Preparation of hydroxyapatite micropatterns for the study of cell–biomaterial interactions†

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A regular hydroxyapatite (HAp) micropattern was prepared combining post-lift-off photolithography, surface grafting of negatively charged chemicals, and simulated body fluid incubation. The chemical micropatterning of the bioceramics on glass was thus successfully generated. The resulting regular HAp microarray was confirmed by scanning electron microscopy, transmission electron microscopy, energy dispersive X-ray spectroscopy, X-ray diffraction, Raman spectroscopy, and Fourier transform infrared spectroscopy. A chemical micropattern of cell-adhesive bioceramic microislands on a non-fouling background was obtained after the glass background was passivated by polyethylene glycol. Culture of mammal cells (MC3T3) on the micropattern illustrated that the cell localization on the HAp microislands was well achieved. The present work affords a methodology of micropatterning of bioceramics for the study of interactions between cells and bioceramics.

Introduction

Understanding of cell–biomaterial interactions is a key fundamental topic in the biomaterials research and development. Recently, surface patterning has been paid much attention in the study of cell–biomaterial interactions1,2 due to its advantages in decoupling various geometric and chemical cues that influence cell behaviors, such as the cell size,3–5 cell shape,6–8 cell–cell contact,9–11 substrate stiffness12 and surface properties.13–19 While micropatterning of polymers and metals has been significantly improved,20–22 the preparation methodology of bioceramic micropatterns remains a technical bottleneck for further study of interactions between cells and inorganic biomaterials.

Hydroxyapatite (HAp), a major component of the bone mineral, has been widely applied as an inorganic biomaterial, especially in artificial bone grafts,23–24 scaffolds for bone tissue engineering25–29 and corresponding protein adsorption.30–33 So far, plenty of preparation methods have been reported to obtain HAp crystals of different morphologies34–37 and to study their effects on cell behaviors.38–44 Yet, there is a lack of a regular HAp or other bioceramic micropatterns with pre-defined microislands on a non-fouling background.

The key is the methodology to prepare a bioceramic microarray on a background of a different chemical. In this paper, we tried to obtain a HAp micropattern on glass. The most popular micropatterning method is photolithography, and especially lift-off photolithography based on a positive photoresist plus chemical deposition.† Since simulated body fluid (SBF) incubation is a common approach to generate solid bioceramics,45 our first choice is the naive combination of lift-off photolithography and SBF incubation, as schematically presented in Sample-1 preparation in Fig. 1. Here, a modified SBF (mSBF) with an over-saturated solution was suggested by us to accelerate the precipitation of bioceramics, but it is not essential for the feasibility of bioceramic micropatterning. Sample-1 did not lead to sufficiently selective precipitation of bioceramics on the pre-defined microislands and the substrate background; the precipitated HAp was sparse and easy to detach from the substrate. Stimulated by that HAp prefers to precipitate on negatively charged surfaces,45 we put forward the second approach, as also indicated in Fig. 1. While dense HAp microlayers were observed in the region of the pre-defined microislands, the background was also covered by bioceramics in Sample-2 with the reasons discussed later. Eventually, we designed a route combining photolithography, surface grafting of negatively charged chemicals, mSBF incubation, and post-lift-off; the regular micropattern of HAp on a background of a different chemical was achieved for the first time, as demonstrated in the preparation of Sample-3 in Fig. 1.

The present paper is aimed at publishing this post-lift-off combinatory strategy to successfully prepare HAp micropatterns. The sizes and shapes of the HAp microislands were pre-defined by masks used in photolithography. After the glass background was passivated with poly(ethylene glycol) (PEG),
mammal cells were found to be well localized on HAp microislands.

Materials and methods

Materials

A positive photoresist (RZJ-304) and a corresponding developer (RZX-3038) were purchased from Suzhou Ruihong Electronic Chemicals Co. Ltd., China. CH$_3$O(C$_2$H$_4$O)$_n$–9(CH$_2$)$_3$Si(OCH$_3$)$_3$ [M-PEG-Si(OMet)$_3$] was a product of Gelest Inc., USA. Thio-glycolic acid, phalloidin–TRITC, 4',6-diamidino-2-phenylindole (DAPI) and mouse monoclonal antivinculin (primary antibody) were from Sigma-Aldrich Co., USA. Minimum essential medium-alpha (MEM-a, Gibco®), trypsin–EDTA (Gibco®), and Alexa Fluor 488-conjugated goat anti-mouse IgG (secondary antibody, Invitrogen™) were bought from Life Technologies Co., USA. Fetal bovine serum (FBS) was purchased from Biochrom AG, Germany. Phosphate buffer saline (PBS, pH 7.4, HyClone™) was from Thermo Fisher Scientific Inc., USA. All the other chemicals were of analytical grade and used in the experiments without further purification treatment. Milli-Q water was supplied by Milli-Q Integral 5 (Merck Millipore, Germany).

Preparation of gold micropatterns

Gold micropatterns on glass substrates were prepared via photolithography. Glass slides were pretreated first, including cleaning by piranha solution (volume ratio of H$_2$SO$_4$–H$_2$O$_2$ 3 : 1) for 30 minutes (caution: operator should be really careful of the violent reaction), washing by Milli-Q water three times, drying by nitrogen stream, baking at 100 °C for 30 minutes to remove water completely, and cooling down to room temperature. Then the slides were spin-coated with the positive photoresist (eight droplets were added dropwise on slides spinning at a rate of 3500 rpm for 20 seconds), baked at 100 °C for 20 min to dehydrate glass, exposed under ultraviolet through a predesigned mask (around 25 seconds), developed by a corresponding developer, washed with Milli-Q water and baked again at 120 °C for 30 minutes. After the slides were cooled down to room temperature, they were ion-sputtered with chromium and then gold, as shown in Fig. 1(a).

Preparation of HAp micropatterns

Three technical routes were put forward in this study to prepare HAp micropatterns. Sample-1 was prepared according to the
procedure presented in Fig. 1(b). The positive photoresist on glass was removed via acetone under ultrasonic treatment (pre-lift-off). Then, the glass substrates were immersed in mSBF at room temperature for 2 hours. The mSBF used by us is composed of Na\(^+\) 400.0 mmol L\(^{-1}\), K\(^+\) 2.0 mmol L\(^{-1}\), Ca\(^{2+}\) 10.0 mmol L\(^{-1}\), Mg\(^{2+}\) 2.0 mmol L\(^{-1}\), and PO\(_4^{3-}\) 4.0 mmol L\(^{-1}\) with pH 4.0–5.0. Next, NaHCO\(_3\) powders were added into mSBF to adjust the pH to around 6.0–7.0 to trigger the HAp deposition. The incubation lasted for 12 hours at 37 °C. Finally, the glass slides were rinsed with Milli-Q water and desiccated in a vacuum dryer.

The preparation of Sample-2 is similar to the preparation procedure of Sample-1, but before immersing the glass substrates in mSBF, samples were incubated in a thioglycolic acid solution (2 mmol L\(^{-1}\)) for 4 hours, as shown in Fig. 1(c).

The preparation of Sample-3 is schematically presented in Fig. 1(d). The glass slides were immersed in thioglycolic acid solution first, then immersed in mSBF, and later triggered with NaHCO\(_3\) powders to initiate HAp precipitation. Afterwards, the positive photoresist on glass was post-lift-off, and the samples were rinsed with Milli-Q water and desiccated in a vacuum dryer.

**Characterization**

After gold was sputtered on samples using an Ion Sputter (E-1045, Hitachi High-Technologies Co., Japan), the surface topographies and elemental compositions of the HAp micropattern were characterized by field-emission scanning electron microscopy (FE-SEM, S4800, Hitachi High-Technologies Co., Japan) in conjunction with energy dispersive X-ray spectroscopy (EDS, QUANTAX 400, Bruker Co., Germany). HAp scratched off from the glass slides was further characterized via X-ray diffraction (XRD, D8 ADVANCE, Bruker Co., Germany), Raman spectroscopy (LabRam-1B, HORIBA Jobin Yvon Ltd., France), Fourier transform infrared spectroscopy (FT-IR, Nicolet 6700, Thermo Fisher Scientific Inc., USA), and analyzed by field-emission transmission electron microscopy (FE-TEM, JEM-2100F, JEOL Ltd., Japan).

**Cell culture on HAp micropatterns with a PEG-passivated background**

A mouse osteoblastic cell line MC3T3 was purchased from the Shanghai Cell Bank in China. MC3T3 cells were cultured in culture medium composed of 90% MEM-z supplemented with 10% FBS in the incubator at 37 °C with 5% CO\(_2\) and 95% humidity. Upon reaching about 90% confluence, cells were detached using trypsin–EDTA and delivered to the next passage.

Before cell seeding, the glass background of the HAp micropattern was covalently grafted with a monolayer of M-PEG-Si(OMet)\(_3\) to prevent non-specific cell adhesion.\(^{13}\) Briefly, HAp micropatterns were pretreated with oxygen plasma (DT-03, Suzhou OPS Plasma Technology Co. Ltd, Jiangsu, China) for 15 minutes, then they were immersed in M-PEG-Si(OMet)\(_3\) solution (6 mmol L\(^{-1}\) in dehydrated toluene, triethylamine as the catalyst). The reaction was kept in a nitrogen atmosphere at 60 °C for 12 hours. Later, HAp micropatterns were fully rinsed with toluene and then ethanol to remove residual reagents.

The HAp micropattern with a PEG-passivated glass background was sterilized by 70% (v/v) ethanol solution for 30 minutes, replaced by PBS and then MEM-z each for 5 minutes three times. Cells were seeded at a density of 10 000 cells per cm\(^2\). After cells were seeded for 2 hours, the culture medium was refreshed. The cells were then cultured for 24 hours.

**Observation of cell adhesion on HAp microislands**

After 24 hour culture, F-actins, vinculins and nuclei of cells were fluorescently stained. Briefly, cells on HAp micropatterns were thoroughly rinsed with PBS, fixed with 4% paraformaldehyde at room temperature for 15 minutes and rinsed with PBS for 5 minutes three times. Later, cells were permeabilized with 0.1% Triton X-100 solution for 5 minutes and rinsed with PBS for 5 minutes three times. Next, vinculin was stained first. Cells were incubated in 1% primary antibody solution overnight at 4 °C, rinsed with PBS for 5 minutes three times, then incubated in 1% secondary antibody solution at room temperature for 2 hours, rinsed with PBS for 5 minutes three times again. Afterwards, F-actin was stained. Cells were incubated in 1 mg mL\(^{-1}\) phalloidin–TRITC solution at room temperature for 30 minutes, rinsed with PBS for 5 minutes three times. Finally, the nucleus was stained. Cells were incubated in 2 mg mL\(^{-1}\) DAPI solution for 10 minutes and thoroughly rinsed with Milli-Q water several times.

The stained cells were observed under an inverted microscope (Axiovert 200, Zeiss, Germany). The bright-field and fluorescence micrographs were captured using a digital CCD camera (AxioCam HRC, Zeiss, Germany).

**Results and discussion**

**Preparation and observation of HAp micropatterns**

The preparation procedures of three samples of HAp on glass are schematically presented in Fig. 1. In the photolithography process, clean glass slides were spin-coated with a positive photoresist, baked, exposed under ultraviolet through a predesigned mask, and developed. Later, glass slides were ion-sputtered with chromium and then gold. Here we introduced chromium between glass and gold, because an integrating layer of chromium is helpful for avoiding the detachment of gold off glass.\(^{14}\) It was also confirmed in this study (data not shown).

In the preparation procedure of Sample-1, the photoresist was pre-lift-off, then glass slides were incubated in mSBF. After that, the HAp deposition process was triggered by the addition of NaHCO\(_3\) into mSBF; this process lasted for 12 h. HAp was found to be deposited sparsely. In the preparation procedure of Sample-2, the photoresist was pre-lift-off, then glass slides were thiolated with thioglycolic acid on gold and later incubated in mSBF. After that, the process of HAp deposition was triggered by the addition of NaHCO\(_3\) into mSBF. After 12 h, HAp was deposited densely. For the preparation of Sample-3, glass slides were thiolated with thioglycolic acid on gold surfaces and then incubated in mSBF. Next, the HAp deposition process was triggered by NaHCO\(_3\). After 12 h, the photoresist was post-lift-off. HAp was deposited densely in pre-defined microdomains in a form of a regular microarray.
FE-SEM images of the three samples are shown in Fig. 2. HAp was precipitated on both gold and glass regions, and more HAp was precipitated on gold than on glass in Sample-1, as shown in Fig. 2(a). However, HAp was not precipitated densely on gold microislands. In the preparation of Sample-2, negative surface charges on gold microislands were introduced by treatment with thioglycolic acid based on thiol–gold bonds.48 Then, HAp was densely precipitated, as shown in Fig. 2(b). However, the dense HAp layer not only precipitated on gold microislands but also on the glass background.

The process of HAp precipitation from SBF includes mainly initial HAp nucleation and HAp crystal formation. In the preparation of both Sample-1 and Sample-2, the initial nucleation was affected by surface roughness. Homogeneous nucleation took place in the mSBF solution, and some nucleation centers might be adsorbed on the gold microisland and the glass substrate. We found that the surface roughness of the gold microisland was larger than that of the glass background, as confirmed by our atomic force microscopy measurements with data shown in Fig. S1 in the ESI.† As a result, heterogeneous nucleation on surfaces was easier on the gold microisland than on the glass substrate, and more HAp was precipitated on the gold than on the glass in Sample-1. However, HAp was not densely precipitated, and the selectivity on the regions of gold microislands and the glass substrate was not good, as shown in Fig. 2(a). Surface charges significantly influence heterogeneous nucleation on surfaces, so that one can greatly enhance the HAp formation on surfaces in SBF.25,26,46 Due to the negative surface charge in the preparation of Sample-2, the enhancement of HAp precipitation was significant, not only on gold microislands but also on the glass background, as shown in Fig. 2(b). The unsatisfying contrast might be attributed to non-specific physical adsorption of thioglycolic acid on the glass as well as on the gold, besides the thiol–gold bonding on the gold microislands. So, the introduction of surface grafting of thioglycolic acid enhanced the HAp precipitation just in an uncontrollable way.

Fig. 2 FE-SEM images of (a) Sample-1, (b) Sample-2 and (c) Sample-3 (HAp micropattern). The left column shows low-magnification images of the three samples. The electron beam scanned from left to right in imaging. The right column shows the high-magnification images of the squared areas indicated by the dashed lines in the left column.
A photoresist can shield and thus protect the background in gold sputtering, and plays an important role in making the final regular gold microarray. So we further applied such a protection in the preparation process, as schematically presented in Fig. 1(d). Here, the photoresist was not lifted-off until completion of the HAp precipitation, and we thus called it “post-lift-off” in order to distinguish it from the conventional lift-off, which is called “pre-lift-off” in this study as indicated in Fig. 1. Besides, thioglycolic acid was still used to enhance HAp precipitation. Finally, after the post-lift-off operation, Sample-3 (HAp micropattern) was successfully prepared. HAp was precipitated densely and precisely on gold microislands, as demonstrated in Fig. 2(c). The thickness of the HAp layer was approximately 600 nm, according to the FE-SEM observation of the cross-section of the HAp micropattern (image not shown).

Hence, Sample-1 had less-dense HAp microislands and an unclean background; Sample-2 achieved dense HAp, yet no significant contrast of surface HAp between microislands and their background; Sample-3 exhibited dense HAp microislands on a clean background, as illustrated in Fig. 2. Based on different pattern designing in masks used in photolithography, it is easy to design HAp micropatterns of different microisland sizes and shapes. Some experimental results are demonstrated in Fig. 3.

Composition analysis of HAp microlayers

For the HAp micropattern, the element compositions of HAp on gold microislands were analyzed by EDS. Characteristic X-rays of calcium and phosphorous elements are shown in Fig. 4(a). Although the EDS detector was focused upon the HAp microislands, the signals of gold and silicon elements were also detected due to the penetration of X-rays through the HAp coating. The XRD pattern in Fig. 4(b) shows peaks of 2θ at 25.9° and 32.9°, the characteristic peaks of HAp. Compared to the single crystal of HAp through high temperature sintering, these peaks were not very sharp, indicating the relatively low crystallinity. The Raman spectrum shown in Fig. 4(c) demonstrates peaks of symmetric stretching of the phosphate group in the region of 950–960 cm⁻¹ representing the crystalline HAp, and also corresponding antisymmetric stretching in 1040–1090 cm⁻¹, and
bending in 430–460 cm\(^{-1}\) and 575–610 cm\(^{-1}\). In the FT-IR spectrum shown in Fig. 2(d), bands at around 562, 602 and 1034 cm\(^{-1}\) are attributed to bending of the phosphate group.\(^9\)

HAp grains scratched from the micropatterns were also observed by TEM. A typical FE-TEM image is shown in Fig. 5(a). A high-resolution FE-TEM image of a single HAp grain is shown in Fig. 5(b), in which the interplanar spacings correspond to the crystalline lattice planes (100) and (002). In summary, the HAp micropattern was composed of numerous HAp grains of relatively low crystallinity aggregated together to form HAp microislands on the background of glass.

![Fig. 5](image_url) (a) FE-TEM image and (b) high-resolution FE-TEM image of the HAp layer on the micropattern.

![Fig. 6](image_url) Schematic graph of a regular HAp microarray with a non-fouling background and cell adhesion on the HAp micropattern.

![Fig. 7](image_url) Optical micrographs of MC3T3 cells on the HAp micropattern. (a) Bright-field image of an HAp microarray on a background of the passivated glass. (b) Fluorescence image of cells on the HAp micropattern with F-actin (red), vinculin (green) and nucleus (blue) stains. The dashed lines indicate the borders of the microislands. (c) High-magnification fluorescence images of cells on an HAp microisland.
Cell adhesion on HAp micropatterns

We further examined cell adhesion on HAp micropatterns. Before cells were seeded, the glass surface was passivated with a linear poly(ethylene glycol) monolayer to make a non-fouling background, which can prevent cell adhesion as illustrated in Fig. 6. Briefly, the HAp micropattern was treated by oxygen plasma; then the glass background was covalentlygrafted with M-PEG-Si(OMe)3. PEG is confirmed to be an ideal material to realize potent and persistent cell-adhesion resistance, and it has been reported in studies of other kinds of micropatterns that cells can be well localized on adhesive microislands with PEG as the background.4–9

A mouse osteoblastic cell line MC3T3 was employed to investigate the cell adhesion on the micropattern. After culturing of cells on the HAp micropattern with the non-fouling background for 24 hours, the F-actins, vinculins and nuclei of the cells were fluorescently stained. The stained cells were then observed using an inverted optical microscope. It was confirmed that cells adhered just on the HAp microislands, as illustrated in Fig. 7.

Most mammalian cells are adhesion dependent. Cell adhesion is, in many cases, the first biological event of adhesion-dependent cells after seeding on biomaterials. The initial adhesion influences the following cell behaviors such as cell migration, proliferation, and differentiation. In order to elucidate the cell-biomaterial interactions, it is important to precisely control cell adhesion. Through designing different mask patterns in photolithography, one can achieve regular HAp microislands of different shapes, areas and spatial arrangements to control cell adhesion. If micropatterns on a nonfouling background can be fabricated to localize cells, many factors could be excluded or decoupled such as the cell shape, cell density and soluble factors, as reviewed very recently mainly based upon surface patterning.1 The successful fabrication of HAp micropatterns in this study might stimulate further fundamental research of interactions between cells and inorganic biomaterials. Further adjusting the HAp preparation conditions to obtain different HAp crystal sizes, morphologies and crystallinities, one can investigate many more factors that influence cells on bioceramics. Therefore, taking advantage of micropatterning to decouple various geometric and chemical cues, interactions between cells and HAp might be elucidated unambiguously.

Conclusions

We successfully developed a methodology which combines post-lift-off photolithography, surface grafting, and mSBF incubation to prepare HAp micropatterns. Mammal cells were well localized on the HAp microisland with the non-fouling background passivated by PEG. Since micropatterns with precise microisland shapes and spatial arrangements can be easily fabricated via photolithography, the present methodology paves a way to investigate interactions between cells and bioceramics in a controlled way.

Acknowledgements

The authors are grateful for the financial support from the NSF of China (grants no. 21034002, no. 91127028, and no. 51273046), the Chinese Ministry of Science and Technology (973 program no. 2011CB606203), and the Science and Technology Developing Foundation of Shanghai (grant no. 13XD1401000).

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