Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Acta Biomaterialia 3 (2007) 151–161
Acta BIOMATERIALIA
www.actamat-journals.com

# Controlled polymerization chemistry to graft architectures that influence cell-material interactions

Robert P. Sebra [a], Sirish K. Reddy [a], Kristyn S. Masters [b], Christopher N. Bowman [a,c], Kristi S. Anseth [a,b,*]

[a] *Department of Chemical and Biological Engineering, ECCH 111, CB424, University of Colorado, Boulder, CO 80309-0424, United States*
[b] *Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80309-0424, United States*
[c] *University of Colorado Health Sciences Center, Biomaterials Research Center, Denver, CO 80262, United States*

Received 10 November 2005; received in revised form 5 July 2006; accepted 19 July 2006

**Abstract**

Acrylate monomers were photografted from polymer substrates to create cell responsive chemically and biologically active surfaces that manipulate cell response. Three monomers, polyethylene glycol monoacrylate (MW 375 g/mol) (PEG375A), a monomeric extra-cellular matrix protein, and a cell-cleavable fluorescent monomer, were spatially photopatterned from a base substrate. The base substrate consisted of a dithiocarbamate (DTC) functionalized urethane diacrylate/tri(ethylene glycol)diacrylate copolymer and was shown to non-specifically support NIH 3T3 fibroblast cell adhesion. The DTC-containing polymer was further modified by grafting PEG375A to demonstrate selective blocking of cell-material interactions. Next, acrylated collagen type I was patterned onto polymer substrates to further promote specific cell interactions (i.e. by presenting cell-adhesive moieties). Hydrophilic PEG375A grafted patterns were shown to prevent 3T3 fibroblast adhesion to polymer in spatially grafted regions, while biologically active acrylated collagen type I promoted cell-surface interactions. Collagen type I was grafted at varying densities (0–7.5 pmol/grafted square), and the extent of cell adhesion and spreading were evaluated for each of these graft densities using fluorescence microscopy. Finally, methacrylated carboxyfluorescein diacetate (CFDA) was synthesized and photografted onto a cell-adhesive substrate as a cell sensing mechanism. The acetate groups found in the structure of CFDA cleave in the presence of cells. This cell-responsive substrate results in fluorescence indicative of acetate-group cleavage associated with cell interactions that occurs in patterned regions on polymer surfaces. Collectively, the results herein show the utility and application of a spatially and temporally controlled photografting process for designing cell responsive polymer surfaces.
© 2006 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

*Keywords:* Biomaterials; Photopatterning; Grafting; Cell adhesion; Bioconjugation

## 1. Introduction

The development of functional biomaterials that control cellular adhesion, spreading, proliferation, and differentiation has greatly impacted fields related to tissue engineering [1–3], biosensing [4–10], and biomedical research [11]. Directed cell attachment and differentiation, influenced by polymeric scaffold surface properties, greatly impact the ability to form organized tissue structures that include one or more cell types. The two major approaches for controlling cell behavior on surfaces include modification of physical features (e.g., surface topology or geometry) [12,13] or the introduction of biochemical functionality [14–16]. Chemical modification is especially useful in promoting or preventing cell processes on surfaces that would otherwise elicit non-specific or uncontrolled responses.

Researchers have used a variety of surface modification chemistries to manipulate cell-material interactions. Self-assembled monolayers (SAMs) [15,16], direct surface

* Corresponding author. Address: Department of Chemical and Biological Engineering, ECCH 111, CB424, University of Colorado, Boulder, CO 80309-0424, United States. Tel.: +1 303 492 3147.
*E-mail address:* kristi.anseth@colorado.edu (K.S. Anseth).

1742-7061/$ - see front matter © 2006 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2006.07.010

conjugation techniques, such as coupling through amine, thiol, or phenyl azido groups [2,14,17–19], and direct cross-linking of biological and chemical functionalities that influence cell-material interactions [1–3,20] are all accepted ways of controlling cell processes on surfaces. For example, Whitesides et al. established the microcontact lithographic technique for patterning chemical cues to control cell function on surfaces [21–23]. Other researchers have used hexaethylene glycol and methyl-terminated SAMs to pattern glass, silicon, and poly(dimethylsiloxane) (PDMS) substrates for controlled cellular adhesion and spreading [24–27]. Surface exposure to the micropatterns of extracellular matrix proteins like fibronectin led to the attachment of endothelial cells to methyl-modified regions, while background ethylene glycol patterned regions remained resistant to protein adsorption and subsequent cell adhesion [24–27]. Patterning methods, such as those using SAMs, have been established to spatially control cell position and distribution on glass and metal surfaces; however, SAM chemistries are substrate-limited. Recently, chemistries for the modification of polymer materials such as poly(ethylene glycol) (PEG), polystyrene, and PDMS have been developed to better control cell adhesion on these cell culture substrates [13,14,16,18–20].

The direct conjugation of proteins and other bioactive functional groups has also been demonstrated using thiol, carboxyl, amine, *N*-hydroxy succinimide (NHS), and meth(acrylate)-terminated sequences, which are readily incorporated into copolymer networks [1,3,28]. Specifically, RGD [2] and fibronectin [20] have been covalently immobilized to PEG hydrogel networks to influence osteoblast and fibroblast adhesion and function. Nuttelman et al. demonstrated the attachment of fibronectin onto the surfaces of mesoporous poly(vinyl alcohol) hydrogels to influence fibroblast adhesion and migration on these gels [20]. However, retaining biological function of surface immobilized proteins is difficult. Although it is commonly known that conjugation chemistry impacts ligand activity and specificity, recent evidence also suggests that ligand spacing, concentration, clustering, and connectivity also play important roles in determining the extent of cell adhesion to a surface [8,29–32]. To circumvent limitations regarding reduced protein surface mobility, density, and activity when using direct conjugation and monolayer chemistries, techniques for covalently grafting proteins from biomaterials surfaces in a controlled fashion are of great interest.

Research related to the grafting of chemical functionalities (e.g., the cell-adhesive RGD peptide sequence) that affect cell adhesion has been reported in the literature, yet studies related to controlled grafting of peptides and proteins from polymer surfaces remain few. Matsuda and coworkers grafted cationic chains of poly(acrylamide) from the surfaces of poly(benzyl *N*,*N*-diethyldithiocarbamate-co-styrene) and phenyl azido coated poly(ethylene terepthalate) to show the influence of densely grafted surface charge on cell adhesion [14,33]. The results presented using this photochemical technique pioneered the use of photochemistry to pattern regions of grafted chemical functionality in order to control cell adhesion spatially on polymer surfaces. Expanding on this concept, we previously utilized quasi-living radical photopolymerizations to modify polymer substrates, including the use of dithiocarbamate (DTC) based photoiniferters, which were pioneered by Otsu [34]. The substrates formed through this DTC-based, iniferter-mediated living radical photopolymerization have previously been extensively characterized and their ability to reinitiate and subsequently graft reactive vinyl species upon exposure to ultraviolet (UV) light has been demonstrated [35–38]. Our previous work using controlled radical photopolymerization chemistry has shown the retention of protein activity when grafting conjugated antibodies to create hydrophilic surface tethers [8,32]. Furthermore, the impact of antibody graft architecture and surface orientation on biological activity and specificity was demonstrated in biosensing, improving conventional surface-based detection sensitivities and assay speed [8,32]. The same design principles of controlled radical polymerization apply in designing surface immobilization chemistries and architectures for proteins that impact cell adhesion and function.

In this study, we demonstrate the versatility of this controlled photografting technique to graft acrylated chemical and biological functionalities that impact cell interactions with polymer substrate surfaces. Utilization of the spatial and temporal control of the photoinitiated grafting process afforded a simple method to control cell-material interactions through designing dense, surface immobilized, and biologically active protein tethers. Aside from LRP's integral role in designing grafted architectures with spatial and temporal control over protein orientation, LRP also offers a means for improving the stability of grafted proteins. Through LRP attachment chemistry, proteins have been oriented in a fashion that facilitates more accessible, mobile sites for interactions with cells and/or other proteins [8,32]. The covalent nature of LRP grafting chemistry also reduces the risk of protein desorption, as experienced when using physisorption-based attachment methods. Further, more dense tethers can be developed through utilization of the temporal control allotted by the LRP technique [8,22].

Specifically in this research, a substrate material that supported cell adhesion through non-specific protein interactions was modified with hydrophilic PEG grafts to block cell-material interactions. Additionally, collagen type I grafts promoted a specific pathway for cell adhesion through the use of cell-adhesive peptide moieties within the structure of collagen type I. In addition to controlling cell adhesion, a carboxyfluorescein diacetate (CFDA) monomer was synthesized and grafted onto polymer substrates to yield a cell-responsive substrate. Previously, CFDA molecules in solution were shown to enter the cell through the cell membrane and cleave in presence of esterases to form a fluorescent molecule [39]. Although, the

exact mechanism of the grafted CFDA-cell interactions is not well understood, the tethered CFDA moieties are believed to be cleaved by either the intracellular esterases (when the tether enters the cell membrane) or externally through extracellular esterases secreted by cells. The ability to spatially photopattern these photoreactive molecules with vastly different biological and chemical properties onto a substrate material demonstrates the wide range of triggered cellular responses that can be attained using this technique.

## 2. Methods and materials

### 2.1. Materials

Urethane diacrylate (UDA) Ebecryl 4827 was donated by UCB Chemicals Corp (Smyrna, GA). Tri(ethylene glycol)diacrylate monomer (TEGDA) was purchased from Sartomer (West Chester, PA). Poly(ethylene glycol (375)) monoacrylate and tetraethylthiuram disulfide (TED), and poly(ethylene glycol) MW 700 diacrylate were all purchased from Sigma–Aldrich (St. Louis, MO). Poly(ethylene glycol)-acrylate-*N*-hydroxysuccinimide of MW 3400 (ACRL-PEG-NHS) was purchased from Nektar Therapeutics (Birmingham, AL). The initiator, 2,2-dimethoxy-2-phenylacetophenone (DMPA) was purchased from Ciba Specialty Chemicals (Tarrytown, NY). Collagen type I was purchased from Collagen Corp. (Palo Alto, CA). Primary mouse anti-collagen type I antibody and Fraction V bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO) and goat anti-mouse-HRP conjugated secondary antibody was purchased from MP Biomedical (Irvine, CA). CFDA succinimidyl ester was purchased from Molecular Probes (Eugene, OR).

## 3. Experimental methods

### 3.1. Preparation of surface reactive, polyurethane-tri(ethylene glycol) and poly(ethylene glycol) polymer substrates

Cell adhesive, grafting substrates were prepared from monomer formulations consisting of 48.75 wt.% aromatic urethane diacrylate (UDA) and 48.75 wt.% triethylene glycol diacrylate (TEGDA) mixed with 1 wt.% tetraethylthiuram disulfide (TED) and 1.5 wt.% 2,2-dimethoxy-2-phenylacetophenone (DMPA) initiator. The formulations were sonicated for 45 min and purged with argon gas for 2 min prior to photopolymerization. The substrate was photopolymerized by exposure to a 45 $mW/cm^2$ intensity collimated, broad-range UV light (Hg arc-lamp centered at 365 nm) for 500 s. The contact liquid photolithographic polymerization (CLiPP) technique utilized for the substrate formation enables fabrication of geometrically and functionally complex devices via unique photoinitiation chemistry in conjugation with liquid monomer enclosed within a custom-designed mask aligner tool [40]. These exposure conditions yielded a polymeric network with over 90% double bond conversion. Conversion was measured by monitoring the acrylate double bond absorbance peak using near-IR analysis. Once polymerized, substrate samples were washed in copious amounts of methanol to remove any unreacted species prior to the photografting procedure. Non-cell adhesive substrates were made using the same protocol, but using a 48.75 wt.% poly(ethylene glycol) MW 1000 diacrylate copolymerized with 48.75 wt.% poly(ethylene glycol) MW 700 diacrylate in the presence of 1 wt.% TED and 1.5 wt.% DMPA photoinitiator.

### 3.2. Synthesis of biologically active, acrylated collagen type I

Collagen type I was acrylated by first dissolving the lyopholized protein into 0.1 mL of MES buffer (2-Morpholinoethanesulfonic acid, monohydrate), pH 2.1. Then, this solution was diluted at 2 mg/mL in 50 mM sodium bicarbonate, pH 7.4, to couple the antibody lysine groups with monoacrylated poly(ethylene glycol) *N*-hydroxysuccinimide (ACRL-PEG-NHS, PEG spacer MW 3400). A molar ratio of 0.5 (ACRL-PEG-NHS:$NH_2$) was used. The reaction was allowed to proceed for 3 h at room temperature with shaking. Excess ACRL-PEG-NHS and other reaction byproducts were removed via dialysis against 0.1 M HCl for 24 h, dialysed in deionized water for 24 h (Slide-A-Lyzer, MWCO of 10,000, Pierce), and lyophilized to obtain a solid product. The structure of the acrylated collagen type I monomer is shown schematically in Fig. 1.

The collagen type I tripled stranded structure contains both cell adhesive sequences and lysine groups that readily facilitate bioconjugation chemistry using NHS:$NH_2$ chemistry. After synthesizing acrylated collagen type I, verification of protein acrylation and activity was determined. To ensure that the collagen type I protein was acrylated, it was dissolved into a solution of PEG375A at a concentration of 1 mg/mL and photografted for 900 s. After grafting, an indirect enzyme-linked immunosorbent assay (ELISA) was performed to verify that collagen type I was present on the surface grafted region as compared to a control where non-acrylated collagen type I was used instead. After the appropriate swelling was performed, approximately a 5-fold chromogenic signal, positive for collagen type I, was shown on surfaces patterned with acrylated collagen type I as compared to the control.

Activity of the modified protein was determined by using a standard indirect ELISA protocol for the detection of collagen type I concentrations ranging from ($5.0 \times 10^{-11}$ M–$5.0 \times 10^{-8}$ M) in phosphate-buffered saline (PBS). Briefly, collagen type I and acrylated collagen type I were adsorbed onto Immulon High-Binding 96-well plates overnight at 4 °C at concentrations ranging from 0.5–10 μg/mL of coating buffer (0.1 M sodium bicarbonate buffer, pH 9.4). After aspiration of the coating buffer and unadsorbed protein, wells were washed with 200 μL of wash buffer (PBS + 0.1 v/v Tween-20). Next, each well

Fig. 1. Chemical structure of collagen type I-PEG-acrylate. Degree of acrylation can be controlled using reaction stoichiometry between NHS and amine groups found in the lysine amino acids within the collagen type I protein structure. (DGEA and RGD are peptide sequences that promote cell adhesion.)

was blocked for 2 h at room temperature using 200 μL of blocking buffer (PBS + 5 mg/mL fraction V BSA). After blocking, each well was washed four times with 200 μL of wash buffer. Then, 100 μL of mouse anti-collagen type I in PBS ($5.0 \times 10^{-8}$ M) was added to each well and incubated for 1 h, followed by washing. After washing, 100 μL of secondary GAM-HRP (5 μg/mL) was added to each well for 1 h and incubated. Following appropriate washing, the wells were then tested for antibody activity using a plate reader (Victor$^2$, Perkin–Elmer) at 450 nm to detect activity between surface proteins and the complementary antibodies and enzymatic substrates. Activity of both acrylated and non-acrylated protein was quantitatively analyzed through the use of TMB, a peroxidase substrate. Reaction of TMB with HRP results in a soluble blue product after 30 min, thereby creating a visually detectable chromogenic response in solution. At this point, the reaction between the TMB substrate and the HRP-labeled antibody graft was terminated through the addition of an equal volume of 0.36 N sulfuric acid, turning the TMB solution yellow for quantitative analysis by the spectrophotometer.

### *3.3. Synthesis of methacrylated carboxyfluorescein diacetate (CFDA-MA)*

5-(and-6)-carboxyfluorescein diacetate methacrylate was synthesized by coupling CFDA succinimidyl ester with 2-aminoethyl methacrylate in 50 mM sodium bicarbonate buffer, pH 8.4 at a 1:1 molar ratio of CFDA succinimidyl ester:$NH_2$. The resulting CFDA-MA was lyophilized and stored at 4 °C. Acrylation was verified in a similar fashion as that described for collagen type I above. The CFDA-MA acrylation was demonstrated by copolymerization with substrate material, followed by comparing this conjugated polymer to material copolymerized in the presence of non-acrylated CFDA-MA. Both materials were copiously washed and swelled in PBS to remove unattached, soluble molecules. Negligible fluorescence was observed in washed substrates prepared in the presence of non-acrylated CFDA as compared to ones that had covalently incorporated CFDA.

### *3.4. Surface grafting of chemically and biologically cell-responsive functionalities: modification with PEG375A, collagen type I, and CFDA-MA*

PEG375A was covalently photografted from a polymeric substrate surface using living radical photopolymerization (LRP) chemistry presented in Fig. 2 [37]. Argon purged, deinhibited, pure PEG375A was grafted for 15 min by exposure to a collimated, 45 mW/cm$^2$ UV mercury arc lamp to form functionalized surfaces that prevented cell adhesion. The spatially patterned region of grafted PEG375A was formed by exposure to 45 mW/cm$^2$ intensity UV light for 900 s using photolithographic techniques previously published [8,38,40]. The resultant pattern was washed in 50/50 ethanol/water followed by deionized water for 24 h, respectively.

Acrylated collagen type I was covalently photografted from UDA/TEGDA substrate surfaces, using the same LRP surface chemistry. After dissolving 1–5 mg of acrylated collagen type I in 50 μL of pH 2.0 MES buffer, the solution was mixed with 1 mL of pure PEG375A for 10 min and purged with argon for 2 min before grafting. A patterned region of grafted collagen type I/PEG375A was formed upon exposure of 50 μL of grafting monomer to 45 mW/cm$^2$ intensity UV light for 900 s. The resultant pattern was washed in 50/50 ethanol/water and then in deionized water for 24 h, respectively. Surface concentrations of grafted collagen molecules were calculated based on knowledge of the graft thickness, molecular weight of collagen type I acrylate, and density of the grafted polymer. Specifically, graft layers of 6 μm thickness were used in combination with pre-purchased 400,000 MW collagen for acrylation and a graft density ($\rho$) of 1 g/ml. For example, in the case where we graft 5 mg/ml collagen type I acrylate, the surface density is 7.5 pmol/cm$^2$ ($= ht * \text{conc} * (1/\rho) * (1/\text{MW}) = (6\ \mu\text{m}/1\ \text{m}) * (5\ \text{mg}/1\ \text{ml}) * (1\ \text{ml}/1\ \text{g}) * (1\ \text{g}/4 \times 10^5\ \text{g/mol})$).

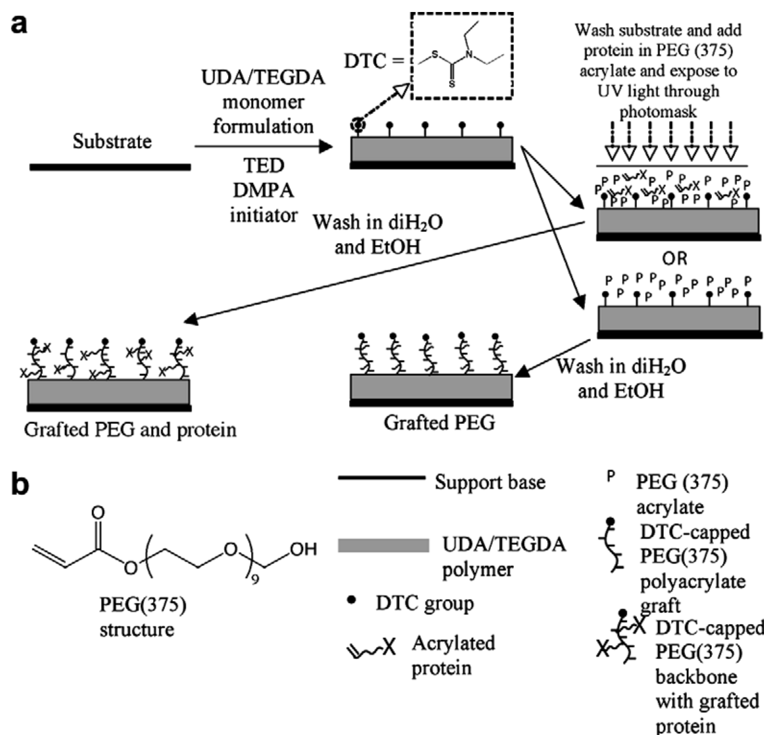


Fig. 2. (a) Grafting scheme using controlled living radical photopolymerization to covalently graft chemical and protein-containing polymer chains from a DTC incorporated polymer surface. Grafted chain length is controlled via UV irradiation time, and spatial control is achieved using photolithographic techniques. (b) Chemical structure of PEG (375) graft monomer.

Finally, to functionalize the surface with CFDA, the monomer (CFDA-MA) was dissolved in 50 μL of DMSO and then added to the UDA/TEGDA substrate monomer at 10 mM concentration for grafting onto the surface of an unmodified substrate layer. CFDA-MA grafted samples were subsequently washed, as mentioned previously.

### 3.5. 3T3 cell seeding and morphology on surface-functionalized substrates

The attachment and spreading of NIH3T3 fibroblasts (American Type Tissue Collection) on PEG375A, CFDA-MA, and collagen type I-grafted polymer surfaces were evaluated. Cells were seeded onto the sterilized polymer surfaces at a concentration of ~30,000 cells/cm$^2$ in Dulbecco's modified medium supplemented with 5% fetal bovine serum (Gibco), gentamicin (Gibco), and PenStrep (Gibco). Substrates were placed in 90 mm tissue culture dishes, and cell-seeded surfaces were incubated at 37 °C and a 5% $CO_2$ atmosphere.

After 24 h of seeding, cell spreading and adhesion on grafted samples were analyzed using a fluorescent calcein stain. 0.5 μL of calcein/mL in PBS was used to make the cell staining solution. Five milliliters of stain solution was used per 90 mm Petri dish to guarantee complete staining of the cells attached to the surface of the sample. After 10 min of staining, samples were rinsed with copious amounts of PBS to remove any free calcein and unattached cells. Samples were analyzed for cell spreading using an optical microscope equipped with a FITC filter for imaging. Cell number and surface coverage per modified square were analyzed using NIH Scion software.

## 4. Results and discussion

The ability to control cell adhesion and function on polymeric surfaces greatly impacts fields related to tissue engineering and biosensor development. Control over cell position on surfaces is important for cell-based screening methods and for a better understanding of both induced and natural cell behavior. In the field of tissue engineering, directed cell attachment and differentiation influenced by polymeric scaffold surface properties greatly affect the ability to form organized tissue structures including those composed of one or more cell types. One of the most challenging aspects of this research is the design of surface architectures that remain active and accessible to influence cell-surface interactions in a highly controlled and specific manner. Here, we present a method for synthesizing and introducing spatially patterned chemically and biologically photoreactive acrylate species with controlled densities on polymer surfaces to control cell orientation, adhesion, and spreading. The ability to graft a variety of functionalities onto these substrates through a controlled radical photografting method is illustrated in the following sections. Demonstrations of enhanced cell adhesion and spreading, as well as cell-activated fluorescence of grafted molecules for sensing, are presented herein.

### 4.1. Surface reactive, polyurethane-tri(ethylene glycol)diacrylate substrates

Before using surface grafted substrates as cell patterning platforms, it is important to understand how cells interact with the unmodified, base polymer substrate. Studies were conducted using 3T3 fibroblasts seeded on the poly(urethane/TEGDA) substrates that were polymerized to above 90% conversion, as shown by monitoring the acrylate double bond peak, found at 1636 $cm^{-1}$, using mid-FTIR techniques. After copious washing to remove any unreacted species, samples were sterilized and then cells were seeded at subconfluent density of 30,000 cells/$cm^2$ and cultured for 24 h. Cells remained viable after seeding, and readily attached to the unmodified substrate surfaces, as evidenced after hematoxylin cellular staining. In Fig. 3a, adherent cells stained purple and the high degree of spreading indicates a high level of protein adsorption of the relatively hydrophobic UDA/TEGDA substrates. To control cell adhesion spatially on this base formulation, photolithographic methods were used to pattern regions of polymer grafts to prevent cell attachment by incorporating PEG-containing grafts.

### 4.2. Spatially patterned chemical functionalities that spatially direct cell attachment

While the UDA/TEGDA copolymer was shown to non-specifically support NIH 3T3 fibroblast adhesion on DTC-functionalized surfaces, preventing cell adhesion with highly resolved spatial control is also advantageous. To demonstrate spatially controlled cell adhesion, LRP chemistry was used to photograft PEG chains from the base substrate surface, thereby preventing 3T3 fibroblast adhesion in selected surface regions. Using contact liquid photolithographic polymerization (CLiPP) photolithographic technique [40], purified, argon-purged PEG375A was grafted from the base formulation by exposure to 45 mW/$cm^2$ UV light for 900 s. The surface attached, hydrophilic chains were shown to prevent protein adsorption and cell adhesion as demonstrated in Fig. 3b, where cells adhered only to unmodified substrate regions (cells stained with hematoxylin). As shown with 200 μm PEG-modified spacing between 200 μm unmodified cell adhesive islands, spatially resolved cell adhesion was achieved using the LRP grafting technique to modify surfaces with covalently attached, PEG chains. PEG-grafted regions prevented cell adhesion with resolution down to 50 μm spacing. While cell adhesive island spacing and geometry seemed to play a role in the interconnectivity of adherent cells between islands, this phenomenon must be investigated more thoroughly. Specifically, various island geometries and spacings between 10 and 100 μm would be interesting to explore, as cell migration and bridging appear to be a function of these parameters and could be monitored over a longer period of cell culture. Also, DTC can be incorporated into non-adhesive PEG substrates to facilitate modification with functionalities that promote cell adhesion on otherwise non-adhesive surfaces (results not shown).

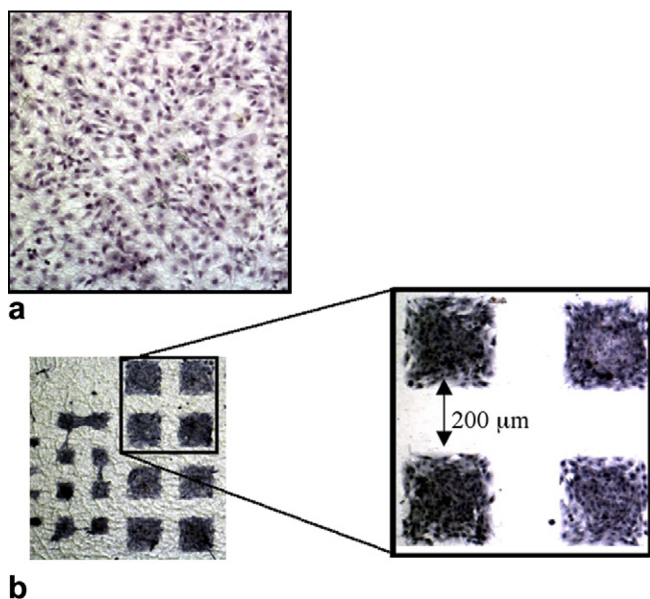


Fig. 3. (a) 3T3 fibroblasts seeded at confluency onto an unmodified UDA/TEGDA polymer substrate. (b) 3T3 fibroblasts seeded at 20,000 cells/$cm^2$ onto PEG grafted polymer substrate surfaces to demonstrate spatial control over cell adhesion. Hematoxylin staining was used in each of the above images where white areas represent PEG-modified regions and purple areas are regions with stained, adherent cells.

### 4.3. Spatially patterned biological functionalities that control cell adhesion and spreading

After modifying proteins, such as collagen type I, with acrylated moieties, the influence of the conjugation reaction on the protein bioactivity must be evaluated to determine if there are negative effects regarding protein activity or conformation. Often, protein activity is compromised due to blocking and/or modification of characteristic biologically active sites. To assess whether the acrylated collagen type I protein retained biological activity, collagen type I antibodies were used to confirm that recognition occurs, post-conjugation. Specifically, an indirect-ELISA was performed using primary mouse anti-collagen type I antibody to recognize both surface immobilized conjugated (i.e. acrylated) and unmodified collagen type I. When secondary antibody was used to quantify these results, the acrylated collagen type I was shown to retain activity, even after conjugation. The ELISA results supporting this claim are shown in Fig. 4. Varying concentrations, ranging from $1 \times 10^{-9}$ to $1 \times 10^{-7}$ M of collagen type I and acrylated collagen type I, were adsorbed to well plates and chromogenically developed using TMB to quantify the assay. No statistical differences were observed in the chromogenic intensity, indicating that the overall biological activity and recognition by collagen type I specific antibodies were not compromised by the acrylation chemistry used here.

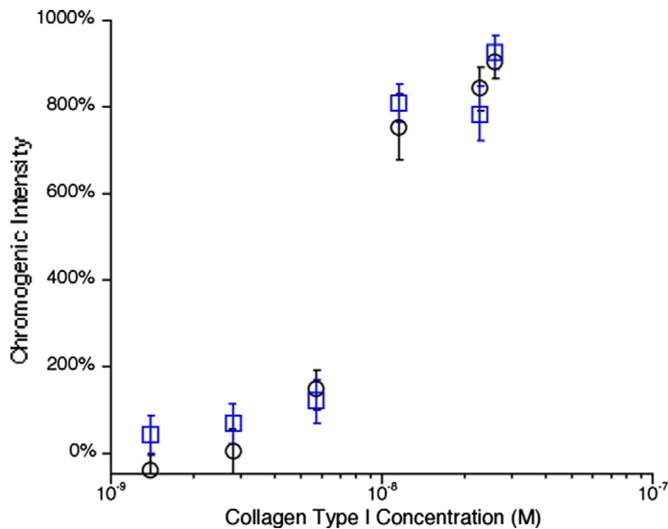


Fig. 4. Assessment of collagen type I bioactivity before and after acrylation chemistry. Chromogenic intensity of indirect ELISA wells when assaying for unconjugated (○) and acrylated (□) collagen type I. The units of $y$-axis are on percentage basis and all values are normalized to a control where collagen type I was not immobilized onto well surfaces.

Cell attachment and function are known to be dramatically influenced by interactions with surface immobilized biologically active functionalities, such as growth factors, peptide sequences, and other proteins. The orientation in which these proteins are modified and attached greatly impacts their surface activity. As discussed previously, acrylated collagen type I was synthesized for grafting from substrate surfaces to introduce a targeted and specific mechanism to modulate cell-material interactions, as well as to provide a method to control these interactions spatially. Specifically, 50 μL of 1–5 mg/mL solutions of acrylated collagen type I in PEG375A was homogenously mixed and photografted in 100 $\mu m^2$ square patterns. The 3400 MW PEG spacer between the graft backbone and pendant collagen type I functionality was chosen to facilitate mobility and accessibility of the protein for cell interactions.

The collagen type I-modified patterns were designed to influence 3T3 fibroblast attachment and spreading onto the modified regions of the UDA/TEGDA polymer substrate through a specific cell adhesion protein interaction.

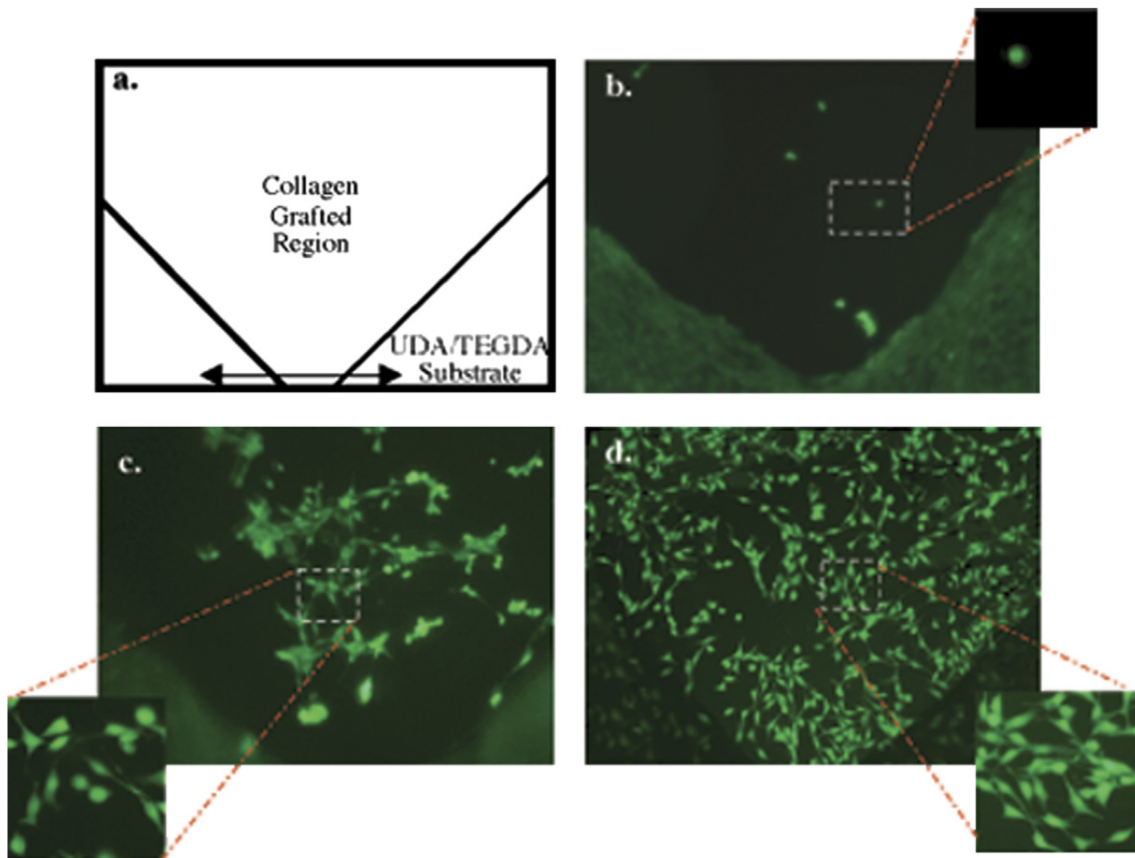


Fig. 5. Fluorescent micrographs of calcein-stained 3T3 fibroblasts seeded at 30,000 cells/$cm^2$ onto substrate surfaces patterned with 100 $\mu m^2$ collagen type I/PEG grafted squares. Fluorescence micrographs of viable 3T3 fibroblasts, seeded on grafted squares constructed using 50 μL of a varying concentration of collagen type I acrylate in PEG375A monomer formulation. (a) An illustration of the photopatterned surface, showing both collagen type I grafted and unmodified substrate regions. (b) Fluorescence micrograph (10×) of cells on PEG-only grafted surfaces, (c) on a square grafted using 50 μL of a 1 mg/mL and (d) a 5 mg/mL collagen type I acrylate in PEG acrylate monomer formulation.

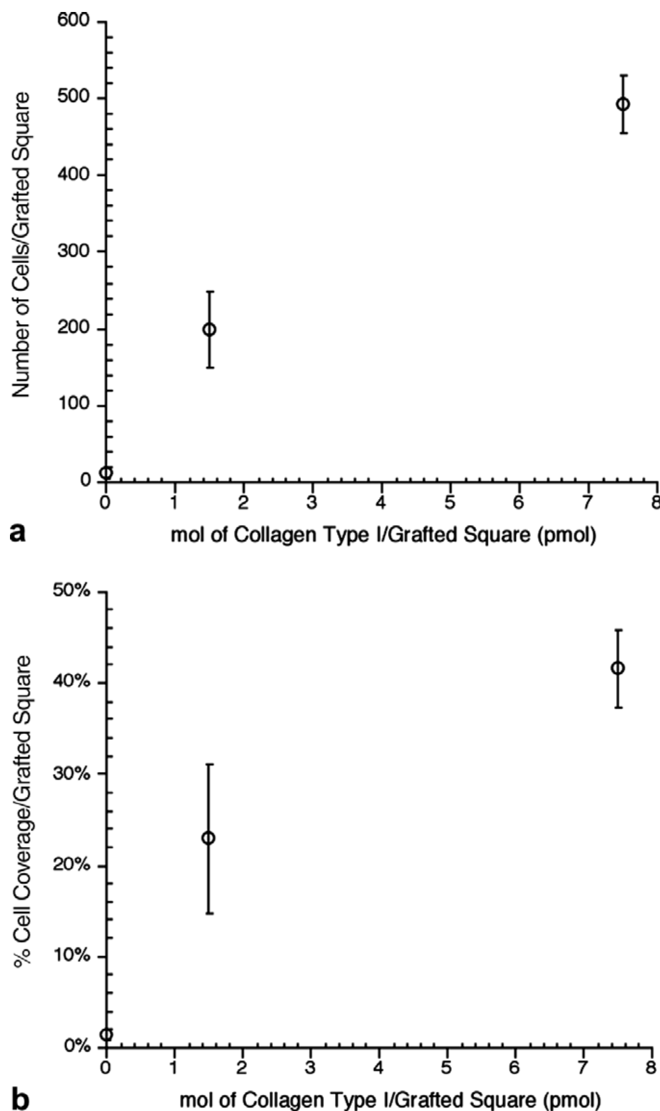


Fig. 6. (a) Cell number as a function of collagen type I concentration. (b) Cell surface coverage per grafted square, as a function of collagen concentration. The control sample is 100% grafted PEG (375) acrylate. Each square has a 100 μm$^2$ surface area.

The LRP surface modification chemistry provides a robust method for introducing protein onto surfaces with controlled density and spatial orientation. As shown in Fig. 5, 3T3s selectively attach to the UDA/TEGDA substrate and only the patterned squares that are modified with collagen type I.

Due to the grafted collagen type I, this attachment occurs despite the fact that PEG is also tethered in these spatial patterns, and occurs through specific interactions with the grafted protein molecules. The calcein-stained cells are adherent and spread on the collagen-modified patterned squares. Control samples, consisting of PEG-only grafted patterns, showed negligible cell adhesion, and the few cells found on PEG-only grafted squares are unable to spread and attach to the surface relative to those found on PEG/collagen type I grafted squares. Furthermore, a range of concentrations of collagen type I acrylate (0, 1, and 5 mg/mL) grafting solutions was used to investigate the role of adhesive ligand concentration on cell spreading. As the surface concentration of collagen was increased from 0 to 7.5 pmol/cm$^2$, both cell number and surface coverage were increased on modified squares, as expected. Photografting with a 50 μL solution of 1 mg/mL collagen type I acrylate in PEG375A led to a maximum of 1.5 pmol of collagen type I available for incorporation per 100 μm$^2$ square, greatly affecting cell adhesion and spreading as shown. When using a 5 mg/mL solution, this value increased to 7.5 pmol of collagen type I available per square. The increase in cell number and surface coverage of collagen-incorporated squares was quantified, and the results in Fig. 6 illustrate a significant increase compared to PEG-only grafted squares. This proof-of-concept research demonstrates the ability to photograft active protein species that can control cell attachment to surfaces. Further work could benefit from the use of grafted proteins, such as laminin or fibronectin, to target a specific cell type amongst a mixed population of cells. Cell sorting and organization on independent regions of the surface could also be achieved with surfaces functionalized with different proteins spatially patterned on the surface of polymeric substrates and scaffold materials.

### *4.4. Cell sensing using spatially patterned functionalities*

Another interesting area pertaining to the design of surfaces that can be activated by cells is that of cell sensing. Often, cell sensing and tracking make use of fluorescent and chromogenic probes or labeled antibodies that achieve cell-sensing capabilities. Using LRP grafting chemistry, moieties containing cell-cleavable groups were grafted from polymer surfaces for introduction into the cell for tracking, sensing, and monitoring. A simple demonstration of this approach was achieved by synthesizing CFDA-MA, which contains ester groups that can be cellularly cleaved, thereby activating fluorescence (Fig. 7).

After complete mixing, the CFDA-MA enhanced monomer mixture was photografted onto the surface of a UDA/TEGDA substrate in 600 μm lines, using the photolithographic methods previously discussed. The grafting process consisted of exposing the CFDA-MA enhanced monomer to a broad spectral range of UV light at 45 mW/cm$^2$ for 350 s. The surface attached CFDA-MA groups appear colorless and non-fluorescent prior to interaction with cells. Four hours after seeding 3T3 fibroblasts at 30,000 cells/cm$^2$, the surface attached CFDA groups fluoresced, indicating cell-interactions and cleavage of the acetate groups on the surface bound CFDA. One possible mechanism leading to this fluorescence could be due to the internalization of the surface grafted CFDA functionalities, which leads to cleavage of pendant acetate groups by reaction with cellular esterases[39] and resulting in cell-internalized carboxylfluorescene useful for sensing cell-material interactions.

Cell adhesion and fluorescence were evaluated via fluorescence microscopy using a FITC filter (ex/em: 488 nm/

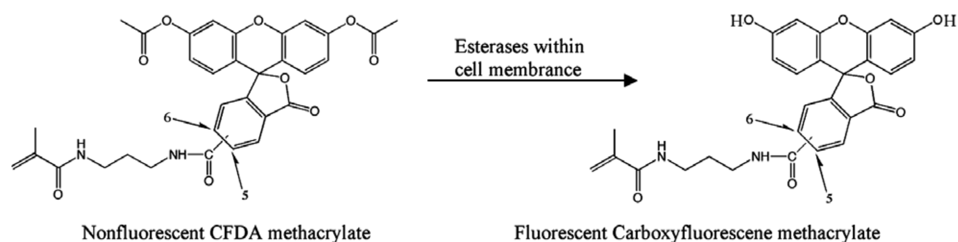


Fig. 7. Chemical structure of monovinyl 5 and 6-carboxyfluorescein diacetate aminomethacrylate. In the presence of esterases CFDA cleaves to form a fluorescent molecule.

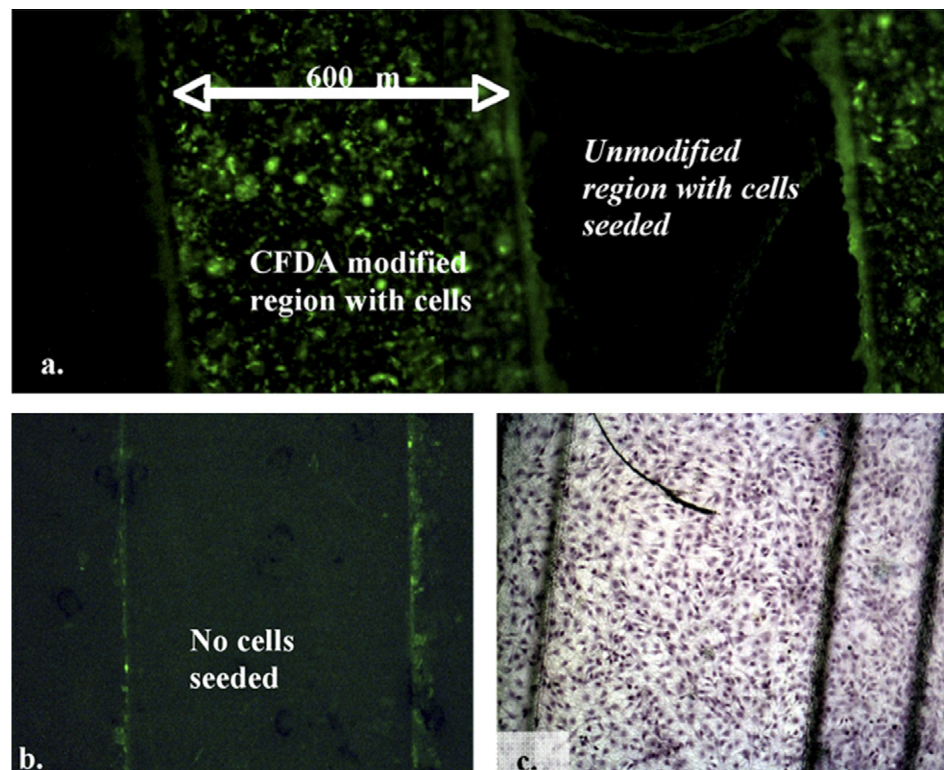


Fig. 8. (a) 3T3 fibroblasts seeded at confluency (20,000 cells/cm$^2$) onto CFDA modified surfaces. Fluorescence only occurs in the cells that were seeded on the 400 μm CFDA-modified stripes. Unmodified regions with seeded cells remain non-fluorescent, as shown. (b) A fluorescence micrograph of the CFDA patterned substrate, in the absence of any cells, only showing insignificant false fluorescence. (c) Light micrograph of cells seeded on the entirety of the patterned surface.

512 nm), and representative micrographs are depicted in Fig. 8. Cell adhesion was uniform across the substrate surface, yet only cells adherent to the CFDA-grafted regions were fluorescent. A control image is shown in Fig. 8b and represents the fluorescence of a CFDA-grafted surface, before cell seeding, showing that background fluorescence is minimal. This experiment demonstrates the ability to graft cell-sensing functionalities from polymeric materials, a concept that can be extended to a variety of other applications including cell tracking, sorting, and detection.

## 5. Conclusions

Polymeric biomaterial modification methods often lack robustness and versatility in immobilizing chemically and biologically active functionalities in a spatially and/or temporally controlled fashion. Furthermore, maintaining the activity of conjugated, surface-attached proteins is often difficult. To overcome these concerns, LRP-based chemistry was used to demonstrate the utility of grafting proteins from UDA/TEGDA polymer surfaces for controlled cell adhesion. When unmodified, the DTC-functionalized surfaces were shown to be cell adhesive using seeded NIH 3T3 fibroblasts. After further grafting of DTC-functionalized polymer substrates with PEG375A, 3T3 fibroblast adhesion occurred on these polymer substrates in spatially grafted regions. While grafting PEG375A prevents cell adhesion due to the hydrophilic nature of PEG, there is often the desire to introduce specific functionalities on non-cell adhesive substrates to regulate cell adhesion through a selective pathway. The ability to graft biologically active protein and peptide cues that selectively induce

cell adhesion and other cell processes (e.g., cell differentiation) would significantly improve biomaterial design. To demonstrate these concepts using the LRP chemistry presented here, collagen type I was acrylated using NHS:$NH_2$ chemistry to synthesize photoreactive protein for surface immobilization through a controlled photopolymerization chemistry. The acrylated collagen type I was shown to be biologically active as determined using an indirect ELISA protocol. Once grafted, collagen type I-incorporated tethers significantly improved cellular adhesion (~24 × increase in cell number/grafted square) and spreading (~21 × increase in cell coverage/grafted square) in areas that were otherwise non-cell adhesive. Finally, grafted CFDA-MA functionalities were shown useful in demonstrating cellular sensing in specific patterned regions on polymer surfaces. The broad range of results shown here recapitulate the versatility of this LRP grafting method as a useful tool in selective surface modification of polymeric biomaterials for improved spatially controlled cell adhesion and patterning on surfaces. This method may provide a route for patterning a variety of other factors to manipulate cell functions such as proliferation, migration, and differentiation.

## Acknowledgement

The authors are grateful for financial support for this research from the DARPA Symbiosys program, AFOSR, Packard Foundation, and Howard Hughes Medical Institute. RPS was also supported by a fellowship from the Department of Education GAANN program.

## References

[1] Burdick JA, Anseth KS. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. Biomaterials 2002;23:4315–23.
[2] Benoit DSW, Anseth KS. The effect on osteoblast function of colocalized RGD and PHSRN epitopes on PEG surfaces. Biomaterials 2005;26:5209–20.
[3] Nuttelman CR, Tripodi MC, Anseth KS. In vitro osteogenic differentiation of human mesenchymal stem cells photoencapsulated in PEG hydrogels. J Biomed Mater Res Part A 2004;68A:773–82.
[4] Borgatti M et al. Separation of white blood cells from erythrocytes on a dielectrophoresis (DEP) based 'Lab-on-a-chip' device. Int J Mol Med 2005;15:913–20.
[5] Liu ZH, Li MY, Cui DF. A novel method for polypeptide design to prepare specific antibody of the peptide and applied to immunoassay. J Immunol Meth 2003;281:17–25.
[6] Mathew FP, Alocilja EC. Porous silicon-based biosensor for pathogen detection. Biosens Bioelectr 2005;20:1656–61.
[7] Miyata T, Uragami T, Nakamae K. Biomolecule-sensitive hydrogels. Adv Drug Del Rev 2002;54:79–98.
[8] Sebra RP, Masters KS, Bowman CN, Anseth KS. Surface grafted antibodies: Controlled architecture permits enhanced antigen detection. Langmuir 2005;21:10907–11.
[9] Xu GX et al. Cell-based biosensors based on light-addressable potentiometric sensors for single cell monitoring. Biosens Bioelectr 2005;20:1757–63.
[10] Zhang FH et al. A self-sampling-and-flow biosensor for continuous monitoring. Electroanalysis 2005;17:668–73.
[11] Liu X et al. Surface engineering of nano-fibrous poly(L-lactic acid) scaffolds via self-assembly technique for bone tissue engineering. J Biomed Nanotech 2005;1:54–60.
[12] Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. Science 1997;276:1425–8.
[13] Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Micropatterned surfaces for control of cell shape, position, and function. Biotech Prog 1998;14:356–63.
[14] Nakayama Y, Matsuda T. Surface macromolecular microarchitecture design: biocompatible surfaces via photo-block-graft-copolymerization using *N*,*N*-diethyldithiocarbamate. Langmuir 1999;15:5560–6.
[15] Mrksich M, Dike LE, Tien J, Ingber DE, Whitesides GM. Using microcontact printing to pattern the attachment of mammalian cells to self-assembled monolayers of alkanethiolates on transparent films of gold and silver. Exp Cell Res 1997;235:305–13.
[16] Mrksich M, Chen CS, Xia YN, Dike LE, Ingber DE, Whitesides GM. Controlling cell attachment on contoured surfaces with self-assembled monolayers of alkanethiolates on gold. Proc Natl Acad Sci 1996;93:10775–8.
[17] Lu ZR, Kopeckova P, Kopecek J. Antigen responsive hydrogels based on polymerizable antibody Fab' fragment. Macromol Biosci 2003;3:296–300.
[18] Massia SP, Hubbell JA. Immobilized amines and basic-amino-acids as mimetic heparin-binding domains for cell-surface proteoglycan-mediated adhesion. J Biol Chem 1992;267:10133–41.
[19] Massia SP, Hubbell JA. Human endothelial-cell interactions with surface-coupled adhesion peptides on a nonadhesive glass substrate and 2 polymeric biomaterials. J Biomed Mater Res 1991;25:223–42.
[20] Nuttelman CR, Mortisen DJ, Henry SM, Anseth KS. Attachment of fibronectin to poly(vinyl alcohol) hydrogels promotes NIH3T3 cell adhesion, proliferation, and migration. J Biomed Mater Res 2001;57:217–23.
[21] Prime KL, Whitesides GM. Self-assembled organic monolayers – model systems for studying adsorption of proteins at surfaces. Science 1991;252:1164–7.
[22] Prime KL, Whitesides GM. Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide) – a model system using self-assembled monolayers. J Am Chem Soc 1993;115:10714–21.
[23] Palegrosdemange C, Simon ES, Prime KL, Whitesides GM. Formation of self-assembled monolayers by chemisorption of derivatives of oligo(ethylene glycol) of structure Hs(Ch2)11(Och2ch2)Meta-Oh on gold. J Am Chem Soc 1991;113:12–20.
[24] Mrksich M, Whitesides GM. Patterning self-assembled monolayers using microcontact printing – a new technology for biosensors. Trends Biotech 1995;13:228–35.
[25] Mrksich M, Sigal GB, Whitesides GM. Surface-plasmon resonance permits in-situ measurement of protein adsorption on self-assembled monolayers of alkanethiolates on gold. Langmuir 1995;11:4383–5.
[26] Jeon NL, Nuzzo RG, Xia YN, Mrksich M, Whitesides GM. Patterned self-assembled monolayers formed by microcontact printing direct selective metalization by chemical-vapor-deposition on planar and nonplanar substrates. Langmuir 1995;11:3024–6.
[27] Xia YN, Mrksich M, Kim E, Whitesides GM. Microcontact printing of octadecylsiloxane on the surface of silicon dioxide and its application in microfabrication. J Am Chem Soc 1995;117:9576–7.
[28] Hubbell JA, Massia SP, Desai NP, Drumheller PD. Endothelial cell-selective materials for tissue engineering in the vascular graft via a new receptor. Bio-Technology 1991;9:568–72.
[29] Danilov YN, Juliano RL. (Arg-Gly-Asp)N-albumin conjugates as a model substratum for integrin-mediated cell-adhesion. Exp Cell Res 1989;182:186–96.
[30] Danilov YN, Juliano RL. Phorbol ester modulation of integrin-mediated cell-adhesion – a postreceptor event. J Cell Biol 1989;108:1925–33.
[31] Maheshwari G, Brown G, Lauffenburger DA, Wells A, Griffith LG. Cell adhesion and motility depend on nanoscale RGD clustering. J Cell Sci 2000;113:1677–86.

[32] Sebra RP, Masters KS, Cheung CY, Bowman CN, Anseth KS. Photografted whole antibodies allow rapid detection of antigens in biologically complex fluids. Proc Natl Acad Sci, submitted for publication.
[33] Matsuda T, Sugawara T. Control of cell adhesion, migration, and orientation on photochemically microprocessed surfaces. Macromolecules 1996;32:165–73.
[34] Otsu T. J Polym Sci Part A: Polym Chem 2000;38:2121–36.
[35] Nakayama Y, Matsuda T. Macromolecules 1996;29:8622–30.
[36] de Boer B, Simon HK, Werts MPL, van der Vegte EW, Hadziioannou G. Macromolecules 2000;33:349–56.
[37] Luo N, Metters AT, Hutchison JB, Bowman CN, Anseth KS. Macromolecules 2003;36:6739–45.
[38] Reddy SSR, Anseth K, Bowman CN. Living radical photopolymerization induced grafting on thiol-ene based substrates. J Poly Sci Part A: Poly Chem 2005;43:2143–4.
[39] Parish C. Fluorescent dyes for lymphocyte migration and proliferation studies. Immuno Cell Biol 1999;77:499–508.
[40] Hutchison JBH et al. Robust polymer microfluidic device fabrication via contact liquid photolithographic polymerization (CLiPP). LAB ON A CHIP 2004;6:658–62.