Alternate Calcification in Microcapillaries for the Fabrication of Hydroxyapatite Films without Light Exposure, Calcination, or Applied Voltage

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Abstract: We discuss an alternate method for calcification in microcapillaries for fabricating calcium phosphate films using silicone molds and calcifying solutions. Calcium phosphate films with a line/space of 5–50 µm were fabricated by controlling the concentrations of calcium chloride and sodium phosphate solutions. Plate-type crystals of hydroxyapatite were grown when the calcium phosphate films were immersed in hydroxyapatite precursors. In the initial stage of hydroxyapatite crystal growth, the c-plane of the crystals was grown parallel to the substrates, and subsequently the growth followed with the c-plane growing perpendicular to the substrates. In narrow capillaries, dendritic structures were formed with a tendency to grow in a direction parallel to the direction of the microcapillaries. This technique is useful in the micropatterning of biocompatible ceramics with a minimized material consumption and a short fabrication time.

Key words: micromolding in capillary, hydroxyapatite, calcification, biomimetics

1 INTRODUCTION

Two- and three-dimensional lithography of soft materials has attracted much attention owing to its application in micro chemical robots in soft robotics¹⁻³. Hydroxyapatite (HAp) and calcium carbonates films have been employed for fabricating cell culture templates and medical diagnosis arrays. Using wet processes, HAp films were fabricated on biocompatibility glasses of SiO₂-CaO patterned by conventional photolithography techniques, which were later immersed in simulated body fluids⁴,⁵. The techniques used for the fabrication of patterned HAp films include electrocataphoresis⁶, laser-pulse⁷, and self-assembly at the air-water interface⁸. HAp precursors containing calcium acetate and sodium dihydrogen phosphate in buffer have been used for the preparation of HAp films without precisely controlling the concentration and mixing ratio, in contrast to the preparation carried out in simulated body fluids⁹. The techniques used for the fabrication of patterned HAp films include electrocataphoresis⁶, laser-pulse⁷, and self-assembly at the air-water interface⁸. HAp precursors containing calcium acetate and sodium dihydrogen phosphate in buffer have been used for the preparation of HAp films without precisely controlling the concentration and mixing ratio, in contrast to the preparation carried out in simulated body fluids⁹. Recently, an alternate soaking process by soaking hydrogel films in calcium chloride and sodium dihydrogen phosphate solutions has been developed for fabricating calcium phosphate films without employing a calcination process as opposed to the fabrication process using biocompatibility glasses of SiO₂-CaO. Applied voltages are not used in this method in contrast to the electrocataphoresis techniques and no laser exposure is employed in contrast to the pulse laser deposition methods⁹⁻¹². However, long fabrication times (several hours per cycle) are required owing to diffusion time of calcium and phosphonate ions migrating in hydrogels.

Previously, we reported an alternate soaking technique for producing wettability-patterned templates using two calcification solutions for performing submillimeter patterning of HAp and calcium carbonate films¹³. Wettability-patterned templates consist of hydrophobic self-assembled monolayers surrounded by hydrophilic solid substrates¹⁴⁻¹⁶. Calcium chloride and sodium dihydrogen phosphate are selectively adsorbed on the hydrophilic regions, resulting in immediate calcification, leading to the selective formation of HAp scaffolds with a reduced material consumption and fabrication time. However, HAp films on the wettability-patterned templates exhibited widths in sub millimeter length scales. The width must be reduced for using the materials in cell culture templates and frameworks in microchemical robots.

In this study, we aim for direct micropattering of HAp...
scaffolds by performing micromolding in capillaries, which is a soft lithography technique. We fabricate HAp scaffolds in microcapillaries of cross-linked polydimethylsiloxane (PDMS) molds by injecting calcium chloride and sodium phosphate solutions, which react to form calcium phosphate films. The HAp scaffolds are then immersed in HAp precursors for growing HAp crystals. The obtained samples are characterized using optical microscopy, scanning electron microscopy (SEM), X-ray diffraction spectroscopy, and energy-dispersive X-ray spectroscopy (EDX).

2 EXPERIMENTAL PROCEDURES

2.1 Materials

Acetone and chloroform (Wako) were used for cleaning the substrates. Calcium chloride, sodium hydrogen phosphate, and methanol (Wako) were used for performing micromolding in capillaries to fabricate HAp scaffolds. Calcium acetate monohydrate (Wako), ammonium phosphate monobasic (Aldrich), and ammonium acetate (Aldrich) were used for the growth of HAp on HAp scaffolds. Glass plates and Si wafers with native SiO₂ were purchased from Matsunami Glass Ind., Ltd. and Furuuchi Chemical Corp. Si molds having different sizes of line/space, pillar array, and hole array were obtained from Kyodo International Inc. Silpot 184 and catalyst Silpot 184 were purchased from Dow Corning Toray to fabricate PDMS molds.

2.2 Fabrication of patterned HAp films

Glass plates and Si wafers were ultrasonicated in acetone and chloroform for 20 min each followed by exposing the substrates to UV-ozone atmosphere for 15 min, which was generated using a PL16-110 UV-ozone cleaner (Sen Lights Corp., Japan). Mixtures of Silpot 184 and its catalyst were poured on the Si molds followed by heating at 110 °C for 60 min to form PDMS molds having two open ends with a line/space pattern. Small droplets of CaCl₂ in methanol were added at the open end of the PDMS molds on the substrates for achieving spontaneous injection by capillary force. After several minutes of spontaneous evaporation of the solvent, small droplets of Na₂HPO₄ in water were added at the other side of the PDMS molds. The molar ratio of CaCl₂ to Na₂HPO₄ was adjusted to 1:1. The PDMS molds were peeled off from the substrates, resulting in the formation of patterned HAp scaffolds. The HAp precursor was prepared by mixing aqueous solutions of 5 mL of 1.3 mol L⁻¹ CH₃COONH₄, 2.5 mL of 30 mmol L⁻¹ NH₄H₂PO₄, and 2.5 mL of 50 mmol Ca(CH₃COO)₂·H₂O in sample bins. The HAp scaffolds were immersed in the HAp precursor at 35°C for 1, 2, or 3 h followed by washing the samples with pure water three to four times. The procedures are summarized in Fig. 1.

2.3 Characterization

Optical microscopic measurements were performed using a BX-60 optical microscope (Olympus, Japan). SEM
and EDX measurements were performed using an S-4200 microscope (Hitachi, Japan). Laser microscopic measurements were carried out using a confocal laser microscope VK-X200 (Keyence, Japan).

3 RESULTS

Calcium phosphate films were fabricated by the alternate method for calcification in microcapillaries by adjusting the concentrations of calcium chloride and sodium phosphate solutions as well as the capillary depth of the PDMS molds. Figure 2 shows the optical microscopic images of the calcium phosphate films fabricated on the glass plates by varying the capillary depth of the PDMS molds as well as the concentrations of the calcium chloride and sodium phosphate solutions. At a concentration of 0.1 mol L$^{-1}$, calcium phosphate films with a line/space of 50 μm are formed irrespective of the capillary depth used. The black regions correspond to the calcium phosphate films surrounded by the surfaces of the glass plates. The optical microscopic images do not indicate any significant effect of the capillary depth on the structures of the films. Regions not containing the calcium phosphate films can be observed in some images. A decrease in the concentration of calcium chloride and sodium phosphate solutions results in the decrease of the amount of calcium phosphate films produced. Thus, micropatterned calcium phosphate films can be prepared by the alternate method for calcification in microcapillaries.

SEM, EDX, and laser microscopic measurements were performed on the patterned calcium phosphate films fabricated by the alternate method for calcification in microcapillaries. Figure 3a shows an SEM image of the calcium phosphate film fabricated with microcapillaries having a line/space of 50 μm and a capillary depth of 10 μm using calcium chloride and sodium phosphate solutions having concentrations of 0.1 mol L$^{-1}$. Porous structures with several micrometers to several tens of micrometers in diameter are observed. Similar structures were obtained when calcium phosphate films were fabricated by the alternate soaking technique on wettability-patterned templates$^{13}$. Figure 3b shows an EDX spectrum of the calcium phosphate films, indicating the presence of Kα signals of Si arising from the substrates, Na arising from sodium phosphate, Ca from calcium phosphate films and calcium chloride, and P from calcium phosphate films and sodium phosphate. The laser microscope image and the profile given in Figs. 3c and d indicate a film thickness of about 400 nm.
HAp films were fabricated on the calcium phosphate films by immersing the prepared calcium phosphate films in the HAp precursors at room temperature. Figures 4a and b show the SEM images of calcium phosphate films immersed in the HAp precursors at room temperature. Plate-type crystals are formed on the calcium phosphate films. The crystal growth rates of a- and b-planes are higher than that of the c-plane of the plate-type crystals of HAp. As shown in the SEM images in Figs. 4c–f, the size of the HAp plate-type crystals and the microcapillary width of the calcium phosphate films increase and the space between the calcium phosphate films decreases with an increase in the immersion time, indicating that the growth proceeds in both the lateral and normal directions of the films. Figure 4g shows an SEM image of the boundary between the substrate surface and the calcium phosphate film after immersing in the HAp precursors. Near the substrate surface, the c-plane of the HAp crystals grows parallel to the substrate surface. In the region 10 μm apart from the boundary, the c-plane of HAp aligns perpendicularly to the substrate surface. In the initial stage of the HAp crystal growth, the growth proceeding with the c-plane parallel to the substrate surface is dominant as the calcium phosphate films are found to be parallel to the substrates. HAp plates were also formed parallel to the substrates in the initial stage of the process reported in our previous study. In the succeeding stage of the HAp crystal growth, an increase in the crystal growth from defects and aggregates on the c-plane of HAp crystals results in an increase in the crystal growth with the c-plane growing perpendicularly to the substrate surfaces, which could be attributed to the diffusion-limited process of calcium phosphate clusters from the solution to the calcium phosphate films.

We performed laser microscopy measurements to estimate the thickness of the HAp films after immersing the calcium phosphate films in the HAp precursors. Figure 5 shows the laser microscope images and the profiles of the HAp films after immersing the calcium phosphate films in
the HAp precursors by varying the immersion time. As shown in Fig. 5g, the film thickness increases linearly with an increase in the immersion time. The concentration of the calcium phosphate clusters in the solution is estimated to remain almost unchanged during the HAp growth by taking into account that the maximum film thickness is more than 0.8 mm, the density of HAp is 3.14 g cm$^{-1}$, and the surface area of the calcium phosphate film is less than 0.125 cm$^2$. This was calculated by multiplying the area of a line in the Si molds with the number of lines, assuming that all of the calcium phosphate clusters were used for growing HAp on the calcium phosphate films. As shown in the SEM images in Fig. 4 and the laser microscopic images in Fig. 5, the increase in the thickness of the film is found to be smaller than that of the capillary width of the HAp film. This also suggests that the growth of the c-plane of HAp crystals dominantly proceeds parallel to the substrate surface in the initial stage, and in the succeeding stage, an increase in the growth of the c-plane perpendicular to the
substrate surface is observed with an increase in the immersion time. We performed EDX spectroscopy measurements for obtaining the elemental map of the calcium phosphate films after immersing in the HAp precursors. The EDX spectrum shown in Fig. 6a indicates the absence of sodium and chloride ions in the calcium phosphate film immersed in the HAp precursors. The Ca/P ratio, calculated by the ratio of intensities of P and Ca signals, is estimated to be about 1.21. The Ca/P ratio close to a value of 1.67 suggests the formation of HAp on the calcium phosphate film. Immersion of calcium phosphate films fabricated by the alternate-soaking technique in the HAp precursors for several days yielded a Ca/P ratio of 1.67, suggesting the formation of HAp films. These results demonstrate that the HAp crystals can be fabricated on the calcium phosphate films produced by the alternate method for calcification in microcapillaries after immersing in the HAp precursors.

The alternate method for calcification in microcapillaries was carried out with the PDMS molds having a line/space of 5, 10, and 50 μm for decreasing the width of the calcium phosphate films. Figure 7 shows the optical microscope images of the calcium phosphate films fabricated using the PDMS molds with a line/space of 5, 10, and 50 μm by varying the concentrations of calcium chloride and sodium phosphate solutions. Calcium phosphate films with a capillary width of 5, 10, and 50 μm were formed on the glass plates. As shown in Figs. 7c and g, dendritic structures are formed on some regions of the calcium phosphate films with a tendency to align along the capillary walls, suggest-
ing that the HAp crystal growth direction can be controlled by adjusting the capillary width. These results demonstrate that the patterning size can be controlled in the order of 100 μm to 5 μm. We are still trying to perform cell cultures on the patterned HAp for controlling the cell structures in micrometer length scales.

4 CONCLUSION

We demonstrate an alternate method for calcification in microcapillaries of the PDMS molds using two calcification solutions for fabricating HAp scaffolds of calcium phosphate films. The calcium phosphate films were formed in the microcapillaries having a line/space from 5–50 μm by tuning the concentrations of calcium chloride and sodium phosphate solutions. HAp crystal films were grown on the calcium phosphate films immersed in the HAp precursors. In the initial stage, the c-plane of the plate-type HAp crystals grew parallel to the substrate surface, and in the succeeding stage, the c-plane grew perpendicular to the substrate surface. In narrow capillaries, dendrite structures were obtained with a tendency to grow parallel to the direction of the microcapillaries.

The alternate method for calcification in microcapillaries enables direct fabrication of HAp scaffolds of calcium phosphate films using wet processes, thereby enabling the environment-friendly fabrication of the HAp films without using conventional calcination techniques or an applied voltage. By varying the calcification solutions, biocompatible ceramic and other functional ceramic films such as calcium carbonate can be produced. The alternate calcification in microcapillaries using soft lithography can be useful in nanopatterning with sub micrometer to several tens of nanometer length scales. This technique is expected to contribute to the fabrication of microarrays for cell cultures and medical diagnosis in bionics as well as in the functional sensing and memory arrays in electronics and photonics.

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References


