Durable, region-specific protein patterning in microfluidic channels

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ARTICLE INFO

ABSTRACT

We present a straightforward, accessible method to covalently pattern proteins in poly(dimethyl siloxane) (PDMS) microchannels. Our approach includes (i) region-specific photografting of a layer of poly(acrylamide) (PAAm) and (ii) bioconjugation of PAAm with a desired protein. The method produces symmetric protein patterns on all channel walls, which have high specificity and pattern fidelity, are compatible with a variety of geometries and exhibit excellent longevity under shear stresses of up to 1 dyn/cm. We demonstrate the generality of the method by creating multi-protein gradients within microfluidic microchannels and by *in-situ* patterning of islands of multiple proteins. Protein activity was observed by the digestion of BODIPY–casein using channels patterned with trypsin.

1. Introduction

Microfluidics, a technology which facilitates formation of an almost unlimited variety of geometries of enclosed microchannels [1], is a useful tool for forming *in vitro* systems that are similar to those found in nature. Microchannels alone are not sufficient for mimicking complex natural systems; a key feature of biological microenvironments is the presence of surfaces bearing heterogeneous (e.g., hydrophobic, hydrophilic, protein coated) functionalities. Thus, there is great interest in combining microfluidics with surface chemistry for a range of different biochemical and biological applications. For example, chemically patterned microchannels have been used in miniaturized biological assays [2] and biosensors [3–5], in the formation of biomimetic 3D structures [6], and the promotion or reduction of cell adhesion in specific regions of microchannels [7–10].

There are a large number of approaches to patterning proteins on microchannel surfaces; however, there are only a few that are rapid, straightforward and accessible to non-experts in the field of surface chemistry (i.e., do not require access to complex instrumentation). Two most widely used fairly straightforward methods are micro-contact printing [7,11–15] and laminar flow of proteins in solution [16–18]. These techniques have numerous advantages; however, their drawback is the reliance on physical adsorption of proteins onto surfaces. Protein patterns formed in this manner tend to desorb from surfaces unpredictably [19], and in addition, are particularly problematic in microfluidic systems operating under flow, as fluid movement promotes desorption of molecules from surfaces.

Here, we report a new straightforward method for *in-situ* covalent patterning of proteins in microchannels. We used region-specific photografting of poly(acrylamide) (PAAm) to PDMS and subsequent bioconjugation of PAAm with a desired protein. Our approach has the following useful features: (i) high specificity and fidelity in protein patterning with high resolution; (ii) the capacity to easily produce a variety of pattern geometries, (iii) the applicability to a wide range of proteins; (iv) excellent longevity under shear stresses of 1 dyn/cm^2; and (v) the ability to create symmetric protein patterns on all four walls of microchannels. We anticipate that this method will be useful for a wide range of applications that require the creation of biological microenvironments in microfluidic systems.

2. Experimental

2.1. Materials

Acrylamide (AAm), benzophenone (BP), 2-butanol, collagen, fibrinogen (FB), fluorescein isothiocyanate (FITC)–casein, immunoglobulin G, Pluronic F-68, sodium dodecylsulfate, sulforhodamine 101 acid chloride (Texas red, TR), tetramethylrhodamine isothiocyanate (TRITC), TPCK treated trypsin and phosphate buffered saline were purchased from Aldrich Canada and used as received. E6638 EnzChek Protease Assay Kit (including quenched, BODIPY-labeled casein) was purchased from Invitrogen (Burlington, ON). Collagen and IgG were conjugated to TR using the procedure described by Brinkley [20] and FB was conjugated to TRITC using the procedure reported by Larsson [21].

2.1.1. Fabrication of microfluidic devices

Microchannels with an average width and height of 800 and 100 μm, respectively, were fabricated in PDMS by soft lithography [22] using a Sylgard 184 Silicone Elastomer Kit (Dow Corning Corp., Midland, MI). PDMS devices bearing networks of microchannels were enclosed by bonding to a glass slide (48 mm x 65 mm), prepared by spin-coating 1 mL of uncured PDMS at 1000 rpm for 1 min and subsequent curing at 75 °C for 2 h. After plasma bonding (550 mTorr in air, 90 s), devices were incubated overnight in an oven at 75 °C to return the PDMS surface to a hydrophobic state. The devices were examined using a microscope equipped with fluorescence illumination (Olympus BX51) and a camera (Olympus U-CMAD3) and Image Pro Plus software (Media Cybernetics, MD, USA).

2.1.2. Patterning poly(acrylamide) in microchannels

As a first step in patterning proteins onto surfaces, PDMS microchannels were modified with PAAm via region-specific photoinitiated graft-polymerization [23,24]. A modification solution containing 17 wt% AAm, 9.7 wt% 2-butanol, 0.3 wt% BP, 0.3 wt% Pluronic F-68 and 72.7 wt% deionized water was prepared. The channels were filled with the modification solution and then exposed to UV-radiation (3 min, 200 mWatt/cm^2) through a photomask at a distance of 15 cm from the lamp, and then rinsed in 2 mL of deionized water. The photomask was printed using a 4000 dpi printer on a transparency film (Norwood Graphics, Toronto).

2.1.3. Patterning proteins in microchannels

Protein patterns were formed in PAAm-modified microchannels in three different geometries: single-protein patches, two-protein gradients, or three-protein arrays. In each case, proteins were delivered to the channels in a patterning solution comprising 0.05 M phosphate buffer (pH 7.0) containing 5 mg/mL 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC), 0.46 mg/mL sulfo-N-hydroxysuccinimide (sulfo-NHS) and 1 mg/mL of the particular protein to be patterned. For single-protein patches, the entire network of channels was filled with a patterning solution containing FITC–casein and incubated under static conditions for 120 min. For two-protein gradients, patterning solutions containing two different proteins (FITC–casein and TR–collagen) were pumped through two inlets of a Y-junction at 0.1 mL/h for 3 h [25]. For three-protein arrays, patterning solutions containing three different proteins (FITC–casein, TR–IgG, and TRITC–FB) were pumped through three inlets of a five-inlet microchannel network at 0.1 mL/h for 3 h. During this time, deionized (DI) water was pumped through the other two inlets, such that the water acted as a barrier between the protein solutions [16]. Following protein patterning, microchannels were rinsed with 2–5 mL of DI water.

2.1.4. Characterization of protein patterns

Functionalized PDMS surfaces were evaluated by Fourier Transform Infrared Radiation (FTIR) spectroscopy, ellipsometry, and fluorescence microscopy. FTIR spectra were generated using a Hyperion 15x reflection microscope with left exit detection (Bruker, Canada). Signals from the background and samples were averaged over 32 scans in the range from 2000 to 500 cm^{-1} with a resolution of 4 cm^{-1}. Ellipsometry data were collected using a GETEST instrument (Sopra, France) scanning from 0.62 to 6.2 eV at 75°. Pattern fidelity was evaluated by comparing fluorescent images of microchannels to bright field images of the photomasks using Image Pro Plus. The total number of fluorescent (device) or clear (photomask) pixels were counted and expressed as a ratio (fluorescent/clear).

Fluorescence intensity measurements were used to characterize the longevity of protein patches formed in microchannels. In these experiments, the devices (bearing 5 cm long channels), were formed either by covalently attaching FITC–casein to a 500 μm-long PAAm patch (as described above) or by non-specific adsorption to the walls (as a control). In the latter, the microchannel was filled and incubated for 2 h with a 2 mg/mL solution of FITC–casein in 0.05 M phosphate buffer (pH 7.0) and subsequently rinsed with 2 mL of DI water for 4 min. To test the longevity of protein attachment to microchannel surface in the microfluidic experiments, the channels were flushed with DI water at 0.1 mL/h, and fluorescent images were collected after 0, 0.5, 1, 2, 6, 12, 24, 48, 72 and 96 h. To account for photobleaching of FITC, we used as a reference a microfluidic device with covalently patterned FITC–casein under static conditions (i.e., no flow). To process the data, in each image collected from channels subjected to flow, the fluorescence intensity was summed over the area of the pattern and divided by the intensity of the corresponding image collected under static conditions. The data for each condition were then normalized to the initial intensity of the pattern of interest (after 0 h).

To evaluate two-protein gradients and three-protein arrays in microchannels, multiple fluorescent images were collected using different filter sets and pseudo-colored using Image J software (National Institute of Health, USA). Combined images for two-protein gradients were formed by overlaying two separate images using Image J software. Intensity profiles for gradient images were generated using Image Pro Plus and normalized to the average background fluorescence. The thickness of the line used during this scan was 1 pixel.

2.1.5. Digestion assay

Microchannels with an average width and height of 800 and 100 μm respectively were patterned with patches of trypsin 100, 200 or 300 μm long. As a control, microchannels were patterned with a 100 μm-long length of PAAm, but not

conjugated with trypsin. Intramolecularly quenched BODIPY–casein (25 μg/mL) in PBS buffer (100 mM, pH 7.8) was flushed through the channels at a rate of 0.01 mL/h via a syringe pump. Fluorescence was detected 500 μm downstream from the protein (or control) patches by laser-induced fluorescence using an inverted microscope (Olympus IX-71) mated with an argon ion laser (Melles Griot, Carlsbad, CA). The 488 nm laser line (20 mW) was focused into the channel using an objective (60x); the fluorescent signal was collected by the same lens and filtered optically (536/40 nm band-pass and 488 nm notch filter) and spatially (500 nm pinhole), and imaged onto a photomultiplier tube (Hamamatsu, Bridgewater, NJ). PMT current was converted to a voltage using a picoammeter (Keithley Instruments, Cleveland, OH) and then collected using an A–D converter and a PC running a custom LabVIEW (National Instruments) program. Four different channel/patch combinations were evaluated per condition.

3. Results and discussion

3.1. Patterning microchannels with proteins

The first step in the new patterning procedure is region-specific grafting of PAAm to PDMS, which is initiated by exposure of PDMS to UV-irradiation. In this method, a benzophenone molecule (the photoinitiator) forms a radical, which abstracts a hydrogen atom from the PDMS surface (Fig. 1A). An acrylamide group then reacts with this surface radical, initiating the process of free-radical polymerization of the monomer, attached to the surface (Fig. 1B). A competing reaction may also take place: a benzophenone radical may abstract a hydrogen from the acrylamide monomer, which will result in the formation of free (non-grafted) PAAm in the solution. If this reaction occurs, the product would be removed by rinsing the channel with DI water after UV-exposure. (We note that termination of free PAAm molecules by reaction with a radical created on the PDMS surface will also lead to the formation of grafted PAAm layer). The average thickness of a dry grafted PAAm layer formed in this manner was approximately 200 nm. In the second step, we attached protein to an amide group (Fig. 1C). A carboxylic acid on the protein (e.g., casein) reacts with a molecule of EDC, creating an attractive leaving group for the formation of an imide bond between the protein and the PAAm.

To examine the reactions occurring during the patterning process in steps A–C (Fig. 1), FTIR spectra were acquired for a bare PDMS substrate, a PDMS substrate modified with grafted PAAm, and a PDMS substrate modified with PAAm and coupled to FITC–casein. In Fig. 1D, the spectrum of PAAm grafted to PDMS shows distinct peaks at 1666 and 1620 cm^{-1}, which correspond to the amide 1 and amide 2 stretches in PAAm [26]. Following covalent protein attachment to the PAAm layer, the spectrum shows a peak at 1650 cm^{-1}, which we attributed to the α-helix portion of casein [27].

To evaluate the fidelity of protein patterns formed in this manner, we compared a bright field image of the photomask used in the patterning procedure to the fluorescent images of a microchannel patterned with FITC–casein. Fig. 2 shows two images: (A) a mask with three 100 x 100 μm square openings and (B) a PDMS microchannel modified with FITC–casein. The pattern transfer fidelity was excellent, with a ratio of fluorescent pixels in the channel to clear pixels in the photomask of 1.05 ± 0.08.

To characterize the longevity of covalently attached and passively adsorbed patterns, we monitored fluorescence intensity in the course of 96 h-long experiments while purging the channels with DI water at a flow rate of 0.1 mL/h. Fig. 2C shows the average normalized fluorescence intensities of the FITC–casein patterns plotted as a function of time. The fluorescence intensity decreased over time for both types of patterns; however the decrease in intensity was substantially larger for the physically adsorbed protein. For example, after 24 h, the intensity of physically adsorbed protein decreased by 80%, and after 96 h it had decreased to 10% of the initial levels. In contrast, covalently attached FITC–casein retained 85% of its original fluorescence during the first 24 h, and
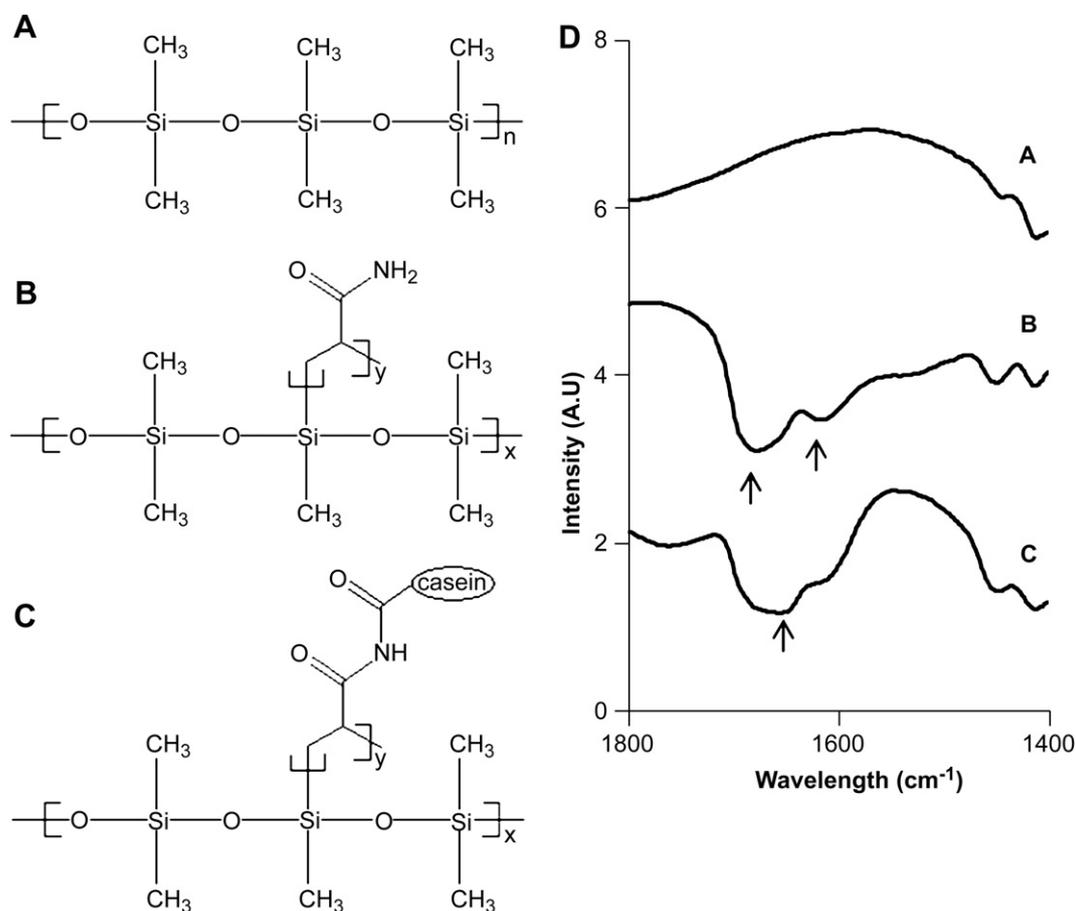
Fig. 1. Structure of PDMS (A), PDMS with a grafted layer of PAAm (B), PDMS with a grafted layer of PAAm that has been conjugated with FITC-casein (C), and the FTIR sprecta of each of those layers (D). The arrows at 1620/1666 cm-1 and 1650 cm^{-1} correspond to amide stretches in PAAm and the α-helix of casein, respectively.

70% after 96 h. Because of the high stability of covalently attached protein patterns, we anticipate that the new method will be advantageous for applications requiring that proteins remain attached to microchannels for several days, e.g., for on-chip culture of slowly proliferating primary cells [25].

In the next step, we explored two potential applications for the patterning method: the generation of surface-gradients of protein density and the generation of arrays of different proteins in microfluidic channels.

3.2. Covalently attached protein gradient

Spatial gradients of surface-attached proteins are useful for studying cell attachment and spreading, haptotaxis, and embryonic development [28–31]. As depicted in Fig. 3A, we generated spatial chemical gradients using a microfluidic device geometry similar to those reported previously (e.g., see reference [29]) to split-and-recombine two reagents (in this case: FITC–casein and TR–collagen). In contrast to previous work (in which gradients were
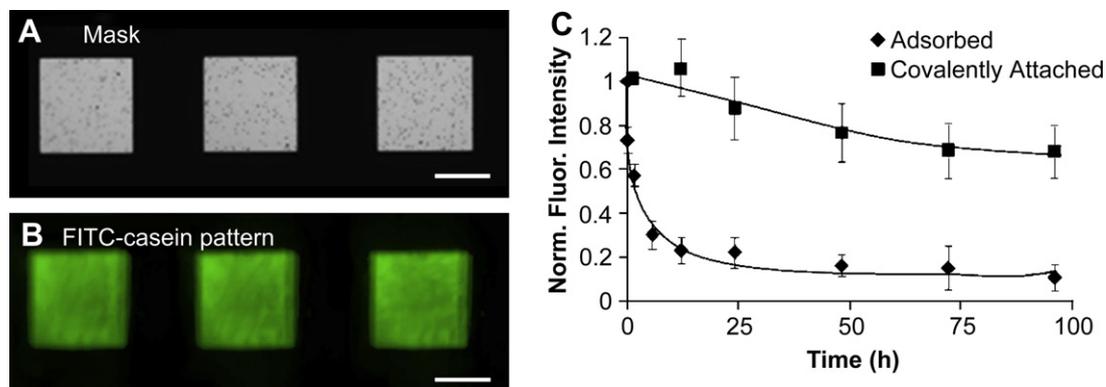
Fig. 2. Pictures of a UV photomask (A) and the resulting pattern of FITC–casein (B) in a microchannel. The scale bar is 75 μm. (C) Normalized fluorescence intensities of covalently attached (■) and adsorbed (◆) FITC–casein in microchannels during flow as a function of time.
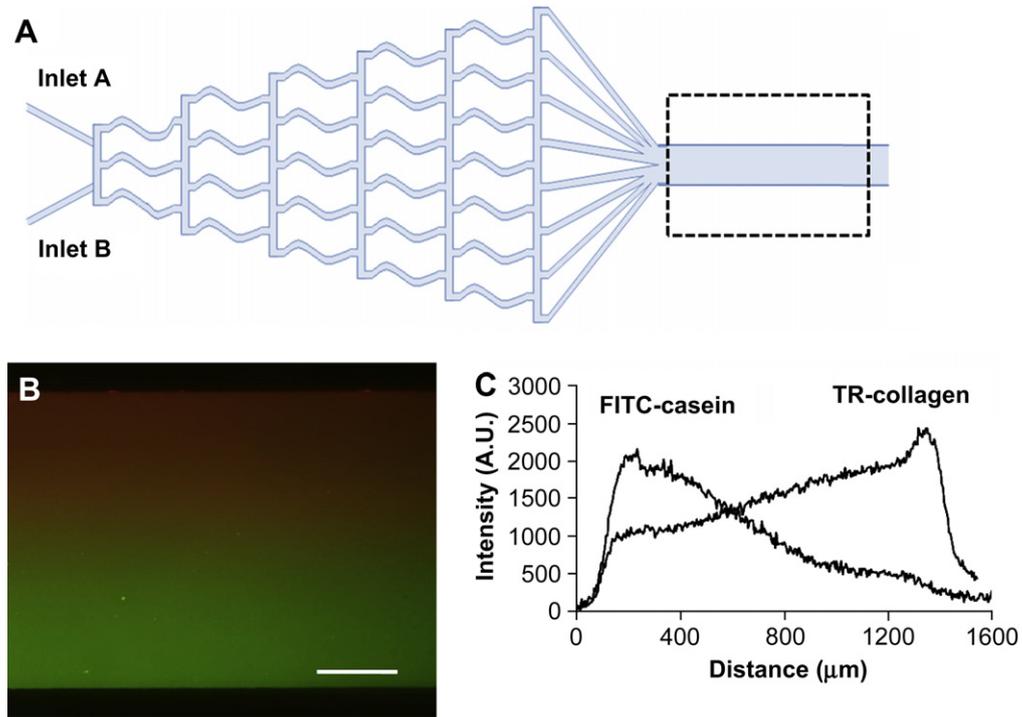
Fig. 3. (A) Schematic of the device used for producing gradient patterns. The dotted line indicates the location of the PAAm pattern. Fluorescent image (B) of FITC–casein and TR–collagen in the device. Scale bar is 300 μm. (C) Variation in fluorescence intensities of two proteins, plotted as a function of microchannel width.

formed by physical adsorption onto the surface), in our work, the two proteins were covalently attached to the surface by conjugating them to PAAm.

To characterize gradient protein patterns in the microchannel, we collected images of the fluorescence intensities of FITC–casein and TR–collagen and fluorescence line profiles across the width of

the channel (Fig. 3B and C, respectively.). These data demonstrate that a gradient of each protein exists across the width of the central channel. The position of the intercept of the line profiles could be tuned by varying the flow rate ratios of two solutions containing FITC–casein and TR–collagen or the concentration of these solutions.
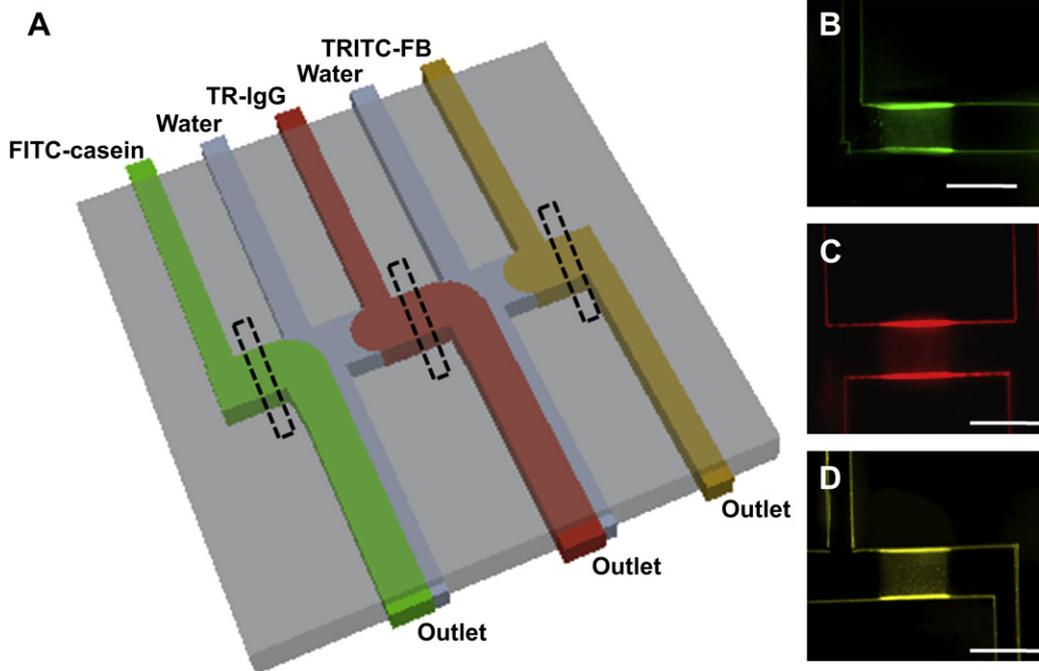
Fig. 4. (A) Schematic of the device used to form an array of protein patches in a network of microchannels. The dotted lines indicate the regions of the channel grafted with PAAm. Fluorescent images of FITC–casein (B), TR–IgG (C), and TRITC–FB (D) attached to PDMS channels. Scale bar is 250 μm. Images B, C, and D were generated from the same device.
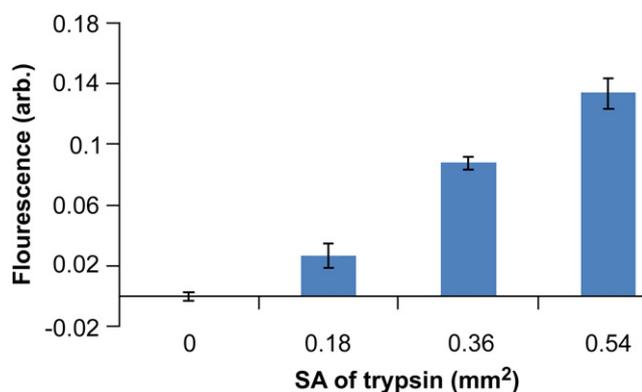
Fig. 5. Fluorescence intensity of digested BODIPY-casein as a function of trypsin-modified surface area (SA). Four measurements were collected per condition, and error bars represent ±1 S.D.

In subsequent experiments (data not shown), the temporal decrease in the normalized fluorescence intensity of gradients of covalently attached proteins was significantly reduced relative to that observed for physically adsorbed gradients of the same proteins. The use of covalently attached proteins ensures that the surface densities remain consistent throughout each experiment.

3.3. Arrays of different proteins

Biological micro-environments are characterized by surface chemistry heterogeneity [32], and arrays of protein patches in microfluidic devices have been used for biological assays, enzymatic reactors, and biosensors [33–36]. Here, we report implementation of region-specific covalent patterning of microchannels with three different proteins. As shown in Fig. 4A, laminar flow was used to deliver different proteins to different regions in the device [16].

Using the method described above, we coupled three different proteins to a network of microchannels: FITC–casein (23 kDa), TR–IgG (150 kDa), and TRITC–FB (340 kDa). Optical fluorescence microscopy images of the patterns of each of these proteins attached to the microchannels are shown in Fig. 4B–D. For each of the proteins evaluated, the ratio of fluorescent pixels observed in the channel to clear pixels in the photomask was 1.25 ± 0.20. The distribution of intensities between patches is small, with the ratio of fluorescent pixels of FITC–casein: TR–IgG: TRITC–FB being 1.15: 1: 1.05. We anticipate that this technique will be useful for rapidly patterning precise and robust arrays of proteins.

3.4. Protein activity

The activity of proteins patterned using the techniques described here was evaluated by forming patches of immobilized, patterned trypsin to digest intramolecularly quenched BODIPY–casein, a reporter that becomes progressively more fluorescent as it is digested. Microchannels were patterned with trypsin in segments 100, 200 or 300 μm-long. As a control, additional microchannels were modified with PAAm (100 μm long) but not conjugated with trypsin. The BODIPY–casein reagent was purged through the channels, and laser-induced fluorescence was used to probe the extent of digestion in solution. As shown in Fig. 5, the fluorescence intensity scales linearly with the increase in surface area of the patterned trypsin, and no activity is observed in microchannels not containing the enzyme. This confirms that proteins conjugated to microchannels via PAAm are active and can be used for on-chip assays and analyses.

4. Conclusion

We have introduced a straightforward, versatile technique for *in-situ* patterning of proteins in PDMS microchannels. This technique is a two-step process, comprising photografting of PAAm to the channel wall and EDC-coupling of the desired protein to the PAAm. Protein patches formed using this technique are active and resistant to detachment under flow. This feature is particularly useful for applications involving two or more proteins that must maintain their position over the course of an experiment, and we speculate that it will be a useful tool for creating microfluidic conditions that mimic biological microenvironments.

Acknowledgements

Appendix

Figures with essential colour discrimination. Figs. 2–5 in this article may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.09.040.

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