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Attachment of hydrogel microstructures and proteins to glass via thiol-terminated silanes

Jeong Hyun Seo, Dong-Sik Shin, Priam Mukundan, Alexander Revzin*

Department of Biomedical Engineering, University of California, Davis, CA 95616, United States

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ABSTRACT

Micropatterning strategies often call for attachment of non-fouling biomaterials and immobilization of proteins in order to create biosensing surfaces or to control cell–surface interactions. Our laboratory has made frequent use of hydrogel photolithography – a micropatterning process for immobilizing poly(ethylene glycol) (PEG) hydrogel microstructures on glass surfaces. In the present study we explored the use of thiolsilane as a coupling layer for both covalent anchoring of hydrogel microstructures and covalent immobilization of proteins on glass. These new surfaces were compared to acryl-silane functionalized glass slides that allowed covalent attachment of gels but only physical adsorption of proteins as well as surfaces containing a mixture of both functional groups. We observed comparable attachment and retention of hydrogel microstructures on acryl and thiol-terminated silanes. Ellipsometry studies revealed presence of significantly higher level of proteins on thiol-functionalized glass. Overall, our studies demonstrate that thiol-silane functionalized glass surfaces may be used to create complex micropatterned surfaces comprised of covalently attached hydrogels and proteins. This simple and effective surface modification strategy will be broadly applicable in cellular engineering and biosensing studies employing hydrogel micropatterns.

1. Introduction

Immobilization of biomolecules is an important aspect in developing biosensor and surfaces for cell/tissue engineering. Covalent attachment of biomolecules, surfaces may be functionalized with a variety of chemical groups for example NH_2—, SH—, COOH—, NHS ester and epoxide groups [1]. The most common way of introducing these functional groups onto a glass or oxide surface is via self-assembly of silanes [2–5].

In addition to their use for biomolecule attachment, silane coupling agents may also be used for anchoring biomaterial constructs to surfaces. For example, acrylate-terminate silanes have been used by several groups for attachment of hydrogel micropatterns to glass and silicon surfaces [6–8]. Our lab as well as other groups has utilized poly(ethylene glycol) (PEG) hydrogel micropatterning to create non-fouling domains on the surface and to control cell–surface interactions.[9–14] In these experiments, cell attachment to glass regions not covered by PEG gel was promoted by physical adsorption of matrix proteins such as collagen or laminin. In other studies, antibodies against cell surface antigens and secreted cytokines were immobilized within hydrogel microwells to capture cells and locally detect secreted cytokine molecules [15]. Therefore, cellular engineering and biosensing applications would benefit from surfaces that allow robust anchoring of hydrogels and immobilization of proteins.

Various strategies may be pursued to enable attachment of biomaterials and proteins on the same surface. Koh and coworkers in a series of publications described the use of nanoporous membranes or electrospun polymers as substrates that allow non-covalent immobilization of hydrogels and high loading of proteins [16,17]. These surfaces are quite promising for cell cultivation and sensing, however, are somewhat complex to prepare and may not be suitable for all applications. Other reported approaches involved co-assembly of two different silane molecules (acrylate- and thiol- or amine-terminated silanes), one bearing gel anchoring and another protein immobilization chemistries [18,19]. This approach has been used by us recently to create hydrogel microwells for capturing immune cells and detecting secreted cytokines [19]. Covalent immobilization of immunoassay components led to 4-fold improvement in sensitivity of detecting cell-secreted cytokines. However, we reasoned that an optimal coupling layer should avoid the use of multiple silane types and should employ the same functional groups for gel attachment and protein binding. The use of single silane type avoids the amount of optimization required for the surface modification. For example, alkoxysilanes are water reactive so that the age of individual silanes needs to be taken into

* Corresponding author. Tel.: +1 530 752 2383.
E-mail address: arevzin@ucdavis.edu (A. Revzin).

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account prior to modification. The use of single silane also ensures functional uniformity of the surface whereas mixture of silanes may hypothetically result in distinct domains of functional groups.

In this paper we explored the use of mercaptopropyl trimethoxysilane (abbreviated to thiol-silane throughout this paper). Thiol-terminated silanes have been used widely in conjunction with hetero-bifunctional crosslinkers for covalent immobilization of proteins on glass [20,21]. In addition, there have been recent reports of photo-initiated thiol-acrylate reaction used to create biomaterial matrices [22,23]. However, attachment of acrylated hydrogels to thiol-terminated surfaces has not yet been demonstrated to the best of our knowledge. This paper demonstrates that self-assembly of thiol-silane on glass provides an excellent surface coating for attachment of hydrogel microstructures and covalent immobilization of proteins (Fig. 1). Given increasing use of hydrogel microstructures and microwells for cell culture, analysis and biosensing the surface modification protocol described here will be widely applicable.

2. Materials and methods

2.1. Materials

Glass slides were obtained from VWR (West Chester, PA). 3-(acryloxypropyl) trimethoxysilane (acryl-silane) was purchased from Gelest, Inc. (Morrisville, PA, USA). 3-Mercaptopropyl trimethoxysilane (MW 196.34) (thiol-silane), poly(ethylene glycol)diacrylate (PEG-DA, m.w. 575), 2-hydroxy-2-methyl-propiophenone (photoinitiator), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), methanol, *N,N*-dimethylformamide (DMF), *N,N*-diisopropylethylamine (DIPEA), toluidine blue O, and anhydrous toluene (99.9%) were purchased from Sigma–Aldrich (Saint Louis, MO). N-hydroxysuccinimidyl 9-(3-maleimidopropionyl)-amido-4,7-dioxanonanoate (MAL-dPEG2-NHS ester) and N-d-biotinamido-N′-(3-maleimidopropionamido)-4,7,10-trioxatridecane-1,13-diamine (biotin-dPEG3-MAL) were purchased from Quanta Biodesign, Ltd. (Powell, OH). For brevity, these compounds are referred to in the text as 'biotin linker' and 'NHS linker', respectively. Alexa Fluor-546 conjugated streptavidin, Alexa Fluor-488 conjugated neutravidin, and neutravidin were purchased from Invitrogen (Carlsbad, CA). PE conjugated anti-CD4 Ab was purchased from R&D systems (Minneapolis, MN). Heparin was purchased from Cellsus Ins. (Cincinnati, IA). High molecular weight poly(ethylene glycol)diacrylate (m.w. 3.4 kDa) (high m.w. PEG-DA) was purchased from SunBio Inc. (Anyang, Korea). Sylgard 184 poly(dimethylsiloxane) (PDMS) and curing agent were purchased from Dow Corning (Midland, MI).

2.2. Silane modification of glass slides

Glass slides were sonicated in ethanol for 10 min to remove bulk contaminants. Immediately prior to silanization, glass substrates were treated in an oxygen plasma chamber (YES-R3, San Jose, CA) at 300 W for 5 min. For thiol-silane assembly, substrates were immersed in thiol-silane diluted to 0.1% (v/v) in anhydrous toluene for 16 h. A similar procedure was followed for acryl-silane with only a 16 h incubation time required. For surfaces modified to give a 'mixed' silane layer, trimethoxy acryl- and thiol-silanes were prepared to give a concentration of 0.1%, v/v for each silane in anhydrous toluene for the longer 16 h incubation time. All silane modification procedures were conducted in a dry nitrogen-purged glove bag to minimize atmospheric moisture. After incubation, slides were rinsed with fresh toluene, dried under nitrogen and baked at 100 °C for 1 h.

2.3. Fabrication and staining of PEG hydrogel micropatterns

Photolithographic patterning of PEG hydrogel microstructures was conducted as previously described. Briefly, a prepolymer solution containing PEG-DA and photoinitiator was spin-coated onto silanized substrates using Spintech S-100 (Redding, CA) operated at 850 rpm for 4 s. The PEG-DA prepolymer layer on glass substrate was then exposed to UV light at a sample-to-source distance of 6 cm (giving ~60 mW cm^{-2}) through a chrome-sodalime photomask for 0.5 s using UV OmniCure series 1000 light source (EXPO, Mississauga, Ontario, Canada). PEG-DA exposed to UV became cross-linked to itself as well as to acryl and thiol groups on the surface while unexposed regions remained unpolymerized and were easily dissolved in DI water.

An alternate formulation of PEG hydrogel containing heparin was also prepared, allowing for staining by toluidine blue O following previously reported protocols [23]. In this case, hydrogels were formed using a high molecular weight PEG-DA (m.w. 3.4 kDa) in combination with a thiolated heparin molecule. The prepolymer solution was spread using a glass coverslip and then exposed to UV for 3 s at a sample-to-source distance of 3 cm. Toluidine blue O staining of these heparin-containing hydrogel microstructures was performed as described by Gosey et al. [24] After staining, heparin-containing hydrogel micropatterns were visualized using optical microscopy (Zeiss Axiovert 40, Carl Zeiss, NJ, USA). The toluidine blue O staining appears as a deep purple.

2.4. Protein immobilization on silane-modified surfaces

Protein immobilization on uniform and micropatterned surfaces was characterized by ellipsometry and immunofluorescent staining. Ellipsometry measurements were made using a Stokes ellipsometer LSE (Gaertner Scientific Corporation, Skokie, IL) and proceeded as follows. Silicon substrates containing acryl, thiol or acryl/thiol functional groups were prepared by silanization protocols identical to those described above. Subsequently, each silane-modified substrate (thiol, acryl, and mixed) was incubated with 50 mL of biotin linker in the presence of 100 mM DIPEA in DMF for 2 h, then washed with DMF and methanol, and dried using nitrogen gas. As the next step, silicon pieces were immersed in a solution of 20 μg/mL streptavidin in PBS for 30 min, washed with PBS buffer and DI water. Ellipsometry measurements were made after each step in the surface modification protocol, assuming refractive index of 1.45. Thickness was measured in three locations of the surface to produce an average thickness per substrate. Three different silicon chips ($n = 3$) were characterized for each surface type.

Immobilization of proteins in PEG hydrogels proceeded as follows. PEG gels were micropatterned on thiolsilane-modified glass and then incubated for 2 h in a 50 mM solution of NHS or biotin linker in DIPEA (100 mM) and DMF. Subsequently, surfaces were immersed in biotinylated anti-CD4 PE (25 μg/mL). Glass slides containing acryl-silane were micropatterned and modified in an identical manner. To develop a fluorescence signal, micropatterned surfaces were immersed in neutravidin-Alexa 488 (10 μg/mL). Samples were imaged using a confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss, Inc., Thornwood, NY).

2.5. Stability of PEG-hydrogel adhesion on different surfaces

In order to assess stability over time, PEG-hydogel micropatterns were fabricated on thiol-, acryl-, and mixed-silane functionalized surfaces under conditions described above. The stability test was carried out with surfaces containing circular PEG-hydrogel micropatterns (diameter: 50 μm, center-to-center: 80 μm). Samples were then incubated at 37 °C in a 5% CO_2 incubator in DMEM cell culture media to test stability of PEG adhesion. The number of

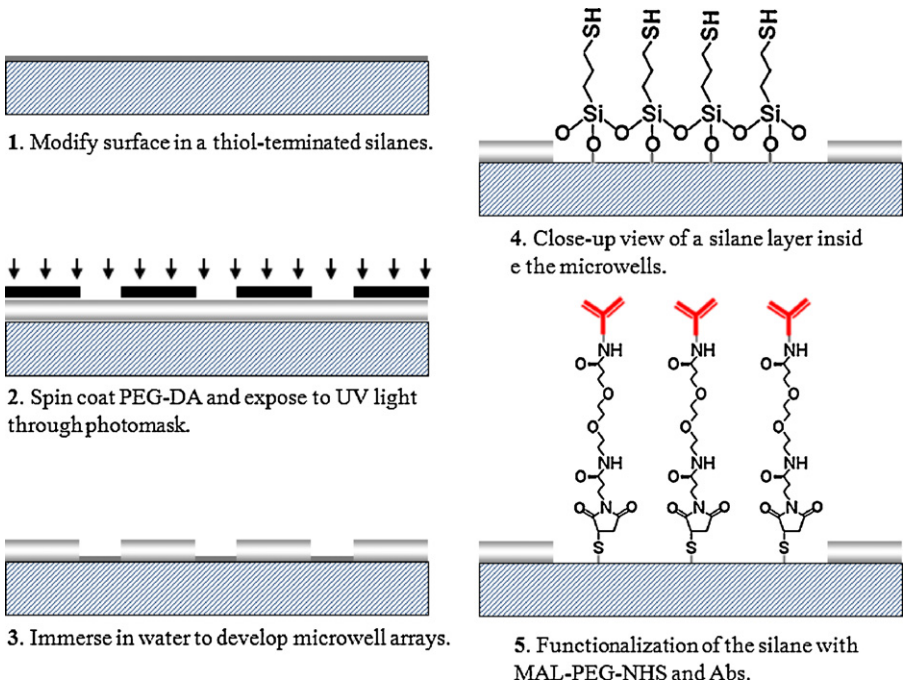

Fig. 1. Process flow diagram for hydrogel micropatterning and protein attachment. PEG-hydrogels were directly fabricated on thiol-silane surfaces using photolithographic methods. Thiol groups remain on the surface of the unpolymerized regions, allowing for covalent linking of proteins.

detached hydrogel microwells was counted daily under a microscope. Stability was obtained from the equation below:

$$\%\,\text{Stability} = \frac{N_{tot} - N_d}{N_{tot} \times 100}$$

where N_{tot} = total number of hydrogel microwells and N_d = number of detached microwells.

3. Results and discussion

In our previous work, self-assembled monolayers with terminal acryl groups were used for anchoring PEG-DA micropatterns onto oxide surfaces [8,14]. In this approach, the acryl groups of the PEG prepolymers were able to crosslink with acryl groups imparted on the surface through silane chemistry in the presence of a radical-promoting photoinitiator following UV exposure. This covalent anchorage was necessary to prevent the detachment of the hydrogel from the surface upon swelling in water. Without this surface anchorage, the PEG-DA molecules become crosslinked to themselves but do not covalently bond to the surface and delaminate within minutes of immersion in water. On the contrary, hydrogels anchored covalently remain attached for days or weeks. Despite the advantages in promoting hydrogel adhesion, acryl groups are not conducive to covalent attachment of proteins and thus biomolecules have typically been immobilized only by physisorption.

In the previous effort to surmount this issue the glass surface was modified in a mixture of acryl- and thiol-terminated silanes to create a mixed layer containing functional groups for gel anchoring (acryl) and protein immobilization (thiol) [19]. While this method proved successful, we reasoned that simplest and most optimal surface modification should involve a single functional group that supports both PEG-hydrogel micropattern adhesion and protein immobilization via covalent linkage. With this in mind, we noted that thiol groups have been shown to react with vinyl (or acryl) groups in the presence of radicals under UV exposure [25–27], and surmised that hydrogels containing acrylated PEG should react with thiol-silane becoming anchored on the surface. Additionally, these thiol groups provide a practical means for covalent coupling of proteins via commercially available bi-functional linkers containing maleimide functionalities.

3.1. Surface modification and protein immobilization

In order to verify the suitability of thiol surfaces for protein immobilization, we examined protein adsorption to three different silane modified surfaces: thiol-silane, acryl-silane, and mixed-silane (a 1:1 molar ratio of the thiol- and acryl-silanes). After silane modification, the surface was incubated with biotin linker followed by streptavidin. Ellipsometry measurements made after each surface modification step are summarized in Fig. 2. Of particular note are following observations. As expected, functionalization with biotin linker did not result in a thickness change for acryl-terminated surface which did not contain

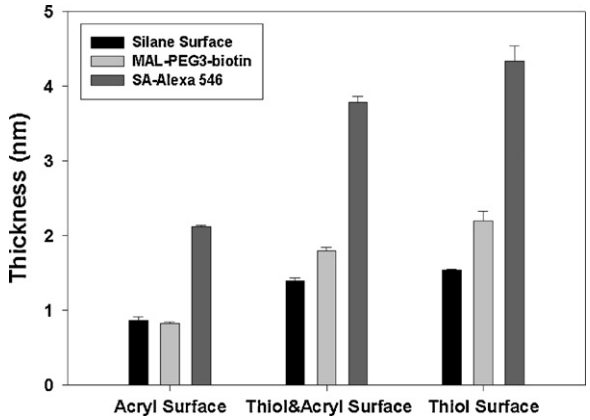

Fig. 2. Characterization of surface modification using ellipsometry. Adsorption of linker (Mal-PEG-NHS) caused a thickness change of ~0.5 nm on mixed-silane and thiol-silane surfaces, but no difference on acryl-terminated silane. In addition, significantly more streptavidin adsorption was observed on thiol-terminated silane (2.14 nm layer) vs. acryl-silane (1.25 nm layer).

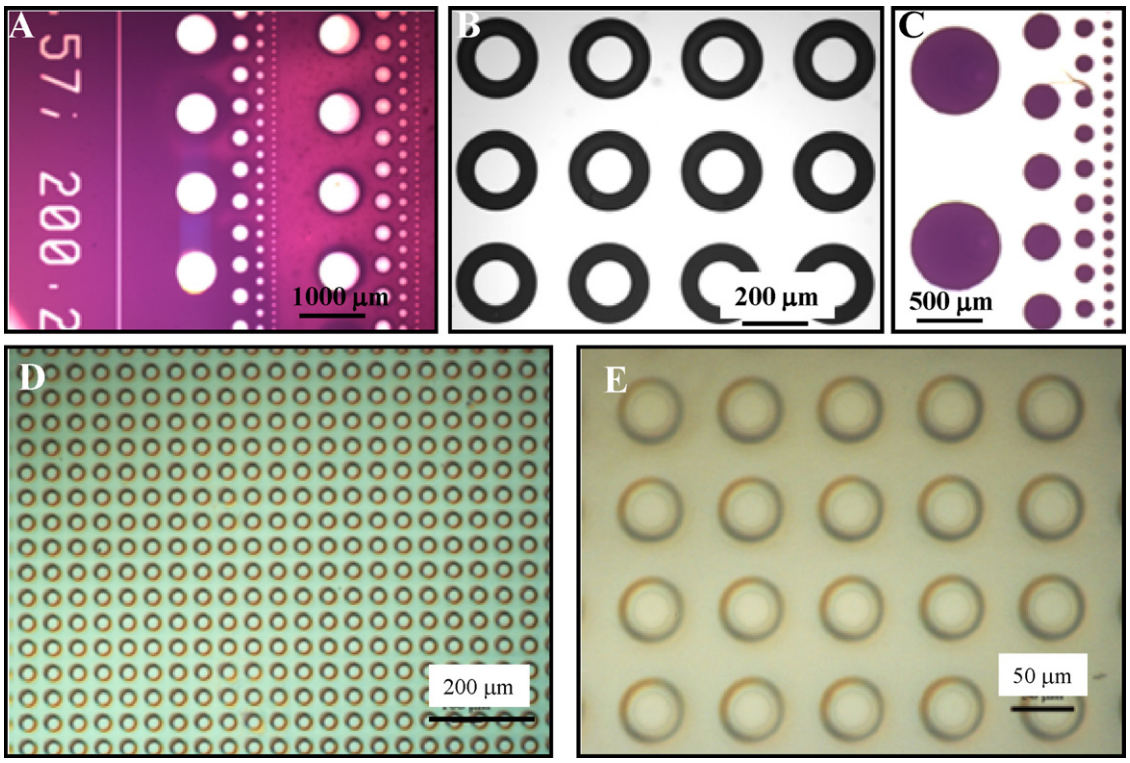

Fig. 3. Micropatterning of hydrogels on thiol-silane-modified glass surfaces. (A–C) Heparin/PEG hydrogels stained with toluidine blue and (D–E) PEG hydrogels microstructures.

maleimide-reactive groups. Biotin linker did adsorb onto mixed- and thiol-terminated silane layers showing thickness change of 0.4 ± 0.07 and 0.65 ± 0.13 nm for thiol and mixed-silane surfaces respectively. Subsequent adsorption of streptavidin resulted in thickness change of 1.25 ± 0.02 nm for acryl-silane, 1.99 ± 0.16 nm for mixed-silane and 2.14 0.08 for thiol-silane. Higher levels of streptavidin on thiol-silane surface compared to acryl-silane demonstrate benefits of covalent protein immobilization. While protein layer was thicker on thiol-silane compared to mixed-silane layer the difference was not statistically significant.

3.2. Anchoring of hydrogel microstructures on thiol-silane modified surfaces

The second key function of interest for our surfaces is that of hydrogel anchoring. In order to test the capability of these thiol-silane surfaces to anchor hydrogels, two formulations of PEG hydrogel were examined after photo-crosslinking as described above. One formulation resulted in the formation of a gel from a low molecular weight PEG-DA (0.575 kDa) liquid prepolymer while the other utilized an aqueous solution of higher molecular weight PEG-DA (3.4 kDa) in combination with thiolated heparin. Both of these formulations have previously been used for PEG microstructure fabrication [19,23]. Structures resulting from each formulation are shown in Fig. 3. Heparin-containing gels have been stained purple using toluidine blue O for ease of visualization. These results demonstrate that PEG-hydrogel microstructures can be fabricated in a variety of shapes and sizes on thiol-silane functionalized surfaces (without any surface acryl groups present).

To characterize gel attachment to the thiolated surface, we examined the stability of the PEG-DA hydrogel over time in aqueous solution. PEG-hydrogel microstructures where fabricated onto thiol-, mixed-, and acryl-silane functionalized surfaces and then incubated in DMEM cell culture media for up to 5 days at 37 °C. The stability was quantified by calculating the ratio of the number of remaining hydrogel microwells to the total number of hydrogel microwells (Fig. 4). Hydrogel microwells on the thiol-silane and mixed-silane surfaces started detaching on the third day (77% and 82% stability, respectively) and detached entirely on the fifth day. Hydrogel microstructures on acryl-silane surfaces persisted through the 3 days of observation and showed 66% stability on the fifth day. Although the PEG microstructures anchored to thiol-silane surfaces were not as stable as those anchored to an acryl-silane surfaces, the thiol-silane anchored hydrogels were durable for up to 3 days. This timeframe is sufficient for applications in biosensing and cell monitoring where these constructs have been employed in the past [13,15,28,29].

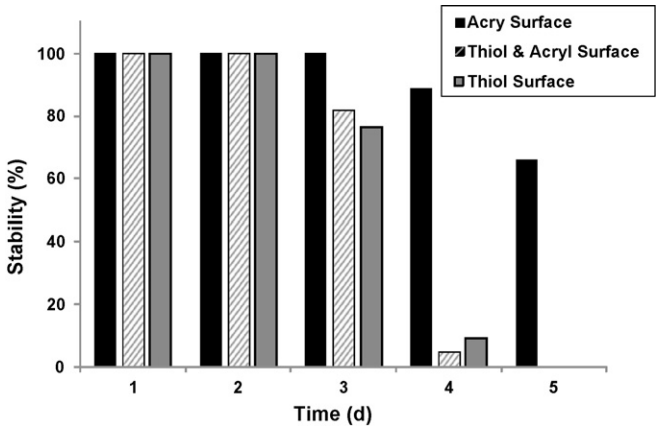

Fig. 4. Stability of hydrogel micropatterns on thiol-, mixed-, and acryl-silane functionalized surfaces in aqueous solution. The stability was quantified by calculating the ratio of the number of remaining hydrogel microwells to the total number of hydrogel microwells.

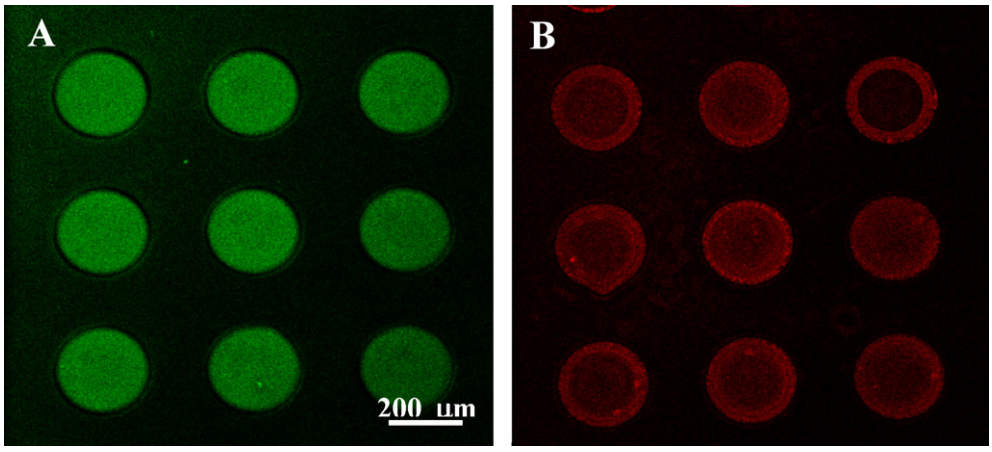

Fig. 5. Fluorescence images of protein adsorption in PEG hydrogel microwells. Individual wells are 200 μm in diameter. (A) Neutravidin-Alexa488 immobilized inside microwells modified with thiol-silane and MAL-PEG3-biotin linker. (B) Anti-CD4-PE adsorbed in microwells modified with thiol-silane and Mal-PEG-NHS linker.

3.3. Characterization of protein micropatterns with fluorescence

As next step in this study, we proceeded to demonstrate immobilization of both proteins and gels on thiol-functionalized surfaces. Hydrogel microwells were fabricated onto glass substrates modified with acryl- and thiol-silane layers. The PEG-DA microwells were observed to exhibit high pattern fidelity and anchor effectively to the substrate. These samples were then functionalized with biotin linker and subsequently incubated with neutravidin conjugated to Alexa Fluor 488. Bright fluorescence was observed in the thiol-silane anchored microwells while only minimal fluorescence was observed in the acryl-silane microwells (Fig. 5A).

The thiol-maleimide chemistry affords the use of a wide variety of hetero-bifunctional crosslinker molecules. Taking advantage of this, we have also demonstrated the utility of these surfaces for antibody capture. By replacing the biotin linker with NHS linker, we were able to capture fluorescently labeled antiCD4 antibodies in thiol-silane microwells. Results shown in Fig. 5B confirm the effective capture of these antibodies. Taken together with the neutravidin capture data above, these studies confirm that the presence of PEG hydrogel microstructures did not interfere with the ability of the underlying surface to immobilize proteins via commercially available linker molecules.

4. Conclusions

In this study, we have demonstrated a method to immobilize both PEG-DA hydrogel microstructures and proteins using a single surface functionality. To achieve this result, we modified glass substrates to contain thiol-terminating functional groups using a commercially available silane. These thiol groups served to anchor hydrogel microwells via thiol-ene reactions and were further useful in covalently linking to the attachment sites inside the microwells via bi-functional maleimide linkers. In addition, PEG attachment on pure thiol surface was stable up to 3 days. Ellipsometry measurements revealed that a considerably higher density of protein was immobilized on thiol modified surfaces and in thiol-silane microwells compared to acryl-functionalized surfaces. Importantly, we also demonstrated that model proteins, neutavidin and anti-CD4 Ab, could be immobilized inside the gel microwells constructed on thiol-silane-modified glass. The strategy of employing thiol-silane in conjunction with hydrogel micropatterning on glass has broad utility for covalent protein attachment and should have applications in cell/tissue engineering and biosensing.

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