and cultured in the prepared scaffolds. Cell proliferation, differentiation and biomineralization are characterized to demonstrate the suitability of the scaffolds for the digit bone tissue engineering application.

1. Introduction

The geometric structures of scaffolds at several size levels influence cell adhesion, viability, proliferation and differentiation [1]. At macro-scale level (mm and above), it is desirable for scaffold to assume the shape of the defective part of the tissue/organ to help the neo tissue organize into the needed three-dimensional (3D) structure [2–5]. At the micro-scale level, structural parameters such as pore size (usually ranging from 50 to 1000 μm), pore shape and porosity have to be controlled so that living cells can grow throughout the entire scaffold, and nutrients and metabolic wastes can be readily transported into or out of the scaffold [6–9]. At an even smaller scale (nm), surface topography influences cellular behaviors such as adhesion and differentiation [10]. To meet these criteria, a unique phase-separation process was developed to fabricate interconnected polylactide scaffolds with nanofibrous (NF) matrix [11–14]. The superior properties compared to scaffolds without NF features (also called solid-walled scaffolds), have been proven in a series of studies [11,15–18]. Significantly larger amounts of serum proteins can be adsorbed onto the NF scaffold, facilitating cellular adhesion and growth [4,19], making NF scaffolds likely advantageous for a variety of tissue engineering applications. Particularly for applications in bone tissue engineering, the results suggest that NF scaffolds can better promote osteoblast differentiation and biomineralization. Runt-related transcription factor 2 (Runx2) protein and bone sialoprotein (BSP) mRNA are expressed at higher levels in osteoblastic cells cultured on NF scaffolds, compared to cells cultured on solid-walled scaffolds. It was also found that biomineralization was enhanced substantially on NF scaffolds as confirmed by von Kossa staining and transmission electron microscopy [15]. The enhanced differentiation of osteoblastic cells on NF matrix was found to be associated with the RhoA/ROCK signaling pathway [20].

Although the importance of controlling scaffold geometries is now recognized, few existing technologies are capable of fabricating NF scaffolds with accurate anatomical shape, tunable inner pore size and controllable interpore connectivity. The electrospinning technique is probably the most intensively studied fabrication technique for polymer nanofibers [21–25]. Great efforts have been made to fabricate nanofibers using various polymers and to understand how to control the diameter of fibers. However, it is difficult to use the electrospinning technique to create inner micropores and macroscopic shapes. That is partially why electrospun fibers are often used only in the form of mats, limiting their wide applications in tissue engineering.

* Correspondence to: Peter X. Ma, Department of Biologic and Materials Sciences, 1011 North University Ave., Room 2211, The University of Michigan, Ann Arbor, MI 48109-1078, USA. Tel.: +1 734 764 2209; fax: +1 734 647 2110.
E-mail address: mapx@umich.edu (P.X. Ma).

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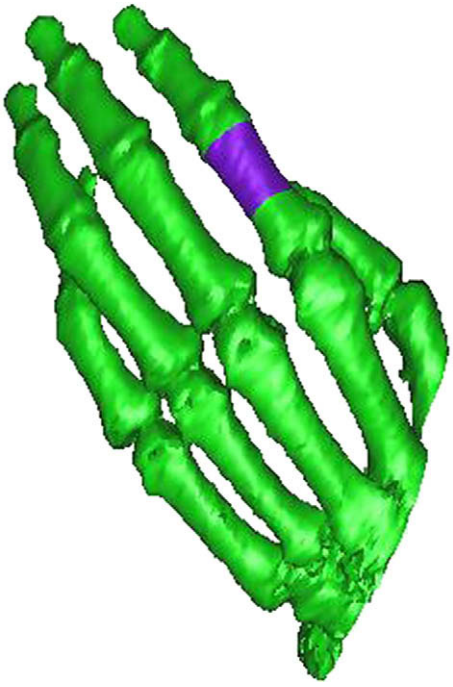

Fig. 1. An image of a female left hand bones (purple color indicates the defective part).

In this study, we combined the unique phase separation based nanofiber preparation technique [11–13] with a prototyping technique. Our aim was to fabricate the NF scaffolds with a controlled overall shape, inner pore size and pore connectivity. The overall shape of the scaffold was ultimately controlled by computed tomographic (CT) files, taken from the patient's defective part, so that the scaffold shape matches exactly the specific anatomic shape, such as a proximal phalanx in this study. A 3D printer was used to print a mold for scaffold preparation based on a stereo lithography (STL) file generated from the CT files. The inner pore size of the scaffold was controlled by the size of porogen, paraffin particles that were made with the emulsion method. The size of the paraffin spheres was adjusted by varying the emulsion process conditions (i.e. stirring speed and surfactant concentration). The interconnectivity of the inner pores of scaffolds was tailored by heating paraffin spheres for different periods of time. A unique phase-separation process was used to fabricate the interconnected nanofibers, which constitute the pore walls and provide the surface for cell adhesion and growth. Murine osteoblast cell line MC3T3-E1 subclone 26 (MC-26) cells were then seeded and cultured in the prepared scaffolds. Cell proliferation, differentiation and biomineralization were characterized to evaluate the performance of the scaffolds for bone regeneration.

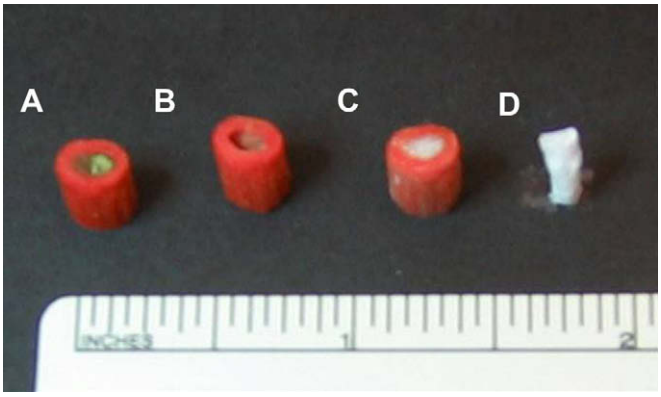

Fig. 2. Fabrication of porous NF scaffolds with an anatomic shape: (A) an image of the mold right after printing, (B) the mold after rinsing away supporting material PSA, (C) mold filled with white paraffin spheres, and (D) the final PLLA NF scaffold.

2. Materials and methods

2.1. Scaffold fabrication

PLLA (inherent viscosity 1.6) was purchased from Alkermes (Cambridge, MA). Printing materials, wax and polysulphonamide (PSA), for 3D mold fabrication were bought from Solidscape Inc. (Merrimack, NH). All solvents, including dioxane, methanol, ethanol, hexane and cyclohexane, were purchased from Fisher Scientific (Pittsburgh, PA).

The CT data were part of a proximal phalanx that was retrieved from the National Library of Medicine's Visual Human Project. CT cross sectional image files were converted into a stereo lithography (STL) file using the software Mimics 8.11 (Materialise USA, Ann Arbor, MI). Based on the STL file, a wax mold was printed in a layer-by-layer fashion using a Modelmaker II (Solidscape Inc.). Paraffin spheres prepared in our lab were poured into the mold and heated at 37 °C for 15 min to bond neighboring spheres together. PLLA was dissolved in a mixture of dioxane and methanol (volume ratio 4:1) to prepare a 9% (w/v) solution. The solution was cast into the mold filled with paraffin spheres. The mold loaded with PLLA solution and paraffin spheres were kept at −20 °C to allow the PLLA solution to phase separate for approximately 2 h. The solvent mixture was then extracted by ethanol at −20 °C and water at 4 °C sequentially for 2 h each. Paraffin spheres and the wax mold were dissolved away with cyclohexane. Thus obtained was a porous PLLA scaffold in the shape of the part of phalanx with inner pore size determined by the size of paraffin spheres.

2.2. Mechanical property measurement

To characterize the mechanical properties of the scaffolds, PLLA NF scaffolds with a regular disk shape were prepared using a Teflon vial as a mold. The scaffold preparation procedure was the same as that described in the above paragraph except using a different mold. The diameter of scaffold disk was 7.2 mm and the thickness was 2 mm. A compressive mechanical test was carried out using a universal testing machine (MTS Synergie 200, MTS Systems, MN). The crosshead speed was 0.5 mm/min. The porosity and mechanical properties were calculated as previously reported [11,14].

2.3. Cell culture and osteoblast differentiation

MC3T3-E1 subclone 26 (MC-26) cells were cultured in ascorbic acid (AA)-free α-modified essential medium (α-MEM) supplemented with 10% FBS, 1% penicillin/streptomycin in a humidified incubator at 37 °C with 5% CO_2. For cell seeding and culture, the scaffolds were sterilized using ethylene oxide and soaked in 70% ethanol solution for 0.5 h under reduced air pressure to allow the ethanol solution to penetrate the scaffold. Afterwards, the ethanol solution was replaced with PBS three times for 30 min each on an orbital shaker (Model 3520, Lab-Line Instruments, Melrose Park, IL) at 75 rpm. Scaffolds were washed with the medium twice for 2 h each, transferred to custom-built Teflon trays, and then seeded with 2×10^6 MC3T3-E1 cells per scaffold. After 48 h of incubation, the cell-scaffold constructs were transferred into 6-well tissue culture plates containing 3 mL of medium per well, supplemented with 50 μg/mL ascorbic acid and 10 mM β-glycerol phosphate, and cultured for specified times. The medium was changed every other day.

For proliferation studies, the harvested cell-scaffold constructs were washed with PBS for 5 min, homogenized with a Tissue-Tearor (BioSpec Products, Inc., Bartlesville, OK), and the DNA content was determined with a fluorescence assay kit from Sigma (St. Louis, MO) [19].

For gene expression studies, real-time PCR was used to detect the amounts of mRNAs encoding bone sialoprotein (BSP) and osteocalcin (OCN). The total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA) with RNase–Free DNase set (Qiagen) according to the manufacturer's protocol after cell-scaffold constructs were mechanically homogenized with a Tissue-Tearor. The cDNA was made using a PCR machine (Applied Biosystems, Foster City, CA) with TaqMan (Applied Biosystems) reverse transcription reagents and 10 min incubation at 25 °C, 30 min reverse transcription at 48 °C, and 5 min inactivation at 95 °C. Real-time PCR was set up using TaqMan Universal PCR Master mix and specific primer sequence for OCN (5′-CCGGGAGCAGTGTGAGCTTA-3′ and 5′-TAGATGCGTTTGTAGGCGGTC-3′) and BSP (5′-CAGAGGAGGCAAGCGTCACT-3′ and 5′-CTGTCTGGGTGCCAACACTG-3′) with 2 min incubation at 50 °C, a 10 min Taq Activation at 95 °C, and 40 cycles of denaturation for 15 s at 95 °C followed by an extension for 1 min at 72 °C on an ABI Prism 7500 Real-Time PCR System (Applied Biosystems) [4]. Target genes were normalized against GAPDH.

For histological analysis, samples were fixed in 10% neutral buffered formalin solution (Sigma), dried through an ethanol gradient, and embedded in paraffin.