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Facile Surface Functionalization of Polystyrene Substrates with Biomimetic Apatite by Utilizing Serum Proteins

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Serum proteins such as human serum albumin and human immunoglobulin G can induce heterogeneous nucleation of hydroxyapatite (HAp) in body fluid conditions, which make it possible for them to act as mediators to deposit HAp on polystyrene (PS) surfaces when they are immobilized on PS surfaces by physical adsorption. The present system for preparation of HAp-coated PS surfaces is simple and conversion of PS chemical structures does not occur, so that it can be applicable to various polymer surfaces having protein adsorption ability.

Surface coating of commonly used polymers with bioceramics is an important research topic from the viewpoint of expanding the potential of these polymers in biomedical applications. Because such polymers are organic molecules, it is preferred to conduct bioceramics coating processes at lower temperatures. Biomimetic deposition of hydroxyapatite (HAp), the major inorganic component in bone, using a simulated body fluid (SBF), a solution mimicking inorganic ion concentrations of human plasma, is a useful system to deposit HAp on various material surfaces.[1,2] It is known that the HAp obtained from this system is so-called bone-like HAp; that is, parts of Ca sites are substituted with other cations such as Mg^{2+} and its crystallinity is lower than that of HAp having stoichiometric chemical composition ($Ca_{10}(PO_4)_6(OH)_2$). These structural characteristics make the HAp highly bioactive.[1,2] Alternating soaking process is also known as a process for HAp deposition.[3] However, HAp deposition on commonly used polymers using these solution-based processes is hardly achieved because these polymers do not have any effective functional groups for heterogeneous nucleation of HAp. Therefore, polymer surfaces are usually functionalized by pretreatments such as radiation or chemical treatments and/or by grafting or coating of other components to enhance HAp deposition.[4] Polystyrene (PS), a commonly used polymer, is widely used as vessels in research in cell biology and biochemistry because of their characteristics such as chemical stability, transparency, and high protein-binding ability. Although hybrid PS–HAp materials have a potential as a hybrid organic–inorganic biomaterial, surface coating of HAp on PS surfaces has not been widely investigated. One example is that plasma treatments were applied to PS substrates before alternate soaking processes.[5] Therefore, low-cost, simple procedures for preparation of HAp-coated PS materials have been strongly desired. In this study, we propose a novel process to introduce functional groups on PS surfaces using serum proteins (Figure 1, left). The effectiveness of this process for HAp deposition is demonstrated.

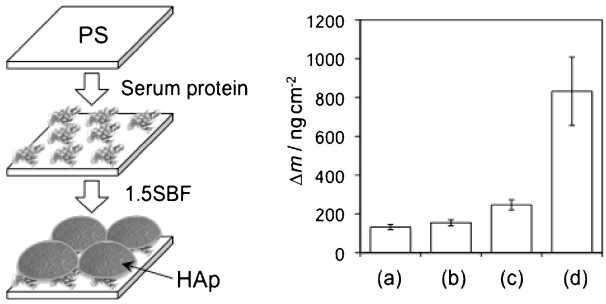

Figure 1. Schematic illustration of this study (left) and binding amount of HSA on PS surface (a) and deposition amount of HAp on HSA-adsorbed PS surface after 30 min (b), 24 h (c), and 96 h (d) immersion in 1.5SBF at 36.5 °C (right). The values were obtained from frequency changes of QCM electrodes monitored in air. Error bars represent standard deviations.

First thin films of PS (atactic, $M_n = 173000$, $M_w/M_n = 1.06$, Polymer Sourse, Inc.) were formed on gold-coated or hydrophobized glass substrates (ca. 10 mm × 13 mm) by spin coating (1000 rpm, 1 min) of PS solutions (10 mg mL^{-1} in chloroform). These PS substrates were immersed in phosphate-buffered saline (pH 7.4) solutions of serum proteins such as human serum albumin (HSA, Fraction V, Sigma) or human immunoglobulin G (hIgG, Sigma) (0.1–20 μg mL^{-1}) for 10 min, followed by rinsing with ultrapure water. FT-IR measurements (Nicolet 380, Thermo Fisher Scientific K. K., single reflection ATR mode) of resulting substrates clarify slight increase of absorption in the amide I region (1690–1640 cm^{-1}) (Figure S1).[6] Energy-dispersive X-ray spectroscopy (EDX, Kevex Sigma) measurements of these substrates' surfaces show the presence of sulfur originating from the proteins (data not shown). In addition, morphological observation of these surfaces using atomic force microscopy (AFM) supports HSA adsorption on PS surfaces (Figure S2).[6] Protein adsorption was also confirmed quantitatively using a quartz-crystal microbalance (QCM) apparatus (9 MHz, U.S.I. Corp.) (Figure 1a). When 10 μg mL^{-1} of HSA solution was used, binding amount (Δm) of HSA on the PS surface was 133 ± 13 ng cm^{-2}, which corresponds 29–77% of surface coverage depending on the binding geometry of HSA.[7] HSA and hIgG bindings on PS surfaces are also supported by previous studies.[8]

These serum protein-adsorbed PS substrates were then immersed in 1.5SBF (Na^+ 213.0, K^+ 7.5, Mg^{2+} 2.3, Ca^{2+} 3.8, Cl^- 221.7, HCO_3^- 6.3, HPO_4^{2-} 1.5, and SO_4^{2-} 0.8 mM,

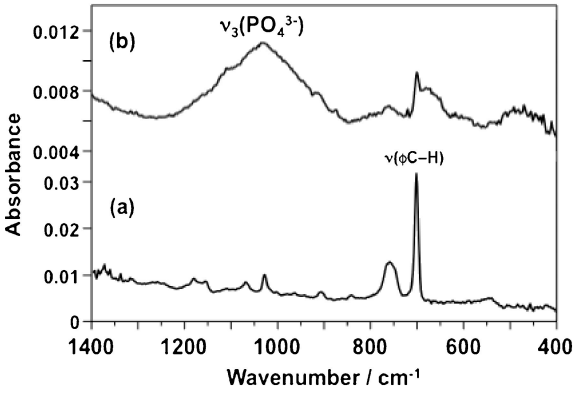

Figure 2. FT-IR spectra of HSA-adsorbed PS surfaces before (a) and after (b) immersion in 1.5SBF.

pH 7.4), a solution having 1.5 times higher ion concentrations than those of SBF and used frequently in biomimetic HAp deposition studies,[1,2] at 36.5 °C. A PS substrate having no protein layer was used as a control. QCM can be used for monitoring HAp deposition on its electrode surfaces under body fluid conditions,[9] and the results clearly show production of deposits on HSA-adsorbed PS surfaces after immersion in 1.5SBF (Figure 1, right). The amount of deposits increased with increase of immersion time, and significant mass increase was observed after 24 h incubation (ca. 110 ng cm^{-2}, (Δm of (c)) − (Δm of (a))). FT-IR measurements of the substrates found that the broad absorption peak in the region of about 1200–850 cm^{-1} its peak top being at 1030 cm^{-1} appeared and that the peaks originating from the substrates decreased (for example, aromatic C–H vending vibration at 760 cm^{-1}) after immersion in 1.5SBF for the HSA-adsorbed PS surfaces (Figure 2). This newly appeared peak was assignable to the stretching vibration (ν_3) of the phosphate (PO_4^{3-}) groups.[10] It is known that this peak splits into a number of distinct peaks for the case of HAp having stoichiometric chemical composition and high crystallinity.[10,11] The broad shape of this peak in the present sample indicates that the obtained HAp deposits had low crystallinity.

Morphological observation of the samples using scanning electron microscopy (SEM, Hitachi S-5000) proved formation of (hemi)spherical deposits on both HSA-adsorbed and hIgG-adsorbed PS surfaces after immersion in 1.5SBF for 24 h (Figures 3a–3c). On the other hand, such deposits were scarcely observed for pristine PS surface (data not shown). Magnified images of these deposits show characteristic morphology similar to that frequently observed in HAp deposited in SBFs (Figure 3b).[3] It seemed that there was a difference in deposition induction ability between HSA and hIgG, although it was difficult to prove it quantitatively from the current experimental conditions. EDX measurements conducted on the SEM specimens revealed that these deposits contain calcium, phosphorus, oxygen, and small amount of magnesium and sodium, in addition to other elements originating from substrates (Figure 3d for HSA-adsorbed PS surface and Figure S3[6] for hIgG-adsorbed PS surface). This indicates that the deposits were calcium-deficient apatite; that is, some of the calcium ions were substituted with Mg^{2+}. It is well comparable to previous reports for the case of HAp deposition of polymer surfaces in SBF and 1.5SBF.[1,2] Finally, thin film X-ray diffraction (Rigaku RINT-2200VL) patterns of HSA-adsorbed (Figure 4) and hIgG-adsorbed (Figure S4)[6] PS substrates after immersion in 1.5SBF for 2 week reveal two distinct peaks at $2\theta = 26$ and 32°, which are assigned to the (002) diffraction line and an envelope of the (211), (112), and (300) diffraction line of HAp, respectively (PDF#09-0432).[13]

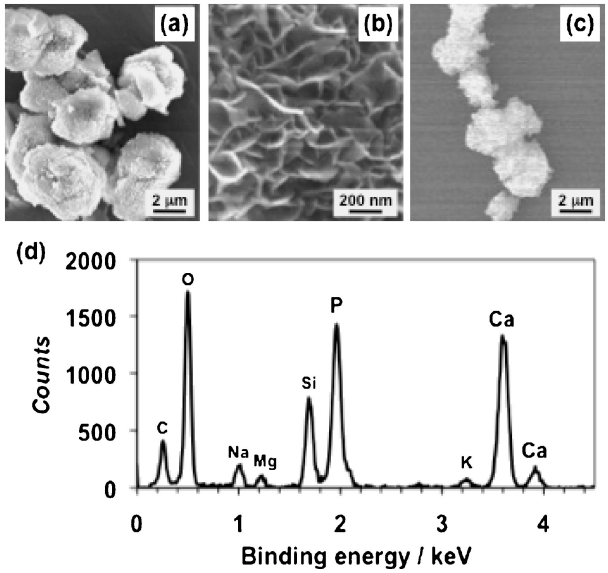

Figure 3. (a–c) SEM images of HSA-adsorbed PS surface (a) and its magnified image (b), and hIgG-adsorbed PS surface (c) after immersion in 1.5SBF. (d) EDX spectra obtained from deposits formed on HSA-adsorbed PS surface. The peak of Si comes from hydrophobized glass substrate.[12]

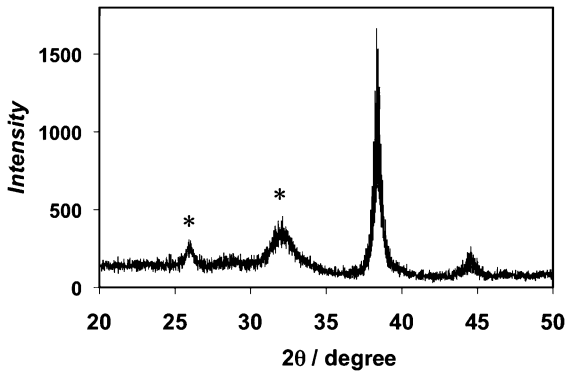

Figure 4. XRD spectra of HSA-adsorbed PS surface after immersion in 1.5SBF for 2 weeks. Peaks indicated by asterisk (*) correspond to those of HAp (see text). Other peaks appeared at $2\theta = 38$ and 44.5° originate from (111) and (200) diffractions of gold[13] in the substrate, respectively.

According to these experimental results, it was found that HSA and hIgG adsorbed on PS surfaces induced heterogeneous nucleation of HAp in 1.5SBF. Since the isoelectric point (pI) of HSA is 4.7,[14] HSA molecule represents negatively charged surface due to dissociation of side chains of acidic amino acids such as glutamic acid under the experimental pH condition. Carboxylic acid anion moieties should be an effective binding site for calcium ions. In addition, it is known that calcium ions bind to serum albumins under physiological conditions.[15]

Calcium ion-bound HSA surfaces can be a trigger to form nucleation sites of HAp. On the other hand, hIgG has a wide p*I* range from 4.35 to 9.95 and main components are basic.[16] Therefore, basic amino acid side chains of hIgG probably act as nucleation sites for HAp deposition. Previous reports show that cationic surfaces such as that represent amino groups have a potential to induce HAp deposition in SBFs.[17] Another possibility is that acidic amino acid side chains still act as nucleation sites in the local environment even when the overall surface charge of hIgG is positive. Such difference in possible HAp nucleation mechanism between HSA and hIgG might affect the size and morphology of HAp deposited on PS surfaces mediated by each protein adsorption layer.

At this moment, complete surface coverage with HAp on these protein-adsorbed PS surface has not been achieved. In the case of longer incubation in 1.5SBF, two HAp deposition mechanisms, one is heterogeneous nucleation initiating from protein surfaces and the other is homoepitaxial deposition initiating from already-formed HAp crystals, are competitive. To make the former process dominant, additional treatments such as immersion of the substrates in $CaCl_2$ solution before immersing in SBFs should be effective according to previous literature.[1,2]

In conclusion, it was demonstrated that serum proteins, HSA and hIgG, have potential to induce heterogeneous nucleation of HAp so that they can act as mediator to produce HAp-coated hybrid surfaces. The present solution-based, bench-top process proceeds under mild conditions and no chemical conversions of polymers (for example, bond cleavages) are involved. Therefore, this is readily applicable to various commonly used polymers having protein adsorption ability to produce novel hybrid polymer–apatite materials. This is also applicable to HAp coating on various shapes of material surface including the inside of vessels such as commercially available cell culture flasks. HAp-coated PS culture flasks can be useful tools for bone cell culturing.[5] As discussed above, the details of the HAp deposition process might be different between HSA and hIgG, which is currently under investigation in our group. The effect of serum proteins, especially albumins, on calcification in vivo has been focused, and relating studies using model systems have been reported.[18] The present results may give an implication to think about the role of serum proteins in biological HAp deposition processes.

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References and Notes

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