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Functionalization of SU-8 photoresist surfaces with IgG proteins

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ABSTRACT

The negative epoxy-based photoresist SU-8 has a variety of applications within microelectromechanical systems (MEMS) and lab-on-a-chip systems. Here, several methods to functionalize SU-8 surfaces with IgG proteins were investigated. Fluorescent labeled proteins and fluorescent sandwich immunoassays were employed to characterize the binding efficiency of model proteins to bare SU-8 surface, SU-8 treated with cerium ammonium nitrate (CAN) etchant and CAN treated surfaces modified by aminosilanization. The highest binding capacity of antibodies was observed on bare SU-8. This explains why bare SU-8 in a functional fluorescent sandwich immunoassay detecting C-reactive protein (CRP) gave twice as high signal as compared with the other two surfaces. Immunoassays performed on bare SU-8 and CAN treated SU-8 resulted in detection limits of CRP of 30 and 80 ng/ml respectively which is sufficient for detecting CRP in clinical samples, where concentrations of 3–10 μg/ml are normal for healthy individuals. In conclusion, bare SU-8 and etched SU-8 can be modified with antibodies by a simple adsorption procedure which simplifies building lab-on-a-chip systems in SU-8.

Additionally, we report the fabrication process and use of microwells created in a SU-8 layer with the same dimensions as a standard microscope glass slide that could fit into fluorescent scanners. The SU-8 microwells minimize the reagent consumption and are straightforward to handle compared to SU-8 coated microscope slides.

1. Introduction

SU-8 is an epoxy-based polymer that is suitable for the production of high-aspect-ratio structures. SU-8 photoresist has excellent mechanical properties, thermal stability, etching resistance and it is chemically stable against several acids and bases [1,2]. Also, SU-8 is highly transparent under near UV and visible light and it is used for optical waveguides [3]. However, SU-8 is auto-fluorescent at several wavelengths such as FITC and Cy3 emission wavelengths [4]. SU-8 polymer has been used not only as a photoresist for microelectronic and micromachining applications but also as a substrate for nanotube vesicle networks, novel colloidosome microcapsules and carbon nanotubes functionalization [1–3,5–7]. SU-8 has a great potential for the fabrication of microelectromechanical systems (MEMS) based sensors and microfluidic devices due to its unique high-aspect-ratio and near vertical sidewalls [8,9]. Therefore, it is of great interest to find new methods for SU-8 surface functionalization especially for bioanalytical applications.

Single stranded DNA can passively be absorbed onto a cured SU-8 surface [4]. Probe densities of about 10 fmol/mm^2 can be obtained using this simple immobilization method. Furthermore, the probes appear to be covalently attached to SU-8 because the functionality of the probes is not reduced by incubation for 10 min in water at 98 °C. Even if it is unclear which type of chemistry provides the link between the DNA and the SU-8 surface, it was hypothesized that exposed epoxy groups could be involved creating the link to DNA.

Recently, Wang Y. and coauthors proved the possibility of UV-mediated grafting of polymers onto the surface of the SU-8 [10]. The SU-8 surface was modified with poly (acrylic acid) and other water-soluble monomers. This method gave an *X–Y* spatial resolution of polymer micropatterns of 2 μm. Controlled protein attachment and cell growth was possible on these patterned SU-8 surfaces. However, to immobilize polymers to SU-8, this method relies on the residual photoacid generator of the SU-8 polymer, limiting the possibility of using it for already fully processed SU-8. Furthermore, an active layer of several microns obtained by this method is too thick for interactions that need to take place as close to the sensor surface as possible. Examples of such sensors are micromachined cantilevers [11,12]. Therefore, the development of a larger number of different functionalization methods for SU-8 surfaces is still necessary.

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Silanization of SU-8 surfaces that were hydrolyzed in the presence of sulfochromic solution and treated with an aminosilane derivative was reported by Manoj Joshi et al. [13]. The surface was subsequently used to immobilize antibodies but no immunoassay was reported.

In this paper we investigate several methods to attach proteins on SU-8 surfaces that resemble surfaces obtained as a part of standard cleanroom processing, such as chrome etchant treated surfaces. Direct adsorption and self-assembled monolayers (SAM) of aminosilanes were used to functionalize the SU-8 surface with IgG proteins. Furthermore, the SU-8 surfaces were characterized by FT-IR spectroscopy, fluorescence techniques and sandwich immunoassays in order to assess protein binding and function.

2. Material and methods

2.1. Materials

Capture antibody (anti-CRP-cAb, monoclonal mouse antibody, clone IgG1) and detector antibody (anti-CRP-dAb, mouse antibody, clone IgG2a) were obtained from Fitzgerald Industries International, MA, USA. C-reactive protein (CRP) was purchased from Scripps Labs, San Diego, CA, USA. Bovine serum albumin (BSA), aminopropyltriethoxysilane (APTES), goat anti-mouse antibody-whole molecule (IgG), glutaraldehyde, isopropanol, toluene, $CaCl_2$, TRIS buffer, physiologically buffered saline (PBS) buffer 10 mM, pH 7.4, mercaptoethanol (ME), ethanolamine (EA) and Tween-20 were obtained from Sigma, Germany. Cy5-amino reactive dye was purchased from GE Healthcare, UK. Skim powder milk was from Arla Foods (Viby J, Denmark). Calf, rabbit and fetal serum were from The State Serum Institute, Denmark. High purity MilliQ water was used to prepare all aqueous solutions. SU-8 and its developer (PGMEA) were bought from Microresist Technology, Berlin, Germany. The SU-8 monomer without photo-initiator was a kind donation from Microresist Technology. Chrome etchant CE 8002-A (CAN), an aqueous solution of cerium ammonium nitrate (13%) and acetic acid (4%) was purchased from Etchant Transene, Inc, USA. All reagents were of analytical grade and utilized without further purification. Standard frosted microscope glass slides (25 mm× 75 mm, 1 mm thick) were purchased from VWR International. Aldehyde coated glass slides were purchased from CEL Associates Inc., Pearland, TX, USA.

2.2. SU-8 films on microscope glass slides

Microscope glass slides were immersed for 3 min in piranha solution (3:1 concentrated H_2SO_4: 30% H_2O_2 by volume) and rinsed extensively with MilliQ water and dried with air stream. Caution: Piranha reacts violently with organic compounds. It should be handled with extreme care. An SU-8 layer with a thickness of 500 nm was obtained by spin-coating the resist at 5000 rpm for 60 s on glass slides. The excess solvent was evaporated by a soft bake for 3 min at 60 °C and 2 min at 90 °C. The SU-8 was exposed to UV light with a dose of 450 mJ/cm^2 and post-baked for 3 min at 60 °C and 2 min at 90 °C. The glass slides were developed 2 min in PGMEA and then rinsed with isopropanol. SU-8 coated glass slides were used without washing when taken out from the cleanroom. The 500 nm film resulted in a less than 20% increase in the background fluorescence signal compared to the glass slides.

For the FT-IR spectra, SU-8 samples were prepared by spin-coating of SU-8 on Si wafers and following all the steps described above.

2.3. Fabrication of SU-8 microwells

A fluorocarbon (FC) coating was deposited by an advanced silicon etch device (ASE, Surface Technology Systems, Newport, UK) on a clean Si wafer following a method published elsewhere [14]. A 4.5 μm thick layer of SU-8 2005 was spin-coated onto the FC-layer and soft-baked for 10 min at 60 °C and 10 min at 90 °C on a hotplate. Standard UV-exposure with a dose of 120 mJ/cm^2 at a wavelength of 365 nm was used to define the area of the well-plates. The exposure was followed by a post-exposure-bake for 10 min at 60 °C and 15 min at 90 °C to cross-link the exposed photoresist. The non-cross-linked areas were developed with PGMEA for 4 min. A second step of UV-lithography was performed to structure the walls of the microwells. Then a layer of SU-8 2075 with a thickness of 160 μm was processed in a similar manner as described above. The exposure dose for the thick layer was 1080 mJ/cm^2. The two bakes were done for 15 min at 60 °C and 30 min at 90 °C. The development time was kept at 25 min. Finally, the fabricated SU-8 microwells could simply be released from the FC-coated Si wafer using a razor blade. Each SU-8 well-plate consisted of an array of 4 × 10 microwells with dimensions of 2 mm× 2 mm that can hold 0.5–1.0 μl of protein solution.

2.4. Labeling of IgG proteins with Cy5-fluorescent dyes

The detector monoclonal anti-CRP-antibody (anti-CRP-dAb) and the polyclonal goat anti-mouse antibody (Ab) were labeled with Cy5 mono-reactive NHS esters. The label antibodies are referred to as anti-CRP Cy5-dAb and Cy5-Ab respectively. The Cy5-labeling procedures were done according to the manufacturer's instructions at a molar dye/protein ratio of 20:1 in PBS buffer, for 45 min at room temperature, in darkness. Micro Bio-Spin P30 TRIS size exclusion columns (BioRad, Hercules, CA, USA) were used to remove unreacted Cy5 fluorophores. The anti-CRP Cy5-dAb and the Cy5-Ab had 2.5 and 2.4 dye molecules per protein respectively as determined by spectroscopy. The Cy5 labeled anti-CRP-dAb and goat Ab were stored in PBS with 0.1% azide at 4 °C.

2.5. CAN treatment of SU-8 surface

The SU-8 coated microscope slides were incubated for 30 min with the CAN solution and subsequently rinsed with MilliQ water and dried. The slides were used immediately after the treatment since a hydrophobic recovery of the surface was noticed. The contact angle (θ) of the CAN treated surfaces changed from $\theta = 25^\circ$ to 41° in two days. We refer to these surfaces as SU-8 (CAN). The contact angle of the untreated surface was $\theta = 78^\circ$.

2.6. Silanization of SU-8 surface

SU-8 surfaces pretreated with CAN were incubated with 10% APTES in toluene for 1 h, then dried. An aldehyde layer was subsequently been obtained by incubating the surfaces with 10% glutaraldehyde for 30 min. After washing with MilliQ water and drying these slides could be used to link proteins via Schiff base reaction.

2.7. Sandwich immunoassays

Anti-CRP-cAb antibodies (100 μg/mL) in PBS were deposited in 0.5 μl droplets on slides with flat SU-8 surfaces or the microwells and incubated for 2 h. Then the SU-8 coated slides or microwells were rinsed with washing buffer (PBS and 0.05% Tween 20 (v/v)). In the next immunoassay steps, the slides were blocked with 1% BSA (w/v), 1% milk powder in PBS for 1 h if not mentioned otherwise.

CRP and Cy5-anti-CRP-dAb diluted in PBS respectively were incubated each for 1 h with the substrate and subsequently washed with washing buffer. All the immunoassay incubation steps were done at room temperature in a humid chamber.

2.8. Contact angle measurements

A DSA-10 contact angle goniometer (Krüss GmbH, Hamburg, Germany) using de-ionized water under ambient conditions (21 °C, relative humidity: 48–52%) was used for contact angle measurements.

2.9. Fluorescence detection

The samples were scanned using a CCD scanner (Array-WorX, Applied Precision, Issaquah, WA, USA). The exposure time was 0.5 s using the Cy5 channel. Mean values of the fluorescence density were obtained using the CCD scanner analysis software.

2.10. FT-IR measurements

Nicolet 850 FT-IR spectrometer connected with a Nicolet Continuum FT-IR Microscope by Spectra Tech equipped with a MCT/A detector was used to acquire SU-8 surface spectra. The magnification of both condenser and objective was 15×. Each measurement was the accumulation of 5120 scans at 2 cm^{-1} spectral resolution. The spectra were collected in reflection mode. Using the reflection–absorption technique the depth of penetration was 100 nm.

3. Results and discussion

3.1. FT-IR characterization of SU-8 surfaces

The surface of the SU-8 polymer was investigated by FT-IR spectroscopy to understand the strong adsorption of biomolecules and ethanolamine [4,15] to SU-8 and to characterize the influence of the SU-8 process parameters. This information is necessary for making rational surface functionalization. The SU-8 monomer IR spectrum was used to identify the epoxy group vibrations and the occurrence of the hydroxyl group after cross-linking reaction.

In Fig. 1 the peak at 1270 cm^{-1} can be attributed to the epoxy ring breathing vibration (symmetrical C–O–C stretching) from the surface of the SU-8 monomer and the SU-8 polymer [16,17]. This peak is missing in the FT-IR surface spectrum of SU-8 (CAN), and is diminished in the spectrum of bare SU-8. In addition, the SU-8 monomer has two peaks at 861 and 910 cm^{-1}, which can be assigned ring vibrations of monosubstituted, disubstituted epoxydes (at the same C atom or at a different C) or trans epoxydes. These peaks are significantly reduced in the SU-8 spectrum but they were detected when a transmission FT-IR bulk measurement was done (results not shown). This behavior could be explained by the inhomogeneous composition of the SU-8 monomer that contains molecules with various numbers of epoxy functionalities and their different reactivities in the polymerization reaction. Moreover, the cationic polymerization of the epoxy groups can take place by the activated chain end mechanism that creates disubstituted epoxy end chains diminishing the monosubtituted and trans epoxy vibrational peaks in the SU-8 polymer [18]. Therefore, the epoxy groups are still present on the surface of the bare SU-8. The strong passive adsorption of biomolecules on the SU-8 surface, previously reported by our group [4], can be explained by the possible reaction between the epoxy groups on the SU-8 surface and the amino moieties of these molecules.

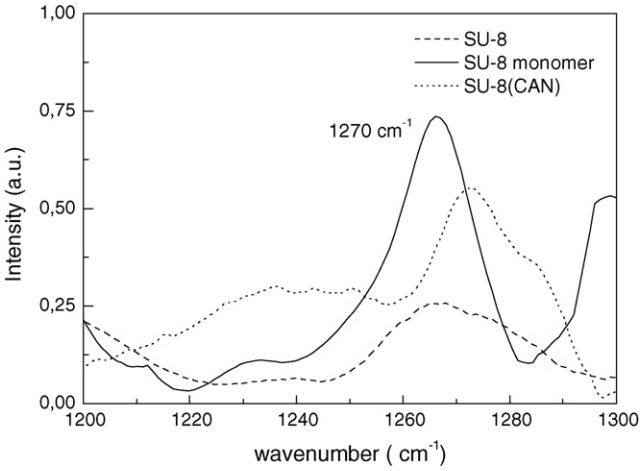

Fig. 1. FT-IR spectra of the surface of monomer SU-8 (solid line), bare SU-8 (dash line) and CAN exposed SU-8 (dot line).

The CAN exposed SU-8 surface is more complex as can be seen in the FT-IR spectrum (Fig. 1). The epoxy groups of the SU-8 surface are transformed into hydroxyl groups but they were also further oxidized by the CAN mixture. It is likely that CAN treated SU-8 surfaces contain a mixture of OH, epoxy and COOH groups [19]. Presence of carboxyl groups on CAN treated SU-8 surfaces was measured using XPS spectroscopy that showed multiple higher oxidation states of C atoms (results not shown). Moreover, CAN treated SU-8 surfaces must be treated as a complex and undefined surface, as SU-8 can undergo hydrophobic recovery relatively fast after CAN treatment as determined here and by others [20].

3.2. Immobilization of polyclonal antibody

A polyclonal Cy5 labeled goat immunoglobulin (Cy5-Ab) was used to compare the degree of immobilization of antibodies on various SU-8 surfaces (Fig. 2). Bare SU-8 could immobilize about 50% more antibodies compared to CAN treated SU-8 (Fig. 3). CAN is known to catalyze the opening of epoxy rings on polymer surfaces [19] and according to the results above some but not all epoxy groups are converted by CAN treatment of SU-8 (Fig. 1). The reduction of epoxy groups on the surface following CAN treatment may explain the lower binding capacity of CAN treated SU-8 compared with bare SU-8. It is likely that any COOH groups present SU-8 (CAN) are unreactive under the assay conditions described here as compared to epoxy groups.

CAN treated SU-8 surface was further treated with aminosilane in order to improve the antibody binding capacity of the CAN treated SU-8 surface. However, aminosilanized CAN treated SU-8 demonstrated the lowest antibody binding capacity (Fig. 3). A likely explanation is that the amine moiety of APTES simply reacts with the remaining epoxy groups on SU-8 (CAN) resulting in an essentially unreactive surface with little Ab binding capacity.

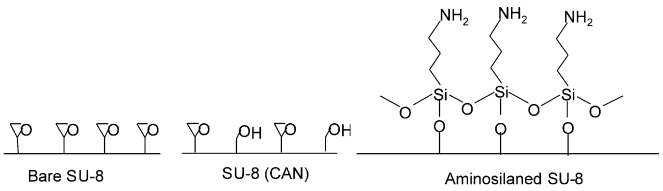

Fig. 2. SU-8 surfaces used in this work. Bare SU-8 surface consisted mostly of epoxy groups on the surface while CAN treated SU-8 displayed a complex set of functional groups on the surface. Aminosilane was used to modify CAN treated SU-8 in order to enhance the IgG protein binding.

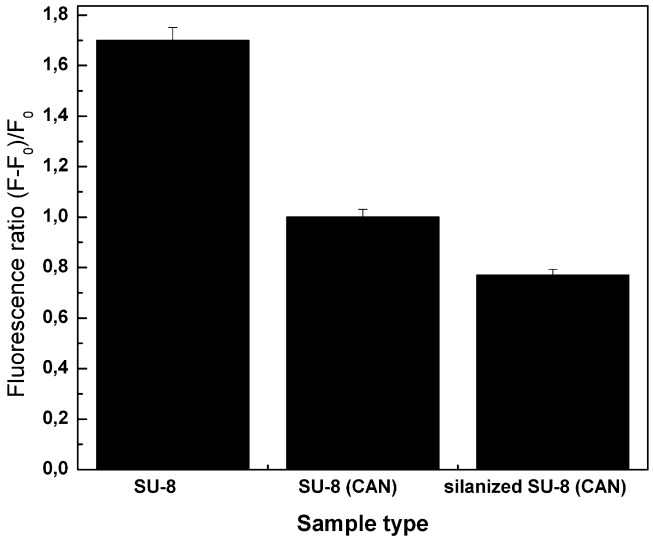

Fig. 3. Fluorescence intensities of Cy5-Ab adsorbed or chemically bound on aminosilanized SU-8. 100 μg/ml polyclonal antibody was incubated for 2 h on SU-8. The fluorescence intensity was normalized to sample background.

3.3. Macro-arrays on SU-8 microwells

Microwells in SU-8 layer with the same dimensions as a standard microscope slide were fabricated in order to simplify optimization of the conditions for the CRP immunoassay. The device was used to minimize reagent consumption during the spotting, incubation, and washing steps and to avoid spotting errors. A schematic diagram of the SU-8 immunoassay-well fabrication process is presented in Fig. 4a–e. The autofluorescence in the Cy5 channel from the bottom of the SU-8 structure is 20-fold lower than that from the walls (Fig. 4f). The edges of the microwells are perfectly sharp and the bottom layers are completely intact after release as can be seen from the SEM image (Fig. 4g). The fabricated SU-8 microwells can hold 0.5–1 μl droplets and the droplets can be deposited with a normal pipette by hand. Fluorescent spots from CRP immunoassays and the microwells are shown in Fig. 4h. The CRP immunoassay results were the same when macro-arrays on microwells or micro-arrays on microscope slides were used. The IgG proteins preserved their function and activity even in the case of micro-arrays when spotted antibody drops dried in between assay steps; conditions that are in contrast with macro-array immunoassays that were performed in humid conditions.

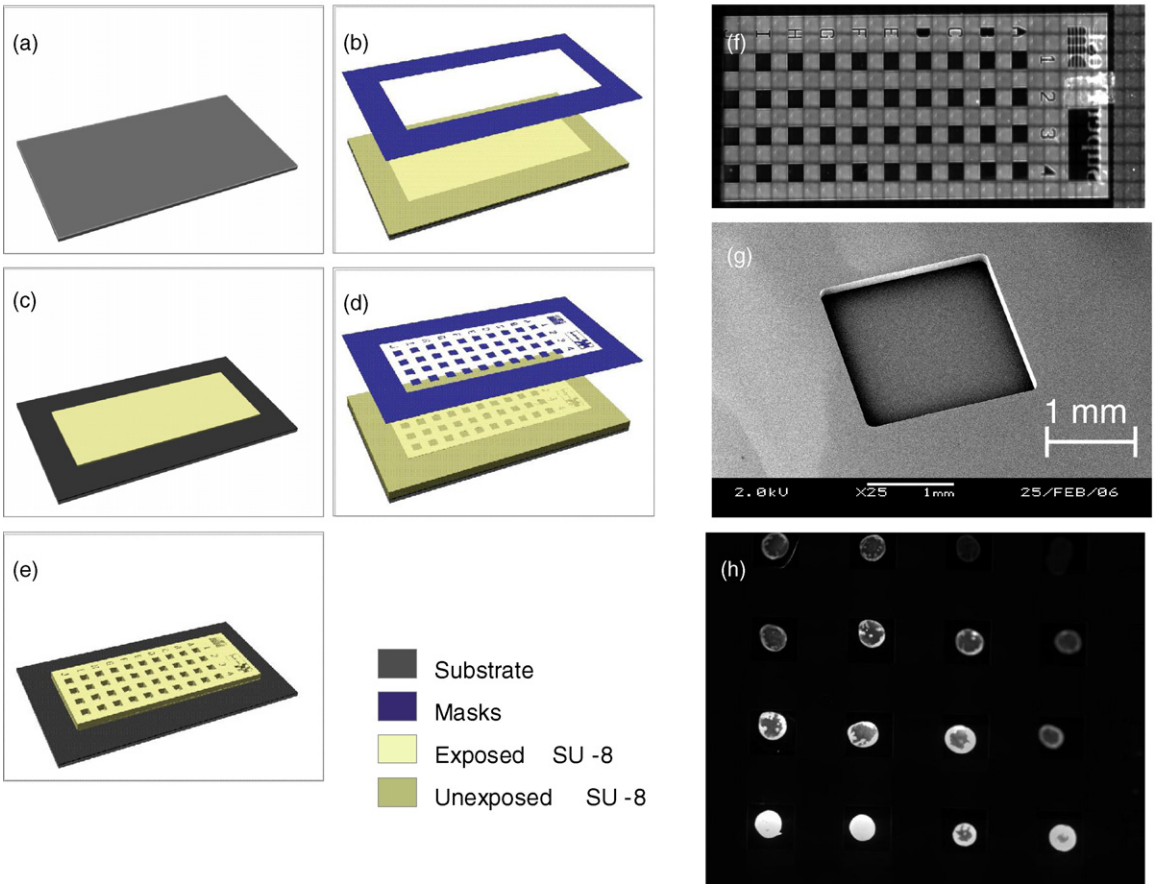

Fig. 4. Fabrication of SU-8 microwells: (a) silicon substrate with fluorocarbon coating, (b) spin-coating of SU-8 and first UV-exposure, (c) development, (d) spin-coating of SU-8 and second UV-exposure, development, (e) micro-well-plate that can be mechanically released, (f) fluorescence image of SU-8 micro-well-plate, (g) SEM-image of one microwell, and (h) CRP-immunoassay on a part of the chip.

3.4. Sandwich immunoassays on SU-8 surfaces

First, the capture antibody (anti-CRP-cAb) concentration was optimized using a constant concentration of CRP (10 μg/ml) and detector antibody (50 μg/ml anti-CRP Cy5-dAb). The fluorescence signals for the immunoassays on a bare SU-8 microwell surface are shown in the Fig. 5a. The maximum signal was obtained when SU-8 was exposed to the capture antibody with concentrations of more than 100 μg/ml. Comparable results were obtained when measured on commercial aldehyde coated glass slides that were micro-arrayed with the same capture antibody (results not shown). Therefore, in all subsequent steps, 100 μg/ml capture antibody was spotted on all types of SU-8 surfaces tested.

Second, the concentration of the detector antibody was optimized using a dilution series from 0 to 100 μg/ml anti-CRP Cy5-dAb. The CRP and anti-CRP-cAb concentrations were kept constant to 10 and 100 μg/ml respectively. 50 μg/ml anti-CRP Cy5-dAb was optimal under the given assay conditions (Fig. 5b). All subsequent immunoassays utilized a detector antibody concentration of 50 μg/ml detection antibody.

Third, the unspecific binding of antibodies to SU-8 was optimized by testing different blocking buffers. PBS buffer supplemented with 1% BSA blocked bare SU-8 surfaces. However, for all CAN treated surfaces this blocking treatment gave high background. Fig. 5c shows the background fluorescence signal of CAN treated surfaces obtained when different blocking buffers were used for the CRP immunoassays using 100 μg/ml anti-CRP-cAb, 10 μg/ml CRP and 50 μg/ml anti-CRP Cy5-dAb. Highly complex protein solution such as fetal, bovine or rabbit serum used as blocking buffers resulted in high background (Fig. 5c). Only a combination of 1% BSA and 1% skimmed milk reduced the background to acceptable levels. All surfaces tested were subsequently blocked with 1% BSA and 1% skimmed milk in PBS buffer.

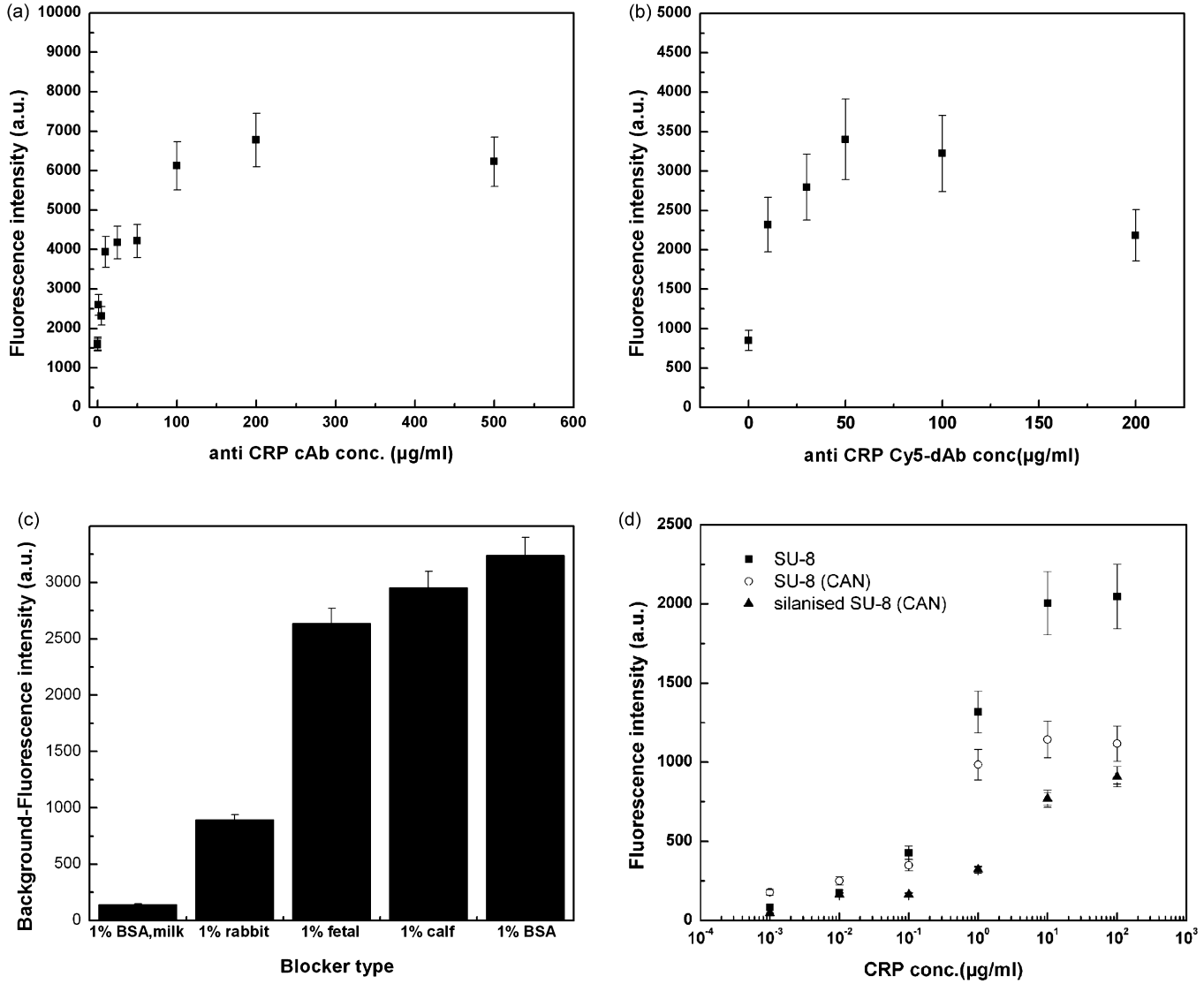

Fig. 5. Sandwich immunoassays on various SU-8 surfaces. (a) Optimization of capture antibody. A dilution series of anti-CRP-cAb was incubated for 2 h on bare SU-8. CRP at a concentration of 10 μg/ml was detected by 50 μg/ml anti-CRP Cy5-dAb. (b) Optimization of detector antibody. Anti-CRP-cAb at a concentration of 100 μg/ml was deposited onto bare SU-8 to capture 10 μg/ml CRP. The concentration of anti-CRP Cy5-dAb was varied. (c) Blocking capacity of several buffers. Immunoassay conditions: 100 μg/ml anti-CRP cAb was spotted onto SU-8 (CAN) surfaces, blocked, incubated with 10 μg/ml CRP and 50 μg/ml anti-CRP Cy5-dAb. (d) Detection limit of sandwich immunoassay performed on different SU-8 surfaces. CRP binding curves on differently treated surface: SU-8 (square points), SU-8 CAN (circles) and aminosilanized SU-8 (triangles). Immunoassay conditions: 100 μg/ml anti-CRP-cAb, 1% BSA, 1% skimmed milk PBS blocking buffer, 50 μg/ml Cy5-anti-CRP-dAb. For all experiments the error bars represent the standard deviation of three experiments. Each dilution was spotted in 4 replicates and the error bars are calculated from 3 consecutive experiments.

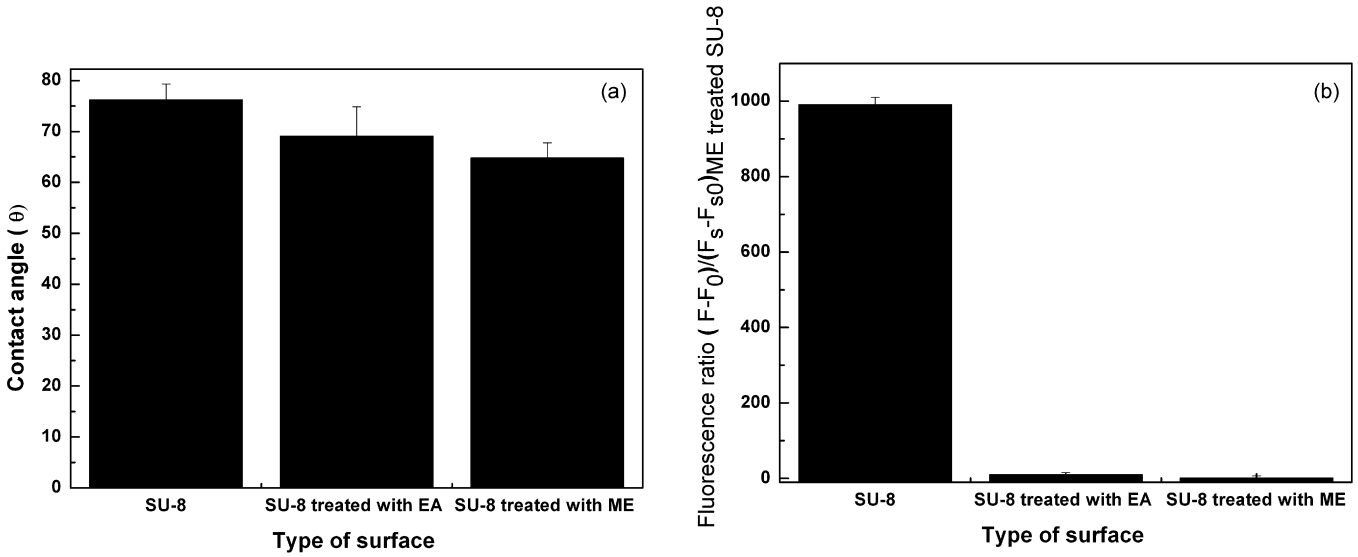

Fig. 6. (a) Water contact angle of SU-8, SU-8 surface reacted with 1 M ME and 1 M EA respectively for 24 h (at pH 7.4). (b) Fluorescence signals when SU-8, ME treated SU-8 and respectively EA treated SU-8 surfaces were spotted with 100 μg/ml anti-CRP-cAb and incubated with 50 μg/ml anti Cy5-dAb.

Using the optimized condition for immunoassays on SU-8, the ability to perform sandwich immunoassays was tested on different SU-8 surfaces. Fig. 5d shows the fluorescent signal obtained on different surfaces after CRP immunoassay. The limit of detection (LOD) for the immunoassays on a bare SU-8 surface was 30 ng/ml CRP similar to sensitive ELISA assays [21]. Incubation of SU-8 with CAN prior to the immobilization of the capture antibody decreased the fluorescence immunoassay signals more than 2-fold and a LOD of approximately 80 ng/ml CRP was achieved. Functionalization of CAN treated SU-8 surface with aminosilane did not improve the immunoassay results (LOD 300 ng/ml CRP). The difference in maximum fluorescence signal between the different surfaces obtained after sandwich immunoassay correlates the capacity of the different SU-8 surfaces to immobilize antibodies (see above). This indicates that maximum signal is limited by the number of immobilized capture antibodies in the immunoassay, being maximal when the polymer surface has epoxy groups.

In order to determine the nature of the chemical bonds between the SU-8 surfaces and IgG proteins used in this study, SU-8 surface was blocked with 1 M ME and EA. After 24 h reaction the contact angle of the SU-8 (76°) decreased to 65° and 69° when incubated with ME and EA respectively (Fig. 6a). The decrease in the contact angle of water droplets on the ME and EA treated SU-8 surfaces proves that the residual epoxy groups on the SU-8 surface can react with both amino and cysteine groups of the IgG protein. The modified ME and EA substrates were followed by contact angle for two weeks, the modification was stable (results not shown).

Bare SU-8 and bare SU-8 blocked with ME and EA were spotted with 100 μg/ml anti-CRP-cAb and incubated with 50 μg/ml Cy5-Ab (see the Materials and Methods section).

The fluorescence signal obtained from the unblocked SU-8 surface was 1000 fold bigger compared with that from the substrate blocked with ME and 100 fold bigger than that from the substrate blocked with EA (Fig. 6b). The ten-fold difference in the fluorescence signal could be explained either by residual epoxy groups that did not react with the EA or by possible unspecific adsorption of the anti-CRP-cAb on the EA modified surface. When the SU-8 surface was blocked with the EA at pH 9.4 and the same immunoassays were performed, the fluorescence signals were 1000-fold lower compared with those from bare SU-8 (results not shown). Therefore, it is most plausible that suboptimal EA blocking of the SU-8 surface at pH 7.4 is the reason of the 10-fold difference in the fluorescence signal of the immunoassays. Nevertheless, since the anti-CRP-cAb contains free lysine groups, the attachment of the protein to the SU-8 surface is certainly covalent.

4. Conclusions

The FT-IR spectra of the SU-8 surfaces showed that there are free epoxy groups on standard processed SU-8. Sandwich immunoassays proved that IgG type proteins performed best when the proteins were immobilized on bare SU-8 which had a relatively well characterized surface consisting of epoxy groups. Transformation of the SU-8 surfaces into amino-modified (aminosilanization) and hydrophilic surfaces (CAN pretreatment) resulted in higher LOD in the immunoassay probably caused by reduced amount of capture antibodies that could be immobilized on these surfaces. The results show that bare SU-8 and CAN treated SU-8 can directly be functionalized with antibodies that can be used for sandwich immunoassays with sensitivities that are relevant for clinical use, which is around 10 μg/ml. The binding of the IgG proteins on the SU-8 surfaces in the immunoassay conditions was proved to be covalent.

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