

Layer-by-layer deposition of hyaluronic acid and poly-L-lysine for patterned cell co-cultures[☆]

Ali Khademhosseini^a, Kahp Y. Suh^b, Jen M. Yang^{b,c}, George Eng^a, Judy Yeh^a,
Shulamit Levenberg^a, Robert Langer^{a,b,*}

^a Division of Biological Engineering, Massachusetts Institute of Technology, 45 Carleton Street, E25-342, Cambridge, MA 02139, USA

^b Department of Chemical Engineering, Massachusetts Institute of Technology, 45 Carleton Street, E25-342, Cambridge, MA 02139, USA

^c Department of Chemical and Materials Engineering, Chang Gung University, Kwei-Shan, Tao-Yuan 333, Taiwan, ROC

Received 13 August 2003; accepted 9 October 2003

Abstract

A novel method for patterning cellular co-cultures that uses the layer-by-layer deposition of ionic biopolymers is described. Non-biofouling hyaluronic acid (HA) micropatterns were used to immobilize cells and proteins to glass substrates. Subsequent ionic adsorption of poly-L-lysine (PLL) to HA patterns was used to switch the HA surfaces from cell repulsive to adherent thereby facilitating the adhesion of a second cell type. The utility of this approach to pattern co-cultures of hepatocytes or embryonic stem cells with fibroblasts was demonstrated. In addition, the versatility of this approach to generate patterned co-cultures irrespective of the primary cell seeding and relative adhesion of the seeded cells was demonstrated. Thus, the proposed method may be a useful tool for fabricating controlled cellular co-cultures for cell–cell interaction studies and tissue engineering applications.

© 2003 Elsevier Ltd. All rights reserved.

Keywords: Patterned co-cultures; Layer-by-layer; Hyaluronic acid; Poly-L-lysine

1. Introduction

Controlling cell–microenvironment interactions such as cell–cell contact and the presentation of extracellular matrix and soluble ligands is important for the development of tissue engineering constructs and in vitro cultures that mimic the organization and complexity of normal tissue architecture [1]. Despite this need, most commonly used cell culture systems lack the complexity and the organization that parallels the in vivo cellular microenvironment. Although co-cultures of two (or more) cell types have been used to better mimic in vivo systems, cell–cell interactions such as spatial signaling and the degree of homotypic and heterotypic interactions within these cultures are not easily controlled [2].

Patterned co-cultures aim to enhance microenvironmental control through spatial localization of multiple

cell types relative to each other. Approaches to generate patterned co-cultures could be categorized into one of three categories. In one approach, photolithographic techniques are used to pattern surfaces with materials that facilitate the selective adhesion of multiple cell types to specific regions. For example, co-cultures of hepatocytes and fibroblasts have been made by the selective adhesion of hepatocytes to micropatterned collagen islands to optimize cell–cell interactions for the functional maintenance of primary hepatocytes [2–6]. In the second approach, cells are delivered to particular regions of a substrate. For example, microfluidic channels were used to pattern multiple cell types to specific regions of a surface [7,8]. Similarly, elastomeric membranes were used to prevent cell attachment to regions of the substrate. Once the membrane was peeled off, the remaining surface could yield to the attachment of the second cell type [7,9–11]. Both of these approaches have certain potential limitations. Photolithographic approaches require selective adhesion of one cell type to the patterned substrate which may limit its application to various cell systems. Also, microfluidic approaches are limited to specific geometries specified by the laminar fluid flow [8] or an intrinsic limit on the

[☆] Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.biomaterials.2003.10.033.

*Corresponding author. Department of Chemical Engineering, Division of Biological Engineering, Massachusetts Institute of Technology, 45 Carleton Street, E25-342, Cambridge, MA 02139, USA.

E-mail address: rlanger@mit.edu (R. Langer).

minimum space between two cell types as determined by the distance between channels or membrane holes [12]. An approach that aims to overcome many of these limitations is through the use of surfaces that can be turned from being cell and protein repulsive to adhesive based on specific stimuli [13–15]. These approaches include the use of electroactive substrates that can be switched from a hydrophilic to a hydrophobic state to promote cell adhesion [13], as well as thermally responsive polymer grafted surfaces [14,15]. Although many elegant switchable surfaces have been developed [16], the fabrication of simple surfaces still requires specialized materials and extensive expertise that may limit their widespread use. Thus, the development of biocompatible, easily applicable and versatile surface engineering approaches that facilitate the fabrication of patterned co-cultures is needed.

One potential method to switch surface properties for such applications is the use of layer-by-layer assembly of polyelectrolytes [17]. Typically such approaches have been used to construct thin polymer films with specific properties [18]. More recently, the layer-by-layer deposition of HA and chitosan has been proposed as a method of repair for damaged blood vessels [19]. Despite these advances, the potential of this approach as a tool for patterning co-cultures has not been investigated.

Here HA and PLL were used to switch surface properties. HA, or hyaluronan, is a polysaccharide containing alternating *N*-acetyl-D-glucosamine and D-glucuronic acid monosaccharide units. As an integral component of extracellular matrix [20], HA is an attractive building block for new biocompatible and biodegradable materials that can be used for drug delivery and tissue engineering [21,22]. HA [23–26] and other polysaccharides [27,28] have been shown to resist the adhesion of many proteins and cells, although not all proteins are repelled by HA [29]. Furthermore HA is negatively charged and can complex with cationic polymers such as PLL, providing for intriguing opportunities to generate surfaces that can be turned from one property to another.

This paper describes a method to pattern two cell types on a surface by using the cell resistant properties of HA. It was hypothesized that the application of a multi-step fabrication would result in initial patterning of a desired cell and/or protein on the exposed adhering substrate. The remaining HA surface could then be converted to cell and protein adhesive through the application of PLL to the surface, which could subsequently facilitate the patterning of the second cell type (Scheme 1). HA was patterned on glass using capillary force lithography [30], followed by adsorption of fibronectin (FN) onto the bare glass substrate. Primary cells were subsequently seeded, which specifically attached onto the FN coated surfaces. Once the cells adhered, HA patterns were complexed with PLL

which switched the patterns from non-biofouling to cell adherent. Secondary cells were subsequently seeded and adhered to the exposed PLL patterns (Scheme 1).

2. Materials and methods

All tissue culture media and serum were purchased from Gibco Invitrogen Corporation, cell lines were purchased from American Tissue Type Collection and all chemicals were purchased from Sigma, unless otherwise indicated.

2.1. Cell culture

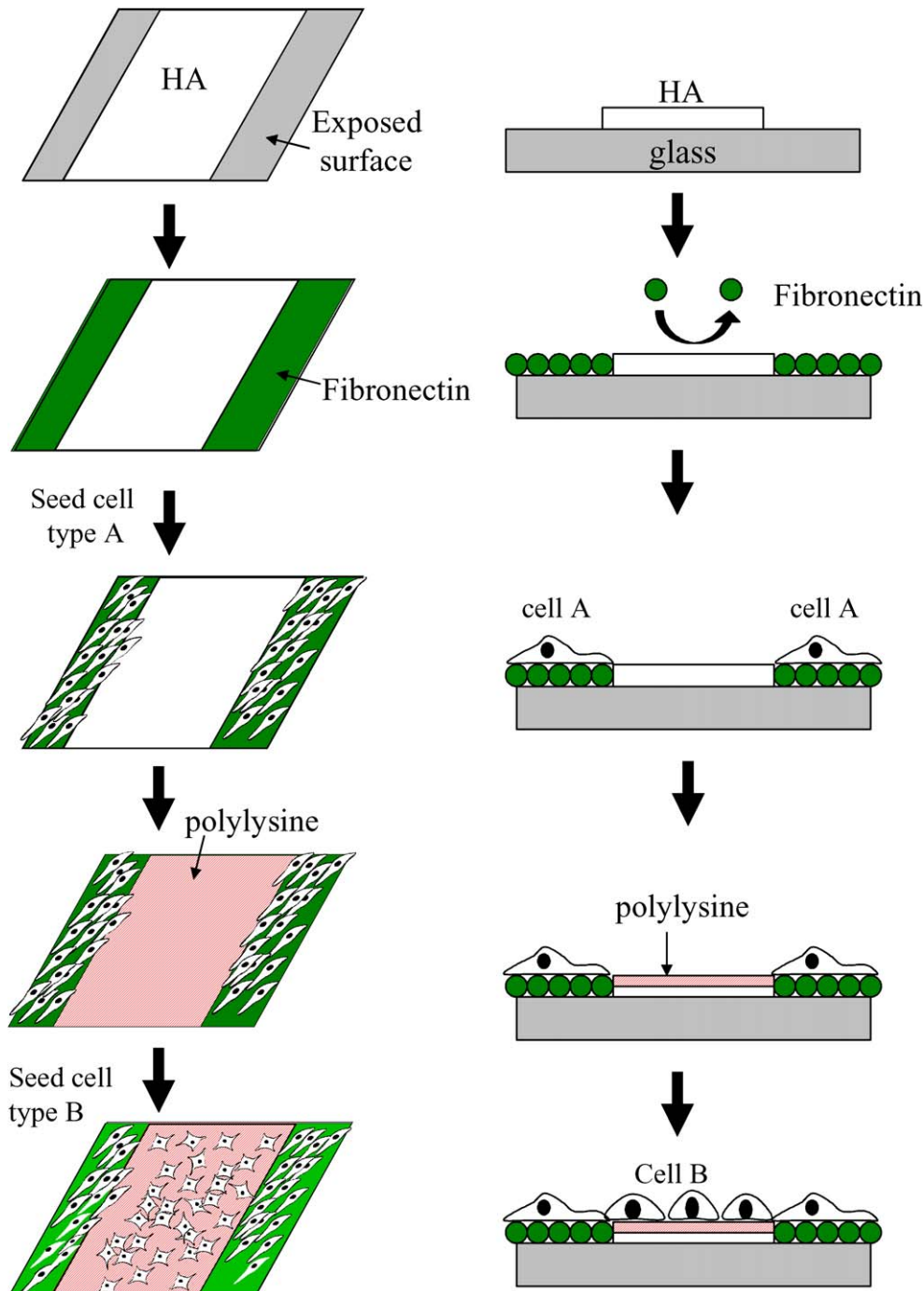
All cells were manipulated under sterile tissue culture hoods and maintained in a 95% air/5% CO₂ humidified incubator at 37°C. NIH-3T3 cells were maintained in 10% fetal bovine serum (FBS) in Dulbecco's modified eagle medium (DMEM). AML12 murine hepatocytes were maintained in a medium comprised 90% of 1:1 (v/v) mixture of DMEM and Ham's F12 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone, and 10% FBS. Confluent dishes of AML12 and NIH-3T3 cells were passaged and fed every 3–4 days. Murine embryonic stem (ES) cells (R1 strain) were maintained on gelatin treated dishes on a medium comprised of 15% ES-qualified FBS in DMEM knockout medium. ES cells were fed daily and passaged every 3 days at a subculture ratio of 1:4. Primary cell patterns were performed with serum supplemented medium specific to the seeded cell type. Depending on the cells, co-cultures were either maintained with medium used for ES cells or AML12 cells.

2.2. Poly-dimethylsiloxane (PDMS) mold fabrication

PDMS molds were fabricated by curing prepolymer (Sylgard 184, Essex Chemical) on silicon masters patterned with SU-8 photoresist. The patterns on the masters had protruding cylindrical holes (ranging in diameter from 15 to 150 μm), which resulted in PDMS replicas with receding cylinders. To cure the PDMS prepolymer, a mixture of 10:1 silicon elastomer and the curing agent was poured on the master and placed at 70°C for 2 h. The PDMS mold was then peeled from the silicon wafer and cleaned with ethanol and acetone prior to use.

2.3. HA coating of surfaces

HA films (Genzyme Inc.) were cast by spin-coating (Model CB 15, Headway Research, Inc.) a solution containing 5 mg HA/ml in distilled water onto



Scheme 1. Schematic diagram of the HA-PLL layering approach to pattern co-cultures. To generate patterned co-cultures, a glass substrate is patterned with HA and subsequently stained with FN which adheres to the exposed substrate. The first cell type is then seeded and allowed to adhere to the FN coated regions. The non-biofouling properties of the HA patterns are subsequently switched by PLL deposition. Secondary cells can then be seeded, resulting in the formation of patterned co-cultures.

silicon dioxide substrates (glass slides or wafers) at 1500 rpm for 10 s. After drying for 12 h at room temperature, the thickness of the resulting film was about 606 nm as measured by ellipsometry (Gaertner Scientific Corp.) and atomic force microscope (Digital Instruments).

2.4. Patterning technique

To pattern surfaces capillary force lithography was used [30]. PDMS molds were washed using soap and water, sterilized in ethanol, dried, and plasma cleaned for 4 min to increase wettability (PDC-001, Harrick

Scientific Co.). A few drops of HA were then spin coated onto a plasma cleaned slide at 1500 rpm for 10 s. The PDMS mold was then placed on the thin layer of HA until it visibly wetted the surface. The mold was left undisturbed for at least 12 h, at which time a visible pattern could be seen.

2.5. Cell patterning

FN was added to the HA patterned glass slides at a concentration of 20 µg/ml in phosphate buffered saline (PBS) for 20 min and then washed. Primary cells at a concentration of 1×10^6 cells/ml were added to the glass slides and allowed to grow overnight. To immobilize PLL, the culture medium was aspirated and replaced by PLL at a concentration of 40 µg/ml for 20 min. The slides were then gently washed to remove cells and then incubated overnight with secondary cells suspended in medium at a concentration of 1×10^6 cells/ml. Cell cultures were analyzed at various times and imaged using an Axiovert 200 inverted microscope (Zeiss). Prior to analysis, glass slides were rinsed with PBS to remove non-adherent cells.

2.6. Cell staining

To stain with PKH26 dye, cells were trypsinized and washed with DMEM medium without serum, and subsequently suspended in a 2×10^{-6} M PKH26 solution of diluent C at a concentration of 1×10^7 cells/ml and incubated for 4 min at room temperature. To stain with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye, cells were suspended in 10 µg/ml CFSE in PBS solution at a concentration of 1×10^7 cells/ml and incubated for 10 min at room temperature. Both staining reactions were quenched with the addition of an equal volume of DMEM supplemented with 10% FBS and washed.

2.7. Protein adsorption

Fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA), FITC labeled immunoglobulin (IgG) and FN were dissolved in PBS at 50, 50 and 20 µg/ml respectively. To test for BSA and IgG protein adhesion, a few drops of the protein solution were evenly distributed onto the patterned substrates and incubated at room temperature for 45 min. To coat with FN, surfaces were dipped into a FN solution for 15 min and subsequently rinsed with PBS. To measure FN adhesion, surfaces were stained with anti-FN antibody for an additional 45 min, followed by 1 h incubation with the phycoerythrin (PE)-labeled anti-rabbit secondary antibody. The patterned substrates were then washed with PBS and analyzed under a fluorescent microscope. All protein staining experiments were done

in triplicate to ensure that multiple pictures were captured. Fluorescent images of various samples were then taken and quantified using NIH-Scion Image viewer. Blank glass slides analyzed under the same light exposure were used as background controls.

2.8. Flow cytometry

AML12, NIH-3T3 and ES cells were stained with propidium iodide (PI) (2 mg/ml) and subsequently analyzed under a FACScan flow cytometer (BD Biosciences). Data was collected and analyzed using the CellQuest software.

2.9. Statistical analysis

The values are represented as average \pm standard deviation. To determine statistical significance we performed one-sided analysis of variance (ANOVA) with $p < 0.05$ considered significant.

3. Results

3.1. Protein and cell patterning of HA coated surfaces

HA films were prepared on silicon dioxide or glass substrates by spin coating. Despite water solubility, a thin layer of HA remained on the patterned surfaces even after extensive washing with PBS. The formation of a stable ultra-thin HA film on the surface was verified by the detection of nitrogen in HA using X-ray photoelectron spectroscopy, as well as ellipsometry and atomic force microscopy measurements, which are described in detail elsewhere [31].

The ability of HA coated surfaces to resist the adsorption of three different proteins was analyzed by quantifying the fluorescent expression of treated glass surfaces. Experiments demonstrated that the adhesion of BSA, IgG, and FN was significantly reduced on HA coated surfaces in comparison to glass controls ($p < 0.001$) (Fig. 1A). This result is supported by previous reports which have indicated that surfaces containing immobilized HA resist protein adsorption [24,31]. In addition, less than 1% of the cells that were seeded onto HA coated surfaces adhered, significantly lower than FN treated controls or glass substrates (Fig. 1B). Interestingly, the addition of FN on HA coated surfaces did not increase the cell adhesion properties of HA, due to low FN adsorption on HA. These results confirm that the non-biofouling properties of HA coatings could be used to repel protein adsorption and cell adhesion.

To test the ability of HA to complex with PLL, HA coated surfaces were treated with FITC-PLL. As illustrated in Fig. 1, FITC-PLL adhered to the HA

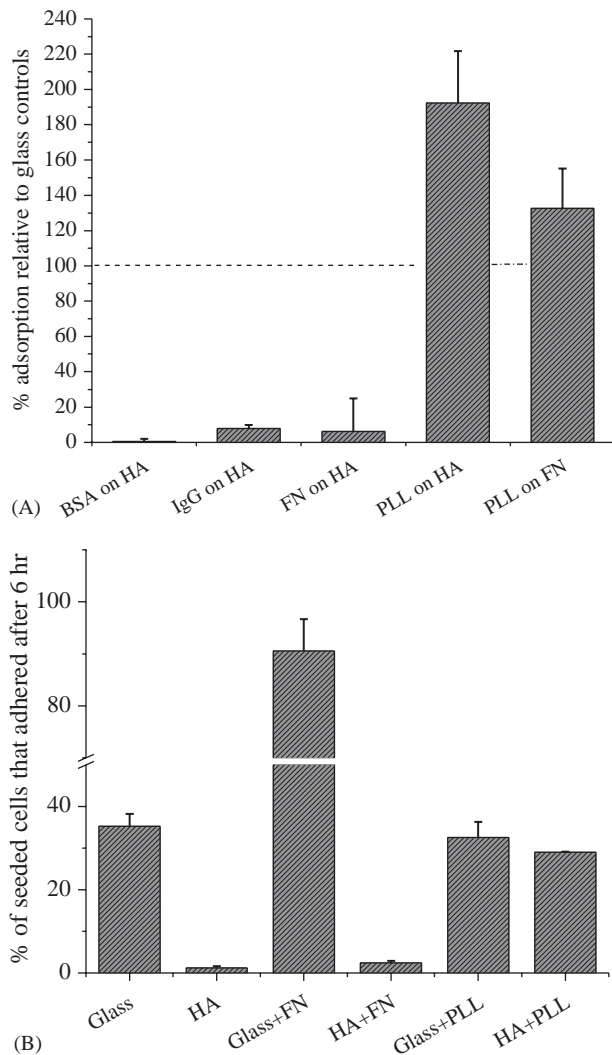


Fig. 1. Protein adsorption and cell adhesion to various surfaces. Figure (A) is the protein adsorption of HA and FN coated surfaces relative to glass controls. BSA, IgG and FN adsorption to HA coated surfaces at significantly lower levels than glass controls ($p < 0.001$). Interestingly, it was found that PLL adsorption to glass and FN coated surfaces were significantly higher than glass controls ($p < 0.05$). Figure (B) is the NIH-3T3 cell adhesion to various surfaces. As it can be seen, cells did not adhere to HA or HA coated surfaces that were FN treated. In contrast, cells adhered to PLL treated HA surfaces at similar levels as PLL treated glass surfaces, indicating that PLL can be used to switch the surface properties of HA.

coated surfaces at significantly higher values than coated glass surfaces ($p < 0.05$). In addition, cells adhered to PLL treated HA coatings at significantly higher values than HA coated controls ($p < 0.05$) (Fig. 1B), further indicating that PLL treatment could reverse the cell and protein resistant properties of HA coated surfaces. In addition, no significant difference was observed between the degree of NIH-3T3 adhesion to glass or PLL treated glass or HA substrates (Fig. 2B), indicating that these cells have the same affinity to attach to PLL or glass surfaces.

To pattern cells and proteins soft lithographic approaches were used [30]. Briefly, a thin film of HA was spin coated onto the substrate and PDMS mold was subsequently placed on top of the film. The dewetting of the polymer film and the capillary force action due to the features of the stamp resulted in the formation of patterned HA. The PDMS stamps were left for 12 h to ensure stabilization of the patterns. HA patterns were visible under light microscopy prior to washing (Fig. 2A) but could not be seen after the surfaces were washed. Thus patterned HA deposited onto the surface could be easily washed away, leaving behind an absorbed layer that maintained the biofouling resistant properties of HA. HA patterns were capable of fabricating arrays of patterned proteins such as FN (Fig. 2B), BSA and IgG (data not shown). To examine the feasibility of the approach to pattern arrays of cells murine embryonic fibroblasts (NIH-3T3), liver (AML12) and embryonic stem (ES) cells were immobilized onto the patterned surfaces that were treated with HA. All three cell lines were patterned on HA surfaces with high consistency and cell pattern fidelity (Fig. 2C–F). In addition, the feature size of the cell patterns could be controlled to produce islands with individual cells ($15 \mu\text{m}$) to those with large multi-cellular aggregates ($> 200 \mu\text{m}$).

3.2. PLL adsorption on patterned protein and cells

To examine the ability of PLL to adsorb to HA patterns, patterned surfaces that were treated with FN were incubated with a solution containing FITC-PLL. As illustrated in Fig. 3A, labeled FN patterns were clearly visible in the shape of grids through adsorption to the exposed substrate. In addition, PLL selectively adhered on HA patterns (square shaped), although lower levels of background adsorption could be observed on FN coated regions (Fig. 3A). These results correlate with the surface binding affinities of Fig. 1, which indicates that both HA and FN coated surfaces adsorb PLL at significantly higher levels than glass controls (1.9 ± 0.3 and 1.45 ± 0.3 times respectively ($p < 0.05$)).

Despite the ability of PLL to adsorb onto FN coated substrates, the feasibility of using PLL is dependant on low adsorption onto cells, as cells cover FN treated regions prior to PLL treatment. To further investigate this phenomenon, the competitive binding of PLL to patterned cells relative to HA surfaces was evaluated. Although flow cytometric staining of FITC-PLL treated cells, indicate that PLL adsorbed to a large fraction of viable cells (71% for ES cells, 74% for AML12 cells and 79% for NIH-3T3 cells respectively), fluorescent images show that PLL adhered to the HA coated regions at detectably higher ratios than cells (Fig. 3B) (other cell types yielded similar images, not shown). This may be

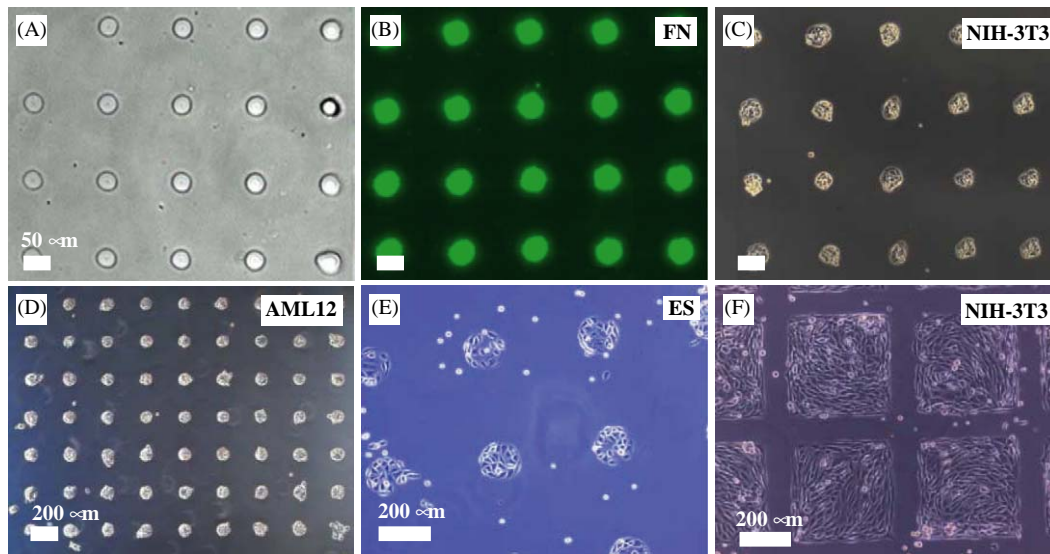


Fig. 2. Cell and protein patterning of HA coated surfaces. Figure (A) is the optical image of an HA pattern on a glass substrate prior to washing, (B) is a fluorescent image of fibronectin adsorption on the surface of (A), and figure (C) illustrates fibroblast adhesion on the surface of (B). Figures (D–F) demonstrate the applicability of the technique for patterning various cell type including AML12 (D), ES (E), and NIH-3T3 cells (C, F) at various length scales.

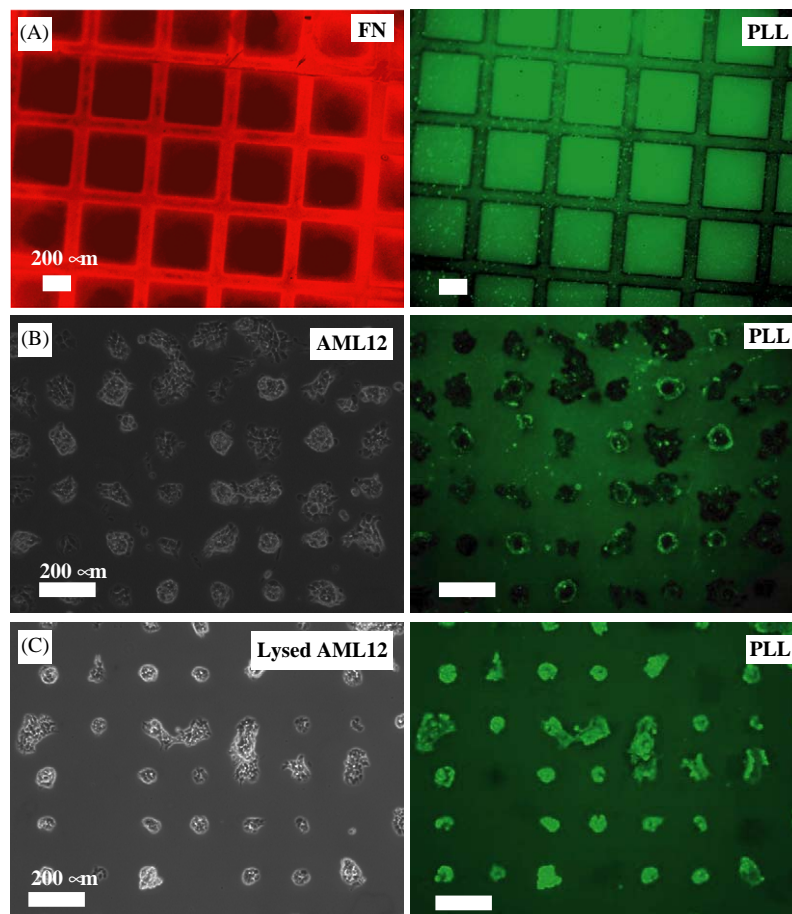


Fig. 3. PLL attachment on patterned HA surfaces and immobilized cells. Figure (A) represents fluorescent images of HA patterned glass slides that were stained with FN (left) and subsequently treated with PLL (right). FN attached on the exposed grids, while PLL selectively adhered to the HA squares. As expected, background staining to the FN coated regions is detectable. PLL selectively adsorbed on HA coated regions in comparison to patterned AML12 hepatocytes (B). Interestingly, if the cells were lysed with 0.4% Triton-X prior to PLL treatment, PLL adhered to the cytoplasmic components of lysed cells (C) indicating that under normal PLL treatment many cells remained viable (NIH-3T3 and ES cells yielded similar images).

attributed to the higher binding affinity of PLL to HA in comparison to membrane bound proteoglycans, glycosaminoglycans and other negatively charged constituents of the cell membrane.

Since high concentrations of PLL have been shown to induce cell toxicity [32], the viability of cells that were treated with PLL was also evaluated. PI staining of NIH-3T3, AML12 and ES cells indicated that >95% of adhered cells that were treated with PLL (at 40 µg for 20 min) were negative for PI, and therefore alive. Furthermore, PLL selectively adsorbed to cells that were lysed with Triton-X, possibly due to the adsorption of PLL to cytoplasmic components of the cell (Fig. 3C). Therefore, the low number of stained cells after PLL treatment shown in Fig. 3B is further evidence that cells remained alive after PLL treatment.

3.3. Patterned co-cultures of ES cells with fibroblasts

To demonstrate the applicability of the patterned co-culture technique, two systems were tested. The first was the co-cultures of fibroblasts with hepatocytes which has been shown to better maintain the differentiated properties of primary hepatocytes in culture [2,33]. The other system was co-cultures of ES cells and embryonic fibroblasts that have been used to prevent the differentiation of ES cells [34]. Random co-cultures of hepatocytes or ES cells with fibroblasts have heterogeneous interactions that lack the control and spatial orientation that is present within the liver or the developing embryo (Supplementary Fig. 1). Thus, patterned co-cultures could potentially provide interesting opportunities to improve these systems.

To generate patterned co-cultures, HA patterned substrates were treated with FN in PBS and seeded with the first cell type and incubated for 24 h. Once primary seeded cells were adhered, patterned substrates were treated with PLL, washed and seeded with the secondary cells. To visualize the co-cultures, each cell type was fluorescently labeled with either a membrane labeling dye (PKH26-red) or cytoplasmic tracer (CFSE-green). At various times after initiation of the co-culture, cells were analyzed under light and fluorescent microscopy.

Fig. 4A is an image of co-cultures of ES cells with NIH-3T3 fibroblast in patterned co-cultures of circular FN islands. Both light and fluorescent images indicate that ES cells were clearly patterned in dense spheroid aggregates against a background monolayer of NIH-3T3 cells. The aggregation of ES cells is due to strong self-interactions that are associated with numerous cell adhesion molecules such as E-cadherin [35]. Fluorescent images confirmed that NIH-3T3 cells were restricted to the HA coated regions and were not seeded on top of ES cells as indicated by the lack of yellow regions on the

aggregates (indicating the presence of both ES and NIH-3T3 cells). ES cell co-cultures remained stable for at least 5 days (Fig. 4A–C). At day 3 (Fig. 4B), ES cell aggregates were ~150 µm in diameter and increased to >200 µm by day 5 (Fig. 4C). In addition, the aggregates remained lodged in place and were not washed away by mechanical agitation and medium replacement. Fluorescent images of the cultures after 5 days confirmed that ES cells had not differentiated into cells with fibroblastic phenotype and were constrained within the spheroid geometry of the aggregate.

In addition, the versatility of the technique to form patterns upon reversing the order of cell seeding was validated. It was found that co-cultures could be established with fibroblasts being immobilized to the circular islands and the ES cells adhering to the remaining regions. These results were confirmed by morphological differences as well as fluorescent staining of the two cell types (Fig. 4D).

3.4. Patterned co-cultures of hepatocytes with fibroblasts

Patterned co-cultures of hepatocytes with fibroblasts were also established. In contrast to the sharp morphological difference that was observed between ES cell and NIH-3T3 co-cultures, hepatocyte co-cultures were morphologically more difficult to distinguish under light microscope (Fig. 5A). Fluorescent images of stained co-cultures validated the formation of patterns. As it can be seen in Fig. 5A, hepatocytes (red) were seeded on 100 µm circular islands, while the fibroblasts (green) were seeded in the surrounding regions. The pattern fidelity of hepatocyte co-cultures was not as sharp as ES cell co-cultures, possibly due to stronger interactions between ES cells within the spheroids which prevented ES cells from migrating into the surrounding regions. In addition, after 24 h a small fraction of NIH-3T3 cells could be seen attached on top of hepatocyte colonies, which could be attributed to higher binding affinity of hepatocytes to PLL in comparison to ES cells. Despite this, patterned co-cultures remained stable for at least 5 days after co-culture initiation (Fig. 5B), although a larger fraction of cells had migrated out of their originally seeded regions in comparison to ES cell co-cultures. It is important to note that since fluorescence intensity of the tracer dye is halved upon each cell division, stained cells in Fig. 5B were less fluorescent in comparison to the original patterns.

To demonstrate that the patterning is independent of the cell type, patterned co-cultures comprised of only hepatocytes (Fig. 5C), ES cells, or NIH-3T3 fibroblasts (data not shown), were generated. It was found that same cells could be used for patterned co-cultures, thus the approach is independent of the differences between primary and secondary seeded cells.

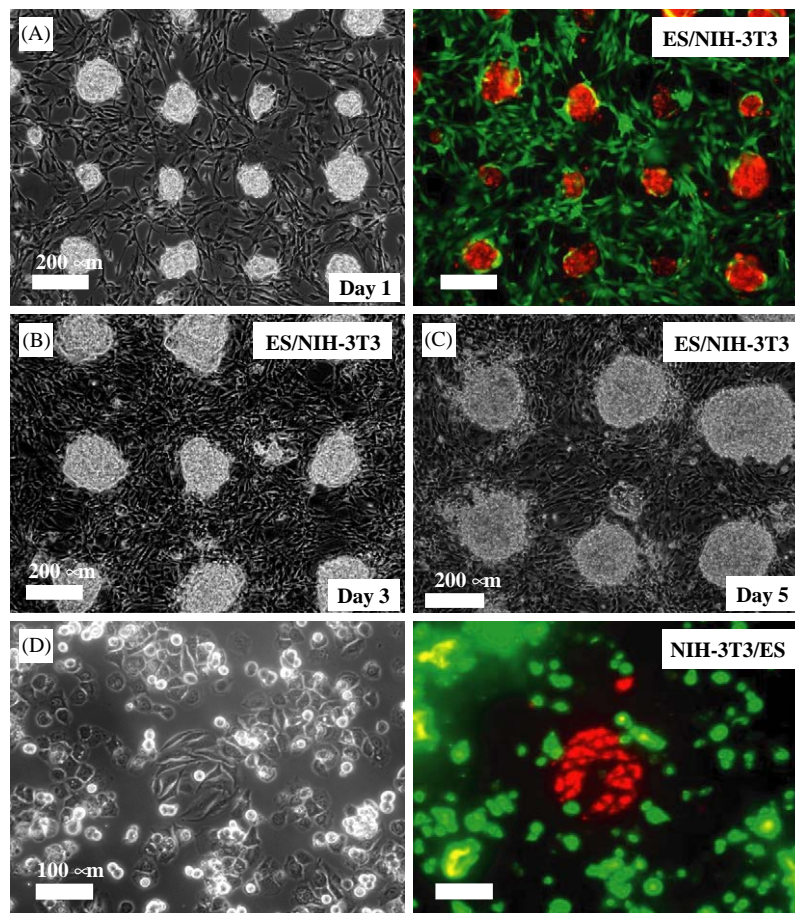


Fig. 4. Patterned co-cultures of ES cells with fibroblasts. Figure (A) represents light (left) and fluorescent (right) images of patterned co-cultures of ES cells (red) with NIH-3T3 fibroblasts (green) after 1 day. Figures (B) and (C) illustrate that co-cultures remained stable for 3 and 5 days respectively. Figure (D) represents the reversal in the order of cell seeding in which NIH-3T3 fibroblasts (red) were initially seeded followed by ES cells as the secondary cells. Therefore the technique is independent from the type of cell initially seeded.

4. Discussion

The application of layer-by-layer deposition of polyionic molecules for cell and protein patterning was demonstrated. There are a number of advantages associated with this technique over previously developed co-culture approaches. For example, unlike approaches that are based on the selective adhesion of two different cell types or exclusion of serum for cell seeding, the approach developed here is not dependent on selective cell adhesion or the absence of serum. In addition, this method provides all the advantages associated with soft lithography, while preventing the use of organic solvents that are used in photolithographic based co-cultures. Furthermore, the approach utilizes the advantages associated with switchable surfaces, yet it does not require changes in temperature or electroactive surfaces that may be difficult to fabricate. Another important advantage with the approach is the availability of the materials used in the co-cultures. Both HA and PLL are commercially available and widely used. Furthermore,

the immobilization of HA or PLL does not require any chemistry or complex techniques. The simplicity of this approach and off-the-shelf availability of PLL, HA and FN, should make this technique easily applicable in biologically oriented labs which may lack the resources required for other patterned co-culture techniques.

Although we have focused on FN and PLL, it is anticipated that the proposed approach would have a wide application in patterning of a multiple proteins on a surface. This is because proteins could be used to directly attach to PLL (Fig. 1A), or attached to biotinylated PLL. The applicability of PLL patterns for subsequent attachment of other polyionic molecules and proteins is currently under investigation.

One of the challenges with the described technique is the use of PLL which has been shown to be cytotoxic at high concentrations. In our protocol we have chosen an optimum PLL concentration in order to modify HA surfaces and minimize cell toxicity. At higher concentrations cells would die while at lower concentrations or shorter exposure times cells would not adhere to the

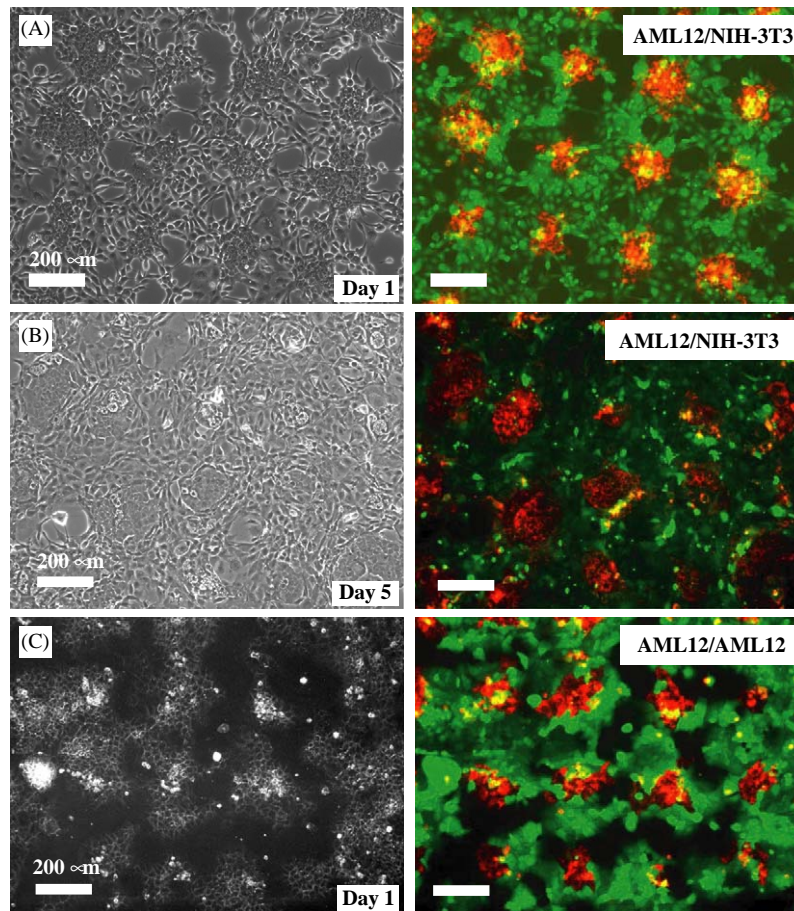


Fig. 5. Patterned co-cultures of hepatocytes with fibroblasts. Figure (A) represents the light and fluorescent images of AML12 (red) in co-culture with NIH-3T3 fibroblasts (green) after 1 day in culture. In addition, the cultures remained stable for at least 5 days (B). Figure (C) demonstrates a co-culture of AML12 (red) cells with other AML12 cells (green), thereby showing that the same cell type could be patterned in co-cultures.

coated regions. The use of other non-toxic adhesion molecules that can adsorb to HA could be potentially beneficial for overcoming this limitation. Another limitation with the current technique is the use of ionic molecules for the switching of surface properties. It is anticipated that this limits the approach to a range of pH values that maintains that electrostatic interactions between the molecules, although for cell culture conditions this should not be a major concern.

We have not analyzed the biochemical behavior of the cells in these cultures. Although previous studies have found that the degree of cell–cell interaction can greatly influence the behavior of the hepatocytes and fibroblast co-cultures [5,6], the role of such interactions in ES cell cultures has not been explored. In addition, since the patterned co-cultures are more homogenous in comparison to random co-cultures (Figs. 3 and 4 and Supplemental Fig. 1), it is expected that they will provide a more uniform cellular behavior. An intriguing possibility with these co-cultures is their use to study ES cell differentiation in presence of other cell types. Detailed analysis of these interactions is a subject of active study in our lab.

5. Conclusions

In conclusion, an approach based on the layer-by-layer deposition of polyionic molecules for patterning of two cell types was introduced. The feasibility of the approach to immobilize PLL and FN on glass substrates was demonstrated. Furthermore, patterned co-cultures of two different cell types were generated for ES cell and hepatocytes in co-culture with fibroblasts. The generation of co-cultures was independent of the selective adhesion of the cell types as well as seeding order. In addition, the co-cultures remained stable for at least 5 days. It is anticipated that with the ever increasing use of soft lithography in biology, this simple approach to pattern co-cultures could potentially provide a valuable tool to study cell–cell interactions, maintain cells in culture and engineer organs for tissue engineering.

Acknowledgements

This research has been supported by NIH (NIH grant # HL60435), NSF (through Bioprocess Engineering

Research Center) and the U.S. Army (through the Institute of Soldier Nanotechnology). AK is partially funded through a post graduate fellowship (PGS-B) from Natural Sciences and Engineering Research Council of Canada. Dr. Yang would like to acknowledge the support from the National Science Council of Republic of China under the Grant of NSC-40212-F and the partial support from Chang Gung Hospital.

References

- [1] Langer R, Vacanti JP. Tissue engineering. *Science* 1993;260:920–6.
- [2] Bhatia SN, Balis UJ, Yarmush ML, Toner M. Effect of cell–cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *Faseb J* 1999;13:1883–900.
- [3] Bhatia SN, Toner M, Tompkins RG, Yarmush ML. Selective adhesion of hepatocytes on patterned surfaces. *Ann NY Acad Sci* 1994;745:187–209.
- [4] Bhatia SN, Yarmush ML, Toner M. Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3T3 fibroblasts. *J Biomed Mater Res* 1997;34:189–99.
- [5] Bhatia SN, Balis UJ, Yarmush ML, Toner M. Probing heterotypic cell interactions: hepatocyte function in microfabricated co-cultures. *J Biomater Sci Polym Ed* 1998;9:1137–60.
- [6] Bhatia SN, Balis UJ, Yarmush ML, Toner M. Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions. *Biotechnol Prog* 1998;14:378–87.
- [7] Ostuni E, Kane R, Chen CS, Ingber DE, Whitesides GM. Patterning mammalian cells using elastomeric membranes. *Langmuir* 2000;16:7811–9.
- [8] Takayama S, McDonald JC, Ostuni E, Liang MN, Kenis PJA, Ismagilov RF, Whitesides GM. Patterning cells and their environments using multiple laminar fluid flows in capillary networks. *Proc Natl Acad Sci USA* 1999;96:5545–8.
- [9] Folch A, Toner M. Cellular micropatterns on biocompatible materials. *Biotechnol Prog* 1998;14:388–92.
- [10] Folch A, Jo BH, Hurtado O, Beebe DJ, Toner M. Microfabricated elastomeric stencils for micropatterning cell cultures. *J Biomed Mater Res* 2000;52:346–53.
- [11] Folch A, Toner M. Microengineering of cellular interactions. *Annu Rev Biomed Eng* 2000;2:227–56.
- [12] Chiu DT, Jeon NL, Huang S, Kane RS, Wargo CJ, Choi IS, Ingber DE, Whitesides GM. Patterned deposition of cells and proteins onto surfaces by using three-dimensional microfluidic systems. *Proc Natl Acad Sci USA* 2000;97:2408–13.
- [13] Yousaf MN, Houseman BT, Mrksich M. Using electroactive substrates to pattern the attachment of two different cell populations. *Proc Natl Acad Sci USA* 2001;98:5992–6.
- [14] Yamato M, Konno C, Utsumi M, Kikuchi A, Okano T. Thermally responsive polymer-grafted surfaces facilitate patterned cell seeding and co-culture. *Biomaterials* 2002;23:561–7.
- [15] Hirose M, Yamato M, Kwon OH, Harimoto M, Kushida A, Shimizu T, Kikuchi A, Okano T. Temperature-responsive surface for novel co-culture systems of hepatocytes with endothelial cells: 2-D patterned and double layered co-cultures. *Yonsei Med J* 2000;41:803–13.
- [16] Lahann J, Mitragotri S, Tran TN, Kaido H, Sundaram J, Choi IS, Hoffer S, Somorjai GA, Langer R. A reversibly switching surface. *Science* 2003;299:371–4.
- [17] Decher G. Fuzzy nanoassemblies: toward layered polymeric multicomposites. *Science* 1997;277:1232–7.
- [18] Vazquez E, Dewitt DM, Hammond PT, Lynn DM. Construction of hydrolytically-degradable thin films via layer-by-layer deposition of degradable polyelectrolytes. *J Am Chem Soc* 2002;124:13992–3.
- [19] Thierry B, Winnik FM, Merhi Y, Tabrizian M. Nanocoatings onto arteries via layer-by-layer deposition: toward the in vivo repair of damaged blood vessels. *J Am Chem Soc* 2003;125:7494–5.
- [20] Mullegger J, Lepperdinger G. Hyaluronan is an abundant constituent of the extracellular matrix of *Xenopus* embryos. *Mol Reprod Dev* 2002;61:312–6.
- [21] Luo Y, Kirker KR, Prestwich GD. Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *J Control Release* 2000;69:169–84.
- [22] Vercurysse KP, Prestwich GD. Hyaluronate derivatives in drug delivery. *Crit Rev Ther Drug Carrier Syst* 1998;15:513–55.
- [23] Pavesio A, Renier D, Cassinelli C, Morra M. Anti-adhesive surfaces through hyaluronan coatings. *Med Device Technol* 1997;8(20–1):24–7.
- [24] Chen G, Ito Y, Imanishi Y, Magnani A, Lamponi S, Barbucci R. Photoimmobilization of sulfated hyaluronic acid for antithrombogenicity. *Bioconjug Chem* 1997;8:730–4.
- [25] Barbucci R, Magnani A, Rappuoli R, Lamponi S, Consumi M. Immobilisation of sulphated hyaluronan for improved biocompatibility. *J Inorg Biochem* 2000;79:119–25.
- [26] Magnani A, Albanese A, Lamponi S, Barbucci R. Blood-interaction performance of differently sulphated hyaluronic acids. *Thromb Res* 1996;81:383–95.
- [27] Frazier RA, Matthijs G, Davies MC, Roberts CJ, Schacht E, Tendler SJ. Characterization of protein-resistant dextran monolayers. *Biomaterials* 2000;21:957–66.
- [28] Osterberg E, Bergstrom K, Holmberg K, Schuman TP, Riggs JA, Burns NL, Van Alstine JM, Harris JM. Protein-rejecting ability of surface-bound dextran in end-on and side-on configurations: comparison to PEG. *J Biomed Mater Res* 1995;29:741–7.
- [29] Miyake K, Underhill CB, Lesley J, Kincade PW. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J Exp Med* 1990;172:69–75.
- [30] Suh KY, Seong J, Khademhosseini A, Laibinis PE, Langer R. A simple soft lithographic route to fabrication of poly (ethylene glycol) microstructures for protein and cell patterning. *Biomaterials* 2004;25:557–63.
- [31] Suh H, Yang JM, Khademhosseini A, Tran TN, Eng G, Langer R. Direct immobilization of hyaluronic acid on solid substrates for cell and protein patterning, submitted for publication.
- [32] Fischer D, Li Y, Ahlemeyer B, Kriegelstein J, Kissel T. In vitro cytotoxicity testing of polyocations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* 2003;24:1121–31.
- [33] Talbot NC, Pursel VG, Rexroad Jr CE, Caperna TJ, Powell AM, Stone RT. Colony isolation and secondary culture of fetal porcine hepatocytes on STO feeder cells. *In Vitro Cell Dev Biol Anim* 1994;30A:851–8.
- [34] Richards M, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol* 2002;20:933–6.
- [35] Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol Bioeng* 2002;78:442–53.