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Amine Accessibility and Chemical Stability of Silver SPR Chips Silanised with APTES via Vapour Phase Deposition Method

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Abstract: *Surface plasmon resonance (SPR) sensor technology has great potential for many applications. In addition, protein immobilisation is a critical step in the production of protein biochips, biosensors, etc. Here, the vapour-phase silanisation with 3-aminopropyltriethoxysilane (APTES) of the SPR response of a thin silver film (50 nm) has been considered. The obtained data were correlated with the morphology, absorbance, stability and water contact angle of the composites to observe the coating mechanism. The identification of an approach for determining the amine accessibility and stability of the silver surfaces that are coated with amino-organic films is the main outcome of this study. The results illustrate that the surface property of the silver layer can be changed using these methods. The evaluation of the amine accessibility verifies that all prepared chips can effectively assist in protein immobilisation on silver-coated slides. Comparing various studied silanisation conditions (different times, temperatures and APTES concentrations), it is concluded that silanisation at 80°C for 30 min is a simple and efficient method for protein immobilisation on silver-coated glass slides, which has potential application in the production of protein chips and biosensors.*

Keywords: SPR, APTES, silver chip, amine accessibility, chemical stability

1. INTRODUCTION

In recent years, there has been a pronounced tendency to use surface plasmon resonance (SPR) in the detection and analysis of chemical and biochemical substances in many important areas such as medicine, environmental monitoring, biotechnology, drug and food monitoring because of its extremely high sensitivity to changes in the optical properties of the surface layers and the ease of their real-time continuous monitoring. To date, most SPR immunoassay systems have been constructed using three layers: glass substrate, metal layer (gold or silver) and bioreceptors. Because of the high stability in aqueous environments, which is required to monitor bio molecular interactions, and the reactivity required for functionalisation, gold has been the dominantly applied in the SPR immunoassay.

However, gold is not the best selection to achieve high-sensitivity SPR sensing. Silver yields a more distinct and dipped SPR spectrum than gold, but this metal suffers from poor chemical stability under chemical reactions.[1,2] This lack of constancy of silver against the atmosphere and different solvents in several steps of biochip production, such as the consecutive cleaning steps and immobilisation of bioreceptors, can cause oxidation and the formation of silver sulphides and silver nitrides and other factors. The formation of these components on the surface of a silver layer can result in interference and decrease the sensor sensitivity.[3]

As previously described, the immobilisation of bioreceptors on the metal layer is necessary for the SPR-based immunoassay. This layer enables the separation of bound and unbound antigens. Many immobilisation techniques have been developed in the past years that are mainly based on the following three mechanisms: physical, covalent, and bioaffinity immobilisation. In physical immobilisation, proteins can adsorb on the surfaces via intermolecular forces, which are mainly ionic bonds and hydrophobic and polar interactions. The drawbacks of the adsorption mechanism are random orientation and weak attachment because the proteins may be removed by some buffers or detergents when the assays are performed. Moreover, the problems relating to mass transport effects and high background signals from nonspecific interactions can result in the false calculation of the kinetic rate constants during real-time SPR measurements.[4]

Biochemical affinity reactions offer a gentle oriented immobilisation of proteins such as the avidin-biotin system,[5] His-Tag system,[6,7] DNA-directed immobilisation[8] and protein A/protein G-mediated immobilisation,[9,10] which provides an important advantage over other immobilisation techniques. However, low loading amounts and the long-term physical instability of the adsorbed antibodies have limited the utility of the affinity-based immobilisation strategies.[11,12]

More often, proteins are covalently bound to the support through a functional group of exposed amino acids. Covalent bonds are mostly formed among the side-chain-exposed functional groups of proteins with suitably modified supports, which results in irreversible binding and produces a high surface coverage.[13,14] This conjugation technique provides a direct and robust coupling of proteins to solid substrates via the formation of physically and chemically stable covalent bonds using various linkers on the solid substrates.

The covalent immobilisation strategies include chemical reactions, such as amine, aldehyde or thiol coupling, on previously formed functional self-assembled monolayers. The most widely used approach to present functional

groups onto thin metal SPR films is based on thiolated organic compounds such as silanes, which spontaneously form self-assembled mono layers on gold surfaces. Silanes have proven to be effective coupling reagents with hydroxyl (OH) functionalised surfaces such as glass and silica and the oxides of aluminium and silver.[15] Tri-functional silanes such as 3-aminopropyl triethoxy silane (APTES) are used as a coupling agent to covalently bind proteins to inorganic surfaces.[16]

Thus, the thickness, morphology, and conformation of APTES are of great interest, and careful studies have been performed regarding the structure of APTES at interfaces.[17] APTES forms 2D layers on a mica surface, which is stabilised by lateral cross-linking.[18] The water contents of the check result in the hydrolysis of alcohoxy groups in APTES and the production of siloxane triols, which enable the silanes to react with one another and with the glass surface to form a polymeric network on the glass surface.[19,20] Further condensation and polymerisation of siloxane triols form a cross-linked polysiloxane network, which is chemisorbed onto the surface to achieve good intrinsic adhesion.[20]

In a previous study, a protocol was disclosed to produce APTES-treated gold films on glass substrates without intermediate chromium or titanium layers; these films had high adherence of the gold-coated layer to the glass substrate and suitable optical properties to be used as an SPR sensor chip.[21,22] To the best of the author's knowledge, there are no reports on the silanisation of the sputtered-silver thin films used as SPR chip until today. From the technological perspective, the effect of silanisation on the SPR response of the prepared biochip and its stability and protection against the effects of water in either liquid or vapour state are the key factors that affect performance. Therefore, it is necessary to develop a low-cost, uncomplicated, and fast procedure that provides the benefits of silver as a metal layer and overcomes its instability for use in the SPR bioassays. The objective of this study is to consider a new SPR biochip based on silver thin film (50 nm) using APTES as a protective layer and cross linker for protein immobilisation.

2. EXPERIMENTAL

The 3-aminopropyl triethoxy silane, H_2O_2 (30 wt%), and H_2SO_4 (94 wt%) were obtained from Merck (Tehran, Iran); 3-aminopropyltriethoxysilane (APTES), bovine serum albumin and glutaraldehyde were purchased from Sigma-Aldrich. Triply distilled water was used in the cleaning steps and in making aqueous solutions.

2.1 Preparation of Chips

The experiments were performed on glass slides, which were cut into pieces (1 × 1 cm^2). To clean and regenerate the surface silanol groups of the glass slides, the slides were treated with a piranha solution (3:1 vol. ratio, H_2SO_4: H_2O_2) for 30 min at 80°C. Then, the cleaned slides were washed with triply distilled water and dried under a stream of nitrogen. Finally, the substrate chips were prepared by thermal evaporating 50 nm of silver onto the cleaned glass slides.

Surface functionalisation was performed using a vapour-phase deposition method with a 200 ml glass bottle as the reaction chamber and under one of the following conditions in freshly prepared solutions:

1. Concentrated vapour-phase deposition (CVPD): 50 ml of the APTES solution was added to the bottle, and the Ag-coated slides were set in the bottle. After sealing, the bottle was placed in an oven and heat-treated at 80°C or 100°C for 30 or 60 min. After salinisation, the prepared biochips were washed with distilled water and dried with a nitrogen flux.
2. Diluted vapour-phase deposition (DVPD): 50 ml of the APTES solution (5% volume ratio APTES to propanol) was used for the silanisation in an identical procedure as that described for CVPD.

2.2 Surface Characterisation

The surface topography of the silane films on the silver-coated glass substrate was investigated under ambient laboratory conditions using a Nanoeducator AFM instrument with a non-contact mode on squares of the samples (2 × 2 μm^2).

The SPR characterisation of the samples was performed using an SPR instrument (Nano SPR), which was assembled according to Kretschmann's prism configuration with a resolution of 0.02°. The absorption spectra were recorded with a UV/vis spectrophotometer. The wavelength range was 400–600 nm.

The contact angle of water on the treated films was measured according to the ASTM D 724-99 test method at room temperature in ambient atmosphere. The mean contact-angle values were obtained on three different measurements at different locations along the sample surface. Prior to the contact-angle measurement, the biochips were rinsed in DI-water and dried under a nitrogen stream.

The NaCl stability test was used to evaluate the chemical stability of the chips during various cleanings, and post-treatment was performed by placing the chips

in 200 cm^3 beakers that contained 2 M aqueous NaCl solution. When the silanised silver layer began to detach, the time was recorded.

The amine accessibility was assayed by immobilising biomolecules onto the bimetallic SPR surface. First, 5% aqueous glutaraldehyde was injected into the system for 20 min at a current rate of 5 ml/min, so that the amino groups on the airfoil of the chip could react with the aldehyde group. Then, the chips were frequently washed using the washing command of the system. Later in this treatment, 0.5 mg/ml BSA was injected into the system for 30 min at the identical flow rate, and 0.1 M NaCl was injected for 1 min at the identical flow rate to wash out the unbound protein.

3. RESULTS AND DISCUSSION

3.1 Topographic Features

To achieve a good SPR response (sharp SPR dip and low reflectance minimum), the thickness of the Ag layer should be maintained in the range of 45∼50 nm. Thus, the sputtered Ag layer thickness in all films was controlled within this range.[23]

Silanisation is a widely practiced procedure to couple bio-molecules to inorganic substrates generally via a subsequent reaction with a cross-linking reagent. The silanisation with APTES forms a self-assembled monolayer (SAM) with reactive amine groups ($–NH_2$). The amine groups then experience further cross-linking.[17]

The topographic features of the sputtered silver on the glass slide surfaces before and after the modification with APTES are revealed in the respective AFM images (Figure 1). A typical AFM image that is obtained from a silver chip with no silanisation is shown in Figure 1(a). The image is notably smooth and clean on a submicron scale. Although the main topographic and structural features are similar for all analysed samples, the differences in size of the silane islands are observed when they are subjected to different conditions (different times, temperatures or APTES concentrations). It is distinctly recorded that the size of the silane domains depends on the silanisation conditions. The average roughness values of the parent sputtered silver layer and prepared chips under different conditions are grouped in Table 1. As previously reported, the AFM images of the silanised surfaces (Figure 1) exhibit similar characteristics. The formation of the APTES layer on the silver surface increased the average roughness of the silver layer. According to Table 1, the surface roughness (R_a) of the silanised silver surface is higher than that of the blank silver surface (6.7 nm) and further increases with the increase in silanisation time and temperature compared to the parent silver surface.[17]

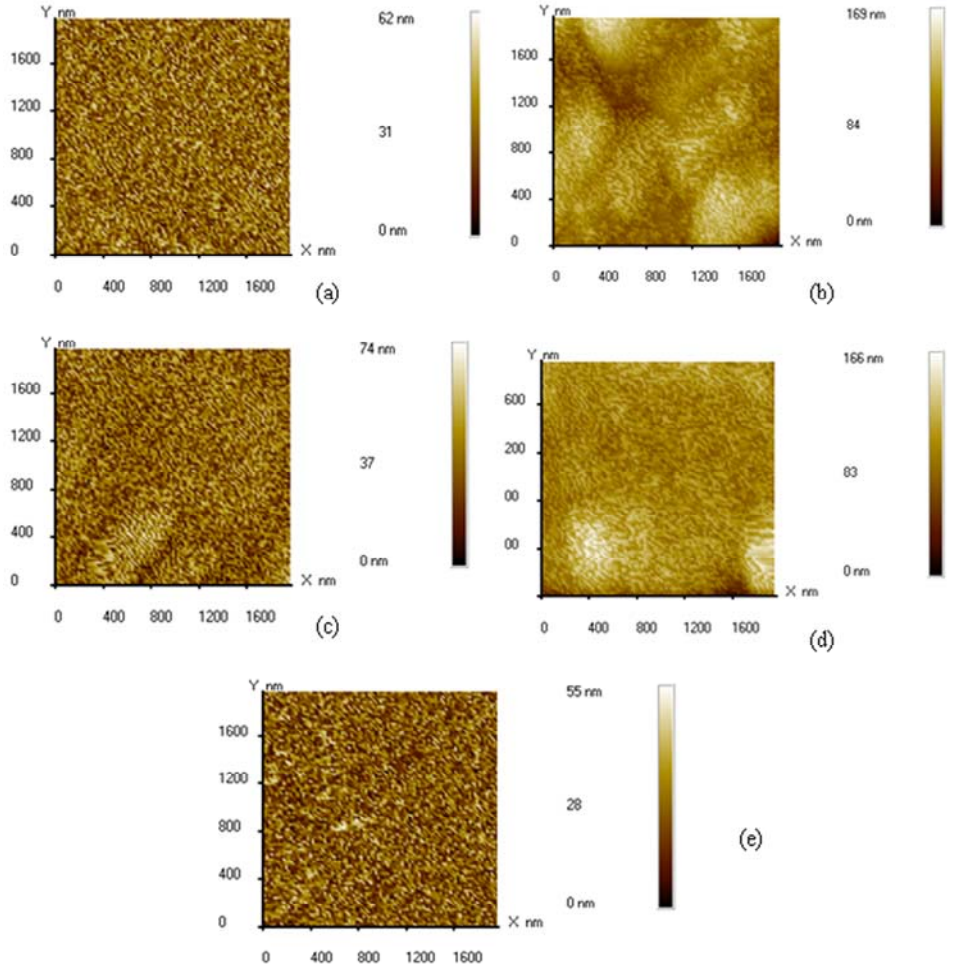

Figure1: AFM images of (a) sputtered 50 nm thin silver film; (b) silanised with APTES by CVPD method at 100°C for 60 min; (c) silanised with APTES by CVPD method for 30 min at 80°C; (d) by DVPD method at 100°C for 60 min; and (e) by DVPD method for 30 min at 80°C.

Table 1: Different physical and chemical characteristic of silanised silver chips.

Conc. of APTES	Time (min)	Temp (°C)	Average roughness (nm)	Amine accessibility (RIU)	NaCl test	Contact angle (°)
5%	60	100	13.91 ± 1.46	No SPR	–	–
	30	100	9.38 ± 0. 70	163	<6min	66.43 ± .76
	30	80	9.72 ± 0.47	218	<1min	62.66 ± .05
100%	60	100	13.73 ± 2.68	No SPR	–	–
	30	100	12.69 ± 0.83	237	<45min	65.13 ± .37
	30	80	9.50 ± 0.36	274	<33min	62.09 1.66

3.2 SPR Analysis

The reflectivity was measured against the incident angle for all prepared chips under various VPD conditions, and the SPR resonance was observed (Figure 2). As shown in Figure 2(a), the resonance angle and reflectivity, which was monitored at the resonance angle, depend on the silanisation conditions. The reflectance minimum (SPR dip) of the 50-nm-thick Ag film approached approximately zero at the resonance angle, as shown in Figure 2(a). Different angle shifts and reflectivity decrements were determined depending on different silanisation concentrations, times and temperatures. The resonance angle increased from 0.08 to 0.44 when the APTES concentrations increased from 5% to 100% (Table 1). In addition to a shift in resonance angle, the force of the resonance minimum decreased from 36.9% to 74.36%, which corresponded to a loss in SPR characteristics. This conflict can be attributed to a change in the actual refractive index of the silver layer when the silane film is deposited on the silver layer.

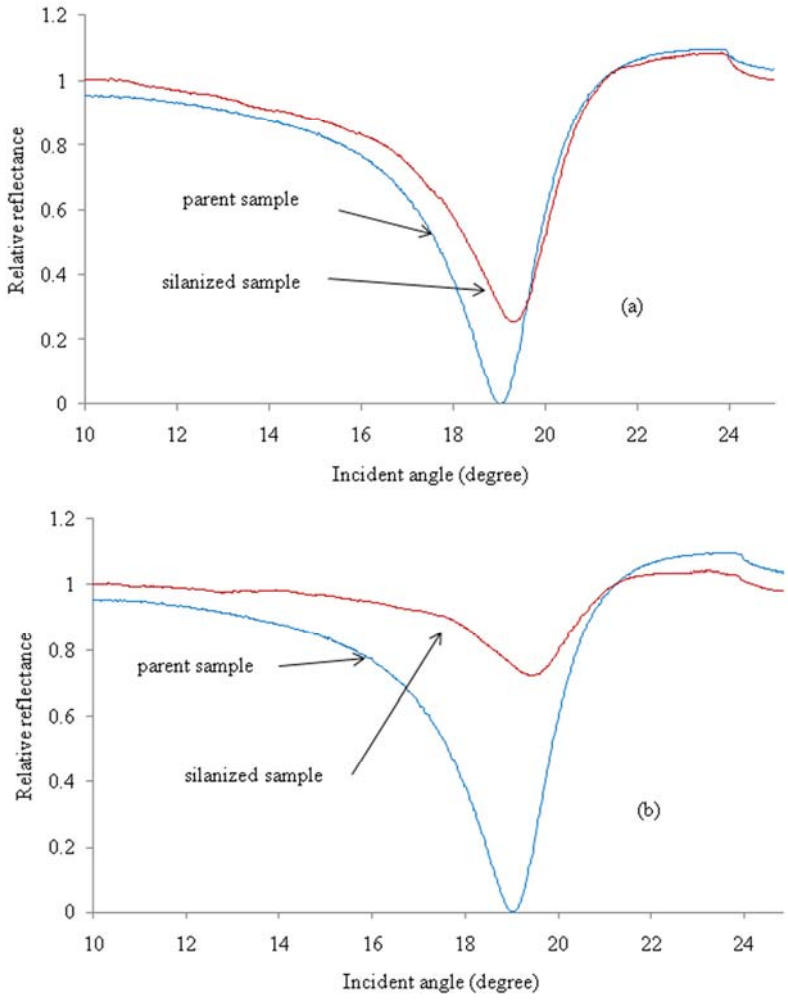

Figure 2: Reflectivity versus incident angle for a 50 nm thin silver film (black line) silanised with APTES (gray line) by CVPD method at 100°C (a) and by DVPD method at 80°C (b) for 30 min.

Furthermore, the heaviness of the silane top layer indirectly affects the resonance angles and reflectivity, which implies that the width of the SPR dip also becomes more spacious with the increase in thickness of the silane layer. The differences of resonance angles before and after silanisation, which correspond to CVPD and DVPD at 100°C and 80°C, are 0.44, 0.14, 0.08 and 0.1 points, respectively. It should be noted that the silanisation by DVPD methods produce a good SPR response with a suitable width of the SPR dip and reflectance. The presented

results show the ability of the silanised chip using a DVPD method for SPR sensors.

3.3 Optical Absorption Spectra

Figure 3 shows the optical absorption spectra of the chips. For the as-deposited samples and the samples that were silanised for 30 min, no absorbance peak was observed, which indicates that during the sputtering process, the sputtered Ag atoms were implanted in the film. For these samples, no absorption peak was discovered because the sputtering method can distribute particles in atomic form, and the optical absorption of Ag particles smaller than 2 nm exhibits no SPR peak. After 60 min of silanisation, the moving pictures displayed an absorption peak at approximately 430 nm, as shown in Figure 3(b). By increasing the silanisation time, Ag particles began to aggregate and react with APTES to result in Ag nano-particles and grain formation.

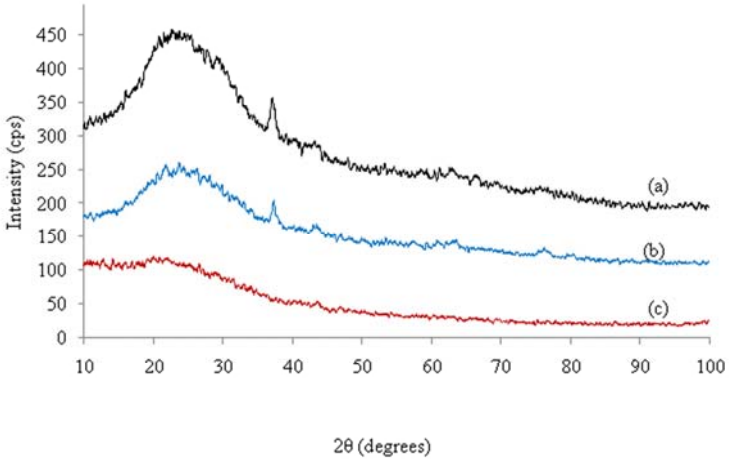

Figure 3: Optical absorption spectra for a 50 nm thin silver film silanised with APTES via vapor phase deposition method at (a) 100°C for 30 min; and (b) for 60 min.

3.4 XRD

The XRD of the parent silver film and that of the films silanised via CVPD at 100°C for 60 min and at 80°C for 30 min are shown in Figure 4. The parent silver film and films silanised via CVPD at 80°C for 30 min showed the XRD peaks at the angles of approximately 36.8°, 42.8° and 62.3°. These peaks can be indexed as the diffractions from the (1 1 1), (2 0 0) and (2 2 0) planes of cubic silver. No peaks were observed in the XRD patterns that were developed from the silane top

layer. The results clearly illustrate that Ag is crystallised into polycrystalline in the pure samples. However, films silanised via CVPD at 100°C for 60 min do not contain the peaks of the parent silver film, which indicates an amorphous structure.[23]

3.5 Contact-angle Measurements

The wettability is a notably important property of surfaces that strongly depends on both surface chemical composition and geometrical microstructure. The surface of the glass slides is notably hydrophilic, with a water contact angle of 31.1º, whereas the contact angle significantly decreased to16.62º after the piranha treatment because of the incorporation of –OH groups onto the surface. Sputtering of a silver layer on the piranha-treated glass slides increased its contact angle to 81.85º, and the surface became hydrophobic. Further functionalisation of the silver-sputtered surface with APTES decreased the contact angle value to approximately 60°–66° (Table 1), which indicates the successful functionalisation of the surface with APTES. The APTES-treated silver is more hydrophilic than the silver surfaces because of the presence of aminopropyl segments on the silanised surface. These results are consistent with the results of our previous works.[21,22]

3.6 Amine Accessibility

The presence of reactive primary amine groups because of the presence of surface APTES on the silver substrate can be distinguished from the SPR response of covalently coupled bovine serum albumin as a reliable model. The use of the SPR response has been demonstrated to be a notably powerful instrument to determine the accessibility of amine-reactive sites on biochip surfaces. The SPR response from BSA, which was chemically bonded to the prepared biochip under various vapour-phase silanisation conditions, is shown in Table 1. The silane films contained identical amounts of amine in their surface regardless of the conditions in which they were produced. The amine accessibilities of the silane films that were produced in the aforementioned conditions are approximately similar. This table also demonstrates that the silanisation with 5% concentration silane produced the smallest quantity of amine. These answers reveal that all of the chips can bind to biomolecules.

3.7 Stability Tests

For SPR sensors, the modified chips must withstand severe chemical treatments in some cases. One example is the generation of surface silanol groups, which are required for silane-coupling chemistry using a brine treatment.

Here, the effect of a salt solution on the stability of the silver chips was investigated. A simple test was used to verify the stability of the silver films (Table 1). When the sample was immersed in a brine solution, the sputtered silver film onto the glass substrates was rapidly removed from the chip. When the chips were prepared with 5% silane, the detachment of the silver layer occurred after only 6 min; with 100% silane, the stability was higher, as the detachment occurred after 33 min. This variation can be attributed to the change in thickness and structure of the SiO_x film.[21]

4. CONCLUSION

It has been shown that stable silica layers on thin silver films can easily be produced using the vapour-phase technique. The resulting materials were characterised using SPR and AFM measurements. The Ag/SiO*x* substrates were stable in NaCl solution. Comparing various studied silanisation conditions (different times, temperatures and APTES concentrations), it is concluded that silanisation at 80°C for 30 min is a simple and effective method to perform protein immobilisation on silver-coated glass slides, which has potential applications in the production of protein chips and biosensors.

5. REFERENCES

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