Spatial control over cell attachment by partial solvent entrapment of poly lysine in microfluidic channels

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Abstract: We demonstrate spatial control over cell attachment on biodegradable surfaces by flowing cell adhesive poly (D-lysine) (PDL) in a trifluoroethanol (TFE)–water mixture through microfluidic channels placed on a biodegradable poly (lactic acid)–poly (ethylene glycol) (PLA–PEG) substrate. The partial solvent mixture swells the PLA–PEG within the confines of the microfluidic channels allowing PDL to diffuse on to the surface gel layer. When excess water is flowed through the channels substituting the TFE–water mixture, the swollen PLA surface collapses, entrapping PDL polymer. Results using preosteoblast human palatal mesenchymal cells (HEPM) indicate that this new procedure can be used for facile attachment of cells in localized regions. The PEG component of the PLA–PEG copolymer prevents cells from binding to the nonpatterned regions.

Keywords: microfluidic, cell binding, tissue engineering, solvent entrapment

Introduction

Microfabrication technologies have the potential to facilitate control over the organization of cells (Patel et al 1998; Michel et al 2002; Tien and Chen 2002; Hyun et al 2003; Tan and Desai 2003; Raghavan and Chen 2004; Lin et al 2005; Rhee et al 2005). Geometric control over cell binding on biodegradable substrates is essential for engineering highly organized tissues with precisely defined cellular architectures (Chen et al 1997; Bhatia et al 1998). Spatial organization of cells is also essential for cell-based sensors, cell culture analogs, controlling cell-to-cell interactions, and for developing new understandings of how populations of cells react to spatiotemporal cues (Raghavan and Chen 2004; Flaim et al 2005; Rhee et al 2005; Tourovskaia et al 2005). Poly (lactic acid) (PLA) and related members of the poly (alpha hydroxy acids) polymer family have gained wide acceptance for applications in drug delivery and tissue engineering but lack functional groups that would allow for covalent functionalization of cell adhesive moieties (Quirk et al 2000; Salem et al 2001). One strategy that has been developed for overcoming this is to surface engineer PLA by physically entrapping functional modifying species onto the surface (Quirk et al 2000; Quirk, Briggs, et al 2001). This is an approach that has previously been used to modify the surfaces of polymers such as poly (ethylene terephthalate), poly (methyl methacrylate), poly styrene, and poly (vinyl acetate) (Desai and Hubbell 1991, 1992). This modification is achieved by mixing a miscible mixture of solvent and nonsolvent for the polymer with the surface-modifying species. When the polymer is exposed to the partial solvent mixture, it causes rapid polymer gelation at the surface. The surface-modifying species can then diffuse into the gel layer of the polymer. When a large excess of nonsolvent is then added, the gel layer collapses, entrapping the surface-modifying species in the substrate. This methodology can be used to prepare both cell adhesive and cell repellant

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substrates (Desai and Hubbell 1991, 1992; Quirk et al 2000; Quirk, Briggs, et al 2001; Quirk, Davies, et al 2001).

Here we report on a new method for binding cells in spatially defined regions using both cell adhesive and cell repellant cues. Poly (D-lysine) (PDL), a cationic polymer reported to promote cell attachment, is entrapped on the surface of a degradable polymer substrate in the spatially defined confines of microfluidic channels. This method of microfluidic networking has found utility in a variety of applications from patterning of proteins (Delamarche et al 1997), cells (Patel et al 1998), and nanowires (Salem et al 2004).

Materials and methods

Mold fabrication

To fabricate the mold, the prepolymer was cured (Sylgard Silicone Elastomer 184, Dow Corning, Rockford, Illinois, USA) on a patterned master which was prepared by spin coating 250 μL photoresist (SU8) onto a silicone wafer for 55 seconds at 2500 rpm, solvent-baked at 100°C for 100 seconds, and then exposed to UV light (11mJ cm^{-2}) from a mercury vapor lamp. The exposed resist was developed in a 4:1 mixture of deionized water, and after drying with nitrogen, the patterned master was then hard-baked for 25 minutes at 125°C. The elastomeric mold with the negative imprint on it was peeled off and washed several times with ethanol, hexane, and deionized water.

Synthesis of poly (lactic acid)–poly (ethylene glycol) (PLA–PEG)

To synthesize PLA–PEG, water impurities were first removed from the MeO-PEG-OH (MW, 2000, Sigma-Aldrich, St Louis, MO, USA). This was achieved by dissolving the m-PEG (200 mg) in toluene (70 mL) and refluxing with a Dean-Stark trap and a condenser. 70% of the toluene was removed by distillation. The polymer was then isolated on a rotary evaporator. Residual solvent was removed by drying the polymer under vacuum for 2 days. To prepare the PLA–PEG, lactide (Purac Biochem bv, Gorinchem, The Netherlands) was graft polymerized onto the MeO-PEG-OH. First, glassware was silanized by rinsing with a 5% methyltrichlorosilane solution in toluene, rinsing with acetone, and then leaving overnight to dry at 130°C. Then a 50-mL round-bottom flask was charged with MeO-PEG-OH (0.2 g). DL-Lactide (2.1 g) was transferred into the round-bottom flask, diluted with 10 mL toluene, and heated to 60°C until the contents went into solution. $Sn(Oct)_2$–toluene (0.1 g in 1 mL) was then added and the reaction brought to reflux at 110°C for 4 hours under argon. Following this, the reaction vessel was equipped with a Dean-Stark trap and any remaining solvent was removed by vacuum rotary evaporator. The remaining viscous material was heated to 140°C; this melt was then left for 1 hour under argon. The reaction mixture was allowed to cool after which it was dissolved in approximately 10 mL dichloromethane. This polymer solution was then added drop-wise to a cold stirring solution of 100 mL diethyl ether. The final product was isolated by vacuum filtration and lyophilized overnight. Based on ^{1}H-NMR measurements of the integral ratios of the (CH_2-CH_2-O) at 3.51 ppm and the (CH_3, 5.22 ppm) and the (CH, 1.53 ppm) from the lactic acid units, the MW was calculated to be approximately 23000.

Immunofluorescence analysis of cell attachment

Immunofluorescent analysis was performed by fixing cells in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes. The cells were then rinsed in 1x PBS followed by permeabilization for 8 minutes in 0.5% Triton X-100 in PBS. Following rinsing with 1x PBS, cells were stained with phalloidin-RITC (1:500) for 45 minutes at 37°C after which the samples were washed in 1x PBS for 5 minutes, rinsed in deionized water for 1 minute and mounted with Flourosave (Sigma). The PLA disks were viewed on an Olympus CKX41 (Leeds Precision Instruments, Minneapolis, MN, USA) microscope equipped with epifluorescence optics and a 10x objective (total magnification 100x). Measurement of cell attachment was carried out by counting absolute cell numbers, n = 3 per condition. For immunofluorescent analysis, 3 random sites per PLA disk were observed.

Statistical analysis

Comparisons in cell attachment were statistically analyzed using a one-way ANOVA with a Tukey's multiple comparison test.

Results and discussion

Figure 1 shows schematically our approach for binding cells in spatially defined regions. First, Poly DL lactic acid (PLA, Sigma, MW 75 000–120 000) discs of approximately 25 mm by 0.22 mm were prepared by melt pressing the PLA between two glass slides. The PLA disks were then uniformly shaved to 2.1 cm in diameter to fit in 12-well plates. PDL solution was prepared by dissolving PDL (Sigma, MW 30–70 K) in a 10%/90% v/v TFE/water at 0.01%, 0.02%, 0.05%, 0.1%, 1%, 5%, and 10% w/v concentrations. TFE was selected as

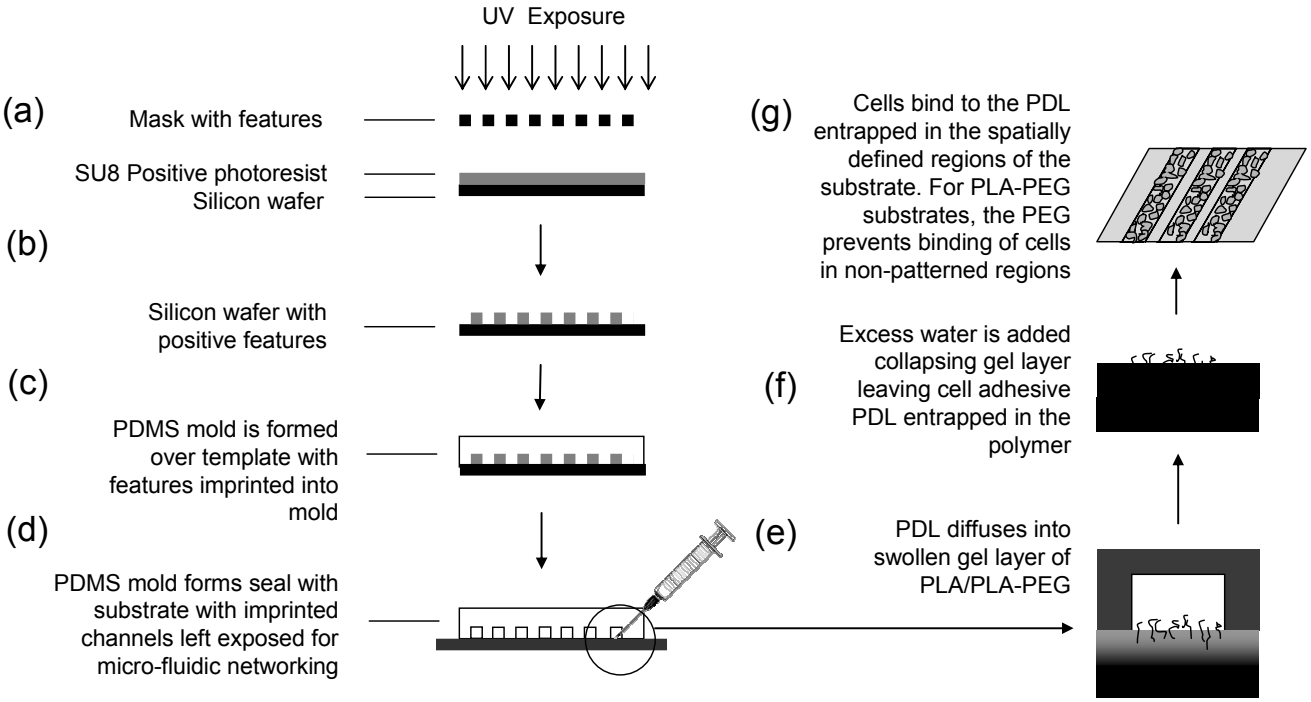

Figure 1 Schematic of localized partial solvent entrapment of PDL on a degradable polymer substrate.
Abbreviations: PDL, poly (D-lysine); PDMS, poly (dimethylsiloxane); PLA–PEG, poly (lactic acid)–poly (ethylene glycol).

a solvent for PLA because it also displays excellent solvent compatibility with poly (dimethylsiloxane) (PDMS) with an S value of 1.01, where S is the swelling ratio determined by dividing the length of PDMS in the solvent by the length of dry PDMS (Lee et al 2003). Selection of the 10%/90% TFE/water ratio was based on the observation that TFE/water concentrations of 30%/70% result in dissolution of PLA, being the dominant polymer–solvent interaction, whilst concentrations lower than 10% TFE produce insufficient gel formation for optimum diffusion of surface-modifying species (Quirk et al 2000)

To determine the concentration of entrapped PDL required for optimum cell attachment, 1mL of PDL in TFE–water was incubated with a PLA disk for 1 minute for each concentration. Twenty mL of excess water (nonsolvent) was then added to collapse the gel layer and entrap the PDL. The PLA disks were placed in 12-well plates and UV sterilized for 30 minutes. Preosteoblast human palatal mesenchymal cells (HEPM 1486; ATCC, Manassas, VA, USA) were grown to confluence in Eagle's minimum essential medium (EMEM) supplemented with Earle's salts, L-glutamine (2 mM), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), 10% fetal bovine serum, and 25 µg/mL penicillin–streptomycin. They were then plated onto the disks in the same culture medium at a seeding density of $1x10^6$ cells. Cultures were allowed to attach for 1 hour before flooding with 1 mL of media. After 2 additional hours, the media was removed from the wells and unattached cells quantitated with the ZM model Coulter counter. Values were expressed as the percent cell attachment on tissue culture plastic. Attached cells were observed by fluorescent microscopy. As illustrated in Figure 2a, the optimum cell attachment observed was at 0.1% PDL which produced a significant increase in cell attachment from 54.4% for PLA with no treatment to 94.2% for PLA with 0.1% PDL treatment ($p<0.001$).

Next, 0.1% w/v PL in 10%/90% TFE/water was entrapped in predefined regions of the PLA substrate using a PDMS mold with channels ranging from 160 µm to 240 µm width. The mold was placed onto the PLA substrate and 1 mL of the 0.1% PDL in 10/90 TFE/water solution was placed at the entrance of the capillaries. After 30 minutes, the channels were flushed with 3 mL deionized water to collapse the gel layers within the channels and remove excess nonbound PDL. This process was repeated 10 times. The PDMS stamp was then removed and the PLA substrate rinsed with excess deionized water several times. The PDL entrapped in the PLA within the PDMS channels act as localized adhesive substrates for cell attachment.

To confirm entrapment of the PDL within the predefined channels, patterned PLA disks were incubated with 5(6)-

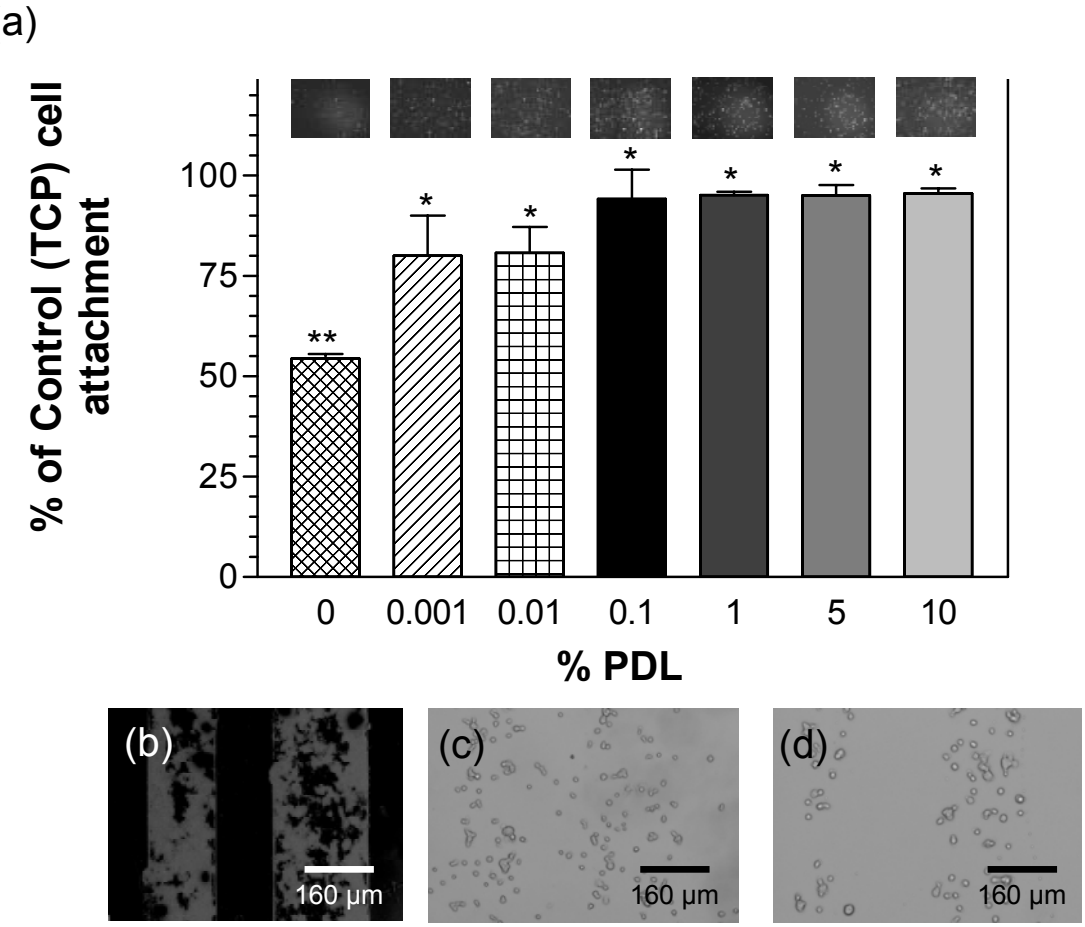

Figure 2 (a) Summary bar chart of HEPM cell attachment assay after 1 hour of incubation on PLA surfaces treated with varying concentrations of PDL (w/v) in 10%/90% TFE/water solutions. The values are expressed as the mean % of control cell attachment (±SD) on TCP with n=3. Statistical analysis was carried out using ANOVA with Tukey's multiple comparison tests. **Control PLA substrates with 0% PDL had significantly lower cell attachment than PLA substrates treated with PDL ($p<0.001$ for 10% PDL, 5% PDL, 1% PDL, 0.1% PDL and $p<0.01$ for 0.01% PDL, 0.001% PDL treatment). Above each bar is a representative image of attached cells stained with phalloidin-RITC. (b) Fluorescent microscopy image of PLA substrates patterned by partial solvent entrapment of PDL and then reacted with NHS-Rhodamine. Representative light microscopy images (objective x10) of HEPM cells attaching to (c) PDL patterned PLA substrate after 1 hour and (d) PDL patterned PLA–PEG substrate after 1 hour.
Abbreviations: HEPM, preosteoblast human palatal mesenchymal cells; PDL, poly (D-lysine); PDMS, poly (dimethylsiloxane); PLA–PEG, poly (lactic acid)–poly (ethylene glycol); TCP, tissue culture plastic; TFE, trifluoroethanol.

carboxytetramethylrhodamine N-hydroxysuccinimide ester (Sigma, NHS-Rhod) in 0.1 M sodium borate buffer (pH 8.5) overnight. The disks were then washed 7 times with excess deionized water and imaged by fluorescent microscopy (Olympus CKX41, Abs 555 nm, Em 580 nm). As seen in Figure 2b, fluorescence from rhodamine is exclusively restricted to the PDL patterned channels. This confirms that PDL can be patterned using the partial solvent swelling procedure and that the free amine groups presented by the PDL could be used for binding a range of moieties from cell adhesive peptides for tissue engineering purposes to other biomolecules such as antigen–antibodies for biosensor applications. The fluorescence observed within the channels was patchy, indicating, phase separation of the PDL which is consistent with previous observations of conditions that swell polymer surfaces for extended periods of times (Quirk et al 2000; Quirk, Davies, et al 2001).

Next, we incubated HEPM cells on PDL patterned PLA substrates in 35-mm dishes at a seeding density of $1x10^5$ cells in 1 mL of supplemented MEM with 10% fetal calf serum. Each sample was incubated for 1 hour and then washed with warmed media to remove unbound cells and imaged by light microscopy (Olympus CKX41). As seen in Figure 2c, cells attached preferentially within the patterned regions but also adhered outside of the patterned region. Given that PLA substrates without treatment display up to 54.4% cell attachment as observed in Figure 2b, it follows that HEPM cells would still be able to bind to PLA regions outside the PDL-patterned regions. PEG is well established for its protein- and cell-resistant properties. To ensure that cells bound to the PDL-patterned regions only, we carried out the microfluidic networking of the PDL in TFE–water mixture on a PLA–PEG (MW 23 000) copolymer substrate. PEG units from the copolymer are presented at the surface

when incubated in aqueous media. When HEPM cells were incubated on PDL-patterned PLA–PEG substrates under the same conditions as the PLA substrates, cells were found to bind specifically within PDL-patterned channels (Figure 2d). The PEG presented by the copolymer was, therefore, minimizing cells from binding outside the channels.

Conclusion

In summary, we have demonstrated spatial control over preosteoblast cell attachment using cell repellant and cell adhesive cues prepared by partial solvent entrapment of PDL in microfluidic channels on a degradable polymer substrate. The free amine groups that are produced in the patterned regions can be used to conjugate a range of biomolecules or cell adhesive species as demonstrated by the reaction with NHS-Rhodamine. The possibility of using functionalized PLA–PEG copolymers (Salem et al 2001) in conjunction with this technique has potential to lead to spatial control over different cell types for optimizing activity in tissues such as liver (Bhatia et al 1998). This technique could also be used for other applications such as guided neuronal growth (Patel et al 1998), controlled bone regeneration (Schneider, English, et al 2004; Schneider, Zaharias, et al 2004), and cell-based biosensors. Finally, this approach could be extended to spatial control over cell manipulation and biomolecule binding on a nanoscale given the capacity of soft lithography and nanofluidic networking to produce features below 100 nm (Zhao et al 1997; Ke et al 2005).

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