Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Nuclear Instruments and Methods in Physics Research B 231 (2005) 413–418
NIM B
Beam Interactions with Materials & Atoms
www.elsevier.com/locate/nimb

Measurement of cell motility on proton beam micromachined 3D scaffolds

F. Zhang [a], F. Sun [b], J.A. van Kan [a], P.G. Shao [a], Z. Zheng [a], R.W. Ge [b], F. Watt [a,*]

[a] *Centre for Ion Beam Applications (CIBA), Department of Physics, National University of Singapore, 2 Science Drive 3, Singapore 117542, Singapore*

[b] *Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore*

Available online 25 March 2005

Abstract

Tissue engineering is a rapidly developing and highly interdisciplinary field that applies the principles of cell biology, engineering and material science. In natural tissues, the cells are arranged in a three-dimensional (3D) matrix which provides the appropriate functional, nutritional and spatial conditions. In scaffold guided tissue engineering 3D scaffolds provide the critical function of acting as extracellular matrices onto which cells can attach, grow, and form new tissue. The main focus of this paper is to understand cell behavior on micro-grooved and ridged substrates and to study the effects of geometrical constraints on cell motility and cell function. In this study, we found that BAE (Bovine Aortic Endothelial) cells naturally align with and are guided along 3D ridges and grooves machined into polymethylmethacrylate (PMMA) substrates. Average cell speed on micro-grooves and ridges ranged from 0.015 μm/s (for 12 μm wide and 10 μm deep ridges) to 0.025 μm/s (for 20 μm wide and 10 μm deep ridges). This compares with the cell motility rate on a flat PMMA surface where the average cell speed is around 0.012 μm/s. In this work we used scaffolds which were directly written with a focused proton beam, typically 1 MeV protons with a beam spot size of 1×1 μm^2.
© 2005 Elsevier B.V. All rights reserved.

PACS: 85.40.Hp; 87.64.−t; 87.22.−q

Keywords: Proton beam writing; 3D scaffolds; Cell motility; Cell guidance

1. Introduction

The new field of tissue engineering can be described as "you start with some building material

* Corresponding author. Tel.: +65 6874 2815; fax: +65 6777 6126.
E-mail address: phywattf@nus.edu.sg (F. Watt).

0168-583X/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.nimb.2005.01.092

(e.g. extracellular matrix (ECM), biodegradable polymer), shape it as needed, seed it with living cells and bathe it with growth factors''. When cells multiply, they fill in the scaffold and make up new three-dimensional (3D) tissues, and once implanted in the body, cells can recreate their intended tissue functions. With blood vessel attachment to the new tissues, the scaffold dissolves, and the newly grown tissues combine with their surroundings. Tissue engineering can also utilize naturally derived or synthetic, engineered biomaterials to replace injured or defective tissues, such as skin, bone, cartilage and even organs. Today, thanks to this realization and the improving research in this area, some of the simpler tissues, for example skin and cartilage, have already been used in clinical applications [1].

The formation of new capillary branches from pre-existing blood vessels is known as angiogenesis. Endothelial cells (ECs) form the inner surface of a blood vessel; they are surrounded with a layer of extracellular matrix (ECM) and an outer layer of smooth muscle cells (SMCs). The efficient control of the angiogenesis process is essential to help organs build up vessels in bioreactors and cure many diseases caused by either the excessive or insufficient blood vessel formation. Therefore it is necessary to understand the behaviour of ECs which line the blood vessels, not only through biochemical signaling, but also the behaviour of cells constrained in a three-dimensional physical environment.

In tissue engineering, 3D scaffolds act as extracellular matrices onto which cells can attach, grow, and create new tissues. The fabricated scaffolds are usually synthetic polymers. It is believed that in the tissue micro-environment, geometric constraints (i.e. a scaffold) can play a key role in determining the endothelial cell behaviour, such as growth, migration, or death [2]. However, very little work has been carried out on the effect of micro-substrate geometry on cell behaviour because of the general unavailability of precise 3D substrate fabrication techniques. The work that has been performed previously was, in the main, dependent on optical lithography in order to pattern surfaces. Optical lithography is a masked surface machining technique that fabricates a shallow pattern in the substrate (usually silicon). In order to extend the pattern to a 3D structure, reactive etching is required. The whole process is relatively complicated, and therefore not widely available to biomedical researchers. Potentially proton beam (p-beam) writing, as the only direct-write 3D micro-machining process, does not need an intermediate masked step or subsequent reactive ion etching. P-beam writing uses a focused MeV proton beam to write structures directly which have precise 3D geometry and vertical side walls [3,4]. We have previously observed that fibroblast cells attach and grow very well directly on the surface of PMMA, and we have also shown that simple ridges and grooves of different sizes in the micrometer range produced by proton beam writing on PMMA substrates can have significant effect on fibroblast cell behaviour [4].

The aim behind this current project is to fabricate various 3D micro-patterns to investigate the effects of different substrate geometry and matrix material on the behaviour of endothelial cells, in particular migration rates and alignment.

2. Experimental

2.1. Scaffold manufacture

All the micro-machining work presented in this project was carried out using the p-beam writing

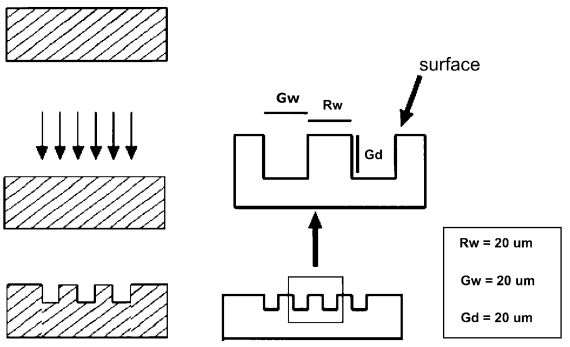

Fig. 1. Schematic representation of the production process of micro-pattern: the dimensions of micro-grooves and ridges are described in terms of groove width (Gw), ridge width (Rw) and groove depth (Gd).

facility at the Centre for Ion Beam Applications (CIBA), National University of Singapore. As shown in Fig. 1, a piece of PMMA, approximately 1×1 cm^2, 2 mm thick, was exposed to 1 MeV protons focused to a beam spot size of 1×1 μm^2. The dose needed for the irradiation by 1 MeV p-beam was 60 nC/mm^2. Generally, the development of the PMMA substrate was processed by the steps described in [5]. Fig. 2 shows an optical photograph of this substrate which has 20 μm wide grooves/ridges at a depth of 10 μm. In order to investigate whether ECs respond differently to various dimensional grooves and ridges, we repeated the same experimental steps to fabricate a second substrate which has 12, 18, 20 μm for width of grooves, ridges and depth respectively.

Endothelial cells require a suitable growth medium in which to survive. For this project, which is to observe cell motility on 3D micro-groove/ridge structures, it is necessary to bond a top housing onto the structure in order to avoid growth medium evaporation. The schematic representation of bonding process is described in Fig. 3. The 20 μm wide ridged scaffold and the 12 μm ridged scaffold were each bonded to a top housing of similar dimensions. A plain PMMA structure was also fabricated in this way to observe the ECs movement on plain surfaces.

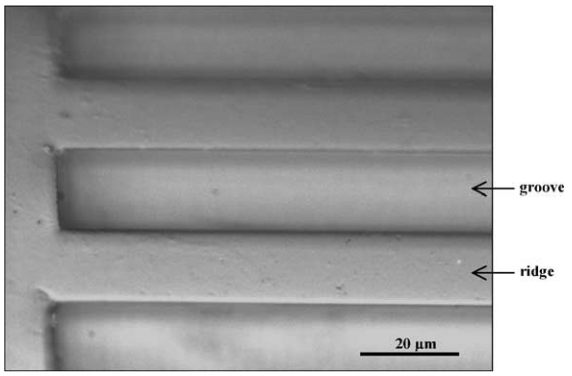

Fig. 2. Plan view of PMMA (2 mm thick) exposed by 1 MeV p-beam (1 μm spot size) and chemically developed.

2.2. Cell culture and migration experiment

Before cell culturing, it was necessary to sterilize each bonded scaffold in UV light for 20 min each side. Next, Bovine Aortic Endothelial (BAE) cells were seeded at the density of

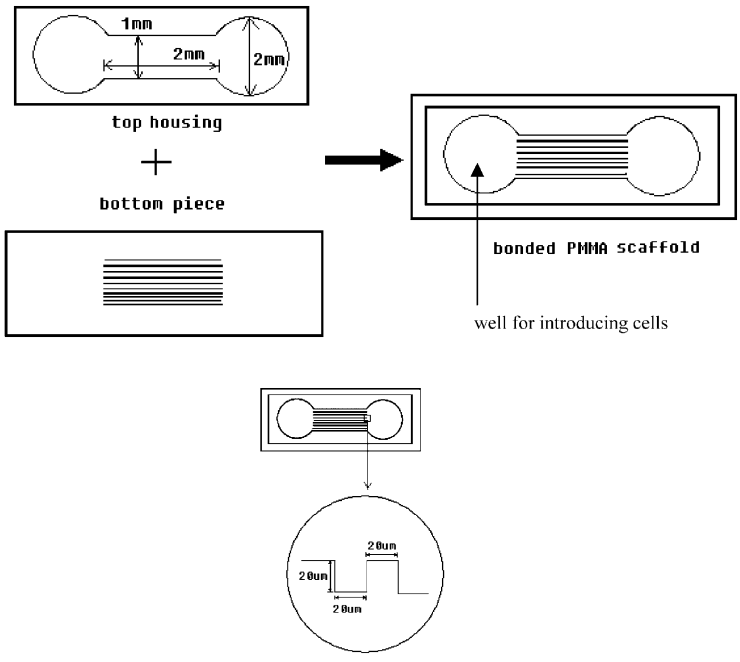

Fig. 3. Schematic representation of the fabrication process for bonding a top housing onto the 3D scaffold.

1×10^5 cells/ml into one of the two wells in each scaffold assembly, located in a culture dish containing growth medium. After the cells had migrated to the ridge/groove area, the dish was moved to an incubator on the microscope. The suitable incubation conditions for cells culture are 37 °C and 5% CO_2 to achieve a PH value around 7. In order to characterize the migratory pattern of cells, digital photographs were recorded by a Zeiss Axiovert 200M Light Microscope. Time intervals between the images were kept at 600 s, which through earlier tests was shown to be an optimal time lapse interval.

BAE cells were respectively cultured on plain PMMA, scaffolds Rw20Gw20Gd10 (ridge width 20 μm, groove width 20 μm, and groove depth 10 μm) and Rw12Gw18Gd10 (12 μm ridges and 18 μm grooves, 10 μm deep). Generally 60 frames were captured to describe the living cells movement in each migration experiment.

3. Results

It is found that on plain PMMA surface BAE cells had a random walk in all directions with a general trend along the connecting slot, see Fig. 4(a). On the grooved and ridges scaffolds, the cells elongated in the direction of channels and were guided by the grooves/ridges, see Fig. 4(b) and (c).

The average cell speed on each of the three structures are listed in Table 1, and were calculated by utilizing image analysis program *IDL*. It can be seen that the cells growing on scaffold Rw20Gw20Gd10 move faster than those on Rw12Gw18Gd10 and flat surface of PMMA.

T-test statistics, with a threshold of 0.05 for significance, were performed to compare the cell speed on Rw20Gw20Gd10 and Rw12Gw18Gd10 with the cell speed on a plain PMMA surface (see Table 2). These two comparisons show that cell speed on Rw20Gw20Gd10 is significantly faster than on a plain surface, while there is no statistically difference between the cell speed on Rw12Gw18Gd10 and on the plain PMMA surface.

The directional movement of each single cell can also be analyzed by the calculated guidance angle between cell migration and the direction of grooves. If the cells are not guided, i.e. random walk, then the average angle is around 45°. On the contrary, if the cells are all aligned and move along the grooves or ridges, the average angle is low [6]. In our case, the guidance angle can be

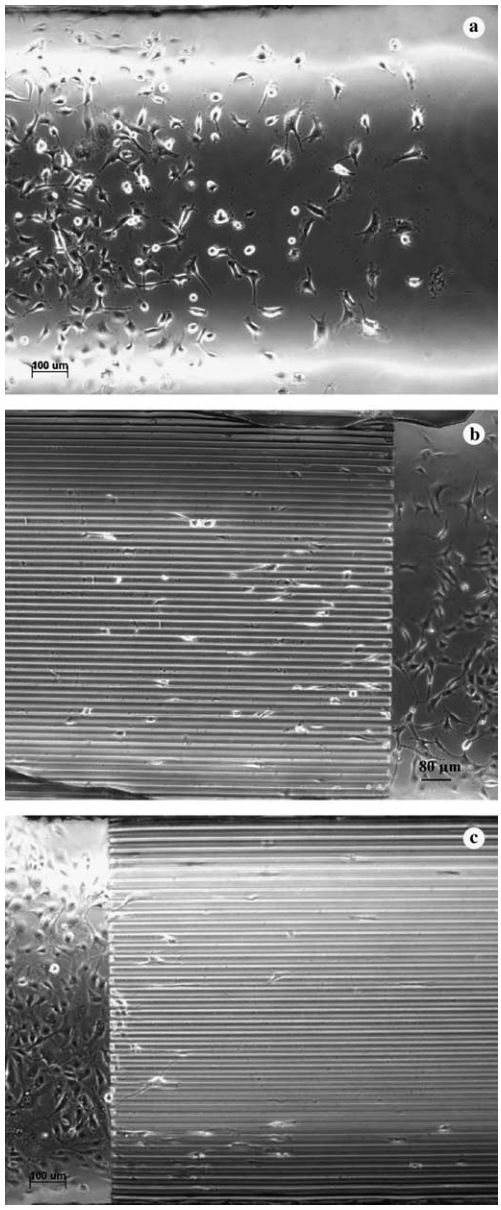

Fig. 4. Image of BAE cell migrating (a) on plain PMMA surface, (b) on Rw20Gw20Gd10, (c) on Rw12Gw18Gd10 (Rw, ridge width; Gw, groove width; Gd, groove depth).

Table 1
Cell speed on plain PMMA surface and grooved/ridged scaffolds (Rw, ridge width; Gw, groove width; Gd, groove depth)

Samples	Width of ridges (μm)	Width of grooves (μm)	Depth of grooves (μm)	Average cell speed (μm/s)
Plain PMMA	0	0	0	0.012 ± (3)
Rw20Gw20Gd10	20	20	10	0.025 ± (8)
Rw12Gw18Gd10	12	18	10	0.015 ± (4)

Table 2
Comparison of cell average speed on plain surface with (a) Rw20Gw20Gd10, (b) Rw12Gw18Gd10 (Rw, ridge width; Gw, groove width; Gd, groove depth)

Average speed on plain surface (μm/s)	(a) Average speed on Rw20Gw20Gd10 (μm/s)	(b) Average speed on Rw12Gw18Gd10 (μm/s)
0.012	0.025 *P* value: 9.686E−5	0.015 *P* value: 0.069

The speed of 10 cells on each substrate were used in each comparison.

observed to be 18° on Rw20Gw20Gd10 and 24° on Rw12Gw18Gd10, indicating that BAE cells are guided more by the Rw20Gw20Gd10 patterns.

4. Discussion and conclusion

Adhesive interactions between cells and the extracellular environment play a crucial role in affecting cell morphology, gene expression, and the rates of cell proliferation [7]. For example, we have shown that cell migration rates vary depending on the various dimensions of micro-grooves and ridges fabricated by p-beam writing.

The internal structure of the cell is responsible for cell movement. The cytoskeleton consists of an internal frame of micro-filaments, micro-tubules and intermediate filaments. The marked cellular behaviour associated with micro-grooves/ridges, such as alignment and elongation, can be well explained in terms of the reformation of the cytoskeleton. Fig. 5(a) displays a schematic of a cell on a flat surface. The micro-filaments as shown are mainly going from the region close to the nucleus towards the periphery of the cell, with only a few micro-filaments being lateral. On the other hand, when the cell is on a grooved/ridged substrate, as shown schematically in Fig. 5(b), the micro-filament bundles form predominately along the direction of the groove [8,9].

From the experiments we can see that cells move faster and are aligned better when confined on 20 μm ridges (Rw20Gw20Gd10) than cells on 12 μm ridges (Rw12Gw18Gd10). This difference suggests that speed of cellular movement is affected by the dimensions of the micro-features on the surface. Endothelial cells change their shape when they are aligned on the grooves and ridges; and in our case it also appears that 20 μm wide ridges stimulate the cells to move faster than either

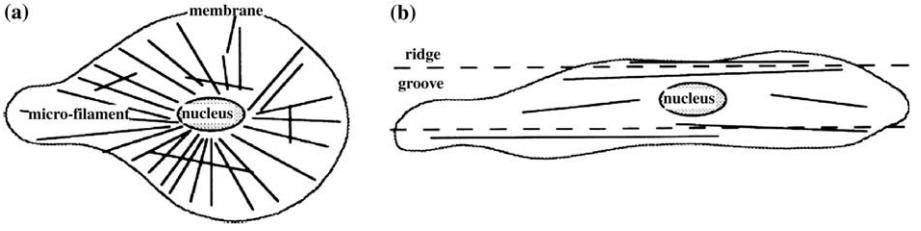

Fig. 5. Schematic view of cytoskeleton. (a) A cell on a flat surface; (b) the reformation of the micro-filaments that happens on the cells is growing on a grooved/ridged scaffold.

on plain surfaces or 12 μm wide ridges. There may well be an optimum geometrical constraint for cell migration enhancement, which will have important consequences in tissue engineering and understanding angiogenesis.

The endothelial cell behaviour studies on the micro-grooved and ridged substrates has also proved that proton beam writing is a very suitable rapid patterning technique which can be used to understand cell response associated with geometrical constraints.

Acknowledgements

The authors thank National University of Singapore for support through the project. We further acknowledge ASTAR for their grant for this project.

References

[1] N. Barrera, Tissue Engineering, www: http://serendip.brynmawr.edu/biology/b103/f00/web3/barrera3.html.

[2] X.F. Walboomers, J.A. Jansen, Cell and tissues behavior on micro-grooved surfaces, Odontology 89 (2001) 2.

[3] J.L. Sanchez, G. Guy, J.A. van Kan, T. Osipowicz, F. Watt, Proton micromachining of substrate scaffolds for cellular and tissue engineering, Nucl. Instr. and Meth. B 158 (1999) 185.

[4] F. Sun, D. Casse, J.A. van Kan, R. Ge, F. Watt, Geometric control of fibroblast growth on proton beam-micromachined scaffolds, Tissue Engineering 10 (1–2) (2004) 267.

[5] S.V. Springham, T. Osipowicz, J.L. Sanchez, L.H. Gan, F. Watt, Micromachining using deep ion beam lithography, Nucl. Instr. and Meth. B 130 (1997) 155.

[6] X.F. Walboomers, W. Monaghan, A.S.G. Gurtis, J.A. Jansen, Attachment of fibroblasts on smooth and micro-grooved polystyrene, Inc. J. Biomed. Mater. Res. 46 (1999) 212.

[7] B. Wojciak-Stothard, A. Curtis, W. Monaghan, K. Macdonald, C. Wilkinson, Guidance and activation of murine macrophages by nanometric scale topography, Exp. Cell Res. 223 (1996) 426.

[8] C.D.W. Wilkinson, M. Riehle, M. Wood, J. Gallagher, A.S.G. Curtis, The use of materials patterned on a nano- and micro-metric scale in cellular engineering, Mater. Sci. Eng. C 19 (2002) 263.

[9] B. Wojciak-Stothard, A.S.G. Curtis, W. Monaghan, M. Mcgrath, I. Sommer, C.D.W. Wilkinson, Role of the cytoskeleton in the reaction of fibroblasts to multiple grooved substrata, Cell Motil. Cytoskele. 31 (1995) 147.