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Mineralization on polylactide/gelatin composite nanofibers using simulated body fluid containing amino acid

Yougang Guo[a], Jinle Lan[a], Caijin Zhang[a], Man Cao[a], Qing Cai[a,b,*], Xiaoping Yang[a,b]

[a] State Key Laboratory of Organic–Inorganic Composites, Beijing University of Chemical Technology, Beijing 100029, PR China
[b] Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, PR China

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ABSTRACT

From physiological point of view, organic–inorganic composite nanofibers are envisioned promising substrates for bone tissue engineering. Biomineralization on polymeric nanofibers using simulated body fluid (SBF) is a common technique to obtain the composite nanofibers. Many factors, however, will affect the nucleation and crystal growth of deposited apatite, such as the additives like amino acids in SBF. In this study, electrospun composite nanofibers consisting of poly(L-lactide) (PLLA, 50 wt%) and gelatin (50 wt%) were soaked in 2.5 times SBF (2.5SBF) for different time periods (1, 2, 3, 5 and 7 days) to perform the biomineralization. Three amino acids (glycine, aspartic acid, or arginine) of different charge characteristics were introduced into the SBF, and their effects on nucleation and transformation of calcium phosphate depositions were systematically investigated. The results revealed that amino acids could take part in the early stage formation of pre-nucleation clusters, leading to different assemblies dependent closely on the feature of amino acid. In comparison with normal 2.5SBF, the presence of amino acid was able to enhance the preferred orientation of hydroxyapatite (HA) crystal along *c* axis and the transformation from amorphous calcium phosphate to hierarchical HA. The incorporation of glycine had promoted the formation of the well-evolved needle-like HA crystals in comparison with aspartic acid or arginine. It was suggested that the addition of amino acids into SBF might be a useful tool to regulate the biomineralizaiton for preparing organic–inorganic composite nanofibers.

1. Introduction

As major component of vertebrates, bone plays important role in providing structural framework, mechanical strength, blood pH regulation and maintenance of the calcium and phosphate ions level for metabolic processes [1]. Its unique properties are from its hierarchical structure and chemical compositions [2]. Bone is a typical complex tissue consisting of nano-hydroxyapatite (HA) and collagen as major portions that HA nanocrystals grow on self-assembled collagen fibrils, forming mineralized structures at scales of several hundred nanometers to macro-size. To restore bone defects by concept of tissue engineering, artificial scaffolds are preferred to be biomimetically constructed in simulating the nanofibrous structure and the organic–inorganic nanocomposite of native bone extracellular matrix (ECM) [3].

Electrospinning of biodegradable polymers including synthetic and natural origins has been widely reported to generate suitable nanofibrous bone matrixes [4,5]. Both MC3T3-E1 osteoblasts and mesenchymal stem cells (MSCs) are able to attach on nanofibers better than on solid-walled scaffolds or flat films [6,7]. The high specific surface area of nanofibrous scaffold enhances the ability of adsorbing proteins, which is vital for cell anchorage. In the issue of achieving high osteocompatibility, incorporation of calcium phosphate (CaP) compounds into the aforementioned nanofibers becomes an important strategy in developing composite nanofibrous substrates [8]. Considering *in vivo* deposition of HA nanocrystals by using collagen fibrils as organic templates, *in vitro* biomineralization in simulated body fluid (SBF) using polymeric nanofibers as templates is envisioned a promising approach to obtain biomimetic artificial ECM for bone regeneration [9,10].

Biomineralization is an extremely complex process, in which, the initial nucleation has strong impact on mineral growth, leading to diversities in Ca/P ratio, morphology, crystal size and structure of final CaP compounds [11–13]. Amino acids were reported able to take part in the initial nucleation during the formation of amorphous calcium carbonate phase, thus causing changes in the subsequent crystal growth orientation and lattice parameters of

* Corresponding author at: State Key Laboratory of Organic–Inorganic Composites, Beijing University of Chemical Technology, Beijing 100029, PR China; Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, PR China. Tel.: +86 10 64412084; fax: +86 10 64412084.
E-mail address: caiqing@mail.buct.edu.cn (Q. Cai).

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the final product [14–17]. Lysine was found able to change surface structure of HA to get more stable interface *via* the adsorption of lysine on the (1 0 0) lattice plane of HA [18]. Aspartic acid could adhere to the (1 0 4) lattice plane of calcite to smooth the edges of crystals [19]. Other researches showed that incorporation of amino acids into CaP mineral systems could affect transformations between different CaP phases [20]. Glysine and glutamic acid were reported able to induce formation of enamel or bone-like apatite, respectively, when they were added into solutions abundant with amorphous calcium phosphate (ACP) [21]. The transformation was suggested being determined by the interfacial tension and energy balance between amino acids and ACP clusters. In these aspects, it was very interesting to perform the study on *in vitro* biomineralization by adding amino acids into SBFs. When electrospun nanofibers were applied as templates to induce apatite formation from amino acid-containing SBFs, therefore, the fabrication of organic–inorganic composite nanofibrous substrates could be very meaningful and important for bone regeneration research.

SBFs used in literatures differed in compositions and concentrations [22]. For biodegradable scaffolds, modified SBFs with higher concentrations (1.5, 2.5, 5 or 10 times SBF, *etc.*) are usually applied to shorten the experimental period in preventing significant material degradation during mineralization [23–25]. Herein, nanofibers consisting of poly(L-lactide) (PLLA, 50 wt%) and gelatin (50 wt%) were electrospun and immersed in 2.5 times SBF (2.5SBF) at 37 °C for different times in this study. The non-woven PLLA/gelatin nanofibers were used because they had been proven hydrophilic and able to remain fiber morphology during SBF soaking in our previous work [26,27]. Amino acids (glycine, aspartic acid, or arginine) with different charge characteristics were added into the 2.5SBF to explore their roles in nucleation and transformation of CaP minerals in simulated biomineralization. Characterized by methods including transmission electron microscope (TEM), scanning electron microscope (SEM), energy dispersive X-ray spectroscopy (EDX), Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD) *etc.*, the occurrence of pre-nucleation, the development of crystal growth and the feature of final minerals were monitored and interpreted. A schematic model of HA nucleation and crystallization was proposed with purposes of providing better understanding and better control on biomimetic preparation of matrixes for bone regeneration.

2. Experimental

2.1. Materials

PLLA (M_w = 100,000) and 2,2,2-trifluoroethanol (TFE) (99%) were purchased from Sigma–Aldrich. Gelatin (type B, from bovine, pH 4.5–5.5, bloom 240–270) was purchased from Amersco Company (USA). All of them were used for electrospinning without any further purification. Both 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 97%), and N-hydroxysuccinimide (NHS, 97%) were purchased from Aldrich Chemical (USA), and used as received. Glycine, aspartic acid, and arginine were all of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (China). All the analytical grade chemicals for preparing SBF were purchased from Beijing Biodee Biotechnology Co. Ltd. (China).

2.2. Electrospinning of PLLA/gelatin composite nanofibers

PLLA/gelatin (1:1 in weight ratio) composite nanofibers were electrospun as previously reported [26]. Briefly, PLLA (0.4 g) and gelatin (0.4 g) were dissolved in TFE (8 ml) overnight at room temperature to get a viscous solution (0.1 g/ml) for electrospinning. The parameters for electrospinning were set as: voltage 14 kV, flowing rate 0.5 ml/h, receiving distance 15 cm, and environmental humidity 30%. Non-woven nanofibrous sheets were obtained by using metal plate as the collector. The as-spun PLLA/gelatin composite nanofibers were crosslinked by EDC/NHS solution at 4 °C for 8 h, followed by washing with DI water and freeze-drying. The freeze-dried nanofibrous sheets demonstrated a thickness around 50 μm.

2.3. Biomineralization in SBFs containing amino acids

SBF solutions were prepared by dissolving salts of NaCl, $NaHCO_3$, Na_2SO_4, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl{\cdot}6H_2O$, and $CaCl_2{\cdot}2H_2O$ in DI water with or without the addition of amino acid (glycine, aspartic acid, or arginine), and buffered to pH value of 7.2 at 37 ± 0.2 °C with Tris–HCl. The concentrations of the ions were 2.5 times of normal SBF, which were 355.0 mM Na^+, 12.5 mM K^+, 7.5 mM Mg^{2+}, 6.25 mM Ca^{2+}, 369.5 mM Cl^-, 10.5 mM HCO_3^-, 2.5 mM HPO_4^{2-} and 1.25 mM SO_4^{2-}, and 2.5 mM amino acid (if amino acid was added). The operating steps for the preparation of SBF were strictly referred to the ISO standard [ISO 23317:2007(E)]. Noticeably, amino acids should be added before the addition of $CaCl_2$ to avoid possible premature precipitation. For simplication, the resulting SBFs were named in terms of 2.5SBF-blank, 2.5SBF-Gly, 2.5SBF-Arg and 2.5SBF-Asp depending on the added amino acid.

PLLA/gelatin nanofibrous film was cut into square pieces with dimension of 10 mm × 10 mm, soaked in various SBFs for different time periods (1, 2, 3, 5 and 7 days) at 37 ± 0.2 °C. SBF solutions were refreshed every other day. At each time point, specimens were retrieved, gently washed with DI water and then freeze-dried for characterizations.

2.4. Nucleation study

To investigate the effect of amino acid on the formation of pre-nucleation cluster (PNC) in solution, drops of aqueous solution were taken out from corresponding SBF after the SBF had been prepared and thermoset at 37 ± 0.2 °C for 8 h. The retrieved solutions were dropped onto carbon-coated copper grids, followed by liquid nitrogen quenching and freeze-drying immediately. The specimens were then observed by high resolution TEM (HR-TEM, FEI G2F30) with an operating voltage at 300 kV.

Based on TEM images, the two-dimensional fractal dimension D_2 (an evaluation of space filling efficiency of aggregate structures) was applied to characterize the pre-nucleation quantitatively. According to literatures [28–30], D_2 parameter was determined by using the definition of $A \propto L^{D_2}$, where A is the projected area of the object (here is the PNC) and L is the maximum length of the projected area of the object. The values of A and L could be acquired from TEM images using ImageJ image visualization software (National Institutes of Health, USA). By plotting log A *versus* log L, the value of D_2 could be obtained from the slope of the curve.

2.5. Characterizations

For morphological observation, the biomineralized PLLA/gelatin nanofibrous pieces were sputter-coated with platinum (30 mA, 20 s) using a sputter coater (E5600, Polaron, USA), and then observed using SEM (Supera55, Zeiss, Germany) at an accelerating voltage of 20 kV. EDX analysis was performed under the same parameters to SEM observation and the exposure time was 180 s. Chemical structures of the biomineralizaiton products were detected by FT-IR (Nicolet 6700, USA) with the wavenumber ranging from 4000 to 500 cm^{-1} at a resolution of 4 cm^{-1}. Crystal structures were evaluated by an X-ray diffractometer (D/Max 2500VB2 + Rigaku, Japan) with a fixed incidence of 1° at a 2θ scanning rate of 10°/min in the range of 10–50° using CuKα