Mineralized Composite Nanofibrous Mats for Bone Tissue Engineering†

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Abstract: Composite nanofibrous mats consisting of poly (L-lactide-co-ε-caprolactone) (PLCL) and collagen type I (COL) were fabricated by electrospinning, and ten times simulated body fluid (10SBF) were employed to mineralize nanofibrous mats. Ball-shaped hydroxyapatite (HA) was deposited on the surface of nanofibrous mats in 1.5 h at room temperature. Human fetal osteoblasts (hFob) were seeded to investigate their proliferation and differentiation on mineralized composite nanofibrous mats. The results showed that hFob grew well on mineralized composite nanofibrous mats and alkaline phosphatase (ALP) activity of hFob on mineralized composite nanofibrous mats at 14 d was much higher than that on untreated nanofibrous mats. Moreover, the expression of osteocalcin of cells on mineralized composite nanofibrous mats was also much higher than those on untreated nanofibrous mats at 7 d and 14 d. This mineralized composite nanofibrous mats may have a great potential for bone tissue engineering.

Key words: *electrospnning*; *nanofibrous mat*; *mineralization*; *bone tissue engineering*

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Introduction

Hydroxyapatite (HA) is one of the main components of natural bone tissues. It has been widely investigated for the applications in bone tissue engineering[1-2]. Simulated body fluid (SBF) is a type of medium with ion concentrations approximating those of human plasma and has the ability to deposit inorganic calcium phosphate on the surface of nanofibers[3]. It was employed to deposit inorganic apatite on the surface of biomaterials. In general, the SBF was based on the recipe from Kitsugi *et al.*[4] It could deposit crystalline apatite on the surface of biomaterial, but at least one week would be taken to precipitate a proper amount of apatite. Five times SBF (5SBF) and ten times SBF (10SBF) were rapid ways to form calcium phosphate layer on biomaterial surface at room temperature[5-6]. A great amount of calcium phosphate could be precipitated just in hours, but flake dicalcium phosphate dehydrate ($CaHPO_4 \cdot 2H_2O$) also formed[6]. Methods have been used to control the mineralization and avoid the formation of flake $CaHPO_4 \cdot 2H_2O$. Andric *et al.* tried to use vacuum and pre-treat the scaffolds with NaOH. They found that the overall mineral precipitation and distribution on scaffolds were improved[7].

Researchers have demonstrated that HA has a positive effect on protein adsorption, signal transduction, cell differentiation, and bone extracellular matrix (ECM) synthesis. Song *et al.* studied the cell growth and differentiation on collagen nanofibers and collagen/HA composite nanofibers[8]. The result showed that the alkaline phosphatase (ALP) activity expressed by MC3T3-E1 osteoblastic cells was higher at day 14 than that on pure collagen nanofibers. Incorporating HA in polymeric matrix could enhance the cell growth and differentiation. And it is promising for bone tissue engineering.

In this study, composite nanofibrous mats were mineralized with 10SBF and a shaker was employed during the mineralization process. We hypothesized that the movement of 10SBF solution would have a great effect on the deposition of apatite on our scaffolds. Human fetal osteoblasts (hFob) were utilized to assess the biocompatibility of the mineralized composite scaffolds. The cellular differentiation and osteocalcin expression also were evaluated.

1 Materials and Methods

1.1 Materials

Poly (L-lactide-co-ε-caprolactone) (PLCL) (75∶25) was purchased from Fine Chemical Sales Carbohydrate Chemistry Team Industrial Research (New Zealand). Collagen type I (COL) was purchased from Koken (Japan). 1, 1, 1, 3, 3, 3-Hexafluoro-2-propanol (HFIP) was obtained from Daikin (Japan). The other regents were purchased from the Sigma-Aldrich Co. (Milwaukee, Wisconsin). All of the materials were used without further purification.

1.2 Fabrication of nanofibrous mats

PLCL and COL were dissolved in HFIP at a weight ratio of 8∶2 for 12 h. The weight concentration of PLCL/COL solution was 8%. The aqueous solutions were filled into a 2.5 mL plastic syringe with a blunt-ended needle. The syringe was located in a syringe pump (789100C, Cole-Pamer, America) and dispensed at a rate of 1.0 mL/h. A voltage of 14 kV from a high voltage power supply (BGG6-358, BMEICO. Ltd., China) was applied across the needle and ground collector, which was placed at a distance of 15 cm.

1.3 Mineralization of nanofibrous mats

Solution preparation recipe (for a total aqueous volume of 1 L) is given in Table 1. The chemicals given in Table 1 are added, in the order written, to 900 mL of deionized (DI) water in a glass beaker. Before the addition of the next chemical, the

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previous one was completely dissolved in water. After all the reagents were dissolved at room temperature, the solution was made up to 1 L by adding the proper amount of water.

Table 1 Stock solution preparation recipe, for a total volume of 1 L

Reagent	Order	Amount/g	Concentration/(mmol·L^{-1})
NaCl	1	58.443 0	1 000
KCl	2	0.372 8	5
$CaCl_2 \cdot 2H_2O$	3	3.675 4	25
$MgCl_2 \cdot 6H_2O$	4	1.016 5	5
NaH_2PO_4	5	1.199 8	10

Before the mineralization of nanofibrous scaffolds, a 600 mL portion of this stock solution was placed into a glass beaker, and a proper amount of $NaHCO_3$ powder was added to raise the hydrogencarbonate ion (HCO_3^-) concentration to 10 mmol/L under vigorous stirring. Then the as-prepared solution was poured into a 4 well plates with nanofibrous mats fixed on its bottom. The nanofibrous mats were mineralized by shaking at 130 r/min for 1.5 h with an Orbital Shaker SO_3 (Stuart Scientific, UK) at room temperature.

1.4 Characterization methods

The morphology was observed with a scanning electronic microscope (SEM) (JSM-5600, Japan) at an accelerated voltage of 15 kV.

Cell proliferation was monitored at 3, 7, and 14 d by MTS assay (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt). The absorbance was read at a wavelength of 490 nm. Tissue culture polystyrene (TCP) was chosen as a control (Control) in this study.

ALP activity was measured by a Phosphatase Substrate Kit (Anaspec Co., USA) at all time points (3, 7, and 14 d). The absorbance was read at a wavelength of 405 nm.

For immunocytochemical analysis, nanofibrous mats with cells were fixed in 4% formaldehyde and exposed to bovine serum albumin (BSA)/phosphate buffered saline (PBS) for a further 30 min at room temperature. After another wash in PBS, the cells were incubated with primary antibodies against osteocalcin (Millipore Singapore Pte Ltd.). Visualization was done after washing in PBS using anti-mouse-fluorescein isothiocyanate (FITC) secondary antibody at room temperature. Finally, the nuclear of cells were stained by 4′, 6-diamidino-2-phenylindole, 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (Invitrogen Corp., Carlsbad, CA) for 30 min. The samples were observed and viewed by laser scanning confocal microscope (LSCM) (Olympus FluoView FV1000, Olympus Corp., Center Valley, PA).

2 Results and Discussion

SEM micrographs of PLCL/COL and mineralized PLCL/COL (M-PLCL/COL) are shown in Fig. 1. The diameter of PLCL/COL was (169 ± 34) nm. After the mineralization of PLCL/COL by 10SBF with shaking for 1.5 h, ball-shaped apatites were deposited on the surface of PLCL/COL (Fig. 1 (b)). They not only grew along the nanofikers, but also had no significant effect on the morphology of nanofibrous mat (Fig. 1 (c)). The structure of nanofibers was maintained. The average diameter of the apatite was (381 ± 60) nm. Moreover, there was no flake-like apatite formed on the surface of composite nanofibrous mats.

Figure 2 shows the proliferation of hFob on PLCL/COL, M-PLCL/COL, and Control at 3, 7, and 14 d, where the cell proliferation on M-PLCL/COL is higher than that on PLCL/COL and Control at day 7 (* $p < 0.05$). At 14 d, no significant difference was found for the proliferation of hFob on all groups. The results indicated that hFob proliferated well on M-PLCL/COL.

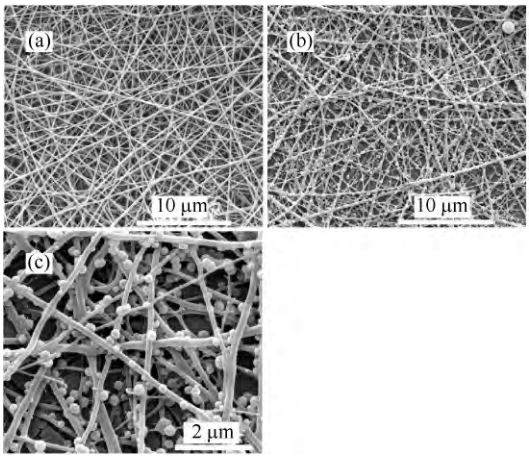

Fig. 1 SEM images of (a) PLCL/COL, (b) M-PLCL/COL, and (c) the higher magnification of (b)

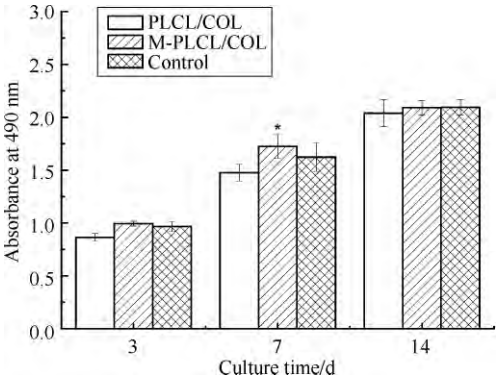

Fig. 2 Proliferations of hFob on PLCL/COL, M-PLCL/COL, and Control

As shown in Fig. 3, ALP activity of hFob increased with the culture times. At 7 and 14 d, ALP activity levels of hFob on M-PLCL/COL were higher than that on PLCL/COL (* $p < 0.05$). It indicated that the ball-shaped apatite had a positive effect on cell differentiation. Mineralized composite nanofibrous mats could improve ALP secretion of cells.

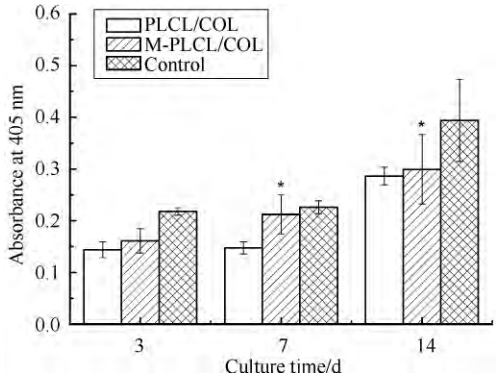

Fig. 3 ALP activities on PLCL/COL, M-PLCL/COL, and Control after 3, 7, and 14 d culture

As shown in Fig. 4, the intensity of the fluorescent indicated the expressions of osteocalcin of hFob on M-PLCL/COL were much higher than those on PLCL/COL at 7 and 14 d.

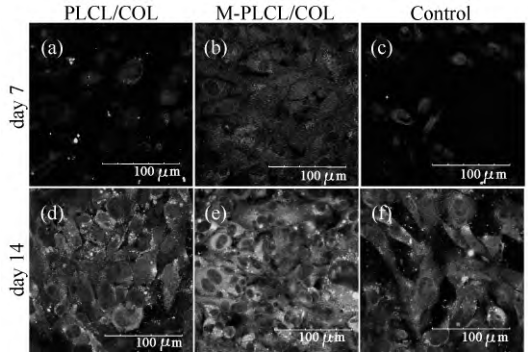

Fig. 4 Osteocalcin stainings of hFob on PLCL/COL, M-PLCL/COL, and Control at 7 and 14 d

For the controlling deposition of apatite, a shaker was employed during the mineralization process. It had great effect on the morphology of apatite formed on the surface of composite nanofibrous mats, which was indicated by SEM results. Ball-shaped apatite with nano-structure grew along the nanofibers. *In vitro* studies showed that mineralized composite nanofibrous mats promoted cell proliferation and differentiation. Our results are in accord with the reported results [9-10].

3 Conclusions

PLCL and COL composite nanofibrous mats were fabricated by electrospinning. Ball-shaped nano-apatites were rapidly deposited on composite nanofibrous mats by 10SBF and they grew along the nanofibers. The porous structure of nanofibrous mats was maintained and the proliferation and differentiation of hFob were promoted by mineralized composite nanofibrous mats. These mineralized composite nanofibrous mats are promising for the applications in bone tissue engineering.

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