Apatite Deposition on Serum Protein-Adsorbed Polystyrene Surfaces under Body Fluid Conditions

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Biomimetic deposition of hydroxyapatite (HAp) on polystyrene (PS) surfaces under body fluid conditions was evaluated. It was found that introduction of adsorption layers of serum proteins such as human serum albumin (HSA) and human immunoglobulin G (hIgG) on PS surfaces using solution casting techniques resulted in formation of HAp deposits on these surfaces when immersed in 1.5SBF, a solution having 1.5 times higher ion concentrations than those of simulated body fluid (SBF). Experimental results including physical characterizations of the HAp deposits support that the ionic amino acid side chains displayed on the surface of these serum proteins induced heterogeneous nucleation and growth of bone-like HAp. Both HSA and hIgG exhibited HAp deposition ability under the current experimental conditions.

Key words: hydroxyapatite, polystyrene, simulated body fluid (SBF), protein, heterogeneous nucleation

1. INTRODUCTION

Development of processes for surface coating of commonly used polymers with inorganic materials is important to fabricate novel organic-inorganic hybrid materials. Polystyrene (PS), a commonly used polymer, is widely used as vessels in researches in cell biology and biochemistry because of their characteristics such as chemical stability, transparency, and high protein binding ability. Therefore surface coating of PS with bioceramics is expected to expand the potential of PS in biomedical applications. Because PS is an organic material, the coating process should proceed at lower temperatures to prevent decomposition or burning out of PS. Biomimetic deposition of hydroxyapatite (HAp, Ca10(PO4)6(OH)2), the major inorganic component in bone and teeth, using simulated body fluids (SBFs) is a useful system to deposit HAp on various materials surfaces [1,2]. SBF is a solution having similar inorganic ion concentrations to those of human plasma. SBF and 1.5SBF, a solution having 1.5 times higher ion concentrations than those of SBF, have been employed to form HAp layers on various kinds of polymers [3–5]. These study revealed that display of ionic functionalites such as carboxylic acid groups and sulfonic acid groups on polymer surfaces is important for inducing heterogeneous nucleation of HAp in SBF and 1.5SBF. If it is also known that the HAp deposits obtained from these systems have bone-like characteristics: i.e., they are partially substituted with other cations (Na+, Mg2+) or carbonate ions, and have relatively lower crystallinity than those of stoichiometric HAp [1–4]. Such characteristics make the HAp deposits high bioactivity. However, HAp deposition on PS surfaces using SBF is hardly achieved because PS does not have any effective functional groups for heterogeneous nucleation of HAp. Therefore only a few examples, including that uses plasma treatments for surface functionalization of PS before deposition of HAp [6], have been reported for HAp deposition on PS surfaces using SBFs and other solution-based processes such as the alternate soaking process [7].

In the present study, we demonstrate a novel system to introduce functional groups for HAp deposition on PS surfaces. Protein layers were formed on PS surfaces by solution casting method as the nucleation site for HAp deposition in 1.5SBF. We chose human serum albumin (HSA) and human immunoglobulin G (hIgG) as the protein because they are the major protein components in human plasma. In addition, the effect of HSA on biological mineralization of calcium phosphates including HAp has been paid attention [8,9]. Experimental results show that such a system actually worked out as we expected.

2. EXPERIMENTAL

2.1 Chemicals

PS (atactic, Mn 173000, Mn/Mw 1.06) was obtained from Polymer Source, Inc. HSA (ALBUMIN, HUMAN, Fraction V) and hIgG (HUMAN IgG, purified immunoglobulin reagent grade) were obtained from Sigma, Co. Ltd. HAp nanopowder used as the standard was purchased from Nacalai Tesque, Inc. All chemicals were used as received. 1.5SBF (Na+ 213.0, K+ 7.5, Mg2+ 2.3, Ca2+ 3.8, Cl- 221.7, HCO3- 6.3, HPO42- 1.5, and SO42- 0.8 mM, pH 7.4) was prepared according to Kokubo’s method [1,2]. Distilled water and ultrapure water used for experiments were prepared using ADVANTEC RFQ20TA and ADVANTEC RFQ414BA, respectively.

2.2 Formation of protein layers on PS surfaces

Firstly PS solutions (10 mg ml-1 in chloroform) were spin-coated (1000 rpm, 1 min) on gold-coated glass
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substrates (ca. 10 mm × 13 mm) to form PS thin films on them. Aliquots (40 µl) of each protein solution (4 µg ml⁻¹ in phosphate-buffered saline, pH 7.4) were then cast on the PS substrates. After 10 min incubation at room temperature, these substrates were rinsed with distilled water and dried by gentle nitrogen flushing.

2.3 HAp deposition on protein-adsorbed PS surfaces
The protein-adsorbed PS substrates were immersed in 1.5SBF for 24 h at 36.5 °C using a dry chamber (AS ONE DOV-750A). The substrates were then rinsed with ultrapure water and dried by nitrogen.

2.4 Characterization
Surface morphology and elemental composition of the resultant samples were evaluated using a scanning electron microscopy (SEM, Hitachi S-5000) and energy-dispersive X-ray spectroscopy (EDX, Kevex Sigma) with an acceleration voltage of 10 kV. In some cases, the specimens were sputtered with Pt-Pd to prevent charge-up. FT-IR spectra of the samples were recorded using a single-reflection attenuation total-reflection (ATR) apparatus (Thermo Fisher Scientific Nicolet 380 combined with OMNI-sampler module).

3. RESULTS AND DISCUSSION
3.1 Formation of protein layers on PS surfaces
To achieve HAp deposition on PS surfaces under body fluid conditions, we utilized water-soluble proteins because they display ionic amino acid side chains, which are expected to induce heterogeneous nucleation of HAp, on their surfaces. As the first example, two kinds of serum proteins, HSA and hIgG, were employed in the present study. It is important to evaluate the effect of these serum proteins, adsorbed on solid substrates, on HAp deposition behavior in 1.5SBF because in vivo HAp nucleation and growth occur in body fluid environment: i.e., in the presence of these proteins.

Both HSA and hIgG are known to adsorb on PS substrates by physisorption [10]. Therefore, we used a simple procedure, solution casting, to form protein layers on PS surfaces. Adsorptions of HSA and hIgG on the PS substrates were evaluated using FT-IR spectroscopy. Although most of the peaks appeared in the spectra originated from PS, slight increase of absorbance in the Amide I region (1690–1640 cm⁻¹), which originating from these proteins (data not shown), were observed after casting of protein solutions. Protein adsorptions on PS substrates were also confirmed using EDX. The peak assigned to sulfur, originating from these proteins, was found only for the PS substrates after protein bindings (data not shown). For these samples, such characteristic peaks in FT-IR and EDX were too weak to evaluate the difference in protein binding behavior between HSA and hIgG.

3.2 HAp deposition on serum protein-adsorbed PS surfaces
When the HSA- and the hIgG-adsorbed PS substrates were immersed in 1.5SBF at 36.5 °C, accumulated semispherical deposits were observed for both surfaces after 24 h incubation (Fig. 1), whereas such depositions were negligible for the case of pristine PS surfaces (data not shown). The deposits formed on the HSA-adsorbed PS surfaces had diameters of about 1–4 µm and tended to appear as aggregate states. On the other hand, smaller sizes (about 0.5–3 µm diameters), less aggregated deposits were observed for the case of the hIgG-adsorbed PS surfaces. Detailed morphologies of these deposits in their magnified image (Fig. 1b) were similar to those of HAp deposited in SBF and 1.5SBF [3,4]. These results support that the HSA- and the hIgG-adsorbed on PS surface can induce heterogeneous nucleation and growth of HAp in 1.5SBF. It is interesting that both HSA, an anionic protein in the condition of 1.5SBF (pH 7.4) (isoelectric point (pI) = 4.7 [11]) and hlgG, a cationic protein (pI = 4.35−9.95 but the main components are basic [12]) can induce HAp deposition under body fluid conditions. If the overall surface charges of proteins determine the accessibility of inorganic ions to protein surfaces in 1.5SBF, calcium ions are more accessible to HSA surface than phosphate ions and they can bind to acidic amino acid side chains such as glutamic acids that displayed on HSA surfaces. On the other hand, phosphate ions are more accessible to hlgG surfaces than calcium ions and they can bind to basic amino acid side chains on hlgG surfaces. In both cases, these events can form nucleation sites of HAp. The difference in their surface charges might affect the size and morphology of HAp deposited on PS surfaces mediated by each protein layer. This point is currently under investigation and will be discussed in our forthcoming reports.

Fig. 1 SEM images of HSA-adsorbed PS surface (a) and its magnified image (b), and hlgG-adsorbed PS surface (c) after immersion in 1.5SBF.
EDX spectra corresponded to the SEM images revealed that these deposits contained calcium and phosphorous, which were not detected for the case of these protein-adsorbed PS surfaces before immersion in 1.5SBF, in addition to small amounts of sodium and magnesium (Fig. 2). The results indicate that the deposits formed on the surfaces of these protein-adsorbed PS substrates were bone-like HAp; i.e., parts of calcium ion sites of HAp were substituted by cationic ions such as sodium ions and magnesium ions. This is well comparable to the case of HAp deposition in SBF and 1.5SBF [1–5]. The Ca/P elemental ratios, or (Ca + substituted cations)/P, are one of the parameters to evaluate the crystal phase of calcium phosphates. For example, the Ca/P ratio of HAp is 1.67 and that of β-tricalcium phosphate (β-Ca3(PO4)2, β-TCP) is 1.5. However, in the case of the HSA-adsorbed PS surfaces, they were unable to be obtained accurately because the peak of phosphorous and that of gold originating from the substrates overlapped each other in the spectra (Fig. 2a). The peak of silicon originating from glass in the substrates was also observed. The influence of the substrates depended on the measuring area in the surface of the specimens. In the case of the result for the hIgG-adsorbed PS surface shown in Fig. 2b, the effect of gold-coated glass substrate seemed negligible. However, the ratio of (Na + Mg + Ca)/P appeared smaller (1.39) than that of stoichiometric HAp (1.67). The results indicated that obtained deposits contained other crystal phase of calcium phosphates, such as octacalcium phosphate (CaH(PO4)2·(H2O)2, OCP, Ca/P = 1.33) and amorphous calcium phosphate (ACP, Ca/P = 1.2–2.2) [13]. Another possible reason is that the intensity of phosphorous might be overestimated due to binding of phosphate ions on hIgG surfaces as the counter anion (surface potentials of the majority of hIgG molecules were positive in 1.5SBF).

Figure 3 shows FT-IR spectra of the HSA- and the hIgG-adsorbed PS substrates before and after immersion in 1.5SBF. The spectra of the surfaces before immersions have peaks originating from PS and proteins in the region of about 800–650 cm⁻¹ (Figs. 3a and 3c); for example, out-of-plane phenyl vibrations (758 cm⁻¹ and 700 cm⁻¹) [14,15]. The peak appeared at 670 cm⁻¹ was assignable to the bending vibration of CO2. The difference in the intensities of the peaks between these two samples probably due to the difference in amino acid sequence and binding amount of the proteins. After immersion in 1.5SBF for 24h, two broad absorption peaks in the region of about 1150–900 cm⁻¹ and 650–500 cm⁻¹ appeared clearly in both the HSA- and the hIgG-adsorbed PS surfaces (Figs. 3b and 3d). In these FT-IR measurements, the decrease of peaks originating from the substrate after immersed in 1.5SBF (i.e., after the formation of deposits on their surfaces) is reasonable because these spectra were recorded by single-reflection ATR mode. The former of the newly appeared peak (1150–900 cm⁻¹) was assignable to the stretching vibration (ν3) of the phosphate (PO4³⁻) groups and the latter (650–500 cm⁻¹) represented the bending vibration (ν4) of the phosphate (PO4³⁻) groups [16]. In the case of standard HAp samples having stoichiometric chemical composition and relatively high crystallinity, both of these two peaks split into a number of distinct peaks (data not shown), same as the case that reported in literatures [16–18]. The broad shape of these peaks indicated that the obtained HAp deposits had low crystallinity or other crystal phase of calcium phosphates co-existed. These FT-IR results also supported that the deposits formed on these serum protein-adsorbed PS surfaces were bone-like HAp.

4. CONCLUSIONS

The results of the present study demonstrate that adsorption layers of serum proteins, HSA and hIgG, on PS surfaces have ability to induce heterogeneous nucleation of HAp in 1.5SBF, which results in formation of bone-like HAp-coated PS substrates. The advantage of this system is that no special equipments such as plasma generators are needed. In addition, no chemical
reaction of PS (for example, bond cleavage) is involved. We only use protein solutions and simulated body fluids. This approach is simple and applicable not only to other proteins but also to other commonly used polymers having protein adsorption ability, to fabricate various HAp-coated polymer materials.

The difference appeared in size and morphology of the HAp deposits between the case of the HSA- and the hIgG-adsorbed PS surfaces is interesting from the viewpoint of biological HAp mineralization. Further investigation along this line is currently proceeding in our group, in addition to detailed characterizations of the HAp deposits, such as more accurate quantification of EDX signals and X-ray diffraction studies.

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