ABCM Symposium Series in Bioengineering, Vol. 1

SOFT LITHOGRAPHIC PATTERNING OF TETHERED EXTRACELLULAR MATRIX (ECM) MACRO-MOLECULES

First Author's Name: Dr Robert Keatch
Institution and address: Division of Mechanical Engineering & Mechatronics, Faculty of Engineering & Physical Sciences, University of Dundee, Dundee DD1 4HN
e-mail: r.p.keatch@dundee.ac.uk

Second Author's Name: Dr Kenneth Donnnelly
Institution and address: Division of Mechanical Engineering & Mechatronics, Faculty of Engineering & Physical Sciences, University of Dundee, Dundee DD1 4HN
e-mail: k.donnelly@dundee.ac.uk

Third Author's Name: Prof. Seth Schor
Institution and address: Division of Cell and Molecular Biology, Faculty of Medicine & Dentistry, University of Dundee, Dundee DD1 4HN
e-mail: s.l.schor@dundee.ac.uk

Fourth Author's Name: Dr Kris Armoogum
Institution and address: Division of Mechanical Engineering & Mechatronics, Faculty of Engineering & Physical Sciences, University of Dundee, Dundee DD1 4HN
e-mail: k.armoogum@dundee.ac.uk

Abstract. *A major impediment to the development of hybrid living/non-living materials is the design of structures that maintain the stability of the interface between cell and substrate. In response to external stimuli, such as physical adsorption, cells often modify the molecules on their cellular surface to promote new biological interactions. Mechanisms must be found to allow living cells to be tethered to biopolymeric materials without compromising either the physiological functions of the cells or the structural integrity of the substrate.*
This study investigates the technique of tethering macromolecules in place. Using soft lithographic methods, micro-patterns of Avidin were created by microcontact printing from a polydimethylsiloxane (PDMS) stamp onto a biotinylated substrate. It is proposed that by biotinylating extracellular matrix (ECM) macromolecules (e.g type I collagen, fibrin, laminin etc.) they may be tethered to biopolymeric substrates thus minimising modification of the surface molecules and producing an in-vivo like model on which cell-subratum interactions may be analysed.

Keywords*: Extracellular matrix, Microengineering, Biomaterials, soft lithographic, biotin/avidin.*

1. Introduction

The past decade has witnessed enormous advances in our understanding of the molecular basis of cell behaviour. For example, scientists, as well as the general public, are well aware of the spectacular success of the human genome project and the consequent burgeoning interest in post-genomics. Tissues, within the body, are multi-component systems consisting of several different cell populations (e.g. interstitial fibroblasts, vascular endothelial cells, lumen endothelial cells, etc.) embedded within a complex biological system containing soluble regulatory factors (cytokines, growth factors, hormones, etc.) and insoluble matrix macromolecules. The complexity of emergent functional interactions between tissue cells and their environment is complicated by the fact that (*i*) the phenotypically distinct cell sub-populations will respond differently to the stimulus (Singhvi et al., 1994, Patel et al., 1998), and (*ii*) this cell response to soluble regulatory factors is not invariant, but is critically modulated by the nature of the extracellular matrix (ECM) (Mrksich, 2000). Previous studies by the authors in this area has revealed that the same soluble regulatory molecule (e.g. - TGF-ß1) can either stimulate or inhibit the migration of the same target cell population as a function of relatively minor changes in the ECM to which the cells are attached (Keatch et al., 2003, Schor et al., 2004) It should further be noted that the temporal and spatial integration of cell behaviour at the tissue level is profoundly affected by the 3-dimensional architecture and the micro-topological features of the supporting matrix, as well as tissue-level parameters (e.g. electrochemical coupling between constituent cells and hydrodynamic shear (Hynes, 1992).

Despite the remarkable recent advances made in elucidating the molecular basis of cell behaviour, and the structure/function of sub-cellular, supra-molecular complexes (e.g. the DNA transcription complex) involved in the regulation of gene expression, cell proliferation, motility and metabolism, it is clear that existing biological techniques are insufficient to adequately model the enormous degree of complexity operating at the tissue level. It is therefore necessary to devise new techniques for fabricating matrices with more *in-vivo*-like characteristics, which will give a more fundamental insight into how cells interact with their extracellular environment.

2. Methods and Materials

2.1 Fabrication of SU-8(2075) micro-patterned template by photolithography.

Using photolithographic techniques adopted from the microelectronics industry very well defined miniature surface topographies were fabricated as follows (Keatch et al.,2000). Standard 3-inch (100) SSP silicon wafers were washed in acetone, rinsed in de-ionised water and dried with a stream of nitrogen. They were baked at 100°C for 3 minutes to remove any traces of water. Approximately 2ml of NANO™ SU-8(2075) (Chestech, Rugby, UK) was poured onto the silicon wafer and spun (1000r.p.m, 30seconds) to produce a planarised layer approximately 250μm thick. The sample was transferred to a level hotplate and baked at 65°C for 5 minutes then at 95°C for 45 minutes to allow the cyclopentanone solvent to evaporate and solidfy the film. The sample was allowed to cool to room temperature and then exposed to near-UV light (365nm) for 25 seconds using an Optical Associates inc. Series 500 mask aligner system.

The sample was post-expose baked to selectively cross-link the exposed portions of the SU-8(2075). A two-step contact hotplate process was used with the sample baked at 65°C for 3 minutes and then at 95°C for 15 minutes. The sample was then allowed to cool to room temperature before being developed in EC solvent (MicroChem Corp, Newton, MA, USA). A two-step development process was used, with the sample first immersed in cyclopentanone (Chestech) for 1 minute with moderate agitation, followed by immersion in EC solvent for 20 minutes again with moderate agitation. The sample was then rinsed in isopropanol and dried under a gentle stream of nitrogen.

2.2 Fabrication of PDMS stamp using soft lithography.

Having produced an accurate master using photolithography, a replica used for transferring the avidin can be cast using a soft elastomer (PDMS). The SU-8(2075) template is used as a mould into which the PDMS stamp is cast using approximately 9 ml of Syndev®1 (BDH Lab Supplies, Poole, UK) mixed with 5 ml of toluene (Fisher Scientific, UK). The PDMS-toluene casting mixture was allowed to cure in a fume cupboard at room temperature for 8 hours. Once cured, the PDMS stamp was carefully peeled away from the SU-8(2075) template, trimmed and mounted on a glass block, which acted as a mechanical support for the PDMS stamp.

2.3 Synthesis of a Biotinylated Surface.

The biotinylated film on which the micropattern could be replicated was synthesised using the following methods:

Avidin-Fluorescein Isothio-cyanate micro-patterning onto Biotinylated films. In order to produce a micropattern on the biotinylated film, the PDMS replica was selectively coated with Avidin and then applied with pressure to the substrate. To achieve this the PDMS stamp was washed with undiluted ethanol, then hexane (Acros), then rinsed with deionised water and finally air-dried at room temperature. A 500μg/ml (1mg in 2ml) solution of Avidin-FITC (Sigma-Aldrich Ltd., Dorset, UK) in distilled water was made up. The mixture was a pale yellow liquid at this concentration.

Avidin-FITC was applied with a sterile dropper onto the regions of interest on the upturned surface of the micro-patterned PDMS stamp. The PDMS stamp was the left in a biological cabinet for 30 minutes. Excess Avidin-FITC was removed with a sterile dropper and transferred to a Sterilin tube for storage at -18°C. Any non-specifically adsorbed Avidin-FITC was then washed away with distilled water and the PDMS stamp again air-dried at room temperature. Once dry, the regions of interest on the PDMS stamp with adsorbed Avidin-FITC were placed in conformal contact with the biotinylated film. Contact was maintained for 5 minutes. The Avidin-FITC patterns were then imaged with an Olympus IX70 fluorescence microscope. FITC has an absorption maximum at 495nm and emits at 520nm.

2.4 Cellular inoculation

To analyse cell adhesion to our micropatterned structure, anchorage dependent cells were selected. These human F_sF_{44} cells were maintained in Eagle's Minimum Essential Medium (MEM) (Sigma) supplemented with 15% (v/v) of 200mM donor calf serum (DCS), 1% (v/v) C-glutamine, 0.5% penicillin and 0.5% streptomycin. The cells were passaged by trypsinisation every 7 days just before reaching confluence; the medium was renewed every 48 hours on average.

2.5 Tethering Collagen Type I to Biotinylated film

In an attempt to analyse the behaviour of cells in more in-vivo like conditions collagen was tethered, in its native conformation, to the stamped Avidin micropattern. Native collagen type I was obtained from a stock solution provided by The Cell and Molecular Biology Unit at Dundee University. To a sterile tube containing 2ml of this solution

(2.2mg/ml, 4ºC), 1mg of Sulfo-NHS-LC-Biotin was added and the mixture shaken well for 2 minutes. The mixture was incubated at 5% CO_2 and 37ºC for 30 minutes, then introduced to a 60mm biotinylated culture dish and micro-patterned with Avidin-FITC. The sample was incubated for 24 hours. F_sF_{44} confluent cells (approx. 1.5 x 10^6) were trypsinised from a stock culture and introduced to the sample, which was then incubated with serum-free MEM for a further 24 hours. The procedure was repeated and the second sample incubated in 15% DCS-MEM for 48 hours.

2.6 Plating of cells onto micro-patterned films

As an intermediated method for determining whether the biotin ligands on the collagen have maintained their reactivity and ability to bind avidin, cells were introduced onto the sample. 90 mm dishes of pre-confluent human F_sF_{44} cells at a concentration of approximately 20x 10^3 cells/cm^2 were used. The medium was removed from the stock culture and the remaining cells washed twice with 4-5 ml Hank's Balanced Salts solution (Sigma). 2ml of trypsin (10X solution of 2.5g porcine trypsin powder/100ml) (Sigma) was added to each 90mm dish and left to incubate at 37ºC for about 8-10 minutes until the cells were seen to round and detach from the dish. 3ml of 15% DCS-MEM per dish were then added to neutralize the trypsin and the mixture pipetted up and down to detach all the cells. The cells floating in the trypsin/DCS-MEM solution were then transferred to Sterilins and centrifuged at 900rpm for 5 minutes. The cells formed a pellet at the bottom of the tubes and the trypsin/DCS-MEM was removed after which 10ml of DCS-MEM per tube was added and the mixture pipetted up and down to re-suspend the cells. The F_sF_{44} cells suspended in DCS-MEM were then transferred to a 90mm dishes previously coated with biotinylated collagen on micro-patterned Avidin-FITC.

3. Results & Discussion

3.1 Microengineering

SU-8 (2000) (cyclopentanone solvent) is an epoxy based negative photoresist. Standard formulations are available which produce a wide range of film thicknesses greater than 200µm (figure 1). This research used SU-8 (2075) resist with a high optical transparency and sensitive to near-UV light. Although the process guidelines are clear much fine- tuning was required to produce structures of sufficient height for soft lithography purposes as well as more complex multiplayer structures (figure 2). Once cured, the SU-8 (2075) structures were highly resistant to the reagents used in soft lithography and cell culture. It also had excellent thermal stability, making it suitable for soft lithographic applications where an elevated temperature is necessary to accelerate curing of the elastomer.

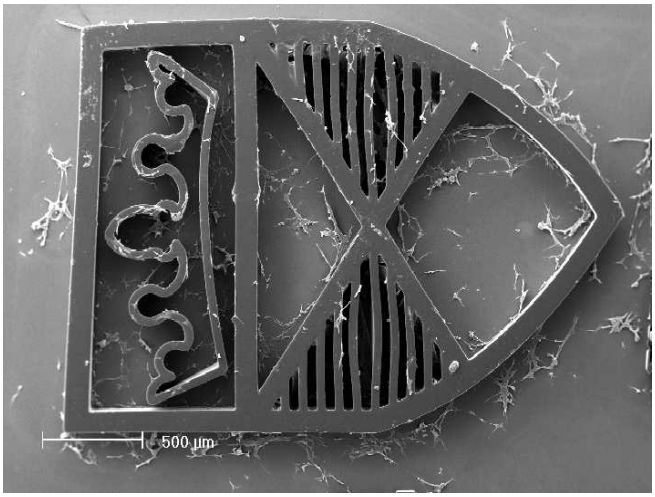

Figure 1: Height of SU-8 (2075) structure > 200um

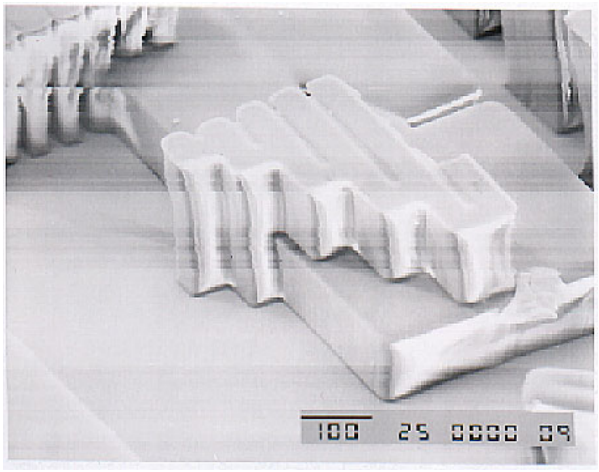

Figure 2: Two-layered micropattern of SU-8 2075

3.2 Soft Lithography

Due to its highly viscous nature the reproduction of micro-patterned features in PDMS was found to be problematic. This was initially resolved by the addition of toluene, as a solvent, to reduce the viscosity of the PDMS. This introduced further complications with the formation of bubbles due to solvent outgassing. However, this could be overcome by curing the PDMS under vacuum thus allowing well defined micro-patterned stamps to be produced as shown in figure 3.

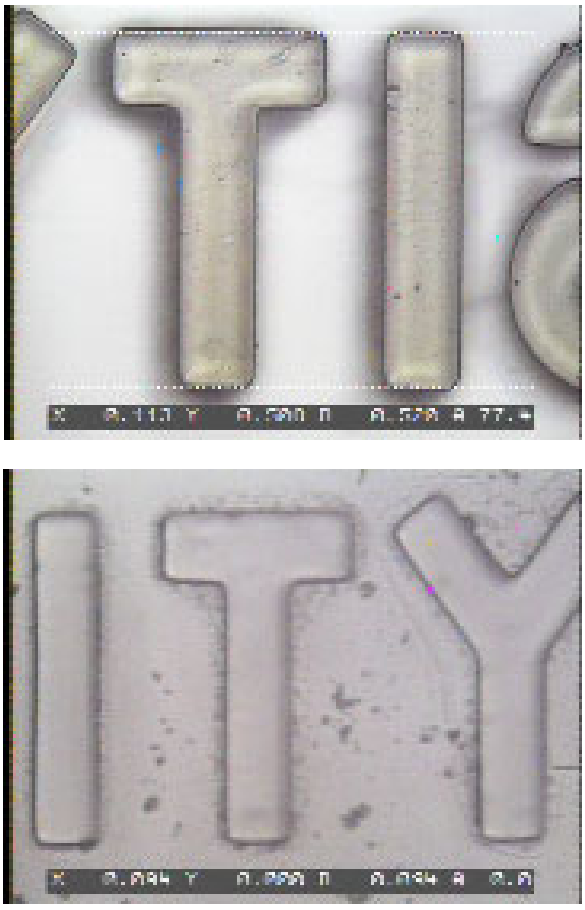

Figure 3: Good feature replication from SU-2075 master (top) to PDMS stamp (below). (100micron wide features)

3.3 Avidin Microcontact Printing

PDMS is biocompatible, permeable to gases, optically transparent and can be used for cell culture. Its elastomeric properties enable it to contact non-planar surfaces conformally and it has the ability to concentrate Avidin onto its surface without irreversibly adsorbing it (Bernard et al., 2001). This allows the Avidin to be released upon contact with the biotinylated surface (Patel et al., 2000). Avidin-FITC adsorption to PDMS was not uniform firstly because the top surface of the elastomer structures was uneven (figure 4) and secondly because the Avidin-FITC itself formed aggregates when the solvent (water) was evaporated.

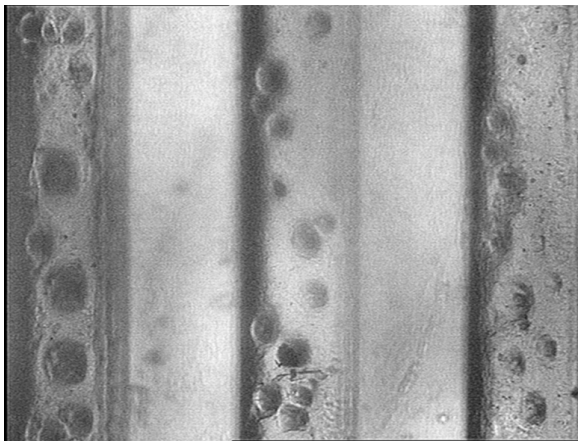

Figure 4: Pitted top surface of 200um PDMS line

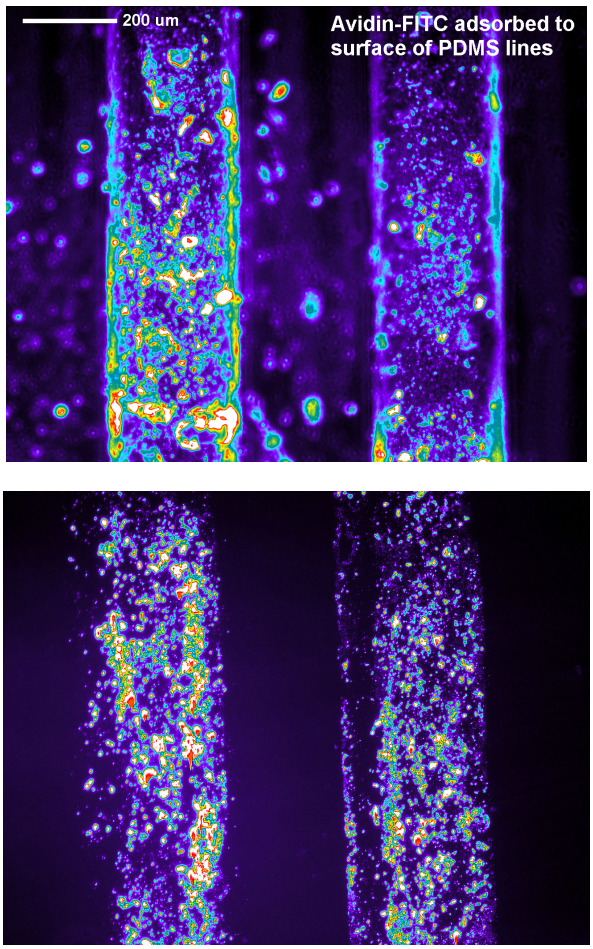

Figure 5: Avidin-FITC transfer of 200um lines on PDMS stamp (above) to biotinylated film (below).

While complete Avidin-FITC transfer was not possible, an acceptable level of greater than 60% was achieved as shown in Figure 5 and further detailed below.

3.4 Determination of optimum Avidin-FITC transfer conditions

The transfer of Avidin-FITC from the PDMS stamp to the biotinylated film was observed under varying conditions. In particular the Avidin-FITC-to-PDMS adsorption time (**A**), the Avidin-FITC-to-biotinylated film release time (**B**) and the PDMS-to-biotinylated film contact force (**C**) were varied. It was found that as 'A' increased from 5 to 30 minutes, Avidin-FITC to biotinylated film transfer improved. The same trend was not apparent as 'B' was increased and an 'optimum' Avidin-FITC-to-biotinylated film release time was 1 minute. A definite improvement in Avidin-FITC-to-biotinylated film transfer was noted as the contact force 'C' was increased from 0.49N to 6.37N. The Avidin-FITC concentration was constant in all experiments at 1 mg/ml.

Figure 6,7 and 8 illustrate the improvement in Avidin-FITC transfer as A, B and C were varied.

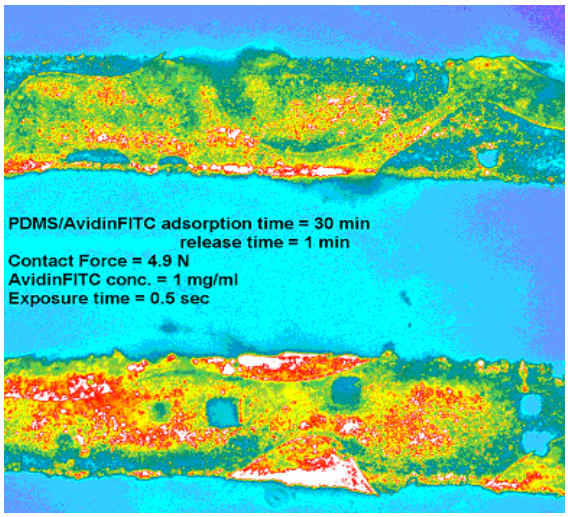

Figure 6: A=30min, B=1min, C=4.9N

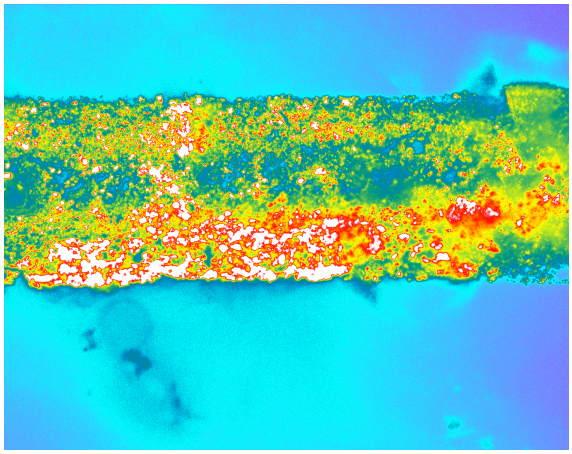

Figure 7: Best Avidin-FITC transfer was at: A=30min, B=1min, C=6.37N

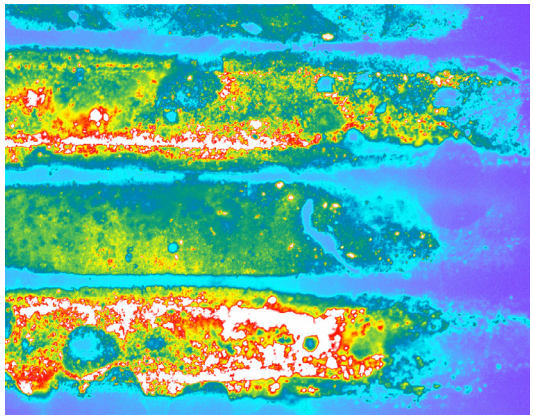

Figure 8: Stamp deformation occurred above C=7.35N (A=30, B=1)

The high affinity between Avidin and biotin should enable rapid formation of a stable complex between the Avidin-FITC and the biotin labelled collagen.

3.5 Cell attachment to tethered native collagen

For the purpose of studying cell behaviour in its more native ECM environment, F_sF_{44} cells were observed on tethered native type I collagen as shown in figure 9.

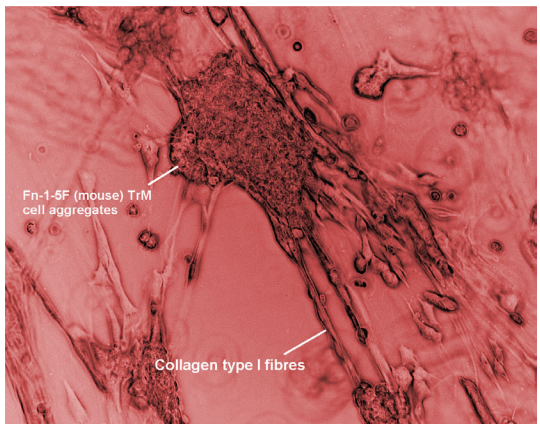

Figure 9: F_sF_{44}cells attached to tethered collagen type I after 48H incubation in 15% DCS-MEM

Conclusions

3.6 PDMS Suitability

PDMS is durable, inexpensive, optically transparent, permeable to gases, biocompatible and its flexibility enables it to contact surfaces conformally. Stamp dimensions, application pressure, deposition time and stamp surface pre-treatment can be optimised for maximum efficiency of Avidin transfer to the PLA-PEG-Biotin films (Branch et al., 1998). The elastomeric nature enables the contact of two surfaces over an extended area on a molecular scale and this is essential for micro-contact printing (Bernard et al., 2001, Delamarche et al., 1997). These factors all combine to make it an ideal soft lithographic tool for cell culture applications. The greatest advantage of PDMS to this study is its ability to concentrate Avidin onto its surface without irreversibly adsorbing it (Singhvi et al., 1994, Kane et al., 1999). This is due to the strength of the non-covalent bond $F_{\mathbf{bind}}$ being greater than the covalent link $F_{\mathbf{chem}}$ (Florin et al., 1994). Therefore, removing the PDMS stamp from the biotinylated film will disrupt the weaker link of the system: $F_{\mathbf{chem}}$ (figure 10).

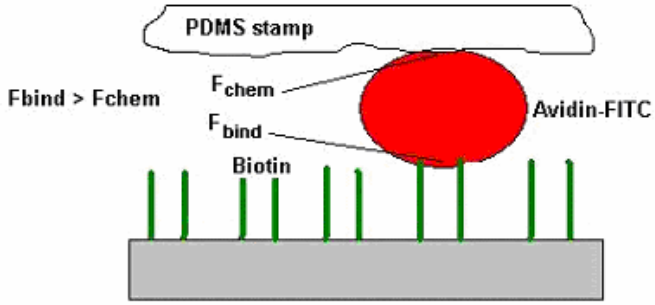

Figure 10: Relative strength of the chemical bonds involved in micro-patterning of Avidin-FITC

3.7 Avidin Transfer

Uneven adsorption of Avidin-FITC to the PDMS stamp was observed. This can be attributed to the surface topography of the microstructures as well as the formation of aggregates of Avidin-FITC on the PDMS surface after evaporation of the solvent. While complete transfer from elastomeric stamp to biotinylated surface was not attained, the degree of Avidin transfer was high enough to generate recognisable micro-patterns. Edge definition of the elastomeric structures was greatly improved by the use of toluene as a solvent to reduce the viscosity at the casting stage. Micro-contact printing of Avidin using PDMS as an elastomeric stamp has shown itself to be simple, reproducible and inexpensive.

3.8 Future Work

Cellular adhesion receptors termed integrins play an important role in the interaction of cells with the ECM during wound healing. Previous studies have found that cells adhere to denatured collagen through the $\alpha_v\beta_3$ integrin and this interaction is inhibited by an RGD containing peptide. It has also been suggested that RGD sites in type I collagen may be masked and that they become exposed upon denaturation of the molecule. Wounding of the ECM may expose RGD sites in collagens that facilitate the interaction of cells with damaged ECM through RGD binding integrins (Davis, 1992).

It is envisaged that tethering biotinylated extracellular matrix macromolecules such as collagen type I in its native and denatured conformation will determine the influence of the RGD motif in the sequence of events leading to wound healing.

4. References

Bernard, A., Fitzli, D., Sonderegger, P., Delamarche, E., Michel, B., Bosshard, H. R., Biebuyck, H., 2001 “Affinity capture of proteins from solution and their dissociation by contact printing”, Nature Biotechnology; 19; pp 866-869

Branch, D. W., Corey, J. M., Weyhenmeyer, J. A., Brewer, G. J., Wheeler, B. C., 1998 “Microstamp patterns of biomolecules for high-resolution neuronal networks”, Cellular Engineering; 36; 135-141.

Davis, G. E., 1992 “Affinity of integrins for damaged extracellular matrix: alpha$_v$beta$_3$ binds to denatured collagen type I through RGD sites”, Biochem. Biophy. Res. Commun. 182; 1025-1031.

Delamarche, E., Bernard, A., Schmid, H., Michel, B., Biebuyck, H., 1997 “Patterned delivery of Immunoglobins to surfaces using microfluidic networks”, Science; 276; pp 779-781.

Florin, E. L., Moy, V. T., Gaub, H. E., 1994 “Adhesion forces between individual ligand-receptor pairs”, Science; 264; pp 415-417.

Hynes, R. O., 1992 “Integrins: Versatility, Modulation and Signalling in Cell Adhesion”, Cell; 69; pp 11-21.

Kane, R. S., Takayama, S., Ostuni, E., Ingber, D. E., Whitesides, G. M., 1999 “Patterning proteins and cells using soft lithography”, Biomaterials; 20; 2363-2376.

R.P. Keatch, B. Lawrenson, M. Finlay, F.B. Lewis, C. Horsfield 2000, “The production of high-aspect-ratio microstructures (HARMs)”, Journal of Fusion Technol., American Nuclear Society, Vol. 38, pp139-142.

Keatch, R.P., Armoogum K., Schor S.L., Pridham M.S., Donnelly K.P., Gribbon C., 2003 “Tethering of Type I Collagen to Retain Native Conformation of Extracellular Matrix”, Int. Journal of Artifical Organs, 26(9), pp855

Mrksich, M., 2000 “A surface chemistry approach to studying cell adhesion”, Chem. Soc. Rev; 29; pp 267-273.

Patel, N., Bhandari, R., Shakesheff, K. M., Cannizzaro, S. M., Langer, R., Tendler, S. J., 2000 "Printing patterns of biospecifically-adsorbed protein", J. Biomater. Sci. Ploymer Edn. 11; pp 319-331.

Patel, N., Padera, R., Sanders, G. H., Cannizzaro, S. M., Davies, M. C., Langer, R., Roberts, C. J., Tendler, S. J., Williams, P. M., Shakesheff, K. M., 1998 "Spatially controlled cell engineering on biodegradable polymer surfaces", The FASEB Journal; 12; 1447-1454.

Schor SL, Schor AM, Keatch RP, Belch JJF. 2004 "Role of matrix macromolecules in the etiology and treatment of chronic ulcers". The Wound Management Manual (ed. B.Y Lee). McGraw Hill, New York, ISBN 0 07 143203 5, pp109-121.

Singhvi, R., Kumar, A., Lopez, G. P., Stephanopoulos, G. N., Wang, D. I., Whitesides, G. M., Ingber, D. E., 1994 "Engineering cell shape and function", Science; 264; 696-698.

5. Responsibility notice

The authors are responsible for the printed material included in this paper.