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Simple surface modification techniques for immobilization of biomolecules on SU-8

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Abstract SU-8, an epoxy based negative photoresist polymer has found wide range of applications in the field of microfabrication based biosensors. SU-8 surfaces need to be modified in order to immobilize bioreceptors. We studied the possibility of grafting desired functional groups by means of simple chemical treatments under normal laboratory conditions. These chemical treatments involve the use of crosslinkers that are expected to react with epoxy groups or hydroxyl groups generated by acid/alkali treatment. Here, a comparison of the results obtained on surface modification using glycine and 11-mercapto undecanoic acid as crosslinkers is presented. Human Immunoglobin G (HIgG) was covalently immobilized to carboxylic acid on SU-8 surface using carbodiimide/succinimide chemistry. The activity of immobilized HIgG was verified by using fluorescence imaging of FITC tagged goat anti HIgG bound to the surface. Fluorescence imaging was used to determine the chemistry best suited to functionalize SU-8 surface for biosensor applications.

1 Introduction

SU8, a multifunctional epoxy derivative of a bis-phenol-A novolac, is a low-cost negative photoresist with excellent mechanical, physical and optical properties after polymerization [1, 2]. Exposed resist has exceptional thermal stability and chemical resistance due to aromatic functionality and a cross-linked matrix [3]. Besides high-resolution patterning in semiconductor industry, SU-8 has been found suitable for fabrication of high-aspect ratio structures in micro-electro-mechanical systems (MEMS). Optical waveguides and micro cantilevers have been fabricated by exploiting its material characteristics to develop low-cost and efficient Lab-on-a-Chip biosensor systems [4]. Boisen and colleagues have reported highly sensitive SU-8 polymer based cantilever biosensors for DNA detection [5]. The deflection due to adsorption of single strand DNA has been found to be six times larger to that of commercially available silicon nitride cantilevers. SU-8 has been found suitable for applications ranging from photonic crystal waveguides for creating compact integrated optical devices to multimode core/cladding waveguides for biosensing [6–8].

In order to realize a biosensor with the help of SU-8 waveguides or cantilevers, desired functional groups are necessary for covalent immobilization of biomolecules. In case of cantilever based biosensors, gold layer coated on SU-8 has been used for selective immobilization of thiolated DNA and also for laser reflection to detect deflection of the cantilever. Optical channels have been modified using layer-by-layer deposition of polyanions and polycations [9]. An idea to use acrylic acid (AAc) as a linking layer has also been proposed [8]. DNA has been immobilized on SU-8 surface by either chemisorption of primary and secondary amine groups in DNA or in the form of Cholesteryl tetraethyleneglycol modified oligonucleotides [10, 11]. The above techniques for immobilization by using chemisorption of biomolecules may not suitable for antibodies as required for immunobiosensors due to steric hindrance of target binding sites. Recently, some protocols

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for immobilization of antibodies have been reported, which involved grafting of amino functional groups on to the surface of SU8 using aminosilanization and ammonia cracking with the help of plasma treatment [12, 13]. However, during the process of silanization, the organosilanes might oligomerize prior to deposition on the surface resulting in uneven surface. Further, a completely-dry argon ambient is required for such processes. Ammonia cracking requires expensive equipment such as hot-wire chemical vapour deposition.

In this study, we attempted to develop a protocol for SU-8 surface modification by simple chemical treatments for grafting desired functional groups. The surface was treated with crosslinker molecules after pretreatment with acid/alkali to cleave epoxy groups and generate hydroxyl groups. Glycine, an amino acid and 11-mercapto undecanoic acid (MUA) were chosen as crosslinkers. Contact angle measurements were performed to investigate changes in the surface hydrophobicity after crosslinking reactions. Human IgG (HIgG) and Goat anti-human IgG (GaHIgG) tagged with FITC were chosen as model bioreceptor molecule and target analyte. Reagent concentrations for effective immobilization of antibodies were optimized with the help of fluorescence image analysis.

2 Materials and methods

2.1 Reagents

SU-8 2002, its thinner and developer solvents were obtained from Microchem, USA. Mercapto undecanoic acid (MUA) was obtained from Sigma chemicals. Glycine was obtained from Sisco Research laboratories (SRL) India. 1-Ethyl-3-[3-dimethyl amino]propyl carbodiimide hydrochloride (EDC) and N-Hydroxy Succinimide (NHS) were obtained from Himedia, India. All the antibodies, buffers and bovine serum albumin (BSA) were obtained from Bangalore Genei, India. All the chemicals and reagents were of analytical grade or better.

2.2 Surface modification methods

SU8 substrates were prepared by using SU-8 2002 as per the standard procedure [14]. SU8 surface was treated with 0.1 M NaOH for 10 min followed by 1 M HCl for 10 min. We expect cleavage of epoxy groups and generation of hydroxyl groups on surface by pretreatment. Two SU8 substrates were taken and each was treated with 10 mM 11-MUA prepared in ethanol and 5 mM glycine in aqueous solution separately. Depending on the availability of epoxy groups, thiol and amine groups may form thioether and secondary amine respectively [10]. In the case of availability of hydroxyl groups, only amines of glycine molecules may bind to the surface through hydrogen bonding and electrostatic attractions. After 2 h samples were washed and activated with EDC:NHS (0.2 M:0.1 M in DI water) for 1 h.

2.3 Immobilization of antibodies

The succinimide activated substrates were incubated in 0.1 mg/ml human IgG (HIgG) solution prepared in PBS for overnight at 4°C. The surfaces were rinsed in PBSTB (PBS + 0.05%Tween 20 + 1 mg/ml BSA) to remove loosely adsorbed HIgG molecules. The presence as well as activity of immobilized HIgG was verified using FITC tagged goat anti-human IgG (GaHIgG) antibodies. The HIgG immobilized substrates were incubated with 0.05 mg/ml GaHIgG (prepared in 2 mg/ml of BSA) for 20 min at room temperature. The surfaces were again rinsed in PBSTB to remove nonspecifically adsorbed antibodies.

2.4 Image analysis

Fluorescence intensity due to the presence of FITC tagged GaHIgG bound to HIgG immobilized on SU-8 substrates was observed under a fluorescence microscope (Zeiss Axioskop-2 MAT) under ×10 magnification. Fluorescence images were acquired by using a peltier-cooled black and white digital camera. Scion Image® software was used to determine the fluorescent intensities of the images. Each pixel in an image under investigation had a gray value between 0 (dark) and 255 (bright). Overall intensity of an image was calculated by subtracting a value that accounts for the background intensity from the mean gray value of an image. The fluorescent signal is proportional to the density of active binding sites for target analyte on any immobilized surface.

3 Results and discussion

3.1 Characterization

Contact angle measurements were performed in order to observe the changes in the hydrophobicity of the surface with each treatment. Measurements were carried out using Digidrop EWS contact angle meter (GBX, France) with water as the solvent. Bare, untreated SU-8 surface was found to have contact angle of 81.8°, which is close to the reported value [15]. We expected a reduction in the hydrophobicity of the surface after acid/alkali pretreatment due to probable cleavage of epoxy groups. But, no significant difference in the contact angle was observed. SU-8

surface modification with MUA also did not result in an appreciable change. However, on glycine treatment the contact angle changed to 71.3°. The improvement in hydrophilicity might be due to the presence of carboxyl groups on surface. Subsequent treatment with EDC/NHS increased the contact angle to 80° due to the presence of succinimidyl groups.

3.2 Effect of surface chemical treatments

A set of three separate samples of SU-8 were taken and two of them were subjected to glycine and MUA treatments and immobilization of biomolecules. The third sample was used as control and HIgG was immobilized through physical absorption. All the three samples were jointly subjected to immobilization of HIgG and testing with FITC tagged GaHIgG. In order to examine the effect of proposed chemical treatments on surface functionalization and immobilization of antibodies, the fluorescence response from the images taken from each of three samples were compared. Any large variations in the fluorescence signal irrespective of chemical treatment, mostly caused by physical absorption onto a hydrophobic surface, were kept under control by using PBSTB washing as described above. Figure 1 shows the response obtained from each chemical treatment. SU-8 samples modified using glycine and control samples gave rise to the highest and lowest fluorescence intensity respectively. Using one-way ANOVA, fluorescent intensities of the glycine treatment were found to be significantly different that of control ($P = 0.025$). This indicates that the application of glycine, under the conditions mentioned earlier, may modify the SU-8 surface to make it amenable for antibody immobilization. It was further observed that the treatment with MUA was slightly less effective than that of glycine. Results obtained from glycine and MUA treatments were not significantly different (One-way ANOVA $P = 0.5$). Since the fluorescence studies using glycine as crosslinker showed better results, all the subsequent experiments were performed with glycine as crosslinker.

3.3 Effect of glycine concentration on immobilization

In order to investigate any dependence of immobilization on crosslinker concentration, SU-8 surface was treated with three different concentrations, 1, 5 and 10 mM of glycine solutions. HIgG molecules were immobilized and subjected to FITC tagged GaHIgG antibodies as described earlier. All the three samples yielded fluorescence signal at a similar levels as shown in Fig. 2. The response of 5 mM glycine was marginally higher than the other two concentrations and hence was chosen for subsequent experiments. However, the difference between the three treatments was found to be statistically insignificant as concluded by a one-way ANOVA test between the treatments ($P = 0.215$).

3.4 Maximization of active binding sites

The number of active binding sites present on any surface is mainly influenced by the kind of functional groups available for binding, solution concentration, incubation time and the iso-electric point of a protein. Succiniimide containing surfaces readily react with amine containing biomolecules at relatively reduced protein concentrations. Hence, HIgG concentration was varied to determine the optimum value with overnight incubation to maximize the IgG binding. Three separate samples were incubated with a concentration of 0.01, 0.05 and 0.1 mg/ml of HIgG in PBS followed by incubation with FITC tagged GaHIgG. Fluorescence intensity was used for relative quantification of

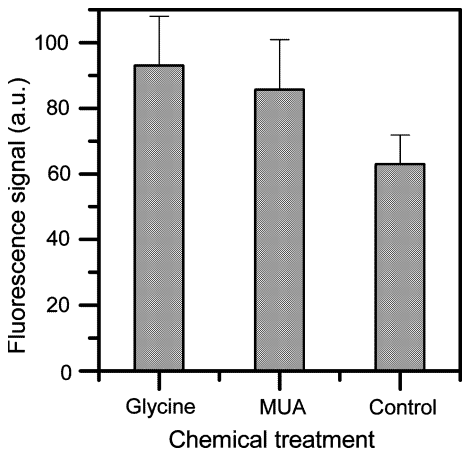

Fig. 1 Fluorescence response obtained due to binding of GaHIgG-FITC to HIgG immobilized on SU-8 surfaces that are unmodified and modified by using glycine and MUA treatments

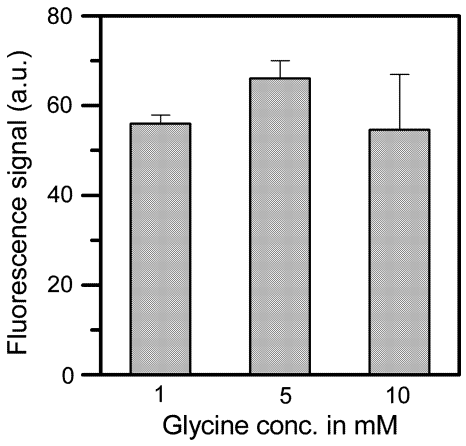

Fig. 2 Effect of glycine concentration on the density of immobilized HIgG. The fluorescence signal obtained from binding of FITC tagged GaHIgG was taken as measure of activity of immobilized protein

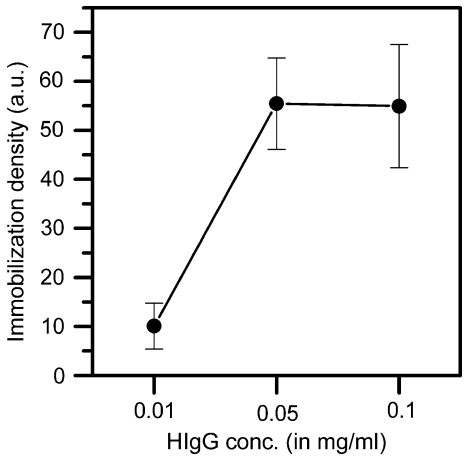

Fig. 3 Dependence of the density of active binding sites of immobilized HIgG on the concentration of protein solution used for immobilization

the activity of the immobilized surface. Although quantification of the number of active binding sites is beyond the scope of this study, from image analysis it may be concluded that HIgG with 0.05 mg/ml is the optimum concentration to achieve maximum density of active binding sites. Figure 3 shows that higher protein concentrations lead to more deviations from the mean density though no significant improvement in the mean was observed.

4 Conclusions

SU-8 surface modification using glycine as crosslinker is an effective means for immobilization of proteins compared to physical adsorption. Optimum protein concentration for a maximum yield in the density of binding sites can be as low as 0.05 mg/ml. Amine containing crosslinkers are a better choice than thiol crosslinker. However, the exact chemical reaction taking place on surface is yet to be explored. Surface analytical techniques such as X-ray photoelectron spectroscopy or FTIR in grazing angle mode can be used to understand the surface chemistry.

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