

Focused ion beam/scanning electron microscopy characterization of cell behavior on polymer micro-/nanopatterned substrates: A study of cell–substrate interactions

E. Martinez ^{a,*}, E. Engel ^b, C. Lopez-Iglesias ^c, C. A. Mills ^b, J. A. Planell ^b, J. Samitier ^b

^a Nanotechnology Platform, Barcelona Science Park, c/ Josep Samitier 1-5, 08028 Barcelona, Spain

^b Nanobioengineering Research Laboratory, Barcelona Science Park, c/ Josep Samitier 1-5, 08028 Barcelona, Spain

^c Scientific Technical Services, University of Barcelona, Barcelona Science Park, c/ Josep Samitier 1-5, 08028 Barcelona, Spain

Abstract

Topographic micro and nanostructures can play an interesting role in cell behaviour when cells are cultured on these kinds of patterned substrates. It is especially relevant to investigate the influence of the nanometric dimensions topographic features on cell morphology, proliferation, migration and differentiation. To this end, some of the most recent fabrication technologies, developed for the microelectronics industry, can be used to produce well-defined micro and nanopatterns on biocompatible polymer substrates. In this work, osteoblast-like cells are grown on poly(methyl methacrylate) substrates patterned by nanoimprint lithography techniques. Examination of the cell–substrate interface can reveal important details about the cell morphology and the distribution of the focal contacts on the substrate surface. For this purpose, a combination of focused ion beam milling and scanning electron microscopy techniques has been used to image the cell–substrate interface. This technique, if applied to samples prepared by freeze-drying methods, allows high-resolution imaging of cross-sections through the cell and the substrate, where the interactions

between the nanopatterned substrate, the cell and the extracellular matrix, which are normally hidden by the bulk of the cell, can be studied.

Keywords: Electron microscopy; Interface; Nanotopography; Osteoblast; Adhesion molecule; Cell morphology

1. Introduction

Knowledge of cell–substrate interactions is nowadays recognised as a crucial factor for understanding the phenomena of biocompatibility, which is a key consideration when developing medical implants or tissue engineering strategies. The influence of topographic structures, in the absence of any immobilised biochemical compounds, has been reported as having significant effects on the cell behaviour. For instance, contact guidance phenomena have been observed when growing cells on micropatterned substrates (Curtis and Wilkinson, 2001; Wilkinson, 1995). Other morphological and conformational changes such as cell elongation or cell polarization have also been described and are summarized in reference (Flemming et al., 1999). From these studies, the effects of substrate roughness and topography on cell adhesion, proliferation, morphology and migration have already been confirmed. On the other hand, with respect to cell differentiation, it has been demonstrated that certain substrate micro-roughness leads to an increase in the rate of cell differentiation (Boyan et al., 2002; Aparicio et al., 2002). Controlling surface topography to promote cell differentiation processes is, therefore, a promising area of research for tissue engineering applications.

When considering the relationship between the size of the topographical patterns and their effects on the cell behaviour, it should be kept in mind that cells are surrounded in their natural environment by nanometric structure through the presence of other cells and extra-cellular matrix (ECM) components. Therefore, it is especially interesting to study the effect of nanoscale topographic

structures on cell behaviour. Indeed, recent studies support the hypothesis that mammalian cells respond to nanoscale features on artificial surfaces (Dalby et al., 2002, 2004; Andersson et al., 2003; Miller et al., 2004; Calvacanti-Adam et al., 2006).

The advances in microelectronics fabrication methods have provided novel techniques for the manufacturing of substrates for cell culturing with well-defined dimensions in the nanometer range. In particular, some of the nanopatterning technologies, originally designed for use with silicon-based materials, can be applied to polymeric materials, some of which have already proven to be useful in biomedical applications. One such technique for nanostructured surface fabrication in polymers is nanoimprint lithography (NIL) (Chou et al., 1995), that has been used to produce structures with lateral resolution better than 10 nm in poly(methyl methacrylate) (PMMA) (Cao et al., 2002). PMMA is a common polymer used in micro and nanofabrication; for example in microelectronics as an electron beam lithography resist or in microfluidics as a substrate material, and has also been shown to be compatible with respect to cell culturing methods (Clark et al., 1990; Wirth et al., in press; Yim et al., 2005).

Another microelectronics fabrication technique is focused ion beam (FIB) lithography. This technique has already proven its applicability in the characterisation of biological samples, as it has previously been used to prepare thin sample lamellae for transmission electron microscopy (TEM) observation (Obst et al., 2005; Kamino et al., 2004). A combination of FIB milling and SEM observation in a dual beam apparatus makes in situ imaging of sample cross-sections possible in well-defined areas. A combination of complementary information, gathered by classical secondary electron images (obtained from the interaction of the sample with a primary electron beam) and novel ion images (obtained from the interaction of the sample with a primary ion beam), can be obtained. Initial results of the combination of these techniques can be found in a recent study of the imaging of cross-sectional views of biological specimens (Drobne et al., 2005). However, to our knowledge, the combination of FIB and SEM techniques has not been applied to the study of cell–substrate

interfaces. When applied to cells cultured on nanostructured substrates, the combined FIB/SEM technique provides in situ high-resolution imaging of the nanopatterned surface, the cell and the extra-cellular matrix, while, at the same time, it makes sectioned cell samples available for post-modification analysis by other techniques, such as traditional SEM or atomic force microscopy (AFM).

However, in order to obtain faithful results using these techniques, high quality sample preparation procedures are essential. Specifically for the study of the cell–substrate interactions, it is very important that the sample preparation method assures the preservation of both the cell and nanopatterned substrate morphologies, as well as preserving as much as possible the structure of the extra-cellular matrix. Sample preparation should minimize stress to the cell and the substrate in order to avoid cell or nanostructure shrinkage, deformation and/or cell fracture or detachment. Deposited metal or carbon layers with thickness in the range of tens of nanometers, commonly used to aid imaging of samples, should be avoided or minimized to ensure that they do not mask the real topography of the cell surface (unless, in this work, localized platinum coating of the sample has been used for cross-sectioning purposes). Here, a freeze-drying sample preparation technique has been applied to the cell–substrate ensemble after basic chemical sample fixation. We then apply novel, combined FIB/SEM techniques for the characterisation osteoblast cells grown on PMMA micro- and nanopatterned substrates. The cell adhesion, cell morphology and cell-substrate interactions have been characterized.

2. Experimental

2.1. PMMA micro-/nanopatterned substrates by nanoimprinting

PMMA (125 μm thick) was purchased from Goodfellow (Goodfellow, UK) and used as received. A nanoimprint lithography technique was used to fabricate free-standing PMMA samples with surface features with lateral dimensions ranging from 10 μm to 200 nm, following a previously reported hot

embossing procedure (Mills et al., 2005). Briefly, this technique uses a micro-/nanostructured silicon mould, covered by a grown silicon oxide and silicon nitride double layer, which is pressed into the bulk PMMA polymer at a temperature of 130 °C and pressure of 30 bar. The moulds were previously treated with a fluoroalkylsilane, (trichloro(tridecafluoro-octyl)- silane, for anti-adhesion purposes, so they can be reused after demoulding. After the nanoimprinting procedure, and prior to cell culturing, the transparent, free-standing PMMA samples were sterilized by gamma radiation (25 kDynes).

2.2. Osteoblast-like (MG63 line) cell culture

MG63 cells were obtained from ATCC (UK). To culture them on the micro-/nanopatterned PMMA substrates, cells were seeded with Dubelco's Modified Eagle Medium (DMEM), supplemented with penicillin/streptomycin, sodium pyruvate, L-Glutamin and 10% fetal calf serum (FCS) (all from Invitrogen, CA, USA). Fresh medium was replenished every day for 4 days.

2.3. Sample preparation for FIB/SEM analysis

Two kinds of sample preparation for the FIB/SEM sample imaging were attempted. Firstly, samples were prepared by conventional critical point drying procedures. The complete protocol involves: chemical cell fixation by 2.5% glutaraldehyde solution, post-fixation in 1% OsO₄ solution, cell dehydration at room temperature via immersion in increasing concentrations of ethanol and finally critical point cell drying in liquid CO₂.

The second sample preparation technique is based on freeze-fixation and freeze-drying in high vacuum, adapting previous knowledge of transmission electron microscopy cryotechniques whereby the conditions required to vitrify the cell water without forming ice crystals are known. This freeze-drying protocol includes chemical cell fixation by 2.5% glutaraldehyde solution before freeze fixation by impacting the sample against a polished copper block cooled by liquid nitrogen using a Cryovacublock (Leica, Vienna). Finally, the sample is freeze-dried at approx. 90 °C and 10⁻⁸ mbar

pressure in a freeze-etching apparatus, BAF 060 from BalTec (Liechtenstein). The result is a complete removal of the water from the cell at temperatures lower than the ones required for water recrystallisation (formation of new ice crystals) in cells. Finally, the freeze-dried samples were coated by rotatory shadowing (136 rpm) at 10^{-8} mbar and approx. 90 °C with a 1-2 nm thick layer of platinum in order to make them conductive.

2.4. FIB/SEM cross-sectioning and imaging

Dual beam FIB/SEM apparatus (DB Strata 235 FIB, FEI Company, The Netherlands) has been used for the sectioning and imaging of the cells immobilised on the polymer substrates. Milling of the cells was achieved using gallium ions accelerated at 30 kV allowing us to perform in-situ cross-sectional analysis of the cells and their interactions with the structured surface. Imaging could be completed using either the ion beam or a high resolution, field-emission scanning electron microscope. The electron and ion beams are mounted at an angle of 52° to each other and are confocal at a fixed sample working distance (5 mm from the SEM column) (Fig. 1). In order to enhance milling efficiency, the sample is tilted 52° so that the gallium ions impact perpendicularly to the substrate while electrons arrive at an angle (52°) to the same point. This configuration allows us to image the sample with the electron or the ion beams, by measuring secondary electron emission. In order to avoid electrical charging of the sample surface, the electrons were accelerated to the target using a low voltage (from 1 to 5 kV).

After choosing a suitable area of the cell to modify, via SEM imaging, the FIB milling of cross-sections of the cell involves a number of steps: (1) In situ ion-beam assisted deposition of a thin platinum layer (approx. 50 nm), which serves as a protective coating for the cell to preserve its surface from the ion milling (Fig. 2a). (2) Rough milling of the bulk material close to the desired observation area using a high ion beam current (3000 pA) (Fig. 2b). This procedure takes approx. 20 min to mill a 10 µm x 10 µm region of 1 µm depth. (3) Fine polishing of the cross-section by successively decreasing the current of the ion beam (from 300 to 30 pA) (Fig. 2c). (4) Cross-section imaging of the modified

area using the electron beam (Fig. 2d). Depending on the size of the region to be characterized, this procedure can take between 30 min and several hours.

In order to improve the milling efficiency of the gallium ion beam, the milling process was carried out while injecting water vapour to the sample through a gas needle (FEI Company, The Netherlands).

3. Results and discussion

3.1. Influence of sample preparation on the FIB/SEM characterization

The low resistivity of PMMA to alcohol exposure in comparison with glass or other hard substrates, caused samples prepared by conventional critical point drying methods showed damage to the micro-/nanostructures. Cell morphology and extra-cellular matrix (ECM) preservation appeared to be acceptable, but the drying method caused very high stress levels in the polymer substrates, causing them to bend which consequently caused the detachment and fracture of the cells immobilised on the surface.

In comparison, the freeze-drying method was successfully applied to the cell-PMMA substrate ensemble. The cell, extracellular matrix and substrate preservation was excellent, as can be seen in Fig. 3, where 200 nm wide structures have been perfectly preserved in a polymer as chemically sensitive as PMMA. The ultra-thin platinum layer (less than 1 nm), deposited during the sample preparation, was thick enough to avoid sample charging when imaging. On the other hand, it was thin enough to avoid masking of the fine details of the cells, such as the very thin phillipodia, protein fibrils, other nonsoluble components and the nanostructures.

The SEM images in Fig. 3 show the elongation and alignment of the MG63 cells cultured on 200 nm wide lines fabricated in PMMA. The cells appeared to have a globular shape with a higher protuberance in the region of the nucleus. The phillipodia extended by the cell on the polymer

surface seem to preferably attach to the edges of the pattern, a phenomenon that has been observed previously (den Braber et al., 1998).

3.2. Cell–surface interaction characterisation by FIB cross-section measurements

Fig. 4 shows the cross-sectional image of a cell cultured on a micropatterned substrate. This image reveals what seem physical contact points between the cell membrane and the patterned substrate, which are normally hidden by the bulk of the cell. This was also confirmed by fluorescence microscopy and the staining of the focal points (not shown here). In this case, with a micropattern consisting of pillars 2.5 μm in diameter and with a 5 μm period, it seems that the cell supports itself by covering the whole of the pillars, which probably serve as anchorage points when cell is migrating. The cell membrane on the underside of the cell forms around the pillars and contacts the polymer on all sides. The space between two consecutive microstructures is large enough to allow the cell membrane to rest on the polymer surface between the structures. A magnified view (Fig. 4b) shows that the cell membrane is not however planar to the polymer surface and instead forms attachment points which define microcavities free from solid components. These microcavities may have been occupied by liquid medium and soluble ECM components before sample drying. These attachment points are separated by distances ranging from 70 to 100 nm, which correlates well with the distance between two integrins on the cell membrane (Calvacanti-Adam et al., 2006; Wojciak-Stothard et al., 1996), and which may explain why cells do not adhere well to nanostructures with spacing of less than approx. 70 nm (Casey et al., 1999).

In the case of the nanopatterned surfaces, two different patterns were studied by the FIB/SEM cross-sectioning method. The first nanopattern consists of 200 nm wide lines, 200 nm tall and separated by 200 nm (width/height aspect ratio = 1:1), the mould of which has been fabricated by e-beam lithography with a procedure described in a previous work (Mills et al., in press). The second nanopattern consists of 300 nm pillars, only 30 nm tall and spaced by 300 nm (aspect ratio = 10:1), the mould of which was fabricated using FIB milling. Fig. 5 shows detailed pictures of the cross-

sectional views of the interface between the patterned substrates and the MG63 cells for both nanopatterns. In the case of the lines (Fig. 5a), it is notable that the cell preferentially sits only on the top of the line structures, producing covered nanochannels between the nanoline structures and the cell membrane. The cell membrane is therefore seen to be unable to enter the nanostructures with this aspect ratio. In-between the cell membrane and the polymer surface, a more continuous layer can be seen (insert in Fig. 5a) that serves as a connecting interlayer. We suppose this layer may be part of the ECM produced by the cell or just proteins adsorbed from the serum medium, and it is used as an interface to attach the cell membrane receptors (integrins) to the components adsorbed by the polymer surface. The cell attachment points follow the periodicity of the pattern and consecutive FIB sections reveal that they are stable along the nanopatterned line structure, as if the adhesion points in the cell position themselves along this line. In fact, adhesion points form focal contacts that link the cell with the substrate, but they are also connected to the actin filaments that form the cytoskeleton (and thus define the morphology) of the cell. As the adhesion sites appear to be organized linearly along the patterned substrate, this may explain why the cells are elongated in shape and aligned along the pattern lines (Fig. 3).

On the other hand, cells grown on the 10:1 aspect ratio nanopatterns are conformal in their attachment with the substrate, with the membrane following the substrate topography (Fig. 5b), and displaying focal contacts again spaced at between 70 and 100 nm. In this case, the aspect ratio of the structures is insufficient to affect the positioning of the cell membrane (by reorganizing the adhesion sites to follow the pattern guides) with respect the polymer surface. In fact, cells cultured on these surfaces looked much more rounded or spindle shape and no alignment with the pattern structure was observed.

4. Conclusion

The combination of focused ion beam (FIB) lithography and high-resolution scanning electron microscopy (SEM) in a double-beam instrument has proven to be a useful tool for the study of cell–

substrate interfaces on cells grown on micro and nanopatterned substrates. This technique allows us to simply choose the desired cell for analysis and achieve high resolution imaging of localized cross-sections of the cell. When using the FIB/SEM technique with samples that have been properly prepared (by means of freeze-drying techniques in high vacuum), it is possible to image the nanopatterned surface, the cell and the non-soluble extracellular matrix components at the same time, thus making possible a study of the relationship between the cell-substrate attachment points, the cell morphology and alignment and the differentiation state of the cells. Extension of this technique, using sequential milling and imaging steps while progressing through the cell, could be envisaged. This would mimic the technique of cryo-sectioning (Dubochet et al., 1988) and would allow a three-dimensional map of the interior cell structure to be produced, a technique that is considered difficult using current microscopy techniques.

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Figure captions

- Fig. 1. Schematic diagram of the configuration of the FIB/SEM dual beam apparatus. The sample is tilted to 52° , so ion milling occurs at 90° to the sample while electron imaging takes place at 52° to the sample.
- Fig. 2. (a) Secondary electron image, taken at 52° angle to the sample while scanning with the ion beam, of an osteoblast-like cell grown on a micropatterned PMMA substrate ($2.5\ \mu\text{m}$ pillars) and coated with a thin platinum layer. (b) SEM image showing the region where the FIB milling is taking place (90° to the sample). (c) SEM image of the cell–substrate interface before the final ion polishing (52° to the sample). (d) SEM image of the cell–substrate cross-section after the final ion polishing (52° to the sample).
- Fig. 3. (a) SEM image of MG63 cells cultured on flat PMMA as a control sample. (b) SEM image (0° to the sample) of MG63 cells cultured on a large array of line nanostructures $200\ \text{nm}$ wide, $1\ \text{mm}$ long and $200\ \text{nm}$ deep. The nanostructures cause the cells to align and elongate. The insert shows a magnified view of a cell on the nanopatterned PMMA substrate.
- Fig. 4. (a) SEM image (52° to the sample) of a cross-section of a cell on a micropatterned substrate: $2.5\ \mu\text{m}$ diameter posts, $1\ \mu\text{m}$ tall and $5\ \mu\text{m}$ period. The dashed line indicates the profile of the pattern on the PMMA substrate. (b) Magnified view of the cell on the micropatterned PMMA, showing the microcavities around the substrate pillars and the cell attachment points on the flat surface areas.
- Fig. 5. SEM images (52° to the sample) of a cross-section of a cell on nanopatterned substrates: (a) $200\ \text{nm}$ wide, $200\ \text{nm}$ tall, $400\ \text{nm}$ period lines and (b) $300\ \text{nm}$ diameter, $30\ \text{nm}$ tall, $600\ \text{nm}$ period pillars. The cell membrane sits only on the top of the lines (aspect ratio 1:1) while the focal contacts follow the smoother surface morphology of the $30\ \text{nm}$ tall-pillared substrate.

Figures

Figure 1

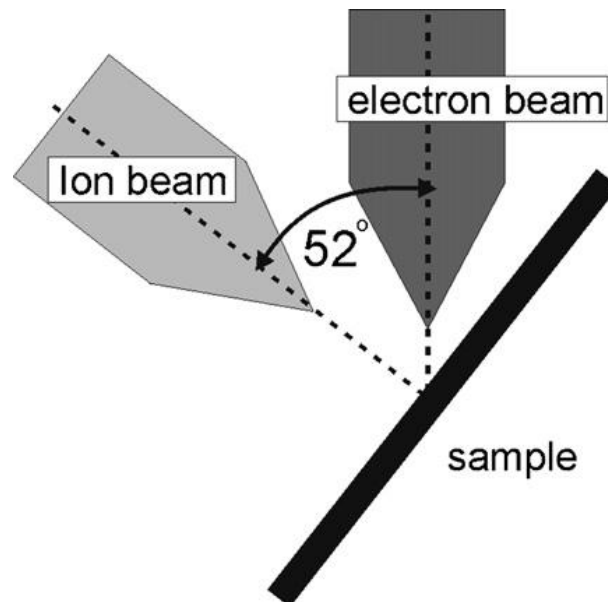


Figure 2

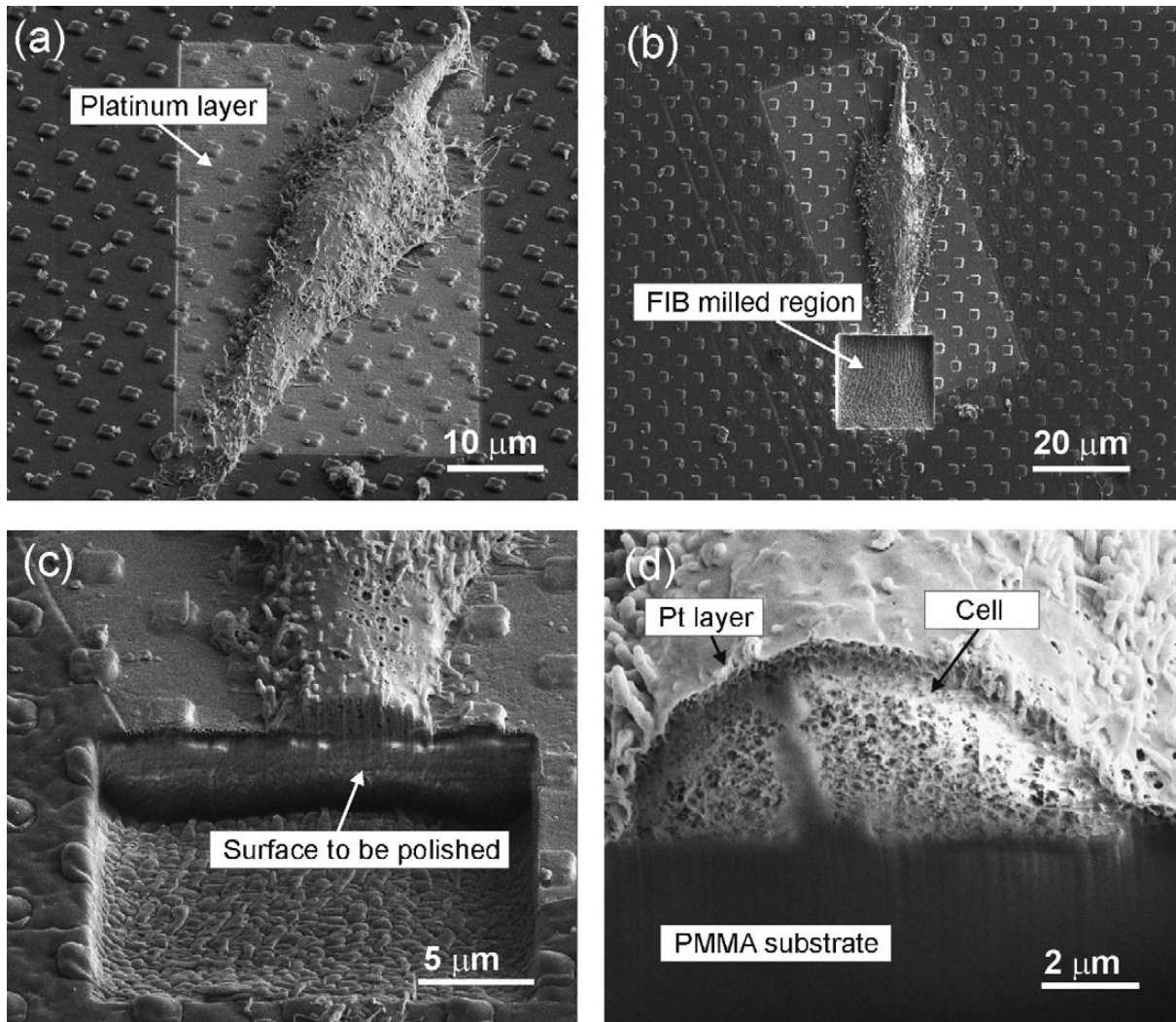


Figure 3

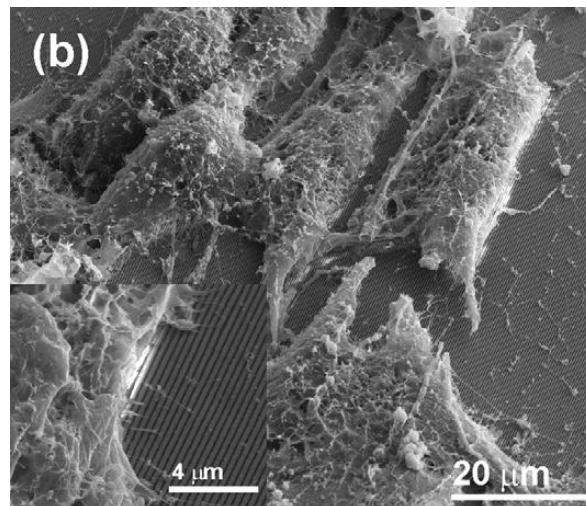
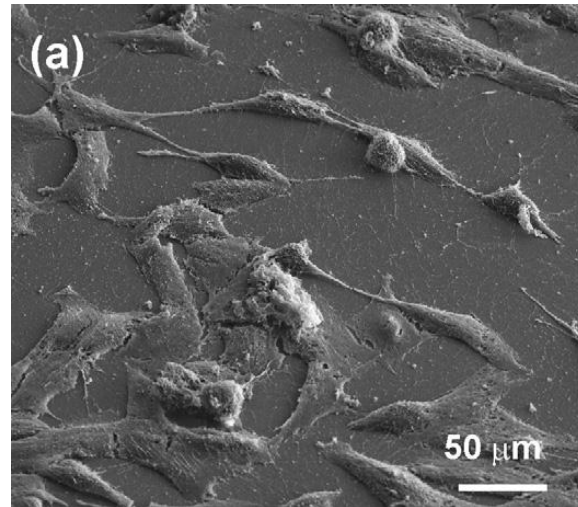


Figure 4

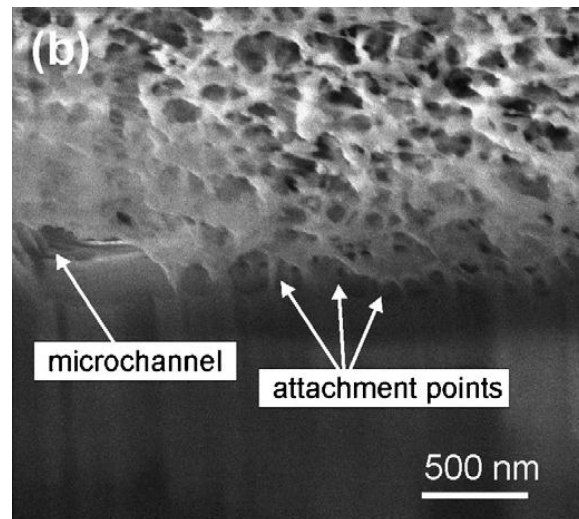
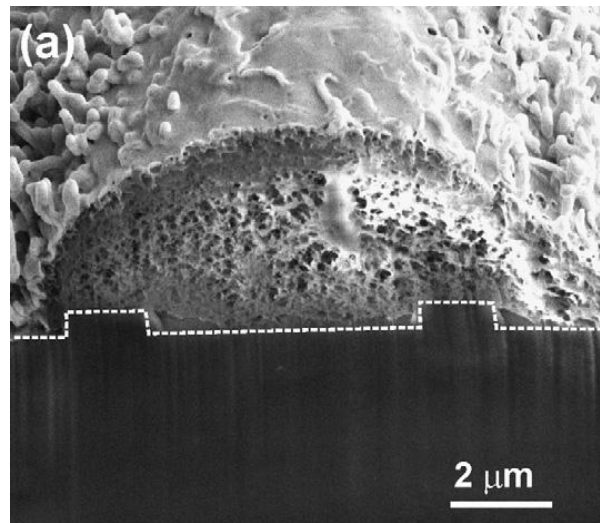


Figure 5

