Formation of Hydroxyapatite Provides a Tunable Protein Reservoir within Porous Polyester Membranes by an Improved Soaking Process

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Biomineralization on porous polyester membranes was examined using an improved alternate soaking process (ASP). The effect of ion migration for the formation of hydroxyapatite (HAp) was shown to be crucial. Ion migration was improved by reducing the surface tension by mixing ethanol into an aqueous solution. The resulting hybrid materials were evaluated in terms of calcium content; structure using scanning electron microscopy (SEM), X-ray diffraction (XRD), and infrared spectroscopy (IR); and protein adsorption. The amount of formed HAp was controlled by the number of ASP cycles and also through the ethanol content of the mixed solvent. As the formation of HAp increased, the formed structure could be verified using SEM, IR, and XRD. Protein adsorption was investigated using albumin, γ-globulin, and fibrinogen, and the amount of adsorbed protein was well-correlated with that of the formed HAp. This result shows that the total amount of the adsorbed proteins can be regulated by the HAp content. In summary, a tunable protein reservoir was formed on a porous polyester membrane.

1. Introduction

Polyesters represent a major class of polymers utilized in the field of regenerative medicine. For example, poly(L-lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers are well-known implantable biomaterials.[1] Though polyesters as is have been frequently used, polyester-based copolymers are also used. For example, isomeric PLA was conjugated with poly(ethylene glycol) (PEG) such that an A−B−A type triblock copolymer was made and it was shown that the PLA segment could form a stereocomplex in an aqueous solution.[2] PEGylation of polyesters have also been performed such that stimuli-sensitive hydrogels were formed, responding to a change in pH and temperature.[3] The swelling properties of polyester hydrogels could also be controlled by a change in polymer architecture and cross-linking agent.[4] Moreover, polyesters may easily undergo post-transformations such that films, fibers, and porous membranes can be made, materials that often are useful in regenerative medicine. To enhance the in vivo performance of polyesters, hybrid techniques are utilized. One example is seen employing polyesters with biobased collagen; the resulting materials could improve the viability of hepatocytes.[5−6] On the other hand, inorganic materials such as hydroxyapatite (HAp) are also used to improve the biofunction of polyester-based biomaterials. Boccaccini et al. systematically summarized a hybrid material composed of polyhydroxyalkanoate and HAp for tissue engineering.[7] Furthermore, Furuzono et al. reported that HAp nanocrystal coating on polyester fabrics improve cell adhesion.[8] These hybrid techniques permit an excellent contact between biomaterials and bioactive molecules such as proteins and cells.

Formation of HAp is made according to different pathways, including mechanochemical−hydrothermal methods[9] and sintering processes.[10] Furthermore, Kokubo reported his pioneering work of HAp formation using a biomimetic process in simulated body fluid (SBF).[11,12] As an alternative wet process, Taguchi and Akashi found an innovative alternate soaking process (ASP).[13] The fundamental protocol was soaking of the substrates in calcium chloride and disodium hydrogenphosphate aqueous solutions alternately.[14−16] The process is applicable to form calcium carbonate from calcium chloride and sodium carbonate aqueous solutions.[17] Moreover, employing a hydrogel for HAp formation was carried out using an electrophoresis approach.[18] The favorable characteristic of this process is a quick and homogeneous HAp formation, because the calcium and phosphate ions could migrate into the hydrogels smoothly. The diversity of HAp formation allows for the preparation of biomaterials composed of organic and inorganic hybrids. This alternate soaking process was shown to be applicable to porous membranes, and Furuzono et al. have already reported HAp formation on a silk fabric.[19] Hydrophobic porous membranes, based on polyesters, are usually not adequate due to insufficient water intrusion. Therefore, an improvement of the ASP is necessary in order to bridge the HAp formation also to porous polyesters. In this study, we overcome this problem by demonstrating how HAp easily may be formed by reducing the surface tension of an aqueous solution used in ASP. More precise, a reduction of surface tension was provided by adding an organic solvent, such as ethanol, into the aqueous solution such that HAp was formed on porous polyester membranes.

2. Materials and Methods

2.1. Materials. Porous polyester membrane was purchased from GC Co., Ltd, Tokyo, Japan, and the product was known as GC membrane for guided tissue regeneration (GTR) in dental implant. The major composition of the membrane was formed by bioabsorbable copolymer, which was composed of glycolic acid and lactic acid. For HAp formation of the membranes, calcium chloride (anhydrous), disodium

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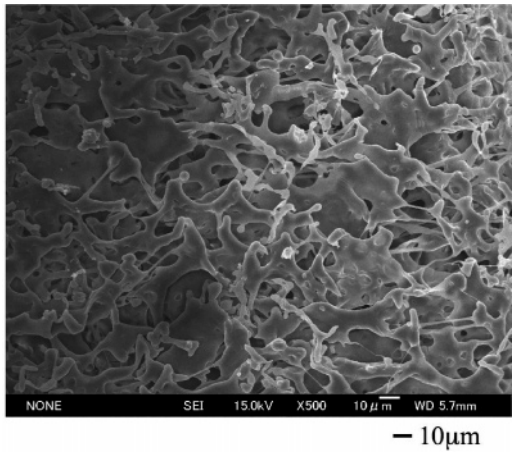

Figure 1. Surface configurations of porous polyester membrane.

hydrogenphosphate, Tris(hydroxymethyl)aminomethane (Tris), and Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethanol (99.5%, Wako Pure Chemical Industries, Ltd.) was used as a part of the solvent to prepare ionic solutions for the alternate soaking process. All reagents were extrapure grade and were used as-received. Ultrapure water was used through the experiment.

2.2. Preparation of HAp on Porous Membrane by Improved Alternate Soaking Process. The HAp formation on the membrane was carried out by an ASP. The fundamental procedure in detail was reported in our previous papers.[13] However, in this study, the protocol was a little bit modified. The calcium chloride and disodium hydrogen phosphate were dissolved in mixed solvent of ethanol and ultrapure water, and their final concentrations were 200 mmol/L and 120 mmol/L, respectively. The alcohol was added to reduce the surface tension of the resulting solution. In this study, the effect of the mixed solvent was investigated for the HAp formation using the ASP. The ASP is as follows. The porous membranes were cut into small pieces (5 mm × 5 mm) and allowed to precondition in ultrapure water for 30 s. Originally, the porous membrane was used for GTR treatment in dental therapy. The membrane has a bioabsorbable function at the implant site, and 5−10 μm pores are formed interconnectively for cell migration, particularly the induction of ligament cells (Figure 1). The membrane was made from copolymer based on the polyester, indicating a hydrophobic interface. Therefore, water intrusion into the interconnective pore was suppressed by the surface tension of the water media. Thus, improvement of the water intrusion, particularly, HAp formation of the membrane using ASP, was necessary. The membrane was soaked into calcium solution and then rinsed in ultrapure water, further soaked into phosphate solution, and then rinsed, indicating one cycle for the ASP. Alternatively, the mixed solvent solution with ethanol was used and the same protocol was carried out. The total amount of ethanol was held at 0, 10, and 25% v/v. The ASP cycles were changed between 1 and 30 cycles. The resulting porous membrane containing HAp was dried at reduced pressure until further characterization.

2.3. Characterization of Hybrid Materials. The HAp formation was evaluated by quantifying the number of Ca ions in the membrane. The Ca ion was easily captured using a chelating agent, and the total amount of calcium ions was evaluated. In general, HAp is soluble in hydrochloric acid (HCl) aqueous solutions, and thus, the resulting membrane was immersed in 1 mL of HCl (1 mol/L). After mild centrifugation, 20 μL of the supernatant was mixed with Calcium E-Test Wako (Wako Pure Chemical Industries, Ltd.). The calcium ions in the supernatant were captured by the chelating reagent (methyl xylenol blue) at alkaline conditions. After the chelate had formed the change in absorbance at 610 nm was monitored by a UV−vis spectrophotometer (U-3010, Hitachi Ltd.). The total amount of calcium ion was evaluated by comparing to a calibration curve. Moreover, phosphorus ions in the supernatant (15 μL) were evaluated using Phosphor C-Test Wako (Wako Pure Chemical Industries, Ltd.). The phosphorus ions in the sample are combined with ammonium molybdate to form phosphomolybdic acid, which is reduced by *p*-methylaminophenol to form molybdenum blue. The change in absorbance at 750 nm was monitored by UV−vis spectrophotometer. Finally, the Ca/P ratio was calculated to estimate the stoichiometric HAp formation.

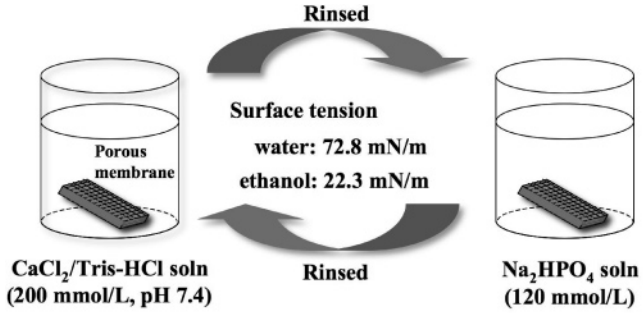

Figure 2. Improvement of the alternate soaking process by addition of ethanol, showing reduced surface tension.

The morphology of the resulting HAp was observed using a scanning electron microscope (SEM, JSM-6700FE, JEOL, Tokyo, Japan). The porous membrane was first lyophilized and thereafter coated with osmium tetraoxide. The SEM observation was carried out using 500× magnification. Moreover, the HAp was also characterized in terms of X-ray diffraction (XRD, RINT In Plane ultraX18, Rigaku, Tokyo, Japan). The X-ray source was Cu Kα, and 40 kV and 200 mA were used for the measurement. The scan speed was 2°/min, and 5−40° was monitored. Alternatively, FT-IR spectra were recorded by the attenuated total reflection (ATR) method by an FT-IR spectrometer (Spectrum 100, Perkin-Elmer Japan Co., Ltd.) ranging from 2000 to 380 cm^{-1}.

2.4. Protein Adsorption. The amount of adsorbed proteins was measured using typical serum and plasma proteins such as bovine serum albumin (BSA, A-8022, SIGMA, MO), bovine γ-globulin (BγG, G-5009, SIGMA), and bovine plasma fibrinogen (BPF, F-8630, SIGMA). The membranes were initially equilibrated by immersion in PBS overnight and then incubated in each protein solution (pH 7.4) for 3 h. The protein concentration was adjusted as 0.9 g/dL (BSA), 0.2 g/dL (BγG), and 0.06 g/dL (BPF). The membrane was immersed into 500 μL of PBS (Dulbecco's phosphate buffered saline, #14200-075, Invitrogen Inc.) to maintain the wet condition. Subsequently, each protein solution was added to the well. The final concentration of the protein was 0.45 g/dL (BSA), 0.1 g/dL (BγG), and 0.03 g/dL (BPF), indicating 10% of the concentration relative to the human body. The incubation time was 3 h. The membrane was rinsed with PBS, and the adsorbed proteins on the membrane were completely removed using 1 wt % of *n*-sodium dodecyl sulfate (SDS, Wako Pure Chemical Industries Ltd.). The concentration of recovered proteins in the SDS solution was determined by using a Micro BCA kit (Pierce, #23235). The change in absorbance at 570 nm was monitored by multiplate reader.

3. Results and Discussion

3.1. HAp Formation onto Porous Membranes. In this study, the mixed water/ethanol solution was used to reduce the surface tension of the media for the ASP and therefore allow easier water intrusion in the membrane. As a comparison, pure water was also used for the formation of HAp. The surface tension of pure water is 72.8 mN/m, i.e., much higher than that of ethanol (22.3 mN/m). The mixture of pure water and ethanol thus has significantly lower surface tension, and the use of such solvent combinations therefore allows easy intrusion into the interconnective pores without any resistance (Figure 2). The HAp formation on the porous membranes was evaluated by quantification of the calcium content. Figure 3 shows the change in the calcium and phosphorus ions content in the membrane by

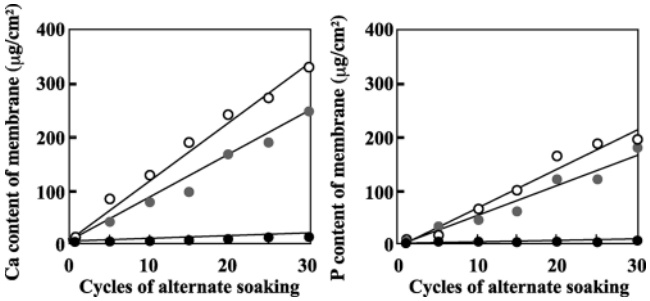

Figure 3. Evaluation of calcium ions (a) and phosphorus ions (b) by changing in alternate soaking cycles: black, 0% v/v; gray, 10% v/v; and white, 25% v/v.

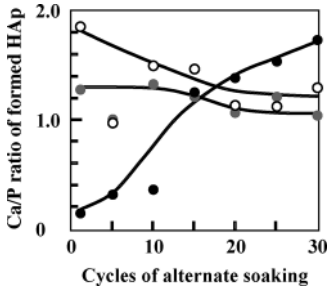

Figure 4. Changes in Ca/P ratio of membranes: black, 0% v/v; gray, 10% v/v; and white, 25% v/v. The stoichiometric Ca/P ratio (1.67) of HAp is indicated as a dash line.

changing the number of soaking cycles. In the case of pure aqueous solutions, the calcium content increased slowly. After 30 cycles, the total amount of calcium content was only 6 μg/cm^2. On the other hand, mixed solvent dramatically increased the formation of HAp. The change in alcohol content was related to the HAp formation. In the case of 10% v/v of ethanol, 250 μg/cm^2 of calcium ions was detected. The HAp formation was well proportional to the number of soaking cycles. Moreover, the resulting porous membrane was slightly tough when it was bending. Particularly, it was clearly observed which contained calcium ions over 200 μg/cm^2. Using a solution with a 25% v/v composition allowed forming even more HAp (Ca ions 330 μg/cm^2). Alternatively, the phosphorus ions were evaluated as shown in Figure 3b. A similar trend was observed. Addition of ethanol was achieved to form more HAp (P ions 200 μg/cm^2, 25% v/v, 30 cycles). From the result, the HAp formation could be controlled by changing the mixed solvent composition and the number of soaking cycles. Moreover, Ca/P ratio was calculated as shown in Figure 4. The stoichiometric HAp shows a ratio of 1.67, which was shown as a dash line in the figure. In the case of pure aqueous solutions, the Ca/P ratio increased with the alternate soaking cycles, and then roughly 1.67 was observed after 25 or 30 cycles of soaking. This result indicated that the resulting HAp slightly changed its crystallinity to form a stoichiometric structure. Perhaps, the resulting amorphous HAp would transform to stoichiometric HAp. On the other hand, using a solution with ethanol allowed formation of the stoichiometric structure, even in the early stages of the alternate soaking cycles (within 15 cycles). However, the Ca/P ratio slightly decreased with increasing the alternate soaking cycles (over 20 cycles). The changes in the Ca/P ratio in ethanol solution were considered to be perfectly formed HAp in the early stage due to the excellent ion intrusion. However, the intrusion efficiency would be gradually low; thus, the formed HAp did not show the stoichiometric number. Taking these results into account, the Ca/P ratio was regulated by ethanol composition and alternate soaking cycles. The fundamental mechanism of the changes in the stoichiometric structure will be reported in a forthcoming paper.

3.2. Characterizations of Resulting HAp on Porous Membranes. Figure 5 shows a microstructure of the membrane in the SEM. In the case of aqueous solutions, the porous membrane was partially coated with HAp particles, even if the number of soaking cycles was 30 times. Though the porous membranes were covered, most of the particles were only located on the surface, and the inner pore was still vacant after the ASP. This observation suggests that suppression of water intrusion prevents formation of HAp to completely cover the membrane. On the other hand, HAp formation at the inner pores was effectively improved by using the mixed solvent (Figure 5c−f). The inner pores were filled with HAp particles, and the effect of the soaking cycles can significantly be observed (Figure 5g). Moreover, the amount of HAp particles increased with ethanol composition. The morphology of the HAp particles was dependent on the solvent used. In general, HAp particles prepared with mixed solvents showed a sharper morphology relative to the aqueous solution. The relationship between the needle shape morphology and their crystalline structure was characterized by using XRD analysis and will be described next.

The crystallinity of HAp is a fundamental property for its biofunction, and the typical sharp peak attributed to the HAp may change according to different preparative conditions. Figure 6 shows XRD patterns of HAp found on the porous membranes. In the case of the aqueous solution, the XRD patterns are halo,

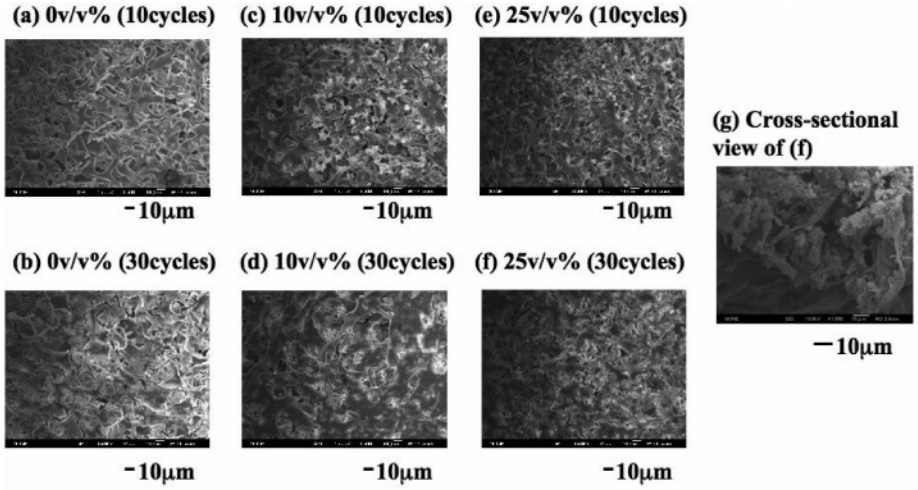

Figure 5. Scanning electron microscope observation of HAp formation onto porous membranes.

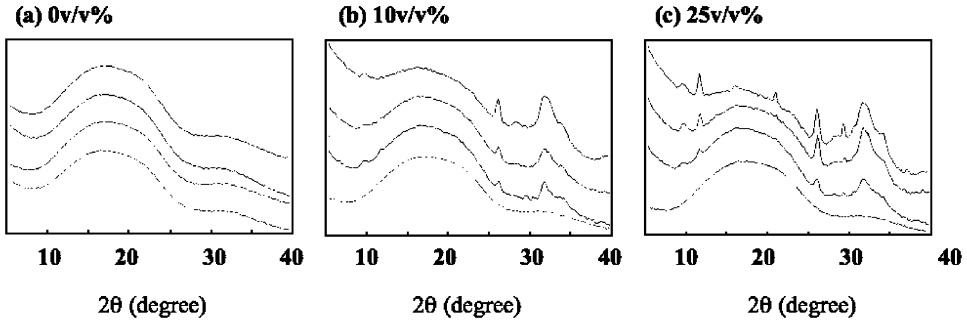

Figure 6. X-ray diffraction patterns of HAp on porous membranes. Ethanol composition was indicated as a−c, and the four lines indicated as 1, 10, 20, and 30 cycles from the bottom.

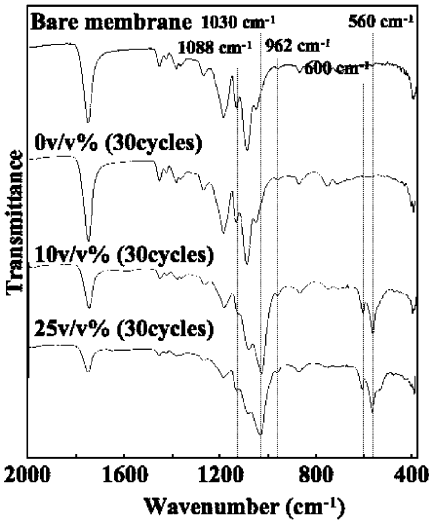

Figure 7. ATR-IR spectra of HAp on porous membranes. The ethanol composition was changed to 0%, 10%, and 25% v/v.

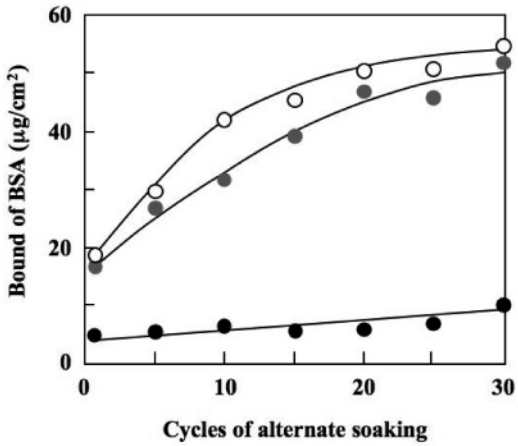

Figure 8. BSA adsorption on hybrid membranes: black, 0% v/v; gray, 10% v/v; and white, 25% v/v.

indicative of an amorphous structure. Though the total amount of formed HAp were significantly lower than that of the mixed solution, the typical HAp peak was not observed, as shown in Figure 6a, even if 30 cycles of the ASP were used. On the other hand, the peaks attributed to HAp were observed in Figure 6b,c. The XRD peaks were attributed to HAp as follows: 25.9° (002), 28.1° (102), 28.9° (210), 31.7° (211), and 34.0° (202). The peak intensity was related to the total amount of HAp on the membrane; particularly, the total number of soaking cycles was a dominant factor. When we related the XRD patterns with the

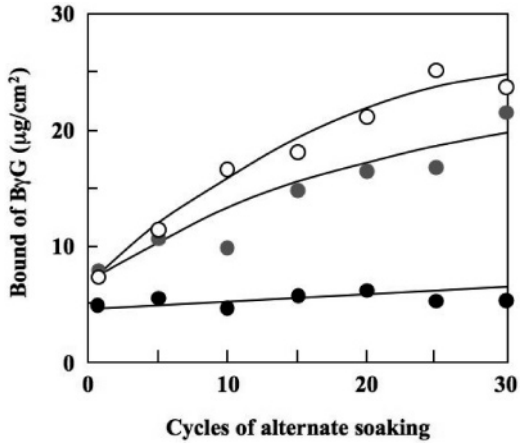

Figure 9. BγG adsorption on hybrid membranes: black, 0% v/v; gray, 10% v/v; and white, 25% v/v.

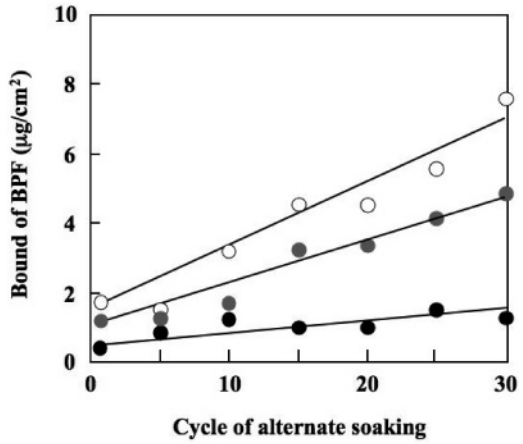

Figure 10. BPF adsorption on hybrid membranes: black, 0% v/v; gray, 10% v/v; and white, 25% v/v.

total amount of HAp, the significant XRD pattern was observed for calcium ion concentrations over 100 μg/cm^2.

Alternatively, the resulting HAp was assigned from the ATR-IR spectra (Figure 7). Typical phosphate peaks (PO_4^{3-}) were observed at 560 (ν4), 962 (ν3), 1030 (ν3), and 1088 cm^{-1} (ν4). Similarly, the OH peak at 600 cm^{-1} was also observed. In this case, the transmittance by IR decreased with increasing the HAp content in the porous membranes. From these results, the resulting HAp was gradually crystalline when increasing the total amount of HAp particles. Therefore, the improved ASP could be used to regulate not only the formation of HAp but also its crystallinity. The next step was to evaluate the biofunction of the formed HAp with a focus on protein adsorption.

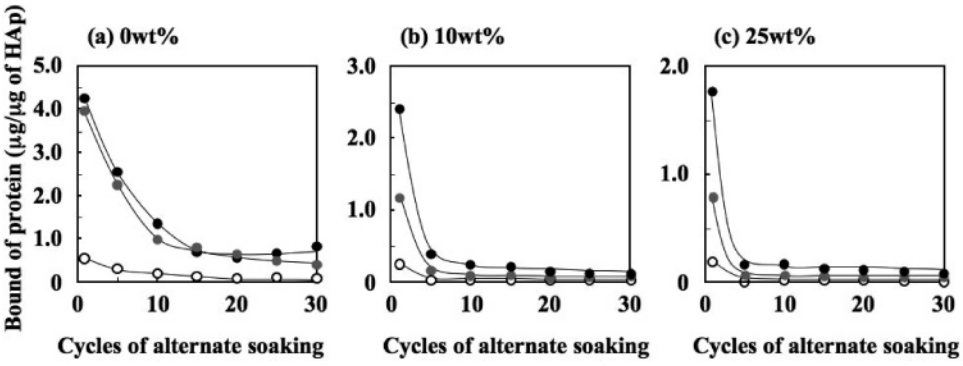

Figure 11. Normalized protein adsorption on 1 μg of HAp particles: black, BSA; gray, BγG; and white, BPF.

3.3. Tunable Protein Adsorption as Biomolecule Reservoir. To replicate the function in vivo we wanted to examine the biohybrid membranes response when in contact with proteins. Major blood serum proteins are albumin, γ-globulin, and plasma fibrinogen, and in this study, these major proteins were used to understand the function between these biomolecules and the formed biomembrane. The resulting protein reservoir on the porous membrane would be capable of utilizing excellent carrier materials for cytokine release for regenerative medicine. Particularly, the formed HAp particles could be easily dissolved under acidic condition (roughly pH 5−6),[20] which is well-observed as an inflammatory response at the early stage of materials implantation. The cytokines carried on the HAp particles would be released with synchronization with HAp dissolution at the acidic pH.

Figure 8 shows the relationship between BSA adsorption and the number of cycles using the ASP. The figure shows a significant difference, in the case of 25% v/v condition, as the bound BSA increases with number of soaking cycles. Particularly, roughly 20 μg/cm^2 of bound BSA was observed after only one cycle. The amount of bound BSA was 4 times larger than that of the aqueous solution. The surface area of the porous membrane was larger than that of bare flat membrane, so the increased BSA adsorption was caused by HAp particles formed on the porous membrane. The BSA adsorption clearly increased with the number of soaking cycles. Moreover, bound BSA was less in the 10% v/v condition. On the other hand, bound BSA showed only 10 μg/cm^2 in the case of aqueous solution, even when 30 cycles of the ASP was used. From these results, BSA adsorption was well correlated to the number of soaking cycles and also to HAp content on the membrane.

The molecular weight of BSA is roughly 66 kDa, which is a bit lower than that of other serum proteins. Therefore, adsorption of BγG and BPF was examined, and their molecular weights are 150 and 300 kDa, respectively. Figures 9 and 10 show the results of adsorption from these proteins. Bound BγG and BPF were well-correlated with the increased number of soaking cycles. Specifically, the total amounts of protein adsorption (BγG and BPF) observed in the case of 25% v/v of ethanol mixed solvent were 24 and 8 μg/cm^2 respectively. Interestingly, the amount of bound proteins was much larger relative to conventional surface protein adsorption (0.90 μg/cm^2 of BSA monolayer adsorption (end-on)).[21] BγG and BPF also showed larger adsorption relative to monolayer adsorption, which was calculated from the surface area of each membrane (BγG 1.85 μg/cm^2 and BPF 1.70 μg/cm^2). The higher protein adsorption on the hybrid membranes is suggested to be an effect of HAp formation, which is a well-known adsorbent. In this study, the total amount of the protein adsorption was normalized by a unit of HAp formation, as shown in Figure 11. In the case of pure aqueous conditions, 1 μg of HAp could adsorb less than 1 μg of protein, if more than 10 soaking cycles were used (Figure 11a). Parts b and c of Figure 11 show the results of ethanol as cosolvent at 10% and 25% v/v. The normalized protein adsorption was below 0.3 μg per 1 μg of HAp, when five soaking cycles were conducted. Taking these results into account, the total amount of protein adsorption was regulated by the amount of HAp formation, indicating a tunable protein adsorption process. Additionally, the amount of bound protein per 1 μg of HAp was roughly correlated with a side-on monolayer protein adsorption (BSA 0.25 μg/cm^2, BγG 0.27 μg/cm^2, and BPF 0.18 μg/cm^2), which was calculated by Lyman et al.[21] The HAp-containing hybrid materials were capable of forming reservoirs of proteins, which suggests that these biohybrids may function as an important scaffold for regenerative medicine. Future studies will focus on the immobilization of important biomolecules such as cytokines, growth factors, and glycoproteins and the resulting bioactivity.

4. Conclusions

Improvement of the ASP for HAp formation within a hydrophobic porous membrane was demonstrated after reducing the surface tension of the ionic solution. The effect of adding ethanol was significant to enhance HAp formation not only at the surface but also within the porous interior. The Ca/P ratio of the resulting HAp was regulated by the ethanol composition and the alternate soaking cycles. In addition, crystallinity of the formed HAp was improved with increasing ethanol content and the number of soaking cycles. The HAp-containing hybrid membranes could adsorb major serum or plasma proteins, and the amount of bound proteins was much larger relative to proteins adsorbed on a flat membrane. The higher protein adsorption resulted after the formation of HAp particles; therefore, these hybrid membranes are of great importance in terms of providing a tunable protein reservoir within a bioabsorbable porous membrane.

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