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Novel nanostructured biodegradable polymer matrices fabricated by phase separation techniques for tissue regeneration

S.-h. Hsu [a,b,*], S. Huang [a], Y.-C. Wang [a], Y.-C. Kuo [a]

[a] No. 1, Sec. 4, Roosevelt Road, Institute of Polymer Science and Engineering, National Taiwan University, Taipei, Taiwan
[b] Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei, Taiwan

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ABSTRACT

Biomimetic nanostructures have a wide range of applications. In particular, biodegradable polymer nanostructures that mimic the subtleties of extracellular matrix may provide favorable cell interactions. In this study, a co-solvent system was developed to configure a thermodynamically metastable biodegradable polymer solution, from which novel nanostructured matrices subsequently formed via wet phase separation (quaternary) or a combination with thermally induced phase separation. Three-dimensional (3D) nanostructured porous matrices were further fabricated by combination with particle-leaching (100–300 μm glucose). The new co-solvent system may generate matrices with reproducible nanostructures from a variety of biodegradable polymers such as poly(D,L-lactide) (PLA), poly(ε-caprolactone) (PCL) and PCL-based polyurethane. In vitro cell culture experiments were performed with mouse pre-osteoblasts (MC3T3-E1) and human bone marrow-derived mesenchymal stem cells (hBM-MSC) to evaluate the osteoinductive potential of PLA nanostructures. The results showed that nanofibrous (<100 nm) membranes promoted the bone-related marker gene expression and matrix mineralization of MC3T3-E1 at 14 days. Nanofibrous 3D matrices seeded with hBM-MSC without osteogenic induction supplements demonstrated a 2.5-fold increase in bone matrix deposition vs. the conventional microporous matrices after 14 and 21 days. Antimicrobial nanofibers were further obtained by plasma-assisted coating of chitosan on PLA nanofibers. This study reveals a platform for fabricating novel biodegradable nanofibrous architecture, with potential in tissue regeneration.

1. Introduction

Biomimetic nanostructures have a wide range of applications due to their unique wetting, adhesive and optical properties [1]. Biodegradable nanostructures can be used to promote tissue regeneration because they mimic the subtleties of extracellular matrix (ECM). For example, the ECM-like collagen nanofibers [2–4] may provide a native environment for improving the attachment, proliferation and compactness of stem cells [5]. However, collagen nanofibers suffer from poor mechanical properties [6]. For this reason, synthetic biodegradable polymers are often used, which include poly(D,L-lactide) (PLA), poly(ε-caprolactone) (PCL) and poly(lactide-*co*-glycolide) (PLGA). When fabricated into two-dimensional (2D) or three-dimensional (3D) nanofibrous matrices, they can promote osteogenic differentiation for different types of cells [7–9].

Current efforts in the fabrication techniques of nanofibers have been focused on electrospinning and phase separation. Electrospinning has been employed in the manufacture of polymeric nanofibers since 1996 [10,11]. The advance in electrospinning provides a fast way to generate various architectures in a variety of polymers. Unfortunately, electrospun nanofibers often have relatively flat topography. It is difficult to make large 3D matrices (scaffolds) by electrospinning, though the collector pattern and design have been continuously improved during recent years [12,13]. Compared with electrospinning, phase separation is a fairly common and time-efficient technique for fabricating scaffolds. Wet phase separation (WPS) is the most common for preparation of polymeric membranes, e.g. in a ternary system a thin layer of polymer solution (polymer/solvent) is immersed in a non-solvent (coagulant). Ma et al. developed a thermally induced phase separation (TIPS) system for the preparation of nanofibrous scaffolds [14,15]. The system involved a polymer, good solvent and non-solvent at a higher temperature (e.g. 60 °C), which then returned to a lower temperature to induce phase separation. The structure of the scaffolds was manipulated by changing the weight ratio between the polymer, the good solvent and the non-solvent. These nanofibrous scaffolds provided a biomimetic cellular environment, which facilitated the proliferation of various types of cells [7,16] and the differentiation of MSC [8,17]. In spite of these advantages,

* Corresponding author at: No. 1, Sec. 4, Roosevelt Road, Institute of Polymer Science and Engineering, National Taiwan University, Taipei, Taiwan. Tel.: +886 2 33665313; fax: +886 2 33665237.
E-mail address: shhsu@ntu.edu.tw (S.-h. Hsu).

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the system may be suitable only for certain types of relatively crystalline and high molecular weight (MW) polymers such as poly(L-lactide) (PLLA) [7,8,14,18–20].

In this study, a polymer/co-solvent system comprising a polymer, a good solvent and a relatively poor solvent was developed to generate novel biodegradable nanofibers. The ratio of the good solvent to the poor solvent determined the stability of the polymer solution. A metastable polymer solution could therefore be achieved at room temperature by regulating this ratio. A non-solvent was then used as a coagulant (i.e. a polymer/co-solvents/coagulant quaternary system) to induce WPS. The system may be combined with cooling (TIPS). Using these techniques, novel nanostructures may be fabricated from various types of biodegradable polymers without the requirement of crystallinity and high MW of the polymer or heating the polymer solution to higher temperatures. Mouse pre-osteoblasts (MC3T3-E1 cells) and human bone marrow-derived mesenchymal stem cells (hBM-MSC) were used to verify the osteoinductive ability of these nanofibrous matrices.

2. Materials and methods

2.1. Fabrication of nanostructured membranes by the new co-solvent system

The system employed two solvents (good and relatively poor) to prepare polymer solution at 25 °C and placed in a coagulant bath (95% ethanol, non-solvent) at different temperatures to induce phase separation and fabricate polymeric membranes with different nanostructures. The system was quaternary instead of the conventional ternary system. A range of parameters including the choices of co-solvents and weight ratios of the components were tested. The processing parameters presented here were optimized from experiments.

PLA (Ingeo 2002D, MW 120 kDa, 4.3% D-lactide content, from NatureWorks, USA) was dissolved in 1,4-dioxane (good solvent) and dimethylacetamide (DMAc, relatively poor solvent) at room temperature with a PLA/1,4-dioxane/DMAc weight ratio of 10/45/45 to form the metastable polymer solution (Supplementary material 1). The PLA solution was cast at 25 °C with a casting knife (thickness 800 μm) on a glass substrate, followed by different procedures to generate membranes with different nanostructures (a total of three nanostructures, one nanospindle and two nanofibrous). The procedures involved WPS or a combination with TIPS.

For fabrication of nanospindle membranes, the cast solution on the glass substrate (800 μm) was immersed immediately in 95% ethanol (non-solvent/coagulant) at 25 °C for 24 h. Membranes were washed with deionized water at 25 °C for 24 h with fresh water, replaced every 6 h, and lyophilized thereafter. In all cases, the top side of a membrane is defined as the side facing the air, and the bottom side of a membrane indicates the side in contact with the glass substrate.

Fabrication of the two nanofibrous membranes is described as follows. To make nanofibrous membranes with fiber diameter ~300–500 nm, the previous cast solution on the glass substrate was immediately placed at −20 °C (gelation temperature) for 6 h to induce phase separation and then immersed in −20 °C 95% ethanol (the coagulant) for 24 h to achieve solvent exchange. The resulting membranes were washed with deionized water for 24 h with fresh water, replaced every 6 h, and lyophilized thereafter. To make nanofibrous membranes with fiber diameter <100 nm, the previous cast solution on the glass substrate was immediately placed at −196 °C (gelation temperature) for 1 h to induce phase separation and then immersed in −20 °C 95% ethanol (the coagulant) for 24 h to achieve solvent exchange. The resulting membranes were washed with deionized water and lyophilized as described earlier.

Two control membranes were also fabricated. One was a microporous membrane, which was made by the polymer/solvent/coagulant system. The solution with a PLA/1,4-dioxane weight ratio of 10/90 was cast at 25 °C with a 800 μm casting knife on glass and immersed immediately in 95% ethanol (the coagulant) at 25 °C for 2 min. The resulting membranes were washed and lyophilized as described. The other was a dense PLA membrane, which was made by placing the single solvent cast solution (PLA/1,4-dioxane at the weight ratio 10/90) in a 30 °C oven for 6 h. The membrane was further dried with vacuum.

2.2. System development and feasibility

For comparison, a more conventional ternary system, which contains a polymer, good solvent and non-solvent, was employed to examine whether the membranes resulting from TIPS had nanostructures. PLA was dissolved in tetrahydrofuran (THF, good solvent) and isopropanol (IPA, non-solvent) at 60 °C for 24 h with polymer/THF/IPA weight ratios of 10/85/5, 10/80/10 and 10/75/15, respectively. All PLA solutions were returned to 25 °C for 30 min. The solutions were cast on glass with the 800 μm casting knife and immersed immediately in IPA (non-solvent/coagulant) at 25 °C for 24 h. Membranes were washed and lyophilized as described.

The co-solvent system was tested on polymers other than PLA. Cast solutions of polymers such as PCL (MW 70–90 kDa, crystallinity ~43%, Sigma, USA), biodegradable poly(ester)urethane (bPU, based on 4,4′-methylene dicyclohexyl diisocyanate (H_{12}MDI) and PCL (MW 100 kDa, crystallinity 40–45%, Industrial Technology Research Institute, Taiwan) and PLGA (lactide:glycolide = 50:50, MW 80 kDa, BioInvigor, Taiwan) with the polymer/1,4-dioxane/DMAc at various weight ratios were prepared. The solutions were cast on glass and immediately placed at different gelation temperatures (−20 °C for 6 h or −196 °C for 1 h) and then immersed in −20 °C 95% ethanol for 24 h to achieve solvent exchange. The resulting membranes were washed and lyophilized as described.

In contrast, various poor solvents such as acetone, dimethyl sulfoxide (DMSO), THF and dimethylformamide (DMF) were used to replace DMAc. Owing to the different extents of polymer solubility in these poor solvents, the weight ratio of polymer/good solvent/poor solvent was optimized from tests for each poor solvent. The solution was cast on glass and immediately placed in 95% ethanol at 25 °C for 24 h or at −196 °C for 1 h to induce phase separation before immersion in −20 °C 95% ethanol for 24 h. The prepared membranes were washed and lyophilized as described.

2.3. Fabrication of 3D scaffolds

Two types of 3D PLA scaffolds were fabricated. One was the nanofibrous scaffold, and the other was the regular microporous scaffold. Preparation of the nanofibrous scaffolds followed that for 2D membranes with some modification. A round mold (diameter 15 mm and height 3 mm) was filled with 2 g glucose particles (size selected 100–300 μm by mesh), where 2 ml polymer solution with a PLA/1,4-dioxane/DMAc weight ratio of 10/45/45 was cast at 25 °C. The final polymer/glucose ratio was 1 g to 1 ml. The mold was placed immediately at −196 °C for 1 h to induced phase separation and immersed in −20 °C 95% ethanol coagulant for 24 h to achieve solvent exchange. The scaffolds were washed and lyophilized as described. The same procedures were applied to fabricate 3D scaffolds from PCL and bPU, but failed to maintain integrity for PLGA.