Apatite-forming ability of bioactive poly(l-lactic acid)/grafted silica nanocomposites in simulated body fluid

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Article info
Article history:
Received 2 December 2010
Received in revised form 1 April 2011
Accepted 1 April 2011
Available online 9 April 2011

Keywords:
Poly(l-lactic acid)
Scaffold
Apatite
Nanocomposites

Abstract
Bioactive PLLA/surface-grafted silica (g-SiO₂) nanocomposite scaffolds were fabricated by solid–liquid phase separation method. And solid PLLA/g-SiO₂ nanocomposite films were prepared by solution casting method. A series of parallel tube-like morphology and internal ladder-like structure of PLLA/g-SiO₂ nanocomposite scaffolds were observed by SEM. The formation of bone-like apatite in the simulated body fluid (SBF) was characterized by XRD, IR, SEM, EDS and weight measurement. The silica incorporation favors the formation of apatite. The growth of apatite with immersion time is found on the surfaces of both the PLLA/g-SiO₂ nanocomposite scaffolds and the films. The potential mechanism is that silanol groups of g-SiO₂ in the nanocomposites serve as nucleation sites for the formation of bone-like apatite crystals.

1. Introduction
Tissue engineering offers a promising new approach to develop and supply a rapidly expanding number of bone substitutes. In this approach, a porous scaffold is needed to serve as a temporary extracellular matrix (ECM) [1,2].

Nowadays, poly(l-lactide) (PLLA) is a promising scaffolding material due to its renewable resource, biodegradability and bio-compatibility. However, in the application of bone substitute, it still has several shortcomings. The mechanical strength and toughness of PLLA is lower than those of natural cortical bones [3]. Additionally it has no ability of osteo-introduction or conduction in vivo conditions [4].

In our previous work, PLLA/surface-grafted silica (g-SiO₂) nanocomposites were developed in order to improve the aforementioned disadvantages of PLLA. The compatibility and adhesion between silica nanoparticles and PLLA were effectively improve by surface-grafting of silica nanoparticles with l-lactic acid oligomer. The introduction of g-SiO₂ nanoparticles in poly(l-lactide) (PLLA) matrix greatly improves the toughness and tensile strength of this material [5].

Silica plays an important role in the biomineralization of many organisms including coral and diatoms [6]. Hench and Paschall [7] have shown the integral role that silica plays in the bioactivity and osteogenic potential of bioglass. The high density of surface silanol groups (Si–OH) that exist on the surface of amorphous silica can induce the formation of biologically active bone-like apatite, giving the bioactivity of PLLA/g-SiO₂ nanocomposites. Lai et al. [8] have reported that silica granules are slowly excreted through urine following the dissolution in vivo. So, there is no reason to worry about silica particles being left behind and accumulating after the preferential degradation of PLLA in vivo.

Therefore, PLLA/g-SiO₂ nanocomposites with good mechanical properties may be used as novel bioactive scaffold for bone tissue engineering. Still, the bioactivity of PLLA/g-SiO₂ nanocomposites should be verified. The apatite-forming ability of bioactive materials in vivo can be reproduced in simulated body fluid (SBF), so one can predict in vivo bioactivity of a material by assessing apatite formation on its surface in SBF [9,10].

In this article, PLLA/g-SiO₂ nanocomposite scaffolds and films were fabricated. The effect of silica nanoparticles on the surface formation of bone-like apatite in simulated body fluid was evaluated. The bioactive PLLA/g-SiO₂ nanocomposites are likely to be applicable to bone substitutes.

2. Experimental procedure
2.1. Materials
PLLA was prepared according to the literature [11]. Its molecular weight (Mn) was about 180,000. Silica nanoparticles with an...
average size of 20 nm were supplied by Guizhou Nanomaterials Engineering Center (China). The surface grafting of silica nanoparticles and the preparation of the PLLA/g-SiO2 nanocomposites have been described in our previous paper [5], which are briefly depicted in Fig. 1. The amount of surface grafted l-lactic acid oligomer on g-SiO2 determined by thermal gravimetric analysis was about 8.3 wt%. All other chemicals were of analytical grade and used without further purification.

2.2. Preparation of PLLA/g-SiO2 films and porous scaffolds

The PLLA/g-SiO2 nanocomposite scaffolds were prepared by freeze-drying method. The PLLA was dissolved in dioxane to make a solution of a desired concentration (from 5 to 10 wt%). Pre-weighed dried g-SiO2 powders were uniformly suspended in the PLLA solution with the help of magnetic stirring and ultrasonic treatment to achieve the g-SiO2 content of 5 wt% in the composite. The solution (10 mL) was transferred into a beaker (30 mL), then the beaker was rapidly transferred into a refrigerator at −20 °C to solidify the solvent and induce solid–liquid phase separation. After 2 h, the solidified solution was transferred into a freeze-drying vessel at −80 °C for 4 days to completely remove the solvent. The porous samples were stored in a desiccator until incubation or characterization. For comparison, the pure PLLA porous scaffolds were also prepared.

Solid PLLA/g-SiO2 nanocomposite films were prepared by solution casting method. g-SiO2 powders were uniformly dispersed in PLLA/chloroform solution (5 wt%) with the help of magnetic stirring and ultrasonic treatment to achieve the g-SiO2 content of 0–40 wt% in the composite. Then PLLA/g-SiO2/chloroform solution (10 mL) was transferred into a Teflon plate. After volatilization of solvent in a vacuum-oven at 40 °C, dried PLLA/g-SiO2 nanocomposite films were obtained.

2.3. Samples soaking in 1.5SBF solution

In the present study, 1.5SBF was used as an incubation solution for bone-like apatite formation on samples’ surface. The concentration of the different ionic species in SBF closely resembles that of human blood plasma. Compared with 1.0SBF (the conventional SBF), 1.5SBF has ionic concentrations 1.5 times to those of 1.0SBF (Table 1) [12]. 1.5SBF was used to accelerate the apatite coating process because of its higher ion concentrations than SBF.

The samples of PLLA/g-SiO2 nanocomposite scaffolds and solid PLLA/g-SiO2 nanocomposite films were soaked in 1.5SBF at 37 °C for different times. The samples were kept in the vertical position inside test tubes (one per tube) containing 20 mL of incubation solution that was renewed every 2 days. After soaking, the samples

| Table 1 Comparison of concentrations (mM) of various ions in human blood plasma, 1.0SBF and 1.5SBF. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Na⁺             | K⁺              | Ca⁴⁺            | Mg²⁺            | HCO₃⁻           | Cl⁻             | HPO₄²⁻          | SO₄²⁻           |
| Blood plasma    | 142.0           | 5.0             | 2.5             | 1.5             | 27.0            | 103.0           | 1.0             | 0.5             |
| SBF             | 142.0           | 5.0             | 2.5             | 1.5             | 4.2             | 148.0           | 1.0             | 0.5             |
| 1.5SBF          | 213.1           | 7.5             | 3.8             | 2.3             | 6.3             | 221.9           | 1.5             | 0.8             |

Fig. 1. Method for preparing of the PLLA/g-SiO2 nanocomposites.

Fig. 2. SEM micrographs of the surfaces of PLLA/g-SiO2 (5 wt%) nanocomposite scaffolds prepared from a 5 wt% solution in dioxane at different original magnifications (A: 50×; B: 100×; C: 300×).

Fig. 3. SEM micrographs of the cross-sections of PLLA/g-SiO2 (5 wt%) nanocomposite scaffolds prepared from a 5 wt% solution in dioxane at different original magnifications (A: 50×; B: 100×; C: 300×).
Fig. 4. SEM micrographs of PLLA/g-SiO$_2$ (5 wt%) nanocomposite scaffolds prepared from 5 wt% (A and B) and 10 wt% (C and D) solution in dioxane at different original magnifications (A and C: 25×; B and D: 200×).

Fig. 5. XRD pattern of PLLA/g-SiO$_2$ nanocomposite films after immersion in 1.5SBF (inset: XRD patterns of PLLA/g-SiO$_2$ nanocomposite scaffolds).
Fig. 6. IR spectra of the apatite particles obtained from surface of PLLA/5% g-SiO2 nanocomposite films after 5 days immersion in 1.5SBF.

were gently washed with distilled water and dried under vacuum for characterization.

2.4. Characterization

The surface microstructures and porous morphologies were analyzed by a field emission scanning electron microscopy (XL30 ESEM FEG, PHILIPS). Element analysis of samples’ surface after soaking in 1.5SBF was performed on energy dispersed X-ray spectrometer (EDS) attached to formentioned ESEM.

The functional groups of the specimens were characterized by Fourier transformed infrared spectroscopy (Bio-Rad FTS135). The particles were scratched from the surfaces of nanocomposite films after immersion in 1.5SBF. The pulverized specimens were mixed with KBr powders and pressed into a disk suitable for IR measurement.

The crystal phases of the specimens before and after the bioactivity test were analyzed by X-ray diffractometry (D/MAX2550, Rigaku).

The mass increase of the films during the soaking in 1.5SBF was measured with an analytical balance accurate to $10^{-4}$ g. The films were dried in a fume hood for 1 week and then vacuum dried at 0.5 mmHg for 24 h before measuring. Three specimens were measured for each sample to obtain the averages and standard deviations. The mass increase was calculated using the following formula:

$$W\% = \frac{100(W_t - W_0)}{W_0},$$

where $W_0$ and $W_t$ are the weights of the sample before and after soaking in 1.5SBF, respectively.

3. Results and discussion

3.1. Microstructures of PLLA/g-SiO2 nanocomposite scaffolds

The PLLA/g-SiO2 scaffolds prepared from solid–liquid phase separation of the PLLA/g-SiO2/dioxane solutions show a series of parallel tube-like morphology (Fig. 2), the diameter of which is in the range 30–100 μm. The internal wall of these channels is also macroporous and shows a ladder-like structure (Fig. 3). Similar
anisotropic tubular morphology with an internal ladder-like structure for pure porous poly(d,l-lactide) and poly(l-lactide) is also reported by Schugens et al. [13] and Ma et al. [14].

Polymer concentration has an effect on the porous structure; the diameter of the tubes obviously decreases when polymer concentration increases from 5% to 10%, as shown in Fig. 4.

### 3.2. In vitro bioactivity of PLLA/g-SiO2 nanocomposite films and scaffolds

Fig. 5 shows the XRD patterns of PLLA/g-SiO2 nanocomposite films and scaffolds with different g-SiO2 contents (0%, 5%, 20% and 40%) after soaking in 1.5SBF solution for different times. For PLLA/g-SiO2 nanocomposite films (Fig. 5A), PLLA matrix maintains its original crystalline structure in 1.5SBF solution and exhibits diffraction peaks located at $2\theta = 14.7^\circ$, $16.7^\circ$, $19.1^\circ$ and $22.3^\circ$ [15,16]. Apatite peaks denoted by “●” symbols are observed to occur in addition to the PLLA peaks after immersion in 1.5SBF. The strongest apatite peak appears at $31.8^\circ$, corresponding to (2 1 1) diffraction planes of apatite [17]. This indicates the formation of apatite crystals on the surface of PLLA/g-SiO2 nanocomposite films. The strength of apatite peaks increases gradually with g-SiO2 content, in particular, the PLLA/40% g-SiO2 composite film shows stronger intensity of apatite peaks after shorter time (6D) soaking in 1.5SBF solution. On the contrary, the pure PLLA film does not show any diffraction peak in the XRD pattern. Inset shows the XRD patterns of PLLA/g-SiO2 nanocomposite scaffolds, the apatite peaks also become more evident with g-SiO2 content. So it can be concluded that the incorporation of g-SiO2 can accelerate the sediment of apatite.

In Fig. 5B, PLLA/g-SiO2 nanocomposite films exhibit stronger diffraction peaks of apatite with soaking time, indicating the growth of apatite on the films.

In order to examine the chemical structure of the particles formed on PLLA/g-SiO2 nanocomposite films, the particles were collected from the surface of PLLA/5% g-SiO2 nanocomposite films after 5 days immersion in 1.5SBF for IR measurement, as shown in Fig. 6. The broad bands around 3430 and 1620 cm$^{-1}$ originate from O–H of water absorbed in the sample [18]. The asymmetrical stretching and bending modes of PO$_4^{3-}$ ion are detected at around 1035, 602, 563 and 467 cm$^{-1}$, respectively [18]. Furthermore, two stretching and an out-of-plane modes of CO$_3^{2-}$ ion are also observed at around 1640, 1420, and 873 cm$^{-1}$, respectively. It means that the PO$_4^{3-}$ sites of the apatite structure are partly substituted by carbonate ions, i.e. the apatite formed was carbonated apatite [19].

Fig. 7 shows the SEM micrographs of PLLA/5% g-SiO2 nanocomposite films after different immersion times in 1.5SBF. Before immersion, the surface of film is relatively smooth, as shown in Fig. 7A. In the lower magnification, one can distinguish a change in particle number and size with immersion time. At higher magnifications (inset), it is possible to detect some small mineral clusters after immersion for 2 day in 1.5SBF. A densification of the mineral layer can be seen after 8 days immersing. After 10 days immersion, a large number of spherical particles are found to almost cover the entire surface.

As a consequence of particle growth from the nuclei formed at different times, there is a wide size distribution. The total mass of PLLA/5% g-SiO2 nanocomposite films increases with soaking time (Fig. 8).

![Fig. 8](image_url) Percentage of weight increase with immersion time for PLLA/5% g-SiO2 nanocomposite films.

![Fig. 9](image_url) SEM micrographs of PLLA/5% g-SiO2 nanocomposite scaffolds after different immersion times: (A, A’) 0 day, (B, B’) 5 days and (C, C’) 10 days. The top and bottom images correspond to different original magnifications (A–C: 100×; A’–C’: 300×).
The growth of apatite with immersion time is also found on PLLA/5% g-SiO2 nanocomposite scaffolds, as shown in Fig. 9. One can distinguish a change in the roughness and appearance of some small mineral clusters after 5 days of immersion. These become more evident with immersing time. After 10 days immersion, apatite layer is observed to completely cover the surface.

The EDS spectra show that the main elements of soaked PLLA/5% g-SiO2 nanocomposite films and scaffolds are carbon, oxygen, calcium, and phosphorus (Fig. 10). Carbon and oxygen could be from both the PLLA and the particles, but calcium and phosphorus could only be from the particles. The Ca/P ratio of the PLLA/5% g-SiO2 nanocomposite films and scaffolds immersed in 1.5SBF for 10 days was 1.56 and 1.58, respectively. Both are less than the stoichiometric ratio 1.67 of apatite.

It is well known that natural bone is a biocomposite of apatite mineral crystals arranged in an organic collagen matrix. As the formed apatite particles were found to have a composition and structure analogous to those in bones and hence called as bone-like apatite. Since they contain carbonate ion substituted at phosphate ion lattice sites, the coatings are therefore calcium-deficient-carbonated apatite exhibiting a lower Ca/P ratio[12].

The surface biomineralization of the bone-like apatite is a precondition of bioactivity[20]. Bone-like apatite has been reported to form on surfaces of various bioactive materials[4,12,21], while is not observed around materials that are not bioactive, like metals and polymers. The bone-like apatite makes the bioactive material favorable to bond to bone chemically.

The potential mechanism of apatite formation on the surface is presented in Fig. 11. Although some silanol groups (Si–OH) have been consumed in the surface modification by grafting l-lactic acid oligomer, a large number of silanol groups are still present on the surface of g-SiO2. As the silanol groups may have negative charge (Si–O−) in the 1.5SBF according to the isoelectric point of SiO2 (around pH 2.5) [4] negatively charged Si–O− on the surface of PLLA/g-SiO2 nanocomposites can first chelate the calcium ions, and then a cluster of amorphous carbonate apatite is formed by assembling further phosphates, carbonate, and calcium ions. The resultant three-dimensional cluster could act as the nucleus for crystals, the cluster can grow spontaneously by consuming the calcium, phosphate and carbonate ions from the surrounding 1.5SBF, since 1.5SBF is already highly supersaturated with respect to the carbonate apatite. With the increase of soaking time, the amorphous carbonate apatite will grow into crystalline carbonate apatite. So the apatite-forming ability is designed to originate from incorporated g-SiO2 in the nanocomposites, the silanol groups serve as nucleation sites for the formation of carbonate apatite crystals.

4. Conclusions

Bioactive PLLA/g-SiO2 nanocomposite scaffolds and films were fabricated by solid–liquid phase separation method and solution casting method, respectively. The PLLA/g-SiO2 scaffolds show a series of parallel tube-like morphology. The internal wall of these channels is also macroporous and shows a ladder-like structure. The PLLA/g-SiO2 nanocomposites can effectively induce the formation of bone-like apatite in simulated body fluid. The apatite-formation ability increases with silica loading in the composites. The growth of apatite with immersion time is found on the surfaces of both the PLLA/g-SiO2 nanocomposite scaffolds and the films. The potential mechanism of apatite formation on the surface is presented.

Acknowledgments

The work was supported by the National Natural Science Foundation of China (Nos. 51003055 and 50973060), the Science and Technology Commission of Shanghai Municipality (No. 08JC1410300), the Shanghai Leading Academic Discipline Project (No. s30107) and Innovation Program of Shanghai Municipal Education Commission (No. 11YZ06). Yuliang Chu and Bo Lu from Instrumental Analysis Research Centre (Shanghai University) are acknowledged for their help in SEM and X-ray diffraction measurement.

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