Methodology of the Microcontact Printing of Fibronectin and Blocking with Pluronic to Control Cell Adhesion

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Abstract

The ultimate goal is to study the response of osteoblasts to shear flow since this is one of the stresses that these cells experience in vivo. Prior to placing the cells in a flow chamber they must be attached to a substrate; the mechanical properties of intracellular structure and the position and size of focal adhesions can be controlled by directing cell spreading on micropatterned substrates through microcontact printing. Both osteoblasts and fibroblasts were used, and the substrates tested included silanized glass, plasma-treated poly(dimethylsiloxane) (PDMS), and polystyrene Petri dishes. An SU-8 mold was constructed, and that was used to fabricate PDMS stamps. Fibronectin (FN) was applied to the substrates through microcontact printing, and Pluronic was used to block adhesion of cells to the uncoated regions. The results showed that polystyrene Petri dishes are the superior substrate, and the stamp that held up the best during microcontact printing had islands that each measured 20 µm across. The micropatterned substrate with cells attached will be placed in a flow chamber, subject to oscillatory fluid flow, and intracellular Ca²⁺ mobilization will be studied.

1. Introduction

Bone has the remarkable ability to respond and adjust to mechanical loading to meet the needs of its environment, and a better understanding of the mechanisms by which it adapts may bring forth better treatments for bone diseases such as osteoporosis. One of the loads that osteoblasts, the bone cells responsible for building bone, experience in vivo is shear stress resulting from oscillatory flow. The response to such stresses can be investigated in vitro by attaching cells to a substrate, placing them inside a flow chamber, and subjecting them to such flow. Their mechanotransduction, the transduction of mechanical signals to biochemical ones, can be detected through the change in intracellular Ca²⁺ concentration, as has been done in previous studies (Jacobs et al. 1998; Batra et al. 2005). Ca²⁺ is an important second messenger within the cell and plays a role in the strengthening of focal adhesions, complexes containing structural proteins that are responsible for linking cells to the extracellular matrix (ECM) (Gallant et al. 2002).

The mechanical properties of intracellular structure can be controlled by directing cell spreading on micropatterned substrates (Chen et al. 2004). Furthermore, the micropatterned substrates act to control the position and size of the focal adhesions so their role in strengthening of attachment can be studied (Gallant et al. 2002). It may be the strengthening of the focal adhesions to the extracellular matrix that results in strengthening of bone.

One way to micropattern surfaces is by microcontact printing, an established type of soft lithography that works by placing the surface into conformal contact with a stamp coated with molecules such as fibronectin (FN) proteins, a natural component of the ECM. The stamp is made from the elastomer poly(dimethylsiloxane) (PDMS). In order to prevent non-specific cell
attachment, the regions of the substrate that are uncoated must be blocked with a molecule such as Pluronic, a surfactant terminating in primary hydroxyl groups.

FN stamping and blocking with Pluronic cannot occur if the surface is either too hydrophobic or too hydrophilic since FN prefers hydrophilic surfaces and Pluronic is preferential to hydrophobic surfaces (Tan et al. 2004). The glass slides must be silanized to increase their hydrophobicity, just as the PDMS surface must be plasma-treated to decrease its hydrophobicity. The hydrophobicity of PDMS is a huge limitation in biological applications in that it limits cell attachment and increases non-specific adsorption of biomolecules. However, it is simple and common to modify the surface of PDMS by O₂ plasma treatment to generate a temporary hydrophilic silicone oxide layer (Wang et al. 2005).

2. Materials and Methods

2.1 Pattern Design

It was intended to have one cell inhabit each “island,” a hexagon comprised of 7 circular regions of fibronectin, each 4 µm in diameter. Using L-edit software, a chrome mask was designed such that the stamp would possess 5 µm tall posts of this pattern. Four stamps were tested: the distance across each island was 20, 28, 36, and 44 µm, and the inter-island spacing was 40 µm for all stamps (Figure 1).

2.2 Master and PDMS Stamp Fabrication

A silicon wafer was placed into a spinner, and then a small amount of SU-8 5 (MicroChem, Newton, MA), a negative tone photoresist, was poured onto the center and allowed to spread almost to the edges of the wafer. It was then spun to a thickness of 5 µm following the instructions given by MicroChem. The wafer was soft-baked on a hot plate first for 2 min at 65°C, then for 5 min at 95°C. The mask was placed over the SU-8 coated wafer and it was exposed to 22 mW/cm² UV light for 6 sec in an OAI near UV system (San Jose, CA). The wafer was post-exposure baked for 1 min each at 65°C and 95°C, and ramped down for 1 min at 65°C. Finally, the SU-8 was developed in SU-8 Developer (MicroChem) for 1 min to expose the features, and the wafer was rinsed in isopropanol and dried with compressed air.

The stamp mold was silanized in chlorotrimethylsilane (Sigma Aldrich, St. Louis, MO) vapor for 10 minutes to allow for easy removal of the PDMS later, and it was placed face up in a Petri dish. PDMS prepolymer base and curing agent from a Sylgard 184 kit (Dow Corning, Midland, MI) was mixed in a 10:1 ratio and stirred vigorously with a rod for 2 min. The mixture was poured over the mold, and once all the bubbles were removed by degassing in a vacuum desiccator, the PDMS was cured in a 60°C oven either for 2 hours or overnight. After cooling, the PDMS was peeled off the mold and the stamps were cut out and imaged using a Leica DM-IRB inverted research microscope (Wetzlar, Germany) with IM50 image manager software.

2.3 FN Stamping and Cell Attachment

The substrates tested included silanized glass slides, O₂ plasma treated PDMS, and polystyrene bacteriological Petri dishes (BD Biosciences, Franklin Lakes, NJ). The PDMS was prepared as described in section 2.2 then plasma treated for 30 sec in a Harrick plasma cleaner/sterilizer. The glass slides were silanized by immersion in 5% dimethyl dichlorosilane (Sigma Aldrich) in dichlorobenzene (Sigma
Aldrich) for 1 min, then rinsed with distilled water and dried with compressed air.

All subsequent steps were performed using aseptic technique under a biological flow hood. Each stamp was cleaned by first spraying with 70% ethanol and then soaking in 70% ethanol for 5 min before drying with compressed air. The stamps were soaked face down in 500 µL of 100 µg/ml FN (Sigma Aldrich) in phosphate buffered saline (PBS) (10X, pH 7.4, Gibco) for 30 min before the FN was applied to the substrates through microcontact printing. The stamps were carefully lowered face down over the surface using forceps and placed in conformal contact with the substrate, using the forceps to apply gentle pressure by tapping before the stamps were lifted off. Some substrate samples had 100 µL of the FN solution applied with a dropper instead of through printing, and after 10 sec the FN was aspirated off. All substrate samples were then immersed in either PBS or distilled water containing a percentage of Pluronic F127 surfactant prill (BASF, Florham Park, NJ) in order to block adhesion of cells to the uncoated regions. Trials were performed with Pluronic concentrations of 0.2% w/v and 5% w/v in PBS and in water, and immersion times of 10 min and 1 hr. The blocker was aspirated away, and the surface was rinsed with PBS and kept wet until cell seeding.

Both MC3T3-E1 mouse osteoblasts (ATCC, Manassas, VA) and 3T3 mouse fibroblasts (ATCC) were used in the experiments. The osteoblasts were subcultured in a complete medium that consisted of 10% v/v fetal bovine serum (FBS) (Gibco) and 1% v/v penicillin-streptomycin (Gibco) in minimal essential alpha medium (α-MEM) (Gibco). The fibroblasts were subcultured in a complete medium of 10% v/v calf serum (Gibco) and 1% v/v penicillin-streptomycin (Gibco) in Dulbecco’s modified Eagle medium (DMEM) (Gibco).

The micropatterned substrates were immersed in the suspensions of osteoblasts or fibroblasts in complete medium and then allowed to incubate at 37°C and 5% CO₂ in an incubator. The cells were viewed through the inverted microscope after 1 hr and 5 hrs of incubation to check for growth and attachment.

Figure 2: Schematic of experimental methods.
3. Results

3.1 Stamp Features and Printing

The stamp features appeared well defined when viewed from above (Figure 3), but SU-8 microcracks in the mold had transferred their pattern to the PDMS and were visible between the islands (Figure 4). There was trouble with many of the stamps collapsing, and it was discovered that the posts on some stamps were actually only small bumps (Figure 5). It was found that the stamps with the smaller dimensions did not collapse as often as the larger stamps. In addition, stamps cured overnight at 60°C were more functional than those cured for only 2 hours.

3.2 Cell Attachment

There was no difference in cell attachment between the two cell lines; both behaved similarly on all substrates. However, the osteoblasts grew more quickly than the fibroblasts.

The cells adhered all over the glass substrate when it was blocked with 5% Pluronic in PBS for 10 min; however, the cells were more spread out and had adhered more to the FN coated regions than to the blocked regions. The result was virtually the same when the surface was blocked for 1 hr. The cells seeded onto the glass blocked with 0.2% Pluronic in PBS for 1 hr also attached extremely well to the FN regions, but
they showed a similar result in that they also attached all over the substrate.

The cells that were seeded onto glass blocked with 5% Pluronic in distilled water for 10 min also attached to all regions of the surface; however, more positive results were found when the blocking time was increased and the substrates were changed. When the surfaces were blocked with the 5% Pluronic in water for 1 hr, it was found that the cells attached to all regions of both the glass and PDMS after 1 hour of incubation. However, given a few more hours the cells on the PDMS surface began to detach, showing some preference for the protein-coated regions. On the polystyrene dishes, the cells attached only to the FN-coated regions.

4. Discussion and Conclusions

4.1 Discussion

It was proposed that excess diffraction during the mold creation was the cause of the short posts on the stamps and resulting collapses, and this could easily be minimized by reducing the exposure time. New masters have been made using reduced exposure times that show excellent promise for molding functional stamps. The ideal exposure time during the fabrication of the master is estimated to be 4 sec, but this cannot be confirmed until the stamps have been made and tested. The new masters also contain fewer SU-8 microcracks as a result of steadier ramping of the temperature during baking. The microcracks were an undesirable effect but not the cause of any significant troubles.

Since the stamps with the small islands held up better regardless of post height, it was proposed that the design of that particular stamp is responsible for its success. All of the posts cover the same area but on the smaller stamp the posts are closer together, so it is expected that that stamp should have better support. This should be considered in future designs. Sagging is known to occur if the distance between the posts is too large (Xia and Whitesides 1998). The stamps cured overnight were most functional due to their rigidity; longer curing times may help to prevent collapsing of the stamps.

Upon experimentation with different concentrations and immersion times for the Pluronic, it was found that 5% Pluronic in distilled water for 1 hr worked best to block cell adhesion.

The results demonstrate that polystyrene dishes are the superior substrate for blocking cell adhesion with Pluronic. This is a preferable substrate to use in the flow chamber compared to PDMS because the low stiffness of PDMS may have an undesirable effect on the flow experiments. The rigidity of the surface to which cells adhere has been found to have an effect on their mechanical state (Chen et al. 2004). However, the polystyrene is less stiff than the glass slides for which the flow chamber was designed, so it may be difficult for the polystyrene to form an effective seal with the flow chamber.

4.2 Conclusions

While microcontact printing to control cell adhesion is conceptually simple, it is a multi-step process in which many problems can occur. Important considerations during stamp creation are exposure time during master fabrication and PDMS curing time. Here, excess diffraction resulting from heightened exposure times during the mold creation was the cause of the short posts on the stamps and the resulting collapses. Longer PDMS curing times created more rigid stamps, which helped to prevent collapses. It was the design of the small stamp that was responsible for its success, and this should be considered in future designs. Non-specific cell attachment proved challenging as well, but polystyrene dishes stood out as the superior substrate for blocking cell adhesion.

4.3 Future Work

The ultimate goal is to learn how osteoblasts can detect the mechanical loading exerted on bone and find how the mechanical signal is being transduced into bio-chemical signals. It is known that bone cells can respond to fluid flow with a transient increase in intracellular Ca²⁺ concentration. The Petri dishes will be cut to fit the flow chamber before micropatterning with FN and attaching the cells. Then the substrate will be placed in a flow chamber and the cells subject
to oscillatory fluid flow (Figure 6), and intracellular Ca\(^{2+}\) mobilization will be observed using the methods of Batra et al. (2005).

![Figure 6: Osteoblasts subject to shear flow: this diagram represents the next part of the experiment. The Petri dish will be cut, and once the osteoblasts are attached to the micropatterned polystyrene, they will be placed in a flow chamber and subjected to oscillatory shear flow similar to what they experience in vivo. Intracellular Ca\(^{2+}\) mobilization will then be studied.](image)

### References


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