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Surface Regeneration of Gold-Coated Chip for Highly-Reproducible Surface Plasmon Resonance Immunoassays

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Abstract

We developed a simple procedure for the complete regeneration of silane-grafted gold surface. This method allowed the reuse of the regenerated gold chip for custom refunctionalization. In addition, we performed highly reproducible immunoassays using these chips over several cycles. The developed procedure was optimized and comprised of consecutive treatments of functionalized Au chip with 12 M HCl for 10 min and 29 W oxygen (O2)-plasma for 5 min. Monitoring and surface characterization of the developed methodology was performed with ellipsometry and Rutherford back scattering. The developed procedure was demonstrated on SPR-based Human Fetuin A (HFA) immunoassay, where the amino groups of APTES-functionalized Au chip were cross linked to the carboxyl groups of anti-HFA antibody using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and sulfo-N-hydroxy succinimide. The APTES-functionalization, anti-HFA antibody immobilization and HFA binding on the regenerated SPR Au chip were highly reproducible over 40 HFA immunoassay cycles.

Keywords: Gold chip; APTES; Surface plasmon resonance; Surface functionalization; Human fetuin A immunoassay

Introduction

Regeneration of the surface immobilized with recognition biomolecules, such as antibody, after immunoassay is very important for expensive bioanalytical platforms such as Surface Plasmon Resonance (SPR), quartz crystal microbalance and micro-cantilevers. This makes the chips reusable thus making it cost-effective. Regeneration is usually done by breaking the antibody-antigen interactions using acidic or alkaline solution [1-4]. The strength of regeneration solution is directly proportional to the affinity of antibody towards its antigen and the stability of the antigen-antibody complex. The chemical composition and functional conformation of an antibody is affected by the ionic strength, pH and chemical nature of regeneration solution. The repeated exposure of an antibody to a strong regeneration solution adversely impacts its functional activity [1,5,6]. Therefore, a regeneration solution that does not affect the affinity and avidity of an antibody should be employed [1]. A wide range of regeneration solutions with varying regeneration efficiencies and effects on the functionality of immobilized antibodies had been reported [7-9]. The determination of an appropriate regeneration solution is a time-consuming and labor-intensive process. However, although a regeneration solution leads to the complete regeneration of an antibody-bound surface, it decreases its activity after few weeks due to storage, contamination, biofouling, protein spreading and other complex effects. Such an antibody-bound SPR chip can no longer be employed for an immunoassay.

In this manuscript we are reporting a novel approach which relies on functionalization of Au surface with APTES [10] regenerating the surface of the used SPR chip rather than focussing on the regeneration of biomolecule interaction. In this new approach, regeneration was achieved by removing the adsorbed aminopropyl triethoxysilane (APTES) layer which will facilitate the complete removal of any immobilized biomolecules. This will tremendously increase the cost-effectiveness of any Au-coated SPR chip as the same chip can be reused over several cycles for performing the same or different immunoassays. The developed and optimized procedure involved consecutive treatments with hydrochloric acid (HCl) [11-14] and O_2-plasma. The complete regeneration of Au-surface of SPR chip was monitored with ellipsometry and Rutherford Back Scattering (RBS). The increase in cost-effectiveness of SPR Au chip was determined by highly-reproducible silanization, anti-HFA antibody binding, and HFA detection in 40 consecutive immunoassay cycles on the same SPR chip after regeneration. The developed regeneration procedure would also be useful for many other biosensor formats that employ APTES-coated surfaces such as microcantilevers, quartz crystal microbalance and gold electrodes.

Experimental Section

SPR Au chip preparation and silanization

The Au chip was prepared following previously mentioned procedure reported by our group [15]. The assembled SPR Au chip was cleaned by treating with piranha solution [3:1 (v/v) ratio of 97.5% H_2SO_4 and 30% H_2O_2] for three minutes followed by extensive DIW washing (at least five times) to remove the traces of piranha. The cleaned chip was then incubated with 2% (v/v) APTES (in 30% (v/v) ethanol) for 1 h at Room Temperature (RT) in a fume hood, which generated a SAM of silane.

Regeneration studies

The functionalized chips were subjected to acid-lysis of siloxane bonds [11-13] by HCl. The acid-mediated lysis based regeneration was optimized using various HCL strengths (5,10,12 and 15 M), and exposure times (5,10,20 and 30 min). The O_2 plasma etching was also optimized at three different plasma powers *viz.* 12, 20 and 29W at different exposure times of 3, 6, 9, 12 and 15 min. Subsequently, the

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combined HCl and O_2 plasma treatment procedure was optimized for Au surface regeneration.

Ellipsometric analysis

The thickness of the APTES layer on Au chip was measured using a Jobin Yvon Horiba UVISEL spectroscopic ellipsometer (Chilly Mazarin, France). The measurements of psi (ψ) and delta (Δ) spectra were conducted at an incident angle of 70° over a wavelength range of 350-1000 nm. The instrument's inbuilt silicon dioxide dispersion layer fitting model was then followed to determine the effective thicknesses of Au on glass and APTES on Au.

Rutherford backscattering (RBS) analysis

The APTES-functionalized SPR Au chip was loaded into the scattering chamber that was maintained under high vacuum conditions. A collimated beam of 2 MeV He^+ ions, generated by a 3.5 MV HVEE Singletron accelerator, was normally incident onto the sample. The ions backscattered at 101.25° scattering angle were collected and their energy was measured by an Ortec Ultra silicon solid-state detector. The resultant spectra were fitted using the SIMNRA software. The total Si areal density for each sample was extracted from the target structure that provides the best fit for the respective spectrum.

SPR-based HFA immunoassay procedure

Anti-HFA antibody (990 µL of 100 µg/mL reconstituted in HBS) was incubated for 15 min at RT with 10 µL of the cross-linking solution containing EDC (4 mg/mL) and sulfo-NHS (11 mg/mL) in 0.1 M, pH 4.7 MES buffer. Later, 100 µL of the resulting EDC-sulfo-NHS activated antibody solution was flown over the silanized Au chip at 10 µL/min to prepare anti-HFA antibody-coated chip. The anti-HFA antibody immobilized chip was then blocked using sulfo-NHS acetate, which reacts to free amino groups on the surface. This was followed by BSA-blocking in order to fill the void space on the chip thus, minimizing non-specific protein adsorption on the surface. Subsequently, the HFA at 0.6 to 20 ng/mL dilutions was assayed on the anti-HFA antibody immobilized chips [15,16] for 40 cycles of regeneration.

Later, the SPR Au chip was regenerated by treatment with 12M HCl for 10 min followed with the 29 W O_2 plasma treatment for 5 min. The regenerated Au chips were reused and analyzed for reproducible immobilization of anti-HFA antibody and HFA detection in consecutive runs for 40 cycles.

Results and Discussion

Various silane-based Au surface modification strategies have been employed for immunoassays; however, their use for SPR applications is fairly recent [16-18]. There are reports claiming the successful regeneration of thiol terminal silane-functionalized Au surfaces [19]. It was observed that strong acid treatment such as HCl can dissolve the siloxane bonds and remove upto the second APTES layer as the first APTES layer on the Au surface was very strongly bound through Au-thiol interactions. However, there was no critical assessment of reusability and functional characterization of SPR Au chip. In addition, HCl may also affect the homogeneity of Au coating. In this report, we have developed a simple strategy, based on consecutive treatments with HCl and O_2-plasma, for the complete regeneration of Au surface as illustrated in Figure 1. The regeneration studies were performed on the Au chips that were (a) functionalized with APTES, and (b) bound to anti-HFA antibody after APTES-functionalization. The thickness of APTES self-assembled monolayer (SAM) and the areal density of APTES on the bare, silanized and regenerated Au chip surfaces were determined by ellipsometry and RBS, respectively Figure 2.

Twenty chips were APTES-functionalized and different regions on each chip (four spots in the corner and one spot in the centre) were analyzed to assess the homogeneity of silane layer. A SAM thickness of 5 nm ± 1 nm was obtained in all the repeats. The thickness was calculated with the instrument software, which generates a differential reflection profile [designated as psi (ψ) and delta (Δ)] for each corresponding layer. The contact angle obtained for the SAM of APTES on Au surface was 59° ± 1°, which is consistent with previous findings [20]. The contact angle obtained for piranha-treated bare Au was 25°, which was in agreement with previous reports that claimed the contact angle of cleaned gold in the range of 0-30° [21]. The regeneration of Au surface by the developed procedure was confirmed by RBS (Figure 2) based on the absence of APTES signal on the regenerated SPR chip. The areal

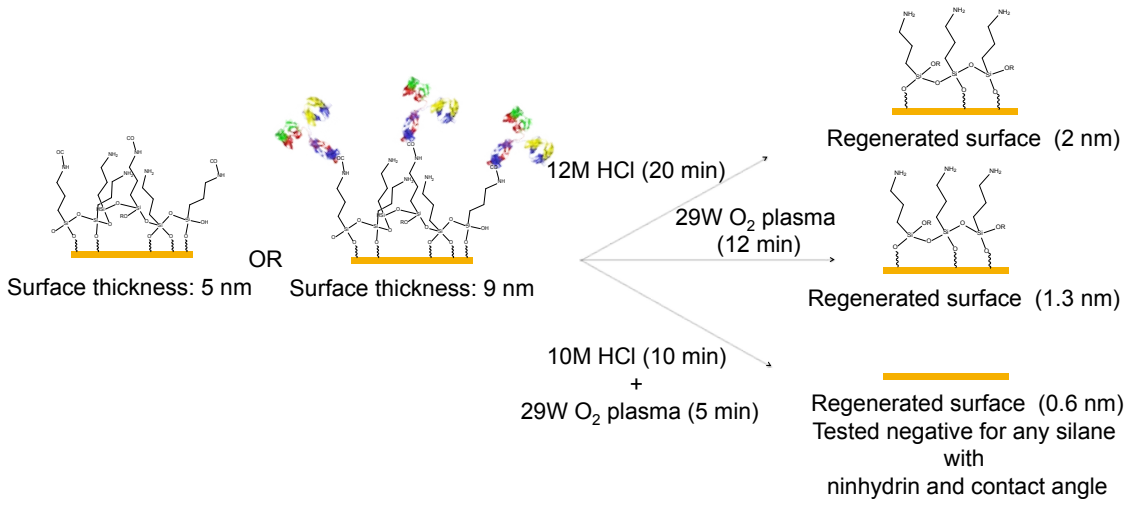

Figure 1: Schematic representation of complete regeneration of the silane-functionalized Au surface by acid and/ or plasmolysis of the siloxane bonds. The surface was characterized with ellipsometry and Rutherford back scattering (RBS) analysis for detection of any residual APTES on the surface.

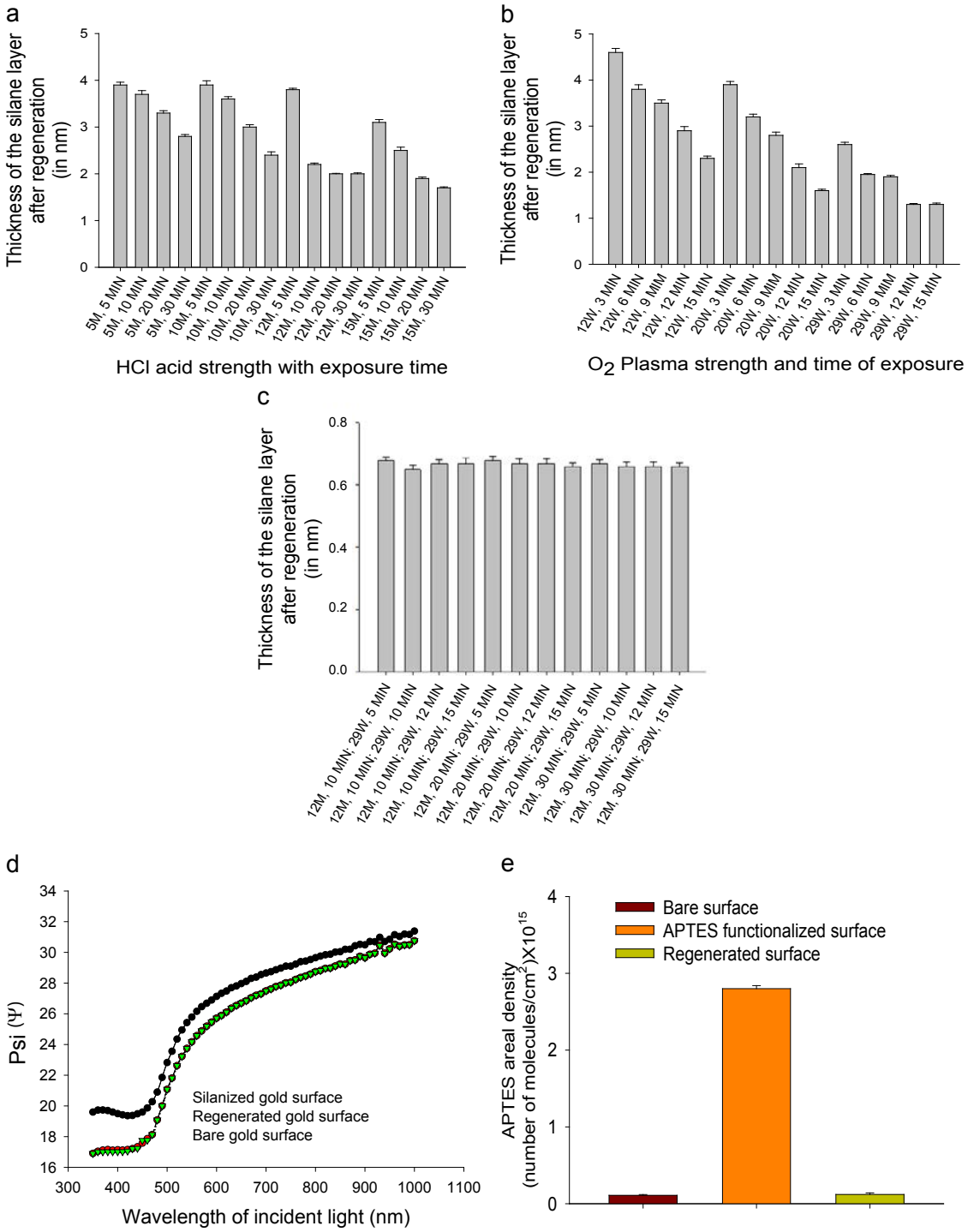

Figure 2: Optimization of surface regeneration procedure (a) siloxane bond lysis at various acid (HCl), (b) various O_2 plasma, and (c) their combined strengths as a function of exposure time (min). Thickness for each set was monitored using ellipsometry as depicted in'd'. (e) Evaluation of areal density of APTES on bare, APTES-functionalized and regenerated Au surfaces by Rutherford back scattering (RBS). Error bars are standard deviation.

density of APTES after APTES functionalization on the regenerated SPR Au chip was similar to that on the fresh SPR chip, which shows the complete regeneration of Au surface. During 40 consecutive HFA immunoassays, the areal density of APTES was highly-reproducible, which demonstrates that the developed regeneration procedure does not affect the surface properties of the Au layer.The developed Au surface regeneration procedure was optimized by determining the appropriate strength and treatment time of HCl and O_2 plasma (individually in the initial stages, and in combination thereafter). Initially the efficiency of regeneration was assessed by treating with 5, 10, 12 and 15 M of HCl for

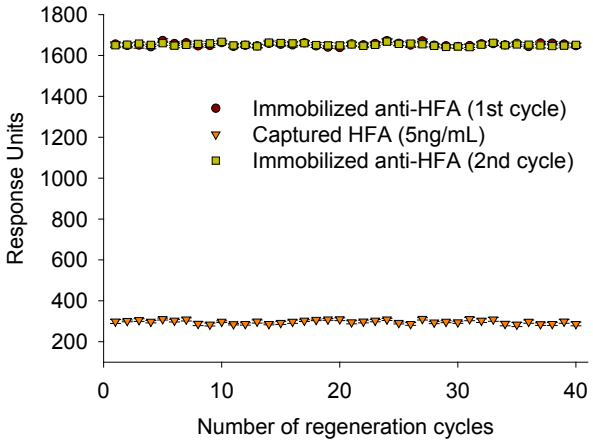

Figure 3: Reproducibility of antibody immobilization and antigen detection on the SPR Au chip, which was reused 40 times after regenerating the Au surface using the developed procedure. This process was mainly cycles of surface functionalization, assay performance, and regenerating the surface followed by repeating the cycle over 40 times. Error bars are %CV.

varied duration i.e. 5, 10, 20 and 30 min. The treatment with 15 M HCl for 30 min was found to be most appropriate for regenerating down to 1.7 nm thickness. However, the acid treatment does not regenerate the surface completely due to the role played by the respective anions generated by acid treatment in the dissolution of siloxane bonds [22]. These anions, such as hydroxyl (OH^-), sulfate (SO_4^{-2}) and chloride (Cl^-), potentially destabilize the siloxane frame work in the presence of water. However, the acidic environment enhances the polymerization of silanes, which is responsible for incomplete regeneration. Therefore, our initial acid-treatment approach was followed by O_2-plasma scrapping/ etching [23].

The O_2-plasma scrapping was done at plasma strength of 12, 20 and 29W for 3, 6, 9, 12 and 15 min such that 29W O_2 plasma treatment for 12min was most effective and regenerated down to 1.3 nm thickness (Figure 2). The O_2-plasma discharges are known to ionize the siloxane bonds present in the liquid phase to the silanol moieties in the vapour phase [23]. However, the same phenomenon might have occurred with the silane coated on the Au surface, as evident from the decrease in the layer thickness.

Finally, the surface regeneration was checked with various combinations of HCl and O_2 plasma. For the initial HCl treatment step, 12 M and 15 M HCl was used for 10, 20 and 30 min; whereas, for the O_2 plasma scrapping, 29W O_2 plasma was employed for 5, 10, 12 and 15 min. The complete regeneration of Au surface was obtained by treating it with 12M HCl for 10 min followed by O_2 plasma scrapping for 5 min, which completely removed the APTES coating. This was confirmed by the absence of silicon peak due to APTES functionalization on the Au surface by RBS. A negative ninhydrin test and a contact angle of 28° cross-validated the absence of silane on the surface.

The regenerated SPR Au chip was reused 40 times for SPR-based HFA immunoassay following the same bioanalytical procedure i.e. APTES-functionalization, anti-HFA antibody immobilization, HFA detection and surface regeneration. The reproducibility of APTES functionalization in each cycle was confirmed by ellipsometry and RBS, as discussed previously. The areal density of APTES after functionalization was consistently uniform in each detection cycle. The immobilization density of anti-HFA antibody and the subsequent detection of HFA was also highly reproducible in each cycle, as confirmed by the SPR response (Figure 3). The percentage coefficient of variance in APTES functionalization, anti-HFA immobilization and HFA detection, during 40 consecutive HFA immunoassays on the same SPR chip after regeneration, were 3-5, 4-6 and 2-4%, respectively. Therefore, the developed Au surface regeneration procedure completely regenerates the Au surface without affecting its surface properties. It increased the cost-effectiveness of expensive SPR Au chip as the same SPR chip can be reused many times without any loss of activity. The developed regeneration is presently being investigated on other potential chemistries, based on caboxymethyl dextran, protein A and thiols, where it may yield similar results.

Conclusion

A simple procedure was devised for the complete regeneration of Au surface in the SPR Au chips that were used for HFA immunoassay. The SPR-based HFA immunoassay involved the functionalization of cleaned Au chip with APTES, followed by covalently binding of anti-HFA antibody, blocking with 1% BSA, and detection of HFA. The regenerated SPR Au chip was effectively reused 40 times for HFA immunoassay with a very high reproducibility at each process step i.e. APTES functionalization, anti-HFA binding and HFA detection. The reproducible APTES functionalization of regenerated Au surface was confirmed by consistent areal density of APTES in RBS analysis, while the reproducible anti-HFA immobilization and HFA detection were determined by their SPR responses. The devised Au surface regeneration procedure will be immensely useful as it will enable the multiple reuse of costly SPR Au chip for cost-effective SPR-based immunoassays.

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