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In our earlier work, a cotton-like biodegradable composite, consisting of poly(L-lactic acid) with siloxane-containing vaterite, has been prepared by electrospinning. In the present work, the fibers skeleton of the cotton-like composites was modified successfully with imogolite, which is hydrophilic and biocompatible, via a dip process using ethanol diluted solution to improve the cellular initial attachment. Almost no change in the fiber morphology after the surface modification was observed. The surface-modified composite showed the similar calcium and silicate ions releasabilities, for activating the osteoblasts, as an unmodified one. Cell culture tests showed that the initial adhesion of murine osteoblast-like cells on the surface of the fibers was enhanced by surface modification.

1. Introduction

Recently, the biodegradable polymers have attracted much attention as scaffolds [1, 2]. Poly(L-lactic acid) (PLLA), one of the common biodegradable polymers, has been reported as a scaffold material for bone reconstruction [3]. Several kinds of inorganic ions released from materials are reported to influence osteogenesis and angiogenesis [4]. Xynos et al. suggested that the ionic products, such as calcium and silicate ion released from Bioglass 45S5, have a stimulatory effect on osteoblast proliferation by increasing availability of insulin-like growth factor-II (IGF-II) [5]. The preparation of composites including inorganic materials, with calcium and silicate species releasing ability, in the biodegradable polymers is one of the important technologies for developing bone-forming biomaterials.

Our group has developed a polymer-ceramic composite material, PLLA/siloxane-containing vaterite (SiV) composite (PLLA/SiV), for the bone-filling material applications. The PLLA/SiV has the calcium and silicate ions releasing abilities derived from SiV [6]. In our earlier works, the PLLA/SiV fiber mats of ~300 μm in thickness, fabricated by electrospinning, showed bone formation by implanting the fiber mats on defects of 8 mm in diameter in the front midline of the calvaria in New Zealand rabbits [7, 8]. We also have prepared the cotton-like PLLA/SiV consisting of fibrous skeletons with open pore structure successfully by modified-electrospinning [9, 10]. The cells penetrated into the cotton-like PLLA/SiV and adhered on the fiber surface, resulting in proliferation inside of the cotton-like structure [10].

We believe that the enhancement of cell adhesion on the fiber surface is a critical factor for improving its biocompatibility. It has been widely accepted that the bone-forming cells prefer to attach on hydrophilic surface than hydrophobic one [11–13]. Nanometer-sized hydrous aluminum silicate, which
is called imogolite, \((\mathrm{HO})_3\mathrm{Al}_2\mathrm{O}_3\mathrm{Si}(\mathrm{OH})\), has tubular structure of \(\sim 2.3\) nm external and \(\sim 1.0\) nm internal diameters [14], with up to several micrometers lengths. Imogolite nanotubes (INTs) have very hydrophilic surface owing to numerous hydroxyl groups on the surface. Ishikawa et al. reported that human and mouse osteoblast-like cells were widely spread on INTs coated on polystyrene cell culture dish, compared with the culture dish [15–17]. INT is one of the potential candidates to modify the fiber surface in the cotton-like PLLA/SiV for enhancing their cellular attachments.

The calcium and silicate ions releasing ability of PLLA/SiV, which has an effect on enhancing the cellular activity [18], is very important for accelerating bone formation on the material. Fujikura et al. suggested that the hydroxyapatite coating layer on fibers of the fiber mats, which were prepared by electrospinning, controlled calcium and silicate ions release from the fibers [19]. The influence of INT modification on the ions releasing ability of the cotton-like PLLA/SiV is considered to be examined.

In our previous report, it has been reported that the cell compatibility of PLLA/SiV fiber mats, especially at the early stage, was improved with INTs coating by electrophoretic deposition [20]. In our preliminary experiments, the electrophoretic deposition method has almost no effect on cotton-like PLLA/SiV. INTs would pass over the cotton-like PLLA/SiV because of the very high porosity of cotton-like structure. The aim of this work is to examine the effective way of PLLA/SiV surface modification with INTs. The PLLA/SiV has hydrophobic surface due to the matrix PLLA. INTs are dispersed in aqueous solution as a result of synthesis. It might lead to difficulty of coating on hydrophobic PLLA/SiV surface. It was considered that, by improving hydrophilicity of the fibrous PLLA/SiV surface temporarily, the modification using dip-coating would be able to be applied effectively. Our idea for improving the hydrophilicity is to treat the cotton-like PLLA/SiV with diluted ethanol. In the present work, the dip-coating method, one of the simplest ways, was investigated for INT modification of cotton-like PLLA/SiV to enhance the cellular attachment.

2. Materials and Methods

2.1. Preparation of Cotton-Like PLLA/SiV. SiV particles, which were spherical particles of \(\sim 1\) \(\mu\)m diameter, containing 2.6 wt% silicon, were prepared by a carbonation method using methanol and \(\gamma\)-aminopropyltriethoxysilane (APTES; Motive Performance Materials, Japan). The detail of the preparation was described in our previous reports [7, 8, 18]. PLLA/SiV was prepared by kneading PLLA (LACIA; molecular weight = \(\sim 140\) KDa, Mitsui Chemicals, Japan) with SiV particles at 200 °C for 10 min. The weight ratio of PLLA : SiV was 7 : 3.

The PLLA/SiV solution for electrospinning was prepared using a chloroform as a solvent; PLLA weight ratio in the solution was 10 wt%. Following our previous report [9], cotton-like PLLA/SiV was prepared by electrospinning in ethanol (Wako Pure Chemicals, Japan) as a collector at room temperature. We have reported that PLLA fiber mats composed of the fiber skeleton \(\sim 10\) \(\mu\)m in diameter allow cellular ingrowth into the gaps between them [21]. The fibers diameter was controlled by electrospun conditions as follows: impressed voltage: 15 kV, collector distance: 300 mm, and solution flow rate: 0.25 mL/min.

2.2. Preparation of INT-Dispersed Aqueous Solutions. The preparation procedure of INTs was described in our previous report [20]. In brief, 18.47 g of aluminum chloride (AlCl3·6H2O; Wako Pure Chemicals, Japan) and 9.20 g sodium silicate (Na2SiO3·nH2O; Wako Pure Chemicals, Japan) were dissolved in 500 g of distilled water, respectively; Si/Al molar ratio of 0.41. sodium hydroxide (NaOH, 1 mol/L; Wako Pure Chemicals, Japan) aqueous solution was slowly added to the above described sodium silicate/aluminum chloride solution until pH of the solution reached 6.8. The resulting samples were separated by centrifugation, and the obtained precipitates were rinsed with distilled water. The centrifugation-rinsing process was repeated three times, and then the aluminum silicate precursors were dispersed in 12 L of distilled water by acidification with hydrochloric acid (HCl, 5 mol/L; Wako Pure Chemicals, Japan). The precursor solution was heated at 95 °C for 4 days. Finally, the dispersed INTs in an aqueous solution with a concentration of 0.087 wt% were obtained.

2.3. INT Modification of the Cotton-Like PLLA/SiV by a Dip-Coating Method. The INT-dispersed aqueous solution was diluted with ethanol to prepare the suspension for the dipping process. The weight ratio of INT-dispersed aqueous solution: ethanol was 10 : 90 or 50 : 50. It is well known that the vaterite of SiV is in the unstable phase in water and easily transforms into calcite [22, 23]. Ethanol was used for dilution because the vaterite is known to be more stable in ethanol than in water [24]. Cotton-like PLLA/SiV was dipped into the suspension and dried in a vacuum chamber at room temperature. The sample, which was modified using 50% diluted suspension, was denoted as PLLA/SiV-INT(50-I). In the case of 90% diluted suspension, the sample was prepared by the dipping process by repeating it 5 times and was denoted as PLLA/SiV-INT(10-5), hereafter.

2.4. Evaluation of INT-Modified Cotton-Like PLLA/SiV. The morphology of cotton-like PLLA/SiV fibers was observed by field emission gun scanning electron microscopy (FE-SEM) (JSM-6301F; JEOL, Japan), incorporating energy dispersive X-ray spectroscopy (EDS) (Vantage, Noran, USA), after coating with amorphous osmium by plasma chemical vapor deposition.

The static water contact angles on the pressed samples, by uniaxial pressing under a pressure of 20 MPa for 5 sec, were measured with a CCD camera using SImage minisoftware. The contact angles were determined from the average of measurements at ten random points per sample (i.e., \(n = 10\)). The crystalline phases of calcium carbonates were analyzed with an X-ray diffractometer (XRD) (PANalytical X′ pert-MPD, Netherlands; Cu Ka: 45 kV, 40 mA).
Each sample of 10 mg was soaked in 10 mL of Tris buffer solution (pH ~7.4) at 37°C for 24 h. The released calcium and silicate ion amounts from samples were measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (ICPS-7000, Shimadzu, Japan). Three specimens at each point were measured (i.e., \( n = 3 \)).

2.5. Cell Culture. Twenty milligrams of each sample was put in a well of 24-well plates and compressed with a silica glass tube of 15 mm outer diameter, 12 mm inner diameter, and 15 mm height. The resultant samples were sterilized using ethylene oxide gas. The samples were wetted by ethanol and then they were rinsed with a culture medium (alpha minimum essential medium, \( \alpha \)-MEM) containing 10% fetal bovine serum. Murine osteoblast-like (MC3T3-E1) cells were seeded onto the samples with a density of 200,000 cells per well and incubated 3 h at 37°C in 5% CO\(_2\) for evaluation of their initial attachment. After the incubation, the samples were rinsed with \( \alpha \)-MEM and then incubated in the culture medium containing the reagent of the Cell Counting Kit-8 (CCK-8; Dojindo, Japan) at 37°C for 2 h. The attached cell number on samples was colorimetrically estimated by measuring the absorbance of the resulting medium at 450 nm (\( n = 3 \)). Differences between the samples were determined by Student’s t-test, with \( P < 0.05 \), which is considered to be statistically significant. The samples after 3 h of the incubation were fixed with 2.5% glutaraldehyde for 40 min at 4°C, dehydrated through a series of increasing concentrations of ethanol, and finally dried with hexamethyldisilazane. The morphology of attached cells on samples was observed by FE-SEM.

3. Results and Discussion

Figure 1 shows the fiber morphologies of cotton-like PLLA/SiV before and after INT modification. The average and standard deviation of 100 parts of electrospun fiber diameters were 12 and 3 \( \mu \)m, respectively. Temperature and relative humidity are known as minor factors in the controlling of fiber diameter [25]. The temperature and relative humidity could affect the certain random fiber diameters of PLLA/SiV. As shown in Figure 1(a), the fibers have numerous pores of submicrometer diameter on their surfaces, due to the volatilization of the solvent during electrospinning. The fiber surface of PLLA/SiV-INT(50-1) was covered partially with the aggregated INTs, as shown in Figure 1(b). On the other hand, as shown in Figure 1(c), the numerous pores on the fiber surface of PLLA/SiV-INT(10-5) were observed; there was almost no change in the fiber morphology between the samples before and after 5 times of the dip-coating using highly diluted solution. INT-containing solution diluted with ethanol is considered to reduce the formation of INT aggregates. The aggregation rate of INTs maybe reduced in lower INTs concentration with an ethanol diluted solution. The EDS analysis (not shown here) exhibited the existence of aluminum, originated from INTs. The amount of detected...
aluminum was $\sim 5.0 \pm 1.8$ at % in PLLA/SiV-INT(10-5). This result indicated that INTs were coated on PLLA/SiV fiber surfaces by dip-coating. The crystalline phase of calcium carbonate (vaterite) in cotton-like PLLA/SiV was identified from XRD analysis; the peak of calcite did not appear. Almost no differences in the XRD patterns of between samples before and after the modification were observed. No conversion of vaterite into calcite occurred during the repeated dip-coating process.

In EDS analysis of our previous report, almost no Al was detected on the fiber of fiber mats by dipping [20]. The hydrophobic surface of PLLA/SiV could be a cause of the difficulty to INT modifying because the INTs dispersed solution was based in water. The surface tension is a decisive factor to determine the wettability of a liquid on the solid surface [26]. Vázquez et al. reported that surface tension of the ethanol aqueous solution decreased with increasing the ratio of ethanol [27]. In case of ethanol diluting, the INTs aqueous solution could get high wettability with PLLA/SiV fibers surface. Thus, dip-coating with ethanol diluted INTs solution could be a useful method for INT modifying of the surface of cotton-like PLLA/SiV.

In contact angle measurement, the water drop penetrated into the INT-modified samples, immediately. In contrast, the contact angle of PLLA/SiV was 105°. INT modification was found to have an excellent effect on the improvement of the fibers in the cotton-like PLLA/SiV, owing to high water-absorption ability of the nanotube structure [28, 29]. As a result, the INT-modification makes it easier for the cell suspension to penetrate into the matrix. That is, we believe that there is no difference in the hydrophilicity between the INT-modified samples. PLLA/SiV-INT(10-5) was discussed to compare it with PLLA/SiV, hereafter.

Figure 2 shows the released calcium and silicate ion amounts from samples. Both samples showed a similar trend on their releasing behaviors, independent of INT modification. It is supposed that the INT modification does not interrupt the calcium and silicate ions release from PLLA/SiV fibers.

Figure 3 shows attached cell numbers after 3 h of incubation on the samples. It is considerable that the great parts of seeded cells were passed through the cotton-like samples because the samples have very high porosity. The attached
Figure 4: SEM images of MC3T3-E1 cells after 3 h of incubation on ((a), (b)) PLLA/SiV and ((c), (d)) PLLA/SiV-INT(10-5). Asterisk indicates the attached cells.

cell numbers on PLLA/SiV-INT(10-5) were almost twice of those on PLLA/SiV. Figure 4 exhibits the cell morphologies on the samples after 3 h of incubation. The cells on the PLLA/SiV were still spherical, as shown in Figures 4(a) and 4(b). In contrast, the cells on PLLA/SiV-INT(10-5) were more spread widely than those on PLLA/SiV. Some of them on PLLA/SiV-INT(10-5) were bridged between adjacent fibers. Aoki et al. reported that the well-spreaded cells adhered stronger and have more affinity to scaffold than round cells [30]. This suggests that the INT-modified cotton-like PLLA/SiV has more favorable surface for cell attachment than PLLA/SiV. Some reports suggest that the protein adsorption on the surface of materials contributes to cell adhesion [31, 32]. In our preliminary experiment, imogolite showed high adsorption ability with cell-adhesive proteins such as fibronectin or vitronectin. The cell-adhesive proteins might be more easily adsorbed on the fiber surfaces of INT-modified cotton-like PLLA/SiV than those of the PLLA/SiV. Therefore, the evaluation on protein adsorption ability of INT-modified cotton-like PLLA/SiV is currently in progress.

4. Conclusions

The fiber surface of cotton-like PLLA/SiV was modified successfully with imogolite nanotubes by a dip-process using ethanol-diluted aqueous solution. There was almost no change in the fiber morphology even after the surface modification. The calcium and silicate ions releasing behavior of the modified sample was almost the same as that of PLLA/SiV. The cell adhesion on PLLA/SiV was enhanced by surface modification with imogolite. Thus, the surface modification may be useful for improving cellular compatibility of biodegradable polymer materials.

Conflict of Interests

The authors declare no competing financial interests.

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