Effect of protein adsorption layers and solution treatments on hydroxyapatite deposition on polystyrene plate surfaces in simulated body fluids

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Abstract We have developed a method to functionalize polystyrene (PS) cell culture plates with hydroxyapatite (HAp) via protein adsorption layers such as human serum albumin (HSA) in simulated body fluids (SBFs). In order to investigate the versatility the method, in this study the effect of protein adsorption layers on HAp deposition on PS plate surfaces in SBF was evaluated. Pretreatments with alternate soaking process (ASP) using solutions containing calcium ions and phosphate ions followed by incubation with SBF for 24 h resulted in HAp deposition on PS plates with adsorption layers of HSA, type I collagen, hen egg white lysozyme, and poly L-glutamic acid, an acidic protein analogue: the deposition behaviors were correlated with adsorption ability and charge state of proteins. We also demonstrated that commercially available tissue culture-treated PS (TCPS) were directly coated with bone-like HAp using the same ASP and SBF processes. Human mesenchymal stem cells adhered and stretched on the HAp-coated

TCPS plates in a similar manner to the case of the HAp-coated PS plates prepared via HSA adsorption layers. The results indicate that the present methods are useful for preparing bone-like HAp-coated cell culture plates that can be utilized function of adsorbed proteins and that are obtainable conveniently and at low-cost.

Graphical abstract

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1 Introduction

Polymer substrates coated with hydroxyapatite (HAp, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) are expected as useful materials in biomedical fields. In coating of polymer substrates with HAp, it is important to prevent deformation and decomposition of polymers. The biomimetic process using simulated body fluid (SBF), a solution with similar inorganic ion concentrations to those of human plasma, has been used to deposition of HAp on material surfaces under mild conditions [1, 2]. In this method, acidic functional groups such as carboxylic acid groups displayed on substrate surfaces

Fig. 1 Schematic illustration of this study. **a** Adsorption layer-mediated HAp deposition on PS plates in SBF, **b** HAp deposition on ASP-treated TCPS plates in SBF

effectively induce the heterogeneous nucleation of HAp in SBFs [3]. These techniques were applied for synthetic polymers with ionic functional groups such as polyamide films containing carboxyl groups [4] and sulfonic groups [5], and natural polymers such as carboxymethylated chitin and gellan gum [6]. HAp obtained from SBFs is a bone-like apatite with high biological affinity [7] due to its small crystallites and a defective structure [8], which means that it is appropriate for uses as biomaterials. HAp depositions on commonly used polymers with these solution-based processes are expected to expand the potentials of their biomedical application: however, these polymers do not have any effective functional groups for heterogeneous nucleation of HAp. For example, polystyrene (PS), a resin widely used as cell culture dishes or plates because it shows high optical clarity and excellent durability. If surface of PS substrates can be modified with HAp, it expands their availability as a cell scaffolds mimicking bone environment and can be used for cell functional analysis and drug screening. Oyane et al. demonstrated that acidic functional groups were generated on the surface of PS substrates by oxygen plasma treatment, which resulted in HAp deposition in calcium phosphate solutions [9]. We have developed methods to functionalize PS substrates with HAp via protein adsorption layers in SBFs [10–13]. Acidic functional groups on side chains of proteins adsorbed on PS substrates induced heterogeneous nucleation of HAp in SBFs. Introduction of adsorption layers of serum proteins such as human serum albumin (HSA) and immunoglobulin G (IgG) on PS substrates resulted in deposition of HAp in 1.5SBF, a solution with ion concentrations that are 1.5-fold higher than those of SBF [10–12]. Furthermore, pretreatment of HSA-adsorbed PS substrates with alternate soaking process

(ASP) using solutions containing calcium ions and phosphate ions [14, 15] resulted in complete coating of the substrates with HAp in conventional SBF [13]. In this case, small nuclei or precursors of HAp were formed on the HSA adsorption layer surfaces by the pretreatment with ASP and then grew in SBF. Using these techniques, HAp-coated cell culture plates were prepared. HAp-coated cell culture plates promoted differentiation of human mesenchymal stem cells (MSCs) to osteoblast comparing to tissue culture-treated PS (TCPS) plates, which indicated that the former more mimicked bone environment [13].

In the present study, in order to investigate the versatility of HAp coating of PS substrate using SBF, the following two points were examined. First, effects of adsorption layers for HAp deposition in SBF were examined using HSA and other proteins (Fig. 1a). In our body, various kinds of specific proteins control biomineral growths [16]. On the other hand, we have demonstrated that HSA, a common serum protein, induced HAp deposition in SBFs [10–13]. We think that the point of the latter systems is that displaying ionic functional groups on the protein surfaces. Therefore we believe that other easily obtainable proteins that are not recognized as the protein relating biomineralization and display ionic functional groups on their surfaces have potential to act as the nucleation sites of HAp in SBFs. If such proteins successfully adsorbed on PS substrates, they can serve as templates for mineralization of HAp. The second one is direct functionalization of commercially available TCPS plates without protein adsorption layers (Fig. 1b). TCPS plates are widely used for culture of adherent cells [17]. They are manufactured by treating PS plates with oxygen-plasma or corona discharge to improve cell adhesions. Such treatments introduce hydrophilic

functional groups on the PS plate surfaces. We tried to utilize these functional groups for convenient HAp coatings.

2 Materials and methods

2.1 Materials

Human serum albumin (HSA), type I collagen from calf skin (COL), lysozyme chloride form from hen egg white (HEWL) were purchased from Sigma-Aldrich, Co., Ltd. Poly L-glutamic acid sodium salt (PGA, MW > 12,000) was obtained from Peptide Institute. Polystyrene (PS, M_w 183,000, M_w/M_n 1.06) was purchased from Polymer Source Inc. All chemicals including those for preparation of SBF were purchased from Nacalai Tesque, Inc. and used without further purification. 24-well PS plates and TCPS plates were purchased from AGC Techno Glass, Co., Ltd. SBF (Na^+ 142.0, K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 103.0, HCO_3^- 27.0, HPO_4^{2-} 1.0, and SO_4^{2-} 0.5 mM, pH 7.4) was prepared according to Kokubo's methods [1, 18]. The distilled water and ultrapure water (18.2 M Ω cm) used in experiments were prepared using Advantec RFD210TA and Advantec RFU414BA, respectively.

2.2 Cell culture

Human bone marrow-derived MSCs with an extended life span through retroviral transduction of hTERT and HPV E7, UE7T-13 cells [19] were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank, National Institute of Biomedical Innovation, and maintained in standard Dulbecco's modified Eagle's medium (D-MEM, Sigma-Aldrich Corp.) supplemented with 10% (vol./vol.) fetal bovine serum (MP Biomedicals, LLC.) (D-MEM(+)) at 37 °C under a humidified 5% CO₂ atmosphere.

2.3 Adsorption of proteins and PGA on PS plates

1 ml of 50 $\mu\text{g ml}^{-1}$ solution of COL in 0.1 M acetic acid and HSA, HEWL, and PGA in phosphate buffered saline (PBS, pH 7.4) were added in each well of 24-well PS plates and incubated for 3 h. Each well was washed with ultrapure water three times.

2.4 Deposition of HAp on protein-adsorbed PS and TCPS cell culture plates

The alternate soaking process (ASP) was applied to protein-adsorbed PS cell culture plates and TCPS plates in order to induce the formation of small precursors of HAp, or other calcium phosphates (CaP) on the plate surfaces [13]. 100 mM CaCl₂ solution in 50% ethanol (vol./vol.) was

added to each well of 24-well PS plates treated with proteins or PGA, and TCPS plates and incubated for 2 min. After washing with 50% ethanol (vol./vol.) twice, 100 mM K₂HPO₄ solution in 50% ethanol (vol./vol.) was added and incubated for 2 min. The set of treatments were performed once, twice or 4 times, respectively. 2 ml of SBF was then added into each well of the plate and incubated at 36.5 °C for 24 h using a dry chamber (DOV-750A, AS ONE Corp.). The wells were then rinsed with ultrapure water and dried by gentle nitrogen flushing.

2.5 Characterization

Water contact angles of the PS treated with solutions of proteins and PGA were collected using water drop contact angle measurement equipment (DM-301; Kyowa Interface Science, Co. Ltd.). 5 μl drops of ultrapure water were placed on ten different areas of each sample and the contact angles (θ /degree) were obtained as the mean $\pm$ standard deviations (S. D.). Amount of proteins adsorbed on PS were quantified using quartz crystal microbalance (QCM) [10, 12]. PS films were formed by spin-coating (4000 rpm, 1 min) of 10 mg ml⁻¹ PS solution in chloroform on QCM gold electrodes of 9 MHz QCM (QA-A9M-AU, Seiko EG&G Co., Ltd.). Gold electrodes spin-coated with PS were immersed in 50 $\mu\text{g ml}^{-1}$ solution of COL in 0.1 M acetic acid and HSA and HEWL in PBS for 3 h. After rinsing twice with ultrapure water and drying with nitrogen, decreases ($-\Delta F$, Hz) in the QCM frequency were measured using a Quartz Crystal Analyzer (QCA922-00, Seiko EG&G Co., Ltd.). In the case of protein adsorption, a $-\Delta F$ of 1 Hz was calculated to correspond to a mass increase (Δm) of 1.08 ng cm⁻² of protein. Elemental compositions of the surfaces of PS plates and TCPS plates were examined using X-ray photoelectron spectroscopy (XPS, JSP-9010MS, JEOL Ltd.) with the X-ray source of MgK α radiation (1253.6 eV). The spectra were obtained at an acceleration voltage of 10 kV and take-off angle of 30°. Elemental compositions of the samples were calculated using the peaks of detailed spectra of O_{1s} and C_{1s}. Obtained data were analyzed using the SpecSurf software (JEOL Ltd.) in order to determine the elemental composition and peak deconvolution of the C_{1s} peaks. The morphologies and elemental compositions of the resultant substrates were evaluated using scanning electron microscopy (SEM, JSM-7001F, JEOL Ltd.) and an energy dispersive X-ray spectrometer (EDX, Kevex Sigma, Noran Instruments, Inc.) with an acceleration voltage of 10 kV. The specimens were coated with Pd-Pt using an ion sputter (MC1000, Hitachi Ltd.) to prevent charge-up. FT-IR spectra were obtained by a single reflection attenuation total-reflection (ATR) method using a Nicolet 380 (Thermo Fisher Scientific, Inc.) combining a Quest ATR accessory (GS-10800, Specac Ltd.). The X-ray diffraction (XRD)

Fig. 2 **a** Water contact angles of PS plates non-treated and treated with human serum albumin (HSA), type I collagen (COL), hen egg white lysozyme (HEWL), and poly L-glutamic acid (PGA). **b** Adsorbed amounts of HSA, COL, and HEWL on the surface of the PS. Values were obtained from frequency changes on QCM gold electrodes monitored in air. Data are shown as the mean $\pm$ standard deviation ($n = 2$). Mass change (Δm) = $-k\Delta F$, $k \approx 1.08 \times 10^{-9} \text{ g Hz}^{-1}$

patterns of the sample scraped from plates using a microspatula were obtained using ULTIMA IV (Rigaku Corp.) with a CuK α source and high-speed detector under the following conditions: tube voltage: 40 kV, tube current: 40 mA, and step width 0.02°. The XRD patterns were obtained after subtraction of the background.

2.6 Adhesion of human MSCs on HAp-coated TCPS plates

The HAp-coated PS plates obtained by utilizing HSA adsorption layers, 2 cycles of ASP, and SBF treatments for 24 h, and the HAp-coated TCPS plates with 2 cycles of ASP and SBF treatments for 24 h were used for cell cultures after sterilization by UV irradiation (254 nm, 9 W, 2 h). 5×10^4 cells in 500 μ l D-MEM(+) were seeded on the HAp-coated PS plates and HAp-coated TCPS plates. After cultured at 37 °C for 7 h, the cells were fixed with 4% formalin (vol./vol.) in PBS containing 10% glycerol (vol./vol.) for 1 h followed by staining with 0.5% crystal violet solution at room temperature for 50 min. These samples were used for microscopic observations.

3 Results

3.1 HAp deposition on protein-adsorbed PS plate surfaces in SBF

First, the effect of protein adsorption layers on HAp deposition were examined using HSA, COL, HEWL, and PGA, an acidic protein analogue. Adsorption of proteins and PGA on PS substrates were estimated from changes of surface wettability and QCM measurement. Although PS treated with all proteins and PGA showed lower water contact angles than that of non-treated PS plates, the degree of the decreases differed depending on the kinds of proteins (Fig. 2a). The results of the quantitative analysis using

QCM indicated that there was no significant difference in adsorbed amount of proteins (Fig. 2b). Then, these plates were treated with ASP twice and incubated with SBF for 24 h. The PS plates with adsorption layers of HSA were completely coated with deposits having plate-like structures (Fig. 3a). For the PS plates treated with COL and PGA, the surfaces were almost covered with deposits but there were some defects indicated by arrowheads (Fig. 3b, d). On the other hand, the surface morphologies of the PS plates treated with HEWL were different from that with HSA. There were smaller numbers of plate-like deposits on HEWL-treated PS plates than HSA-treated ones (Fig. 3c, a).

3.2 Surface characterization of commercial TCPS plates

The surfaces of commercially available TCPS plates and PS plates were characterized by ATR FT-IR and XPS. No significant differences in the ATR FT-IR spectra were observed between TCPS and PS plates (Fig. S1). In the XPS spectra of TCPS plates, the broad peak originating from O_{1s} partly assignable to O–C in carbonate (535.0 eV), O–C in acid and ester (534.3 eV), alcohol and ether (533.6 eV), O=C in ketone (533.6 eV), and acid, ester, and carbonate (532.8 eV) [20, 21] was observed (Fig. 4b). Although trace amount of oxygen was also detected in PS (Fig. 4b), the elemental ratio of oxygen of PS was much smaller than that of TCPS (Table S1). By deconvolution of the peak corresponding to C_{1s} region of TCPS, peaks assignable to C–O, C=O, O–C=O, and O–CO=O [22] were detected (Fig. 4c, d, Table 1). Water contact angle of the TCPS plate was $66.6 \pm 2.8^\circ$ which was much lower than that of the PS plate (Fig. 2, $85.4 \pm 1.1^\circ$).

3.3 HAp deposition on TCPS plates

SEM observations revealed that TCPS plates with 2 cycles of ASP using 50% ethanol as the solvent were completely and homogeneously coated with deposits having plate-like

Fig. 3 SEM images of well surfaces of PS plates treated with HSA (a), COL (b), HEWL (c), and PGA (d) with 2 cycles of the ASP treatments followed by incubation with SBF for 24 h. The arrowheads indicated defects of HAp coating

Fig. 4 XPS survey spectra (a) and detailed spectra of O_{1s} (b) and C_{1s} (c, d) regions of TCPS and PS. The deconvolution of C_{1s} of PS (c) and TCPS (d) were shown. Blue: data, red: deconvoluted peak, and magenta: cumulated peak

structures after 24 h incubation with SBF (Fig. 5a). In contrast, there was no deposition on TCPS plates with a single treatment of ASP after incubation with SBF (data not shown). In the case of the plates with 4 cycles of the ASP,

there were some large deposits on homogeneous layers of deposits with plate-like structures (Fig. 5b). When ultrapure water was used as a solvent of $CaCl_2$ and K_2HPO_4 , there were no depositions (data not shown). The results of EDX

analysis of the deposits with 2 cycles and 4 cycles of ASP treatments after incubation with SBF for 24 h indicated that the deposits contained Ca, P, and O and small amounts of Na and Mg (Fig. 5c, d). The Ca/P elemental ratios of the deposits with 2 cycles and 4 cycles of ASP followed by incubation with SBF were 1.41 and 1.37, respectively. In the FT-IR spectra, peaks assignable to stretching vibrations (ν_3 , 1200–1000 cm^{-1}) and bending vibrations (ν_4 , 650–500 cm^{-1}) of PO_4^{3-} were detected (Fig. 6). Direct examinations of the TCPS plates using an XRD apparatus were difficult because of the small amounts of the deposits; therefore the deposits were collected by scraping the plate surfaces and then evaluated. XRD patterns of the deposits formed on TCPS plates treated with 2 cycles of ASP and incubation with SBF for 24 h showed two peaks at $2\theta = 26$, and 31° , which are assignable to the (002), and (211) planes of HAP (PDF# 00-009-432) [23], respectively (Fig. 7).

3.4 Adhesion of MSCs on HAP-coated TCPS plates

Usability of the obtained HAP-coated TCPS plates in cell cultures of bone-related cells was checked using MSCs. The HAP-coated TCPS plates obtained by utilizing 2 cycles of ASP, and SBF treatments for 24 h, were used. After culture for 7 h, cells seeded on HAP-coated TCPS plates were observable under transmitted light microscopy (Fig. 8a). MSCs attached and spread with a spindle cell morphology on HAP-coated TCPS plate (Fig. 8a). Morphology of the cells on HAP-coated TCPS plates were similar to that on HAP-coated PS plates prepared utilizing HSA-adsorption layers (Fig. 8b). The numbers of the cells that attached to both HAP-coated PS plates were similar (297 ± 40 cells/field, $n = 3$, for HAP-coated TCPS plates and 309 ± 10 cells/field, $n = 3$, for HAP-coated PS plates prepared by utilizing HSA-adsorption layers, respectively).

4 Discussion

In the present study, firstly the effects of protein adsorption layers on HAP deposition were examined using HSA, COL, HEWL, and PGA, an acidic protein analogue. As we expected that adsorption of proteins with ionic functional groups decreased water contact angles of hydrophobic PS

Table 1 Peak deconvolution of the C_{1s} regions of PS and TCPS plates

	C-C	C-O	C=O	O-C=O	O-CO=O	$\pi \rightarrow \pi^*$
PS	91.5	3.2	–	–	–	5.3
TCPS	88.4	6.4	0.7	1.0	1.8	1.7

Fig. 5 SEM images (a, b) and EDX (c, d) spectra of well surfaces of TCPS plates with pretreatments of 2 cycles (a, c) and 4 cycles (b, d) of the ASP treatments followed by incubation with SBF for 24 h. In the insets of a and b, the scale bars of image were 1 μm . In c and d, the peaks of Pd are originated from the Pt-Pd coating in order to prevent charge-up

substrates, PS plates treated with all proteins and PGA showed lower water contact angles than that of non-treated PS plates; however, the degree of the decreases differed depending on the kinds of proteins (Fig. 2a). There was no significant difference in adsorbed amount of proteins among HSA, COL and HEWL (Fig. 2b). The main driving force

Fig. 6 FT-IR spectra of non-treated TCPS (a) and TCPS plates with 2 cycles of the ASP treatments followed by incubation with SBF for 24 h (b). The asterisk shows peaks assignable to stretching vibrations (ν_3 , 1200–1000 cm^{-1}) and bending vibrations (ν_4 , 650–500 cm^{-1}) of PO_4^{3-}

Fig. 7 XRD patterns of deposits on TCPS plates with 2 cycles of the ASP treatments followed by incubation with SBF for 24 h. Peaks indicated by an asterisk correspond to those of HAp (see text)

Fig. 8 Microscopic observation of human mesenchymal stem cells (MSCs) seeded on HAp-coated TCPS (a) and HAp-coated PS plates prepared using HSA adsorption layers (b) after a 7-h culture. Scale bars were 200 μm

for adsorption of HSA on PS are assumed to be hydrophobic interactions between the hydrophobic domains of HSA and hydrophobic surface of PS substrates [24]. According to our previously report [12], amount of HSA adsorbed on PS substrates after 3 h incubation using QCM suggested that the surface coverages of PS with HSA was estimated to be 43–113% assuming monolayer adsorption. Adsorptions of COL on PS were considered to be the ring–ring interactions of the phenyl groups on PS with the rings of the collagen molecule [25]. Jacquemart et al. showed that COL was adsorbed on PS up to 24 h with decrease of water contact angles to 40° through transition state with heterogeneous dendritic or netlike patterns [26]. Our experimental condition (3 h incubation) and values of water contact angles ($62.1 \pm 1.2^\circ$) suggested that the dendritic or netlike structures of COL were formed on PS plate surfaces. As for the adsorptions of PGA on PS, although PGA has ionic side chains at higher density than other proteins, the decrease of the contact angle was much smaller than others. Matsushita et al. demonstrated that PS cell culture dishes were coated with PGA [27]. The hydrophobic interactions between $\text{CH}_2\text{--CH}_2$ moieties of PGA and PS are assumed, whereas it has been reported that the amounts of PGA adsorbed on PS were quite small [28]. Results of water contact measurement indicated that the amounts of PGA adsorbed on PS plate surfaces were smaller than other proteins. On the other hand, HEWL adsorbs on PS substrates by hydrophobic interactions [29] and such adsorptions are stable even in solution with high ionic strength [29].

Pretreatments with alternate soaking process (ASP) using solutions containing calcium ions and phosphate ions followed by incubation with SBF for 24 h resulted in HAp deposition on PS with all kinds of adsorption layers. However, there were differences in the coverage and morphologies of the deposits. As described in our previous report [13], PS plates with adsorption layers of HSA were completely coated with deposits having plate-like structures (Fig. 3a). We have identified that these deposits were bone-like HAp with low crystallinity by characterization

using EDX, XRD, and FT-IR [13]. For the PS plates treated with COL (Fig. 3b) and PGA (Fig. 3d), there were some defects in the deposition layers. Although total charge of COL (isoelectric point (pI): 7.8 [30]) is slightly positive in neutral solution, there are still some acidic functional groups. In PGA, pK_a of carboxylic acid of side chain is 4.9 [31] and these are negatively charged in neutral solution. These negatively-charged acidic functional groups in COL and PGA may be nucleation sites in alternate soaking process. In addition to surface charge, surface morphology of the protein adsorption layers is another considerable factor that affect the morphology of the deposition layers. Regarding COL, Jacquemart et al. demonstrated that, after 2 h incubation, the contact angles of COL-adsorbed surfaces varied in the range of ca. 40–80° depending on the drying process after rinsing of the surfaces [26]. Here network structures of COL on PS surfaces were observed for the samples having relatively higher contact angles. It can be regarded that COL-adsorbed surfaces obtained in the present study had similar network structures: in other words, part of PS surfaces might not be covered by COL molecules. As for PGA, its binding amount on PS surfaces should be smaller than other proteins such as HSA because the driving force of the adsorption is weak, as described above, which indicated that PGA molecules did not cover PS surfaces completely. Therefore, defects in the deposition layers can be attributed to heterogeneity of COL adsorption layers and low adsorption density of PGA on PS plate surfaces.

On the other hand, smaller numbers of plate-like deposition were observed on HEWL-treated PS plate surfaces than HSA-treated ones (Fig. 3c). HEWL (pI: 11.4 [32]) is positively charged in solution of pH 7.4 in contrast to HSA (pI: 4.7 [33]) with negative charges. In the ASP treatments, the well plates were treated with solution containing calcium ions at first, then that of phosphate ions. Therefore, charges of the adsorption layers might affect the formation of small nuclei or precursor of HAp on protein surfaces and accordingly resultant HAp layers after incubation with SBF. From these results, it is suggested that adsorption layers of various kinds of protein or poly-amino acid can be used for HAp deposition on PS plate surfaces using ASP and SBFs. Morphologies, density, and charge state of protein adsorption layers correlated with deposition of HAp thereon. Although HSA with high affinity to PS is the most appropriate as the adsorption layers for HAp deposition on PS plate surfaces in the present system, other protein adsorption layers can also be used for HAp deposition on other polymer substrates with high affinity to the protein. For example, it has been reported that COL were coated on substrate surfaces of polyetheretherketone (PEEK) [34], a super engineering plastic used as orthopedic and spinal implants [35],

therefore, COL layer can be used for HAp coating on PEEK substrate surfaces.

The surface of tissue culture-treated PS (TCPS) plates widely used in culture of adherent cells are more hydrophilic than that of PS plates due to functional groups such as carboxylic acid groups generated by oxygen-plasma or corona discharge treatment. It is expected that these acidic functional groups can be effective for heterogeneous nucleation of HAp in SBFs. The surface of commercially-available TCPS plates were characterized by ATR FT-IR (Fig. S1) and XPS (Fig. 4) and the results suggested that acidic functional groups such as carboxylic acid groups were displayed on the outmost surface of TCPS plates. It was supported by the fact that contact angles of TCPS were much lower than that of PS. There was no deposition on TCPS plate surfaces after incubation with SBF up to 48 h (data not shown). This indicated that the functional groups displayed on TCPS plates were ineffective to induce heterogeneous nucleation of HAp in SBF. Then ASP was used as the pretreatment to form small nuclei or precursors of HAp on the surface of TCPS plates in order to induce HAp deposition in SBF more effectively. After 2 cycles of the ASP, there were deposits on the surface of TCPS plates (Fig. S2). Carboxylic acid groups on TCPS plate surfaces probably acted as binding sites for calcium ions during incubation with a solution containing calcium ions. When the calcium ion solution was removed and a solution containing phosphate ions was subsequently added, calcium ions still bound to the carboxylic acid groups attracted negatively charged phosphate ions from the solution. This event could be repeated by progressing the ASP cycles. As a result, small nuclei or precursors of HAp were generated on the TCPS plate surfaces after 2 cycles of the ASP. We have shown that the deposits formed on HSA-adsorbed PS plates after 2 cycles and 8 cycles of the ASP were HAp and a mixture of HAp and dicalcium phosphate dihydrate (DCPD), respectively [13]. TCPS plates with 2 cycles of ASP using 50% ethanol as solvent were completely and homogeneously coated with deposits having plate-like structures after 24 h-incubation with SBF (Fig. 5a). Their surface morphologies were similar to that obtained the process using HSA-adsorption layers (Fig. 3a). In contrast, there was no deposition on TCPS plates with a single treatment of ASP after incubation with SBF, and there were some large deposits on homogeneous layers of deposits with plate-like structures for the case of 4 cycles of the ASP (Fig. 5b). We have demonstrated that surface roughness of HAp affected proliferation of preosteoblast [13], which means optimization of the preparative condition is important. When ultrapure water was used as a solvent of $CaCl_2$ and K_2HPO_4 , there were no depositions. It may be due to the difference in solubility of the precursors in 50% ethanol and ultrapure water or the acceleration of ion migration in

50% ethanol by reducing the interface tension [36]. The results of EDX analysis of the deposits with 2 cycles and 4 cycles of ASP indicated that the deposits contained Ca, P, and O and small amounts of Na and Mg (Fig. 5c, d). The Ca/P elemental ratios of the deposits (1.41 and 1.37) appeared to be smaller than that of stoichiometric HAp (1.67). These values were within the range of a biological apatite (1.2–1.6) and were similar to those observed for HAp deposited from SBF [37]. In the FT-IR spectra, two peaks assignable to stretching vibrations (ν_3 , 1200–1000 cm^{-1}) and bending vibrations (ν_4 , 650–500 cm^{-1}) of PO_4^{3-} were detected (Fig. 6). The broad shape of these peaks indicated that the obtained HAp deposits had low crystallinity [38]. XRD patterns of the deposits formed on TCPS plates treated with 2 cycles of ASP after immersion in SBF for 24 h indicated that the deposits were mainly consisted of HAp (Fig. 7). From these characterizations, it was suggested that deposits coated on TCPS plate were bone-like HAp.

HAp-coated TCPS plates were observable under transmitted light microscopy after cell culture, which indicated that the HAp layers formed on the TCPS plates were thin enough (Fig. 8a). MSCs attached and spread with a spindle cell morphology on HAp-coated TCPS plate (Fig. 8a) and morphology and number of the cells thereon were similar to that on HAp-coated PS plates prepared by utilizing HSA-adsorption layers (Fig. 8b and the relating description in 3.4). We previously demonstrated that the number of MSCs attached on the latter plates was almost equal to that on TCPS [13]. These results indicated that the surfaces of the TCPS plates were homogeneously coated with HAp as well as the HAp-coated PS plates prepared utilizing HSA adsorption layers, and both HAp-coated plates were useful for cell culture.

5 Conclusion

This study revealed that wide variety of protein adsorption layers can be used for HAp deposition on PS plate surfaces accompanying the ASP treatments and incubation with SBF, whereas surface coverage and morphologies of the deposits reflected adsorption ability and charge states of the proteins. We also demonstrated that commercial TCPS plates were functionalized with bone-like HAp using the same ASP and SBF processes and the resulting plates were useful for MSC cultures. These two types of HAp-coated plates have their own advantages: the biochemical functions or physical properties of the proteins can also be utilized in the plates prepared using the former system, whereas HAp-coated plates are obtainable conveniently and at low-cost in the latter one. We believe that these HAp-coated culture plates can contribute to basic researches and tissue engineering of bone-related cell systems.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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