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Rapid photochemical surface patterning of proteins in thiol–ene based microfluidic devices

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The suitable optical properties of thiol–ene polymers combined with the ease of modifying their surface for the attachment of recognition molecules make them ideal candidates in many biochip applications. This paper reports the rapid one-step photochemical surface patterning of biomolecules in microfluidic thiol–ene chips. This work focuses on thiol–ene substrates featuring an excess of thiol groups at their surface. The thiol–ene stoichiometric composition can be varied to precisely control the number of surface thiol groups available for surface modification up to an average surface density of 136 ± 17 SH nm^{-2}. Biotin alkyne was patterned directly inside thiol–ene microchannels prior to conjugation with fluorescently labelled streptavidin. The surface bound conjugates were detected by evanescent wave-induced fluorescence (EWIF), demonstrating the success of the grafting procedure and its potential for biochip applications.

Introduction

Thanks to their attractive physico-chemical properties, thiol–ene formulations have recently generated considerable interest in micro- and nanofabrication. Harrison *et al.* first suggested the use of commercially available thiol–ene based adhesives (NOA61, NOA68 and NOA81, Norland Optical Adhesives, Norland Products Inc., Cranbury, NJ) for the rapid prototyping of solvent-resistant structures.[1] Kim *et al.* used NOA63 as an UV-curable polymeric material for micro- and nanostructure replica moulding,[2,3] and for the fabrication of hydrophilic microfluidic chips.[4] NOA81 has also been used successfully to create microchannels and microfluidic stickers with adjustable wetting properties and high solvent resistance.[5,6] However, these commercial formulations lack flexibility as they do not offer the possibility of altering the resulting polymeric material's properties. In the thiol–ene polymerization reaction, a monomer containing multiple sulfhydryl functional groups ("thiol") adds to a second monomer featuring multiple alkene functional groups ("ene") to form a crosslinked polymer. Since the reaction proceeds through a step-growth polymerization mechanism, both monomers are consumed in equal amounts and alterations in the reactants' stoichiometry will result in an excess of functional groups at the polymer surface.[7] Good *et al.*[8] examined in detail the impact of monomer functionality and stoichiometry on the material properties of thiol–enes (Young's modulus, strain-at-break) for microfluidic applications. Rydholm *et al.*[9] and Khire *et al.*[10] incorporated a small excess of multifunctional thiol monomers to thiol-acrylate polymerizable formulations in order to control material properties as well as the number of reactive sites available for post-polymerization surface modifications. Khire *et al.*[7] have advantageously used this altered stoichiometry to create thiol–ene polymer substrates in which the excess thiols present on the polymer surface are used as covalent anchoring groups for the attachment of thin polymer films. Ashley *et al.*[11] and Carlborg *et al.*[12] both took advantage of the fact that the thiol–ene reaction is photoinitiated to create microfluidic features and surface patterns by exposing the reactants to collimated light through stencil masks, producing a pinched-flow thiol–ene microfluidic device with complex geometry and small (50 μm) channel dimensions, and patterned red-dye letters (400 μm smallest feature size) on a thiol–ene substrate featuring an excess of thiol groups, respectively. Extra surface thiols have also been used to covalently bond micromoulded thiol–ene polymers to functionalized silicon substrates.[13] The thiol–ene reaction has also recently been used for the functionalization of porous monoliths with dodecyl and zwitterionic functionalities.[14]

The ability to immobilize biomolecules at specific locations on the surface of solid supports is central to many biochip applications. Immobilization of biological recognition species on the surface of optical fibers and waveguides for biosensing applications can be achieved by covalent immobilization, physical adsorption, entrapment within polymer matrices or indirect attachment *via* intermediate biomolecular species.[15] The thiol–ene photoreaction is considered bioorthogonal,[16] *i.e.* it does not interfere with native biochemical processes due to its specificity for alkenes and its robustness in aqueous buffer. Therefore, it is an

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attractive reaction scheme for the attachment of biomolecules inside biochips. Although polymeric substrates (poly(dimethyl siloxane) (PDMS), poly(methyl methacrylate) (PMMA), polyethylene (PET), polycarbonate (PC) and cyclic olefin polymer (COP)) are increasingly used due to their low cost and attractive physico-chemical properties, glass is still one of the most commonly used substrates for the immobilization of protein probes.[17,18] Jonkheijm *et al.*[19] demonstrated the photo-immobilization of alkene-functionalized biotins onto thiol-modified silicon surfaces using the thiol–ene reaction. The thiol–ene reaction allows site-specific immobilization and by using a photomask, line patterns with features as small as 5 μm could be obtained. Further miniaturization of the line patterns down to a width of 650 nm was achieved by laser-writing the patterns on the substrates using a scanning laser source in conjunction with a confocal microscope. However, the immobilization procedure is labor intensive, requiring several steps to yield the desired thiol-terminated surfaces (plasma enhanced chemical vapor deposition and silanization to activate the silicon wafer substrate, attachment of polyamidoamine dendrimers to the silicon oxide surfaces followed by an aminocaproic acid spacer, coupling of the spacer to cystamine followed by reduction of the disulfide groups to yield the desired thiol functional groups). We demonstrate here that microfluidic chips made of thiol–ene can be rapidly and selectively functionalized with biomolecules through the thiol–ene reaction. This is the first example of functionalization of thiol–ene chips with biomolecules. Using photolithography, complex protein patterns can be obtained in a single step on thiol–ene chips.

The suitable optical properties of thiol–ene polymers combined with the ease of modifying their surface for the attachment of recognition molecules make them ideal candidates in many biochip applications. An example is evanescent wave induced fluorescence (EWIF) where detection occurs within a few 100 nanometers of the waveguiding surface.[15] Here, we demonstrate that surface bound biomolecules grafted *via* the photo-initiated thiol–ene reaction can be detected by evanescent wave induced fluorescence (EWIF) inside thiol–ene microchannels.

Experimental

Materials and reagents

Pentaerythritol tetrakis-(3-mercapto-propionate), 1,3,5-triallyl-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), chloroform, sodium dihydrogen phosphate monohydrate, sodium phosphate monobasic, ethylene glycol, Tween-20 and phosphate buffered saline (PBS) were purchased from Sigma Aldrich (Saint Louis, MO, USA). PDMS was prepared from a Sylgard 184 silicone elastomer kit (Dow Corning, USA). Biotin alkyne (PEG4 carboxamide-propargyl biotin) and streptavidin (Sav) Alexa Fluor® 488 and 532 conjugates were purchased from Invitrogen (Grand Island, NY, USA). Darocur 4265 was purchased from BASF Kaisten AG (Hardmatt, CH).

Thiol–ene chip fabrication

PDMS moulds for thiol–ene casting were prepared in a micro-milled PMMA master and cured at 80 °C for 2 hours. The PMMA master's surface had been smoothed by exposing to chloroform vapour (4 min at 25 °C) prior to use.

The bottom part of the chip was made by pouring the thiol–ene monomer mixture into the PDMS mould featuring the patterns for the microchannel and the guiding channel for the optical fibre. The mould was sealed with a glass microscope slide and exposed to UV light (Dymax 5000-EC Series UV curing flood lamp, Dymax Corp., Torrington, CT, 40 mW cm^{-2} at 365 nm) with the microscope slide facing the lamp for two minutes, then turned and exposed for two more minutes through the PDMS. After curing, the PDMS part was peeled gently and the cured thiol–ene remained on the microscope slide. The lids for the chip were made by pouring the thiol–ene in a mould entirely made of PDMS so that after curing (4 min), the thiol–ene lids could be peeled off from the moulds. To minimize auto-fluorescence, no photoinitiator was used. The thiol–ene parts were bonded immediately after production. The thiol–ene parts were warmed up for 10 minutes in an oven at 70 °C and placed in conformal contact. A slight pressure was applied on the chip with a rubber roller to ensure uniform bonding.

Surface characterization

The surface thiol density was quantitated using DTNB in a protocol adapted from Ellman's procedure for quantifying the free sulfhydryl group in solution.[20] Thiol–ene slabs (20 mm × 20 mm × 0.5 mm, 0–120% excess thiols) were immersed in DTNB (0.08 mg mL^{-1} in 0.1 M sodium phosphate buffer, pH 8.0). After 10 minutes, the thiol–ene slab was removed and the absorbance of the solution was measured at 412 nm (UV-1800 Shimadzu UV spectrophotometer, Shimadzu Corporation, Kyoto, Japan). The number of thiols on the surface of the thiol–ene slabs was evaluated from the molar extinction coefficient of TNB (14 150 M^{-1} cm^{-1}).[21]

Surface functionalization

Exposure time optimization. To verify the effect of irradiation time on the attachment of biotin on thiol–ene substrates, biotin alkyne (10 mM PEG4 carboxamide-propargyl biotin in ethylene glycol, no photoinitiator) was drop cast on the surface of the thiol–ene substrates (90% excess thiol) and covered with a microscope slide cover slip. After exposure to UV light (1–10 min, Dymax 5000-EC Series UV curing flood lamp), the substrate was thoroughly rinsed (PBS 0.01 M, pH 7.4, with 0.05% Tween-20). After removal of the unreacted biotin, the surface was incubated with fluorescently labeled Sav (Alexa Fluor 532, 10 μM in 0.01 M PBS, 10 minutes incubation time) and rinsed thoroughly (PBS, 0.01 M, pH 7.4, with 0.05% Tween-20) prior to visualization by confocal microscopy (Leica TCS SP5 confocal microscope, Leica Microsystems GmbH, Mannheim, Germany).

Photochemical surface patterning. The thiol–ene substrates (90% excess thiols) were coated with biotin alkyne (10 mM in ethylene glycol, 3% Darocur 4265) immediately covered with a chromium photomask and exposed to UV light (405 nm laser, 10 s at ~30 mW). The photoinitiator was added to the biotin alkyne solution to accelerate the photografting mechanism. After exposure, the substrates were thoroughly

washed with buffer (PBS 0.01 M, pH 7.4, with 0.05% Tween-20) before incubation with fluorescently labeled Sav (Alexa Fluor 488, 100 μM in 0.01 M PBS, 10 minutes incubation time) and rinsed thoroughly with buffer to remove unbound molecules before visualization on an inverted fluorescence microscope (IX71, Olympus Corporation, Tokyo, Japan).

SITE SPECIFIC IMMOBILIZATION OF BIOTIN INSIDE MICROCHANNELS. The microchannel (90% excess thiol chip, 500 μm wide × 500 μm deep microchannel) was filled with a biotin alkyne solution (10 mM in ethylene glycol, no photoinitiator) before exposing (405 nm blue laser, 60 s at ~30 mW) directly through the microchannel top wall (500 μm thick, 90% excess thiol thiol–ene substrate). After exposure, the microchannel was thoroughly rinsed with PBS before being filled with fluorescently labeled Sav (Alexa Fluor 488, 10–100 μM in 0.01 M PBS, 10 minute incubation time) and rinsed again thoroughly with buffer to remove any unbound Sav. The channels were filled with buffer during EWIF measurements.

Fluorescence and EWIF measurements

Light from a tunable argon ion laser (488 nm, Melles Griot Series 543, Carlsbad, CA) was guided through an optical fiber (FVP400440480, Polymicro Technologies, AZ, USA) placed to form an incident angle of 75° to the normal of the microchannel wall, thereby ensuring total internal reflection of the light in the detection area (Fig. 1). At the channel wall interface the critical angle necessary to obtain total internal reflection is approximately 58° (assuming $n_{solution} = 1.33$ and $n_{thiol-ene} = 1.57$). The use of an incident angle of 75° provided a sufficient margin to neglect any issues stemming from the fact that light diverges as it exits from the optical fiber. Fluorescence and EWIF measurements were recorded on an inverted microscope (IX71, Olympus Corporation, Tokyo, Japan) equipped with a Canon 550D (Tokyo, Japan) digital camera. The microscope magnification was set at 10× (numerical aperture N.A. = 0.3). Images were acquired with an integration time of 2.5 to 5 seconds in 14 bit RAW format. Image analysis was performed with Matlab (MathWorks, Natick, MA, USA).

Results and discussion

Surface characterization

Ellman's reagent (DTNB) was used to evaluate the thiol group density at the polymer surfaces as a function of monomer mixture composition. As schematically illustrated in Fig. 2(a), DTNB produces the yellow colored 2-nitro-5-thiobenzoate (TNB) upon reaction with free sulfhydryls. The reaction can therefore be used to determine quantitatively the number of thiols present at the surface of the thiol–ene slabs. Fig. 2(b) shows that the thiol surface coverage ranges between 0 and 280 SH nm^{-2} for mixture compositions ranging from 0–120% excess thiol functional groups. However, 90% excess thiols was found to be the highest possible ratio practically usable for the fabrication of microfluidic chips and was used in further studies. At higher ratios, the amount of thiol monomers was too high and the material could not cure fully, resulting in the very high number of free thiols shown in Fig. 2(b) for the 120% excess thiol substrates. Fig. 2(b) also shows that after 5 days of storage, the number of thiol groups present on substrates made with 90% excess thiols had reduced from 136 ± 17 to 104 ± 5 SH nm^{-2}. In the presence of oxygen, thiol groups easily undergo oxidation, reducing the number of free thiols available for surface modification. Therefore, thiol–ene chips were always used within 24 hours of fabrication to ensure a maximum of surface reactive groups was present. Fig. 2(b) also shows that the number of surface thiols is affected by exposure to an oxygen plasma reducing the surface thiol density to 60 ± 8 SH nm^{-2} for the 90% excess thiol substrates.

Surface functionalization with biotin

To demonstrate the vast potential of thiol–ene as a substrate material in the fabrication of biochips, the excess thiol groups present at the surface of the thiol–ene chips were used for rapid and selective surface patterning of biotin/Sav conjugates. The thiol–yne reaction (Fig. 3(a)) was used to immobilize an olefin functionalized biotin (biotin alkyne) directly onto the thiol–ene chip surface, without any prior surface modification or the use of a photoinitiator. After removal of the unreacted biotin alkyne molecules, the substrates were incubated with labeled Sav for visualization (Fig. 3(a)). As shown in Fig. 3(b), biotin alkyne

Fig. 1 Thiol–ene chip layout for EWIF experiments.

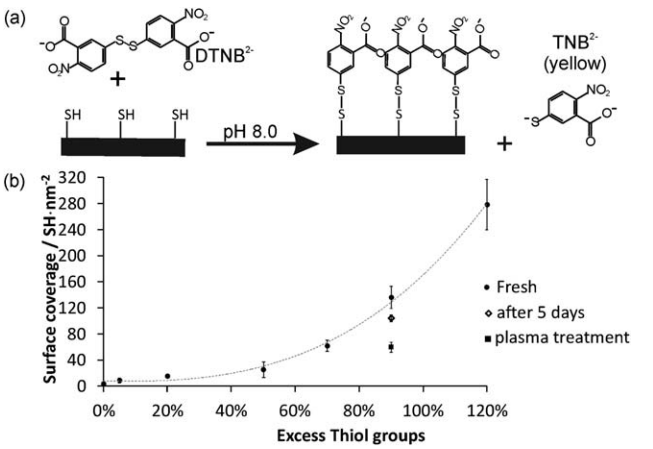

Fig. 2 (a) Determination of sulfhydryl group density with Ellman's reagent. (b) Thiol surface coverage as a function of monomeric composition (●). The substrates containing 90% excess thiol groups were also tested after 5 days of storage (◇) and after plasma treatment (■).

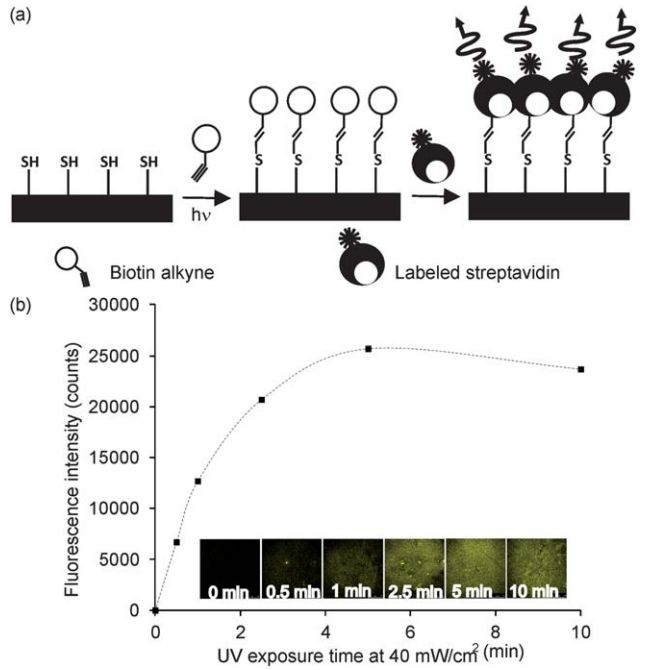

Fig. 3 (a) Photo-immobilization of biotin alkyne at the surface of thiol–ene substrates by the thiol–yne reaction and conjugation with labeled Sav. (b) Immobilization of biotin on 90% excess thiol thiol–ene as a function of UV irradiation with a UV curing flood lamp.

could be immobilized on the thiol–ene substrates without the need for a photoinitiator. The amount of material photochemically immobilized can be varied by modifying the UV exposure time, with maximal coverage achieved in 5 minutes using a UV flood lamp for irradiation.

Photochemical surface patterning was achieved by irradiating the thiol–ene substrate coated with biotin alkyne through a photomask. The micrographs shown in Fig. 4(a) show that a honeycomb pattern (20 μm wide honeycomb cells with 8 μm thick walls) could be replicated with high fidelity with just 10 s irradiation time (blue laser excitation, 3% added photoinitiator). These features are comparable in size to the photomasked biotin/Sav conjugate patterns obtained by Jonkheijm *et al.*[19] on thiol-modified silicon oxide substrates (5–100 μm) and smaller than the red-dye patterns obtained by Carlborg *et al.*[12] on thiol–ene substrates (400 μm smallest feature size). These results demonstrate that microfluidic chips made of thiol–ene can be rapidly and selectively functionalized with biomolecules with results comparable to what is achievable on thiol-modified silicon surfaces without the labor-intensive steps required to yield the desired thiol functional groups on silicon substrates. To avoid surface contaminations and damage to the coated surfaces during the bonding process, it is often preferable to coat the microfluidic chips after bonding. Fig. 4(b) shows that immobilization of biotin in already closed thiol–ene microchannels was also possible. With the photoinitiator being added (3% Darocur 4265), the photografting procedure was successful with 10 s irradiation time (405 nm laser excitation). Fig. 4(c) shows that surface patterning of biotin in an already closed thiol–ene microchannel was also possible. The patterned lines are 30 μm thick and separated by a 10 μm wide gap. In the absence of photoinitiator, the site-specific photografting procedure was successful with 60 s irradiation time using the 405 nm laser excitation. The irradiation time is dependent on the light source used.

Evanescent wave-induced fluorescence (EWIF)

To demonstrate the potential of thiol–ene as a substrate for biochip applications, the patterned biotin/Sav conjugates were quantified by EWIF. As shown in Fig. 5, the EWIF intensity increased proportionally with increasing Sav concentration in the range of 0–60 μM, after which the limit of linearity for the method is reached. The large error bars on some concentrations are attributable to the fact that photografting was achieved by exposing the biotin alkyne through a mask using a laser forming an ~5 mm spot size at the surface of the mask; therefore,

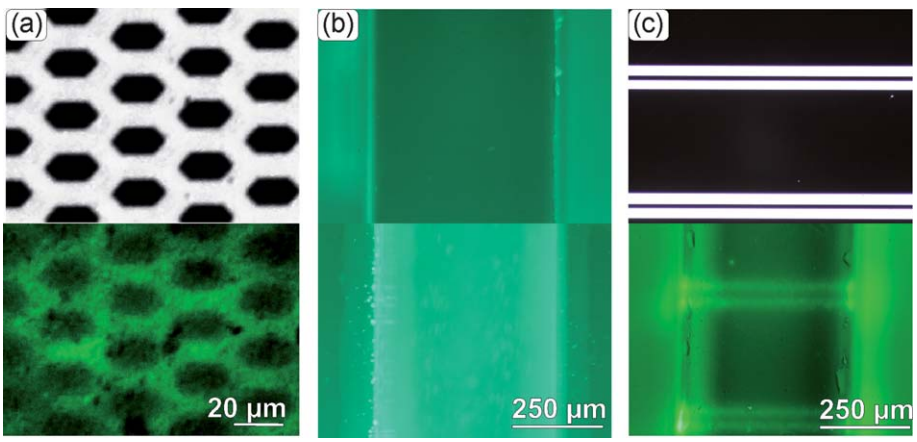

Fig. 4 Site specific immobilization of biotin/Sav conjugates on thiol–ene substrates. (a) Chromium mask (top) and resulting biotin/Sav pattern (bottom). (b) Biotin functionalization inside closed microchannels: thiol–ene microchannel before (top) and after (bottom) the photolithographic grafting of biotin/Sav. (c) Chromium mask (top) and resulting biotin/Sav line patterns formed inside the closed microchannel.

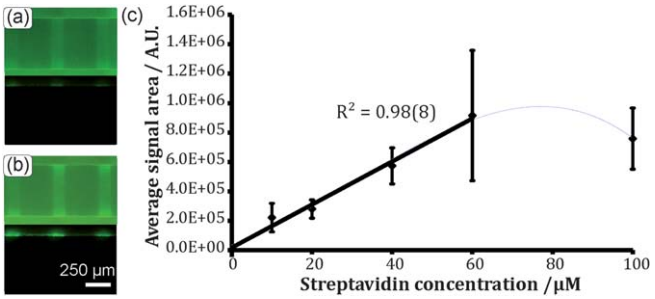

Fig. 5 Detection of labeled Sav conjugated to photochemically patterned biotin alkyne in closed microchannels. Micrographs: biotin patterns incubated with 10 μM (a) and 60 μM (b) labeled Sav detected by fluorescence (top) and EWIF (bottom). (c) Calibration curve for the detection of patterned biotin/Sav by EWIF inside microchannels ($N = 6–12$, error bars shown for 95% confidence interval).

exposure of some bands located towards the edges of the laser spot was uneven. Reproducibility could be improved by the use of a commercial mask aligner. Nevertheless, these results demonstrate how simple (1-step photografting) and rapid (10–60 seconds exposure) the procedure is.

Conclusions

Thiol–ene polymeric substrates offer several advantages that make them an attractive alternative to the more commonly used glass substrates in the fabrication of biochips. In this study, we demonstrated that high thiol surface densities and a good control over the number of surface functional groups available can be achieved by simply varying the thiol–ene monomer mixture ratios. The thiol–yne reaction was further used for the rapid photochemical surface patterning of biotin molecules inside enclosed thiol–ene microchannels with results comparable to what can be achieved on thiol-modified silicon surfaces without the need for any prior surface treatment or modifications. These results demonstrate the vast potential of thiol–ene as a substrate material in the fabrication of biochips where rapid and selective surface patterning of biomolecules is required, such as EWIF.

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