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Surface functionalization of tissue culture polystyrene plates with hydroxyapatite under body fluid conditions and its effect on differentiation behaviors of mesenchymal stem cells

Kazutoshi Iijima[a,b], Ryo Suzuki[b], Ayako Iizuka[a], Hitomi Ueno-Yokohata[c], Nobutaka Kiyokawa[c], Mineo Hashizume[a,b,*]

[a] Department of Industrial Chemistry, Faculty of Engineering, Tokyo University of Science, 12-1 Ichigayafunagawara-machi, Shinjuku-ku, Tokyo 162-0826, Japan
[b] Graduate School of Chemical Sciences and Technology, Tokyo University of Science, 12-1 Ichigayafunagawara-machi, Shinjuku-ku, Tokyo 162-0826, Japan
[c] Department of Pediatric Hematology and Oncology Research, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan

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ABSTRACT

The surfaces of polystyrene (PS) cell culture plates were functionalized with hydroxyapatite (HAp) under body fluid conditions utilizing protein adsorption layers and a pretreatment with an alternate soaking process (ASP) using solutions containing calcium and phosphate ions. Adsorption layers of human serum albumin (HSA) formed on the surface of each well of commercial 24-well PS plates by solution processes. $CaCl_2$ and K_2HPO_4 solutions were alternately added to the wells, the plates were incubated to form the precursors, and this was followed by the addition of simulated body fluid (SBF) and a further incubation for 24 h. These treatments resulted in the surfaces of the PS cell culture plates being completely covered with bone-like HAp. The coating of PS plates with HAp promoted the adhesion of mesenchymal stem cells (MSCs) and maintained cell growth that was as fast as that on tissue culture-treated PS (TCPS) plates. Osteogenic differentiation was greater, whereas adipogenic and chondrogenic differentiation was less in the culture on HAp-coated PS plates than in that on TCPS plates. The present method is useful for preparing HAp-coated PS plates at clean benches without the need for any expensive apparatus. HAp coated on PS plates by this method was a bone-like apatite with high bioactivity; therefore, the present HAp-coated PS plates are promising materials for assays of bone-related cells in the bone remodeling process.

1. Introduction

Polystyrene (PS) has been widely used for cell culture dishes and plates because it is inexpensive, is easily molded into various shapes, and has excellent transparency and physical properties. Research on cell cultures under conditions mimicking the *in vivo* environment has recently advanced in the fields of stem cell biology and drug screening [1]. These strategies are of particular importance for cultures of bone-related cells such as osteoblasts and osteoclasts because they exist in environments rich in inorganic components, mainly hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$), not on PS plates. For example, a culture of preosteoblasts or mesenchymal stem cells (MSCs) on sintered HAp substrates was found to more strongly promote differentiation to osteoblasts, which synthesize bone, than that on tissue culture-treated PS (TCPS) plates [2], [3]. These findings indicate the importance of the use of HAp substrates. However, the synthetic HAp discs used in these experiments are opaque, expensive, and have low mechanical strength. From the viewpoint of biochemical properties, synthetic stoichiometric HAp is not resorbed by osteoclasts, unlike natural bone [4]. Therefore, the preparation of inexpensive and easily manufactured bone-like apatite scaffolds will expand the availability of this cell culture system, which mimics the bone environment.

Simulated body fluid (SBF), a solution with similar inorganic ion concentrations to those of human plasma, and 1.5SBF, a solution with ion concentrations that are 1.5-fold higher than those of SBF, have been used in the biomimetic deposition of HAp on

* Corresponding author at: Department of Industrial Chemistry, Faculty of Engineering, Tokyo University of Science, 12–1 Ichigayafunagawara–machi, Shinjuku–ku, Tokyo 162–0826, Japan.
E-mail address: mhashizu@ci.kagu.tus.ac.jp (M. Hashizume).

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various material surfaces under mild conditions [5–7]. Acidic functional groups such as carboxylate groups displayed on substrate surfaces are known to effectively induce the heterogeneous nucleation of HAp in SBFs [8]. HAp obtained in SBFs is a bone-like apatite with small crystallites and a defective structure [9], and shows high biological affinity and is resorbed by osteoclasts [10]. However, PS does not display effective functional groups for the heterogeneous nucleation of HAp in SBFs. We previously demonstrated that the introduction of adsorption layers of serum proteins such as human serum albumin (HSA) on PS films by solution processes enhanced the deposition of HAp on these surfaces when immersed in 1.5SBF [11,12]. Furthermore, aqueous $CaCl_2$ treatments of protein-adsorbed PS films enhanced the deposition of HAp in 1.5SBF and complete surface coverage was achieved [13].

HAp-coated PS cell culture plates were prepared in the first part of the present study. The above-described process was applied to the HAp coating of commercial PS cell culture plates. In addition, for a more convenient preparation, the effects of a pretreatment with an alternate soaking process (ASP) using a solution containing calcium ions and that containing phosphate ions before their immersion in SBF was investigated (Fig. 1). A previous study demonstrated that the alternate soaking of various polymeric substrates such as polymer hydrogels [14] and hydrophilic polymer-grafted [15] and -coated [16] polymer films in solutions containing calcium ions and phosphate ions resulted in the formation of HAp on the surfaces of the gels and films. The ASP was applied to HSA-adsorbed PS cell culture plates in order to induce the formation of small nuclei, the precursors of HAp, or other calcium phosphates (CaP) on the plate surfaces, which were expected to act as the nucleation site and induce HAp growth in SBF. The effects of these pretreatments on the deposition behavior of HAp were evaluated and plausible mechanisms were discussed.

In the latter part of the present study, we examined a culture of human bone marrow-derived MSCs on HAp-coated PS plates. MSCs have the ability to differentiate into various types of cells in mesodermal lineages, such as osteoblasts, adipocytes, chondrocytes, and myocytes, and are expected to become a cell source for cell therapy and tissue engineering applications [17]. The effects of coating PS plates with HAp on the adhesion, proliferation, and differentiation of MSCs were evaluated.

2. Materials and methods

2.1. Reagents

Human serum albumin (HSA, Fraction V) was obtained from Sigma Co., Ltd. Oil red-O was obtained from Wako Pure Chemical Industries, Ltd. All chemicals, including those for the preparation of SBF, were obtained from Nacalai Tesque, Inc. and used without further purification. Forty-eight-well compatible HAp discs were purchased from 3D Biotek, LLC. The distilled water and ultrapure water (18.2 MΩ cm) used in experiments were prepared using Advantec RFD210TA and Advantec RFU414BA, respectively. 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 method [18,19].

2.2. Cell culture

Human bone marrow-derived MSCs with an extended life span through retroviral transduction, UE7T-13 cells [20] (Japanese Collection of Research Bioresources (JCRB) Cell Bank, National Institute of Biomedical Innovation) were maintained in standard Dulbecco's modified Eagle's medium (D-MEM, Sigma-Aldrich Corp.) supplemented with 10% (vol./vol.) fetal bovine serum (FBS, MP Biomedicals, LLC.) (D-MEM(+)) at 37 °C under a humidified 5% CO_2 atmosphere.

2.3. Preparation of HAp-coated PS cell culture plates

HSA solution (50 μg mL^{-1} in phosphate-buffered saline (PBS), pH 7.4) was added to each well of commercial 24-well PS plates (AGC Techno Glass Co., Ltd.) and incubated at room temperature for 3 h. In the ASP, 100 mM $CaCl_2$ solution in 50% ethanol (vol./vol.) was added to each well and incubated for 2 min. After washing with 50% ethanol (vol./vol.) twice, 100 mM K_2HPO_4 solution in 50% ethanol (vol./vol.) was added and incubated for 2 min. The above treatment was performed twice or 8 times, respectively. Two milliliters of SBF was then added into each well of the plate and incubated at 36.5 °C for 24 h and 48 h using a dry chamber (DOV-750A, AS ONE Corp.). In the 48-h incubation, SBF was replaced with fresh SBF after 24 h. The wells were then rinsed with ultrapure water and dried by gentle nitrogen flushing.

2.4. Characterization

The morphologies and elemental compositions of the resultant substrates were evaluated using scanning electron microscopy (SEM, JSM-7001F, JEOL Ltd. and S-4800, Hitachi 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 (E-1030 and MC1000, Hitachi Ltd.) to prevent charge-up. FT–IR spectra were obtained by a single reflection attenuation total-reflection (ATR) method using a Thermo Fisher Scientific Nicolet 380 combining a Quest ATR modulus (Specac Ltd.). The X-ray diffraction (XRD) patterns of the samples scraped from plates using a microspatula were obtained using ULTIMA IV (Rigaku corp.) with a CuKα 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 after background subtraction were shown.

2.5. Adhesion and proliferation of human MSCs on HAp-coated PS plates

The HAp-coated PS plates obtained by utilizing HSA adsorption layer, 2 cycles of ASP, and SBF treatment for 24 h, were used for a cell culture after sterilization by UV irradiation (254 nm, 9 W, 2 h). In the cell adhesion experiments, 5×10^4 cells were seeded on the surfaces of HAp-coated and non-coated 24-well plates (dispersed in 500 μL D-MEM(+) per well), and were cultured at 37 °C for 7 h. The cell proliferation experiments were conducted using the same conditions, except for 1×10^4 cells being seeded on wells and cultured for up to 72 h. After the predetermined incubation times, the cells were stained with 0.5% crystal violet solution at room temperature for 50 min after fixation with 4% formalin (vol./vol.) in PBS containing 10% glycerol (vol./vol.) for 1 h. The numbers of cells were evaluated by quantifying the amount of DNA in the cells. After washing with PBS to remove unattached cells, cells were collected using trypsin-EDTA (Immuno-Biological Laboratories Co., Ltd.) and dispersed in 1 mL of Tris-EDTA buffer (TE buffer, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The cells were disrupted by ultra sonication for 3 min in a bath sonicator (US Cleaner, ASONE). Cell lysates were mixed with Hoechst33258 (Dojindo Laboratories Co., Ltd.) at a final concentration of 2 μg/mL and their fluorescence intensities were measured using a fluorescent plate reader (ARVO X5, Perkin Elmer, Inc.) with a 360 nm excitation filter and 460 nm emission filter. Calf thymus DNA (Sigma-Aldrich) was used to construct the calibration curve in order to measure DNA concentrations in the samples.