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ScienceDirect
Nuclear Instruments and Methods in Physics Research B 260 (2007) 130–135
NIM B
Beam Interactions with Materials & Atoms
www.elsevier.com/locate/nimb

Growth of osteoblasts on lithographically modified surfaces

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Available online 9 February 2007

Abstract

Here we report about preliminary investigations on developing substrates for culturing osteoblasts, the cells responsible for production of mineralised bone, by lithographically modifying the surfaces of several materials. The proton beam writing system at the National University of Singapore was used to fabricate high aspect ratio structures in PMMA, while two-dimensional low aspect ratio structures were fabricated using conventional electron beam lithography (EBL) and UV lithography (UVL) in SU-8. It was found that oxygen plasma treatment of structured SU-8 surfaces changed the surface layer and significantly improved cell attachment and proliferation. Cells grown on patterned thick PMMA exhibit a remarkable geometry-dependent behaviour.

PACS: 06.60.Ei; 68.37.Hk; 81.15.−z; 81.65.Cf; 87.80.Rb

Keywords: Osteoblast; Bone; Cell growth; Proton beam writing; SU-8; PMMA; Ion-sputtering; Surface treatment

1. Introduction

Bone is natural composite material comprising cells (mainly osteoblasts, osteoclasts and osteocytes) in a mineralized matrix made up mostly of hydroxyapatite and type-I collagen. The matrix has features with sizes ranging from the nanometre striations on collagen [1], that are believed to be important for chemical signalling to trigger production of mineralized bone, to a 3D-random matrix on a macroscopic scale.

Micro and nanofabrication technology can be utilized to produce artificial cell growth substrates with modified surface topology [2–5] for basic studies of the bone development at the cellular and sub-cellular level. It has been shown that the surface geometry (both on nano and micrometre scales) influences osteoblast-like cells' behaviour such as attachment, profileration, orientation, migration, and function [6–9]. A major barrier to understanding bone physiology has been a lack of fabrication techniques for making suitable test structures and growth substrates with geometrically defined features on μm and nm scales. Conventional micro and nanometre scale lithography methods for surface modification used in previous studies (photolithography, colloidal lithography, etching, soft lithography, etc.) are generally limited to rather shallow low-aspect-ratio patterns in polymers and Si. The emerging MeV proton beam writing (PBW) technique [10] offers the possibility to write extremely high aspect-ratio patterns [11] to form 3D multi-layer microstructures [12,13] with vertical side walls and nanometre-scale dimensions [14,15]. Cell behaviour on substratum is not only governed by the surface topography, but also by the chemical properties of the surface. Cells perceive the surface chemistry of their environment via cell membrane-receptors in order to find suitable sites for attachment. If a suitable site possessing the correct chemical signals for attachment is not located, the cell may undergo apoptosis [16]. It follows that

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doi:10.1016/j.nimb.2007.02.008

bio-compatible scaffold and substrate materials must not only be non-toxic but must also present the correct chemical signalling sites for attachment. PMMA is a widely used bio-compatible polymer that is used clinically as a cement for bonding metal prosthesis to bone. SU-8 has been demonstrated to be bio-compatible for different cell types [17–19]. Other key requirements for bio-compatible substrate materials is that they can be sterilized and are stable under long-term exposure to the physiological environment. SU-8 is a convenient resist material for micromachining because it can be deposited in thick (5–100 μm) layers and exposed by long wavelength (360 nm) UV light as well as X-ray, electron and proton beams. However, the long-term stability of SU-8 in cell-culture media is questionable [17,20] as the SU-8 structures tended to lift-off from the substrate surface. In the preliminary investigations reported here we have studied the behaviour and proliferation of murine osteoblasts on micromachined surfaces of PMMA and SU-8 subjected to different lithographic and surface modifications.

2. Materials and methods

2.1. Fabrication of SU-8 samples

The substrates were glass cover slips that could be fitted into an environmental test chamber which enabled imaging in a confocal microscope of live cells during culturing. These were cleaned in chromic acid solution for 5–10 min, followed by intensive washing in deionised water and subsequently rinsed in *iso*-propyl alcohol (IPA), dried in flowing He and baked at 180 °C for 15 min immediately prior to resist-spinning. SU-8 2025 solution [22] was spin-coated at 1000 rpm for 15 s followed by 3200 rpm for 30 s to give a final thickness of 10 μm. Soft-baking was carried out before and after exposure by slowly ramping to 95 °C with subsequent slow cooling to room temperature. The negative pattern (20 μm × 500 μm lines with a 50 μm pitch) on a Ti/quartz glass mask was exposed by UV light contact lithography (UVL) and the patterns developed with SU-8 developer (1-methoxy-2-propyl-acetate solution) [22] for 4 min followed by rinsing in IPA. The optimal exposure time of 100 s was determined by calibration of the Karl Süss MA45 UVL system used.

Some SU-8 coated samples were exposed by electron beam lithography (EBL). In this case to overcome surface charging effects, Si wafers or evaporated 10 nm Ti-layer on glass cover-slip substrates were used. The patterns (500 μm × 20 μm parallel lines with 100 μm pitch) were written with a 2–160 μC cm^{-2} fluence range of 40 keV electrons and subsequently developed in the same manner as above. We also investigated the fabrication of channels bridged by a thin (about 1 μm) membrane by using 10 keV and 40 keV electrons (1.5 and 12 μm ranges in SU-8 [21], respectively), using the same method as PBW for writing multilayer 3D patterns [12,13]. After the development some of the SU-8 fabricated surfaces were treated with O_2 plasma (Plasmalab80Plus from Oxford Instruments, 50 sccm, 60 W, 40 mTorr, 60–90 s). One group of samples was immersed in sterile water and sealed in a sample box in order to overcome the need for an additional sterilization step.

2.2. PMMA Samples

To mimic the chemical signalling from hydroxyapatite, the glass substrates were first sputter-coated at a deposition rate of 0.7 nm s^{-1} to a 50 nm final thickness by Ar ion bombardment of a target of high grade hydroxyapatite powder [26] compressed uni-axially with 2000 N cm^{-2} into a pellet. The sputtering system [27], was operated with 6 kV accelerating voltage and discharge current of 1.5 A. The ion beam impinged on the HA pellet at 45° to the surface normal and the substrate to be coated was placed at 90° with respect to the incoming beam. Prior to the deposition of HA and PMMA, the glass substrates were cleaned as described above. PMMA resist was spin-coated 2500 rpm for 45 s from PMMA A11 solution [23] followed by a soft-bake at 180 °C for 5 min to yield a 3 μm layer. This spin-coating procedure was repeated three times to obtain a 9 μm final PMMA resist thickness.

The proton beam writing facility at the Centre for Ion Beam Applications, National University of Singapore, was used to fabricate channels in PMMA. Patterns of 200 μm long parallel channels of 400 nm or 10 μm width 20 μm pitch and were written using a 400 nm × 1 μm sized beam of 1 MeV protons. The proton fluence corresponded to 160 nC mm^{-2}. To minimise contamination during air transport from Singapore, the samples were developed in Jyväskylä. This was done using a IPA:de-ionized water (7:3) solution for 4–6 min, followed by a double rinse in de-ionized water and dried under helium flow.

In other experiments, a two-phase mixture of 5 wt.% HA powder [26] in PMMA C2 solution [23] was magnetically stirred for 24 h and subsequently spin-coated at 2500 rpm for 45 s onto glass substrates.

2.3. Cell culture

Prior to cell growth the samples were sterilized in 70% ethanol for 40 min. All reagents for the cell culture were from Invitrogen. Murine (mouse) osteoblasts from the MC–3T3 E1 [25] cell line were used. These were grown in a modified Eagle medium supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) in a humidified atmosphere with 5% CO_2 at 37 °C. At 70% confluence the cells were trypsinized and seeded at the density of 5×10^5 cells in 2 ml of culture medium on specified surfaces. After 24 h of cultivation the samples were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Thereafter the samples were permeabilised with 0.5% Triton X-100 in PBS for 5 min. After washing in PBS the actin

network of the cells were labelled with Alexa-488 phalloidin [28] (0.125 U/ml PBS) for 10 min. After extensive washing with PBS the samples were mounted in Mowiol (Calbiochem) including 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma Aldrich; Catalog No. D-2522). Stacks of images were collected with a scanning confocal microscope (Olympus FluoView FV1000) and processed with BioImageXD software [29,30].

3. Results and discussion

3.1. SU-8 Samples

Initial experiments revealed that cleaning in chromic acid was an essential step to avoid detachment of the SU-8 film from the glass substrate in the cell culture medium. Moreover, it was observed only a few living cells attached to the surfaces of SU-8 micromachined structures. It was found that brief RIE with O_2 plasma generally enhanced the adhesion of cells to the surface of these structures produced by UV lithography as illustrated in Fig. 1. The results of the experiments were characterized by a remarkably large variability and general irreproducibility. Cells grown on UV exposed patterns treated with plasma and kept in water did not show any different behaviour to those that were exposed to the plasma without water immersion. Different types of attachment and alignment of the cells with the channels can be seen in Fig. 1.

The results for EBL were quite similar to those exposed using UV light. For thick resists the electron scattering limited the width of line in 10 μm film thickness. Although fluences of 40 keV electrons as low as 2 μC/cm^2 could expose the resist, the structures at this fluence did not exhibit straight side walls. Higher fluences corresponding to 40 μC cm^{-2} were required to produce smooth patterns with nearly straight side walls (Fig. 2). Note the bridge with about 1.5 μm thickness written using 10 keV electrons in this figure. The significance to this is that by combining EBL with PBW narrow channel structures and reservoir volumes that are embedded by a thin (~1 μm) membrane, can be directly written in SU-8 and processed in a single development step, without the need to seal the structure by bonding. This opens a route to produce integrated nanofluidic and mechanical actuator/sensor structures without the need to use low energy (50–100 keV) protons or switch to heavy ions, albeit with the expense of an extra alignment step. Osteoblast-cells did not exhibit preferential growth inside such embedded channels, although they proliferated actively on the Si and Ti substrate surface regions around the patterns. Preferential attachment inside the channels can probably be achieved by internal surface coating, e.g. by collagen.

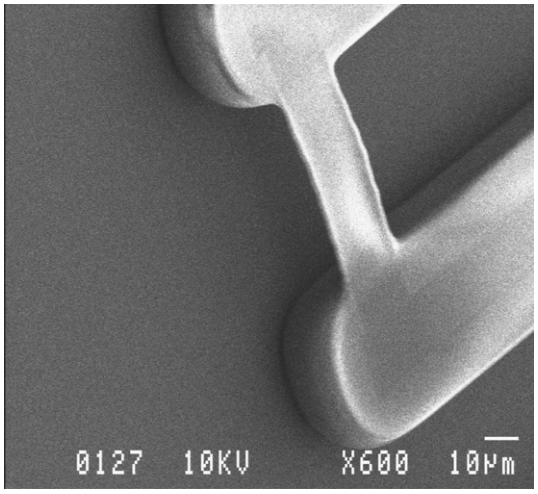

Fig. 2. Pattern written by EBL in SU-8 using 40 keV and 10 keV electrons illustrating the 3D writing capabilities. The membrane bridge, written using 10 keV electrons, is about 1.5 μm thick.

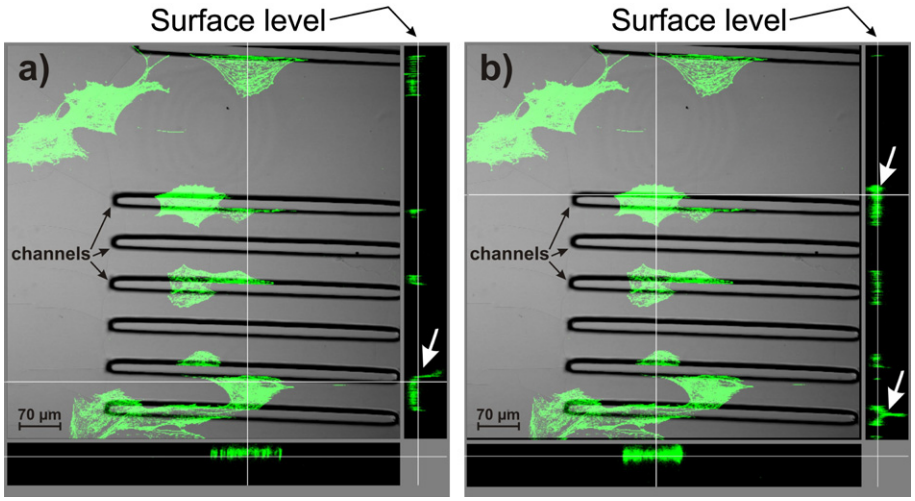

Fig. 1. Plane view of fluorescence confocal microscope images and side view image projections of osteoblasts cultured on SU-8 surfaces that were patterned with 20 μm channels with 50 μm pitch using UV lithography: (a) the arrow points at the attachment of a cell on the channel wall and (b) the arrows point at the cells growing across the channel (top) and inside the channel (bottom).

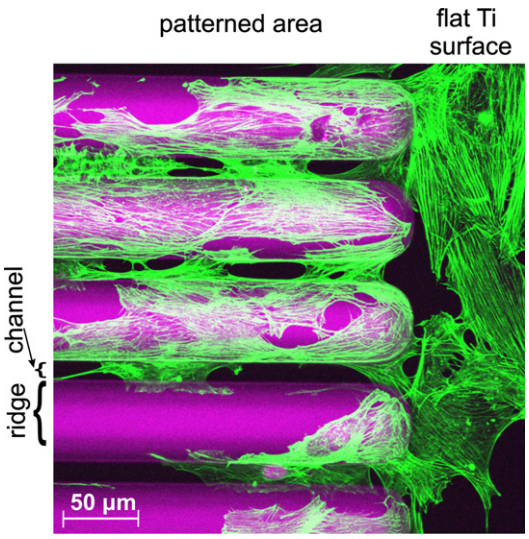

Fig. 3. Osteoblasts cultured on EBL patterned SU-8 on Ti-coated glass substrate. Actively proliferating cells can be seen on the Ti surface (right). The cells growing on the SU-8 patterns organized their cytoskeleton longitudinally (left). The osteoblasts behave similarly on Si substrates, however, their growth activity is reduced compared with Ti surfaces.

On EBL-patterned SU-8 samples, that were kept in autoclaved water after the plasma treatment, the cells behaved differently from cells on structures patterned with UV light. The cells were observed to grow normally on the Ti and Si surfaces outside of the patterned area (Fig. 3). On the basis of the UV lithography results presented above, we expected the cells to grow on the bottom of the open channels where Ti and Si surfaces can be found. Remarkably, we observed that the cells preferred to attach to the ridge regions of the SU-8 channel structures and their walls. When a migrating cell encounters a tall SU-8 structure, it generally established a focal attachment point on the structure and grows up the wall with a clear tendency to reach the upper structure's surface. Some cells attached on ridges forming long extensions that extended along the wall, channel bottom, and continued on the wall of a neighbouring ridge. Such behaviour indicated that topography is an important signal for cell migration and attachment. Moreover, inspection of Fig. 3 reveals that the osteoblast cytoskeleton is organized in such a way that it extends along the ridges. The different cell behaviour for EBL and UVL exposed surfaces might be explained by the fact that on

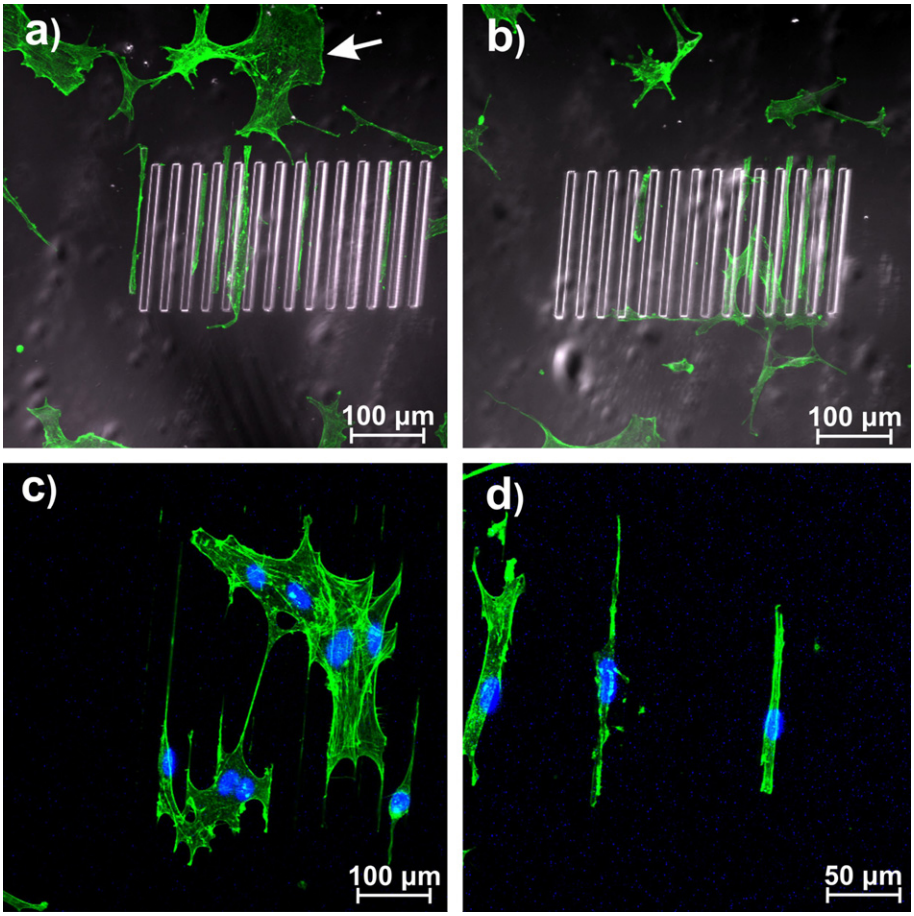

Fig. 4. Fluorescence confocal microscope images of murine osteoblast cell cultured on PBW-machined 9 μm PMMA/50 nm sputtered-HA/glass substrates. (a–b) Wide channels (an arrow points at a typical cell growing on smooth PMMA surface). (c–d) Narrow channels. Blue denotes cell nuclei. (For interpretation of the references to colour this figure legend, the reader is referred to the web version of this article.)

UVL samples the substrate surface was mainly SU-8 with channels machined in it, while for EBL samples most of the surface was Ti, which is highly biocompatible. Similar results were also obtained when the majority of the exposed substrate was Si.

3.2. PMMA Samples

Examination in the confocal microscope of osteoblast cells' focal contact points (data not shown) cultured on the HA/PMMA mixture showed preferential formation of focal attachment points to the HA grains indicating this substance provides chemical signals promoting formation of focal attachment points. Fig. 4 shows osteoblast cells cultured on 9 μm PMMA/50 nm sputtered-hydroxyapatite/glass with wide (10 μm) and narrow (~400 nm) channel structures produced by PBW. It should be born in mind that the sputtered HA films are non-crystalline and their composition depends on the sputtering conditions [24]. The confocal microscope images revealed the patterned regions seen in the Fig. 4(a) and (b) were slightly higher (about 1 μm) than the surrounding region. This effect may be attributed to dissolution of the sputtered HA causing local lift-off of the PMMA in the cell culture media. Different types of cell growth could be directly observed on the substrates. In the upper part of the Fig. 4(a) and (b) the cell growth is typical for cells on smooth PMMA surfaces. Inside the patterned area with wide (10 μm) channels the cells grow along the ridges. The osteoblast cytoskeleton is organized longitudinally along the ridges (not shown). In contrast, for the narrow (~400 nm) channels the osteoblasts grow above the channels with pronounced filopodia extending inside and along the channels (Fig. 4(c) and (d)). It may be seen that the cytoskeleton of the cell body, excluding the filopodia, is characteristic of osteoblasts growth on a flat PMMA surface. A direct comparison between the results shown on Fig. 4(a)–(d) can be made because the cells were grown simultaneously in the same culture medium. The implication of the difference described above is that topography on μm and nm scales has different effect on osteoblast migration and cytoskeleton organization. Although no direct comparison between the results for SU-8 (Fig. 3) and PMMA/sputtered-HA (Fig. 4) can be made, it is noticeable that for wide-channel structures the behaviour of the osteoblasts is quite similar in both cases with cytoskeleton organized along the ridges. This again suggests that surface topography is an important signal governing osteoblast migration and attachment.

4. Conclusions

Two important conclusions can be derived from this preliminary study. Firstly, SU-8 can be used as a substrate material for osteoblast growth if the basic surface of the substrate is made-up of a biocompatible material (such as Ti or Si), the samples are exposed to O_2 plasma and followed by storage in a sterile aqueous environment. Secondly, structures on μm and nm scales govern osteoblast attachment, migration and cytoskeleton organization in remarkably different ways.

Acknowledgements

This work has been carried out under the auspices of the Academy of Finland Centre of Excellence in Nuclear and Accelerator Based Physics (Ref. 213503) and the BOGOTAS consortium. A.S.A.R., T.S., and H.J.W. are grateful for travel support from Magnus Ehrnrooth's Foundation, and L.B.K. for the Swedish Natural Science Research Council. Professor Frank Watt and Dr. Dan White are warmly thanked for helpful discussions and advice.

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