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EFFICIENT PROBE IMMOBILIZATION ON POLY (DIMETHYLSILOXANE) FOR SENSITIVE DETECTION OF PROTEINS

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1. ABSTRACT

Chemical surface modification methods were investigated to activate surface of poly-(dimethylsiloxane) (PDMS) for probe immobilization in enzyme-linked immunosorbent assay (ELISA). The investigations started from dramatization of PDMS surface with (3-aminopropyl)-triethoxysilane (APTES). Amino groups generated by APTES were either derived into carboxyl group by Succinic acid anhydride (SAA), then captured the protein through the heterobifunctional cross-linker, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), or directly reacted with homobifunctional cross-linker, glutaraldehyde (GA). Rabbit IgG, Goat IgG, Human IgA and Rat IgG were covalently immobilized on PDMS by both methods, and examined through well-organized competitive inhibition ELISA. The results demonstrated that both GA and EDC based methods had good covalent immobilization capability, and the EDC method had higher efficiency than the GA method. The competitive ELISA with probe immobilization through EDC had a detection limit of pg/ml level. The method was proved to be applicable for immobilization of different proteins. The topography of the immobilized protein properties was studied by atomic force microscope (AFM), demonstrating that the immobilization by GA had protein conglomeration, resulting in poorer uniformity and lower immobilization efficiency than EDC method. The possible reason is protein inter-molecule crossing linkage by the homobifunctional group of GA. Due to its simplicity, low cost, and high immobilization efficiency, EDC based immobilization method could provide great potential for making ELISA protein chips based on PDMS.

2. INTRODUCTIONS

PDMS is a substrate widely used for construction of plastic microfluidic devices. PDMS offers rapid prototyping, biocompatibility, optical transparency, and gas permeability (1). Multifunctional 3-D PDMS microfluidic devices can be fabricated through inexpensive molding and patterning process (2, 3). However, PDMS has a number of drawbacks that hinder its uses in certain applications. One of them is its hydrophobic property of PDMS, which captures protein or cells by the hydrophobic interaction between biomolecule and PDMS (4). This would lead to fouling of PDMS-based microstructures, even damaging the sensor's function (5). Thus, device performance can be significantly improved by reducing the hydrophobic interaction through surface modification. Methods for surface modification have been reported to generate a hydrophilic surface (6, 7), which include polymer grafting, chemical fictionalization, polyelectrolyte coating, and plasma oxidation. Introduction of reactive groups on the PDMS surface by blending or copolymerizing PDMS with a styrene and acrylonitrile could produce a hydrophilic functional surface (8). Irradiation has been reported for PDMS surface modification, but they involve multiple steps and long preparation time, and could alter the transparent optical properties (9). PDMS surface can be oxidized with oxygen plasma to become hydrophilic since high energy surface is more easily wetted by polar liquids than native, hydrophobic PDMS. However, physical damages of PDMS surface from the method often occur and result in surface "cracks" (10). Further, the hydrophilic surface after oxygen plasma treatment is not stable and can revert to its original hydrophobic status sometimes within an hour (11, 12). Apart from those approaches, adsorption of polymers or biomolecules on the PMDS surface, followed by covalent immobilization of desired biomolecules on the adsorbed material, such as three-layer biotin-neutravidin sandwich coating, polyelectrolyte multilayer has been reported (13-17). However, the method is tedious and not efficient. There is need to develop a simple and efficient method for protein immobilization on PDMS.

Surface modification by a silanization reaction is used in numerous fields of bionanotechnology, especially in the field of biomaterial, where cellular adhesion and proliferation often need to be promoted for better biocompatibility (18-21). Up to date, most of the silanization studies are focused on glass and silicon. In the case of 3-aminopropyltriethoxysilane (APTES) modified glass, the reaction is self-catalyzed by the amine group of APTES (22). The reaction is attractive because it offers a chemical link between the silane and the glass surface, not a physical coating of molecules on the surfaces as in other surface immobilization approaches (22). The covalent immobilization of proteins on PDMS has not been carefully investigated for ELISA applications.

In our work, APTES was used to graft a silane film on PDMS surface. Both homo- and hetero-bifunctional cross-linkers were studied for covalently immobilizing proteins via its amine groups. Due to the abundant amine groups on PDMS-APTES surface, the SAA was used to generate carboxyl groups on the PDMS-APTES surface, and then –COOH group serves as the anchor to tether probe proteins. The immobilization efficiency for different methods was examined through heterogeneous immunoassay, an inhibition competitive ELISA method. The topographies of the solid PDMS surfaces with different immobilization approaches were studied by AFM to further characterize the immobilization effects.

3. METERIAL AND METHODS

3.1. Material

Goat IgG, rabbit IgG, rat IgG, human IgA, Anti-rabbit IgG peroxidase conjugate, monoclonal anti-goat IgG alkaline phosphatase conjugate, monoclonal anti-human IgA (α-Chain specific), anti-Rat IgG peroxidase conjugate, Tris-HCl, anti-mouse IgG peroxidase conjugate, bovine serum albumin (BSA), human serum albumin, chicken serum albumin, alkaline phosphatase yellow (pNPP) liquid substrate, and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from Sigma, USA. 3-aminopropyltriethoxysilane (APTES), succinic acid anhydride (SAA), glutaraldehyde (GA), sylgard 184 silicone elastomer (PDMS monomer) and its curing agent, Blocker^{TM} Casein in PBS and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Pierce, Germany. The deionized water used in all experiments was produced by a water purification system, Q-Grad ®1, from Millipore Corporation, USA. All other used chemicals were of analytical grades and obtained from common commercial supplies.

3.2. Apparatus

Nanoman atomic force microscope (AFM) with a dimension 3100 scanning probe platform from Veeco metrology group, USA was used in studies of the surface topography of immobilization on PDMS surface. GENios plus purchased from Tecan, USA was used as ELISA reader in all competitive ELISA experiments.

3.3. Experimental

3.3.1. Preparation of PDMS-APTES solid-substrate

A curing agent and PDMS monomer from Pierce, Germany, were thoroughly mixed in a 1:10 weight ratio. To remove air bubbles from the precursor mixture, vacuum was applied for 1hour. Then the mixture was cured for 2 hour at 80 □. Rinse the cured PDMS by absolute ethanol. After drying, the cleaned PDMS was immersed in 10% v/v APTES in absolute ethanol for 10 min. at room temperature. The APTES treated PDMS were rinsed by 96% ethanol and followed by air-dry, then heated at 80 □ in a vacuum oven for 2 hours. The resulted PDMS-APTES substrates were stored in a clean box until use for avoiding contamination. The surface modification process to form PDMS-APTES is schematically shown in Figure 1.

3.3.2. Protein immobilization on PDMS

3.3.2.1. Covalent immobilization via EDC

First, SAA was used to modify the APTES for producing carboxyl groups on the surface with succinic anhydride through the following procedure. 100 μg solid

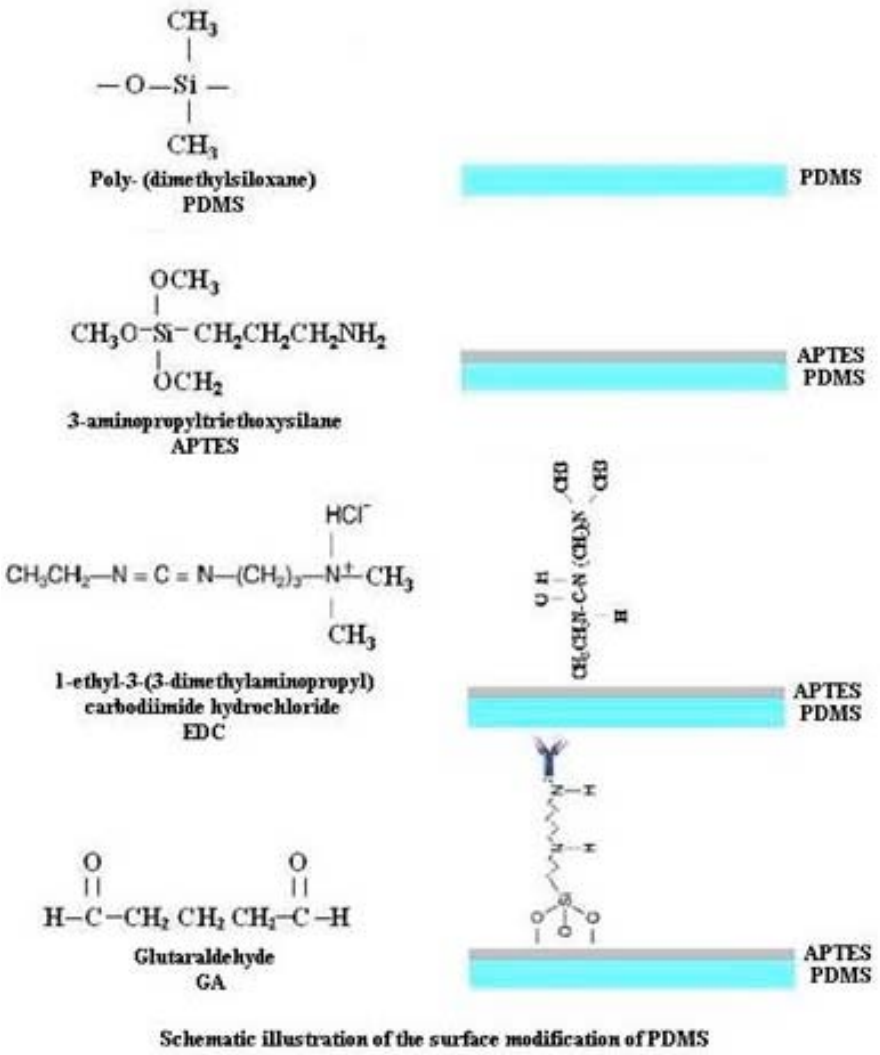

Figure 1. Schematic illustration of surface modification of PDMS.

SAA was dissolved at 2 ml pure water. The pH of the solution was maintained at 6.0 by addition of 3 M NaOH for continuous adjustments while the SAA dissolving. Then PDMS-APTES substrate patch was immersed in the SAA solution. The reaction was allowed for 2 hours at room temperature. Then the patch was rinsed by phosphate-citrate buffer and dried under nitrogen flow. EDC and protein mixture were dropped onto the modified solid surface. The concentration of the EDC used was 20μg/ml; the protein concentrations used in experiments are listed in table 1. The reaction buffer was 0.01M, pH4.6 phosphate-citrate buffer. The cross-linking process was kept for 1 hour at room temperature, and then Tris-HCl buffer was used to stop the crosslink reaction. Immersed the protein coated surface in 1% BSA for either 2 hours at 37□ or overnight at room temperature in attempt to block non-specific protein adsorption. Store protein coated patches in sealed box at 4□ for usage within 7 days.

3.3.2.2. Covalent immobilization via GA

In this approach, APTES treated PDMS surface was modified by GA to covalently immobilize protein on PDMS surface. The amino groups on the surface were activated by 2.5% GA for 1 hour at room temperature. Carefully rinsed the surface by pH 8.0 Tris-HCl buffer, and dried by nitrogen flow. Protein probes in Tris-HCl were added onto the glutaraldehyde-activated surface (table 1). The cross linking reaction took place at room temperature for 2 hour. After washing, the protein-coated patches were surface-blocked by the method described at 3.3.2.1.

3.3.2.3. Passive protein adsorption on PDMS-APTES surface

The protein probe solution was diluted by coating buffer, pH8.0 Tris-HCl to an appropriate concentration. APTES treated PDMS substrate was directly immersed in the probe solution for surface adsorption. The adsorption

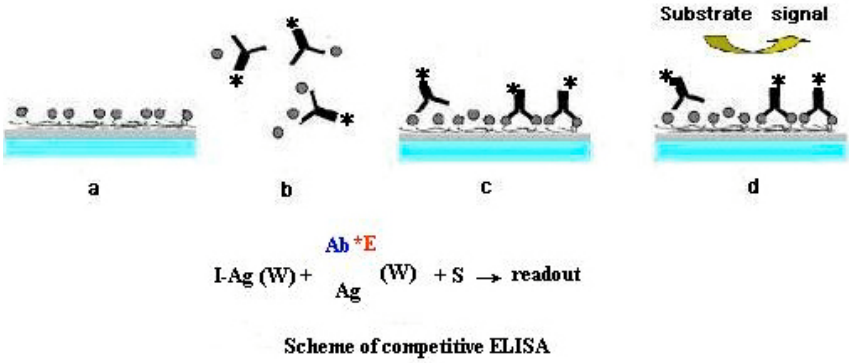

Figure 2. Schematic illustration of surface modification of competitive inhibition ELISA (a) is the protein coated solid surface. (b) The reaction mixture of enzyme labeled antibody and protein. The protein here competes with the immobilized protein for the antigen-binding site. (c) Enzyme labeled antibody react with the immobilized protein; free protein and labeled antibody were washed off. (d) Immobilized enzyme catalyzes substrate developed color. The high the absorbance is, the more the enzyme was immobilized, the less protein in the reaction mixture in Figure 2b.

Table 1. the parameters of the competitive inhibition ELISA

		Rabbit IgG	Rat IgG	Goat IgG	Human IgA
Coating Protein Concentration	GA	11.2 μg/ml	11.1 μg/ml	11.1 μg/ml	20 μg/ml
	EDC	1.12 μg/ml	1.1 μg/ml	1.1 μg/ml	2 μg/ml
	PA	11.2 μg/ml	11.1 μg/ml	11.1 μg/ml	20 μg/ml
Competitive Protein Concentration		From 5.6 μg/ml to 5.6 pg/ml in 10 fold dilution	From 5.5 μg/ml to 5.5 pg/ml in 10 fold dilution	From 5.5 μg/ml to 5.5pg/ml in 10 fold dilution	From 1 μg/ml to 1pg/ml in 10 fold dilution
Enzyme-labeled antibody [1]		1:20,000	1:20,000	1:10,000	1:10,000

[1] The enzyme-labeled antibody in Rabbit IgG, Rat IgG and Human IgA is peroxidase conjugated. In goat IgG, it is alkaline phosphatase conjugated.

reaction was performed for 1 hour at room temperature. Then rinsed the surface by washing buffer (Tris Buffered Saline (TBS) plus 0.05%BSA). Protein-coated surface was then blocked by using the method described in 3.3.2.1.

3.3.3. Immunoassay method

The immunoassay conducted in all experiments is based on the competitive inhibition ELISA. In the competitive scheme, antigen was immobilized on the solid surface. During the test, the sample contains two kinds of proteins. One is the enzyme-labeled antibody, which can specifically react with the immobilized antigen. Another one is the protein as same as the immobilized antigen. Therefore, immobilized protein and the protein in the sample is a competitive pair for antigen binding sites of enzyme-labeled antibody. As the concentration of the competitive protein in the sample increases, the amount of enzyme-labeled antibody, which could be captured by the immobilized protein, decreases. With the addition of the color reagent solution, the amount of captured enzyme-labeled antibody is detected, resulting in an inverse relationship between absorbance and concentration: the higher the absorbance, the less target protein in the sample. Schematic competition method is shown in Figure. 2

3.3.4. AFM characterization

The measured samples were prepared by covalently immobilize protein onto PDMS-APTES solid surface or by passively adsorption onto the PDMS-APTES and nature PDMS solid surface. Those protein immobilization methods were the same as described at 3.3.2 except the blocking step. Operation was taken place in ambient environments. Tapping Mode AFM was operated to investigate the topography of different conjugated PDMS surfaces.

3.4. Data Analysis

Absorbance curves were obtained by plotting absorbance against the logarithm of analyte concentration by Origin 6.0. The inhibition degrees were measured by the inhibition percentage, which was derived from the absorbance, against the logarithm of analyte concentration. The inhibition rate can be expressed as Inhibition percentage (%) = [(An-Ax)/An] ×100% where An is the absorbance of the negative control, in which there is no competitive protein in the reaction system. Ax is the absorbance value of tested spots which correspond to different competitive protein concentration.

4. RESULTS AND DISCUSSION

4.1. Effect of blocking formulations

Blocking reagents were investigated to reduce the non-specific binding on PDMS-APTES surface. Six blocking formulations, 1%BSA, 1% human serum albumin, 1% chicken serum albumin, 0.5% BSA + 0.5 % casein, 3% BSA, and 1% casein, were studied. Inhibition competitive ELISA was performed to evaluate the blocking effects.

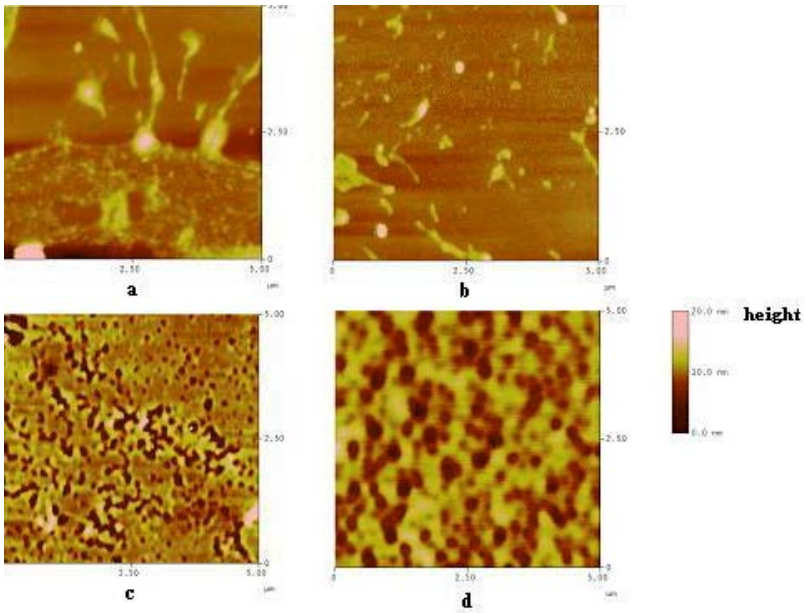

Figure 3. AFM images (5×5 μ m) of PDMS: (a) Tapping mode AFM height images of protein passively adsorbed on to PDMS surface, (b) Tapping mode AFM height images of protein passively adsorbed onto PDMS-APTES surface, (c) Tapping mode AFM height images of GA immobilized protein (rabbit IgG), and (d) Tapping mode AFM height images of SAA/EDC immobilized protein (rabbit IgG).

Experimental results demonstrated that the best blocking capability on PDMS solid-surface was given by 1%BSA in TBS buffer as the blocking formulation. It is known that high temperature accelerates biological interactions, and is often used to reduce the reaction time of biological or chemical reactions. Incubation times for all blocking formulations were tested for either 2 hour at 37^0C or overnight at room temperature, respectively. The results demonstrated that the block experiments with both conditions generated similar blocking performance. Thus, for reducing assay time, the blocking formulations used in all other immunoassay experiments were 1% BSA at 37□ for 2h.

4.2. Optimization of competitive ELISA on PDMS

The saturating conditions were first determined by a non-competitive two-dimensional titration method over a range of concentrations from 0.112 to 1.12 mg/ml. Then, concentrations in a narrow range from 1.12 pg/ml to 112 μg/ml were examined by competitive assays. In all cases, the minimum absorbance (background signal) obtained with excess of analyte was approximately zero. The negative control had no competitive protein in all tests. The parameters of the competitive inhibition curves for each combination tested are shown in Table.1

The influence of the off-spot incubation time of the competitive mixture was first investigated for incubation time effects. Before transferring the competitive mixtures onto the PDMS solid-surface, different off-spot incubation times were studied for 10, 20 and 30 minutes. The experimental results showed that off-spot incubation time had insignificant effect on the assay results. This indicated that the competition degree was mainly determined by the amount of the competitive protein, not by the off-spot incubation time. The second time factor investigated was the time of the competitive mixture reacted with the immobilized probe protein. The reaction times for 15, 30, 45, 60, 75, to 90 minute were tested, respectively. During the tests the optimized enzyme-labeled antibody concentration (1:20,000) was employed and the protein immobilization solution used in all experiments was 11.2μg/ml. During the tests the optimized enzyme tracer concentration was employed while keeping a constant concentration of the target protein that was the same as the immobilized protein on the PDMS surface. Experimental results showed that 45 minutes reaction time had the lowest detection limit for the competitive step.

4.3. Studies of protein immobilization by AFM

The samples for AFM characterization were prepared according to the method described in experiment section 3.3.2. 11.2μg/ml rabbit IgG solution was used for protein immobilization onto PDMS surface. Figure 3 (a) and (b) are the AFM images of protein passively adsorbed onto the solid surface, while figure 3(c) and (d) are the AFM images of protein covalently immobilized onto solid surface. Figure 3(a) shows the tapping mode AFM height image of protein passively adsorbed on to PDMS surface, which was the surface after incubation in 11.2μg/ml rabbit IgG solution for 1h at ROOM TEMPERATURE. Although protein could be observed on the solid surface, but significant conglomeration dominates the whole surface morphology. This is mainly due to the highly hydrophobic PDMS surface that was ready to interact with the hydrophobic groups of the protein. The agglutination of the proteins could either denature or impair the function of PDMS based structure. Figure 3(b) is the result of protein passive adsorption onto APTES-PDMS surface. Apparently, there is no significant protein immobilization

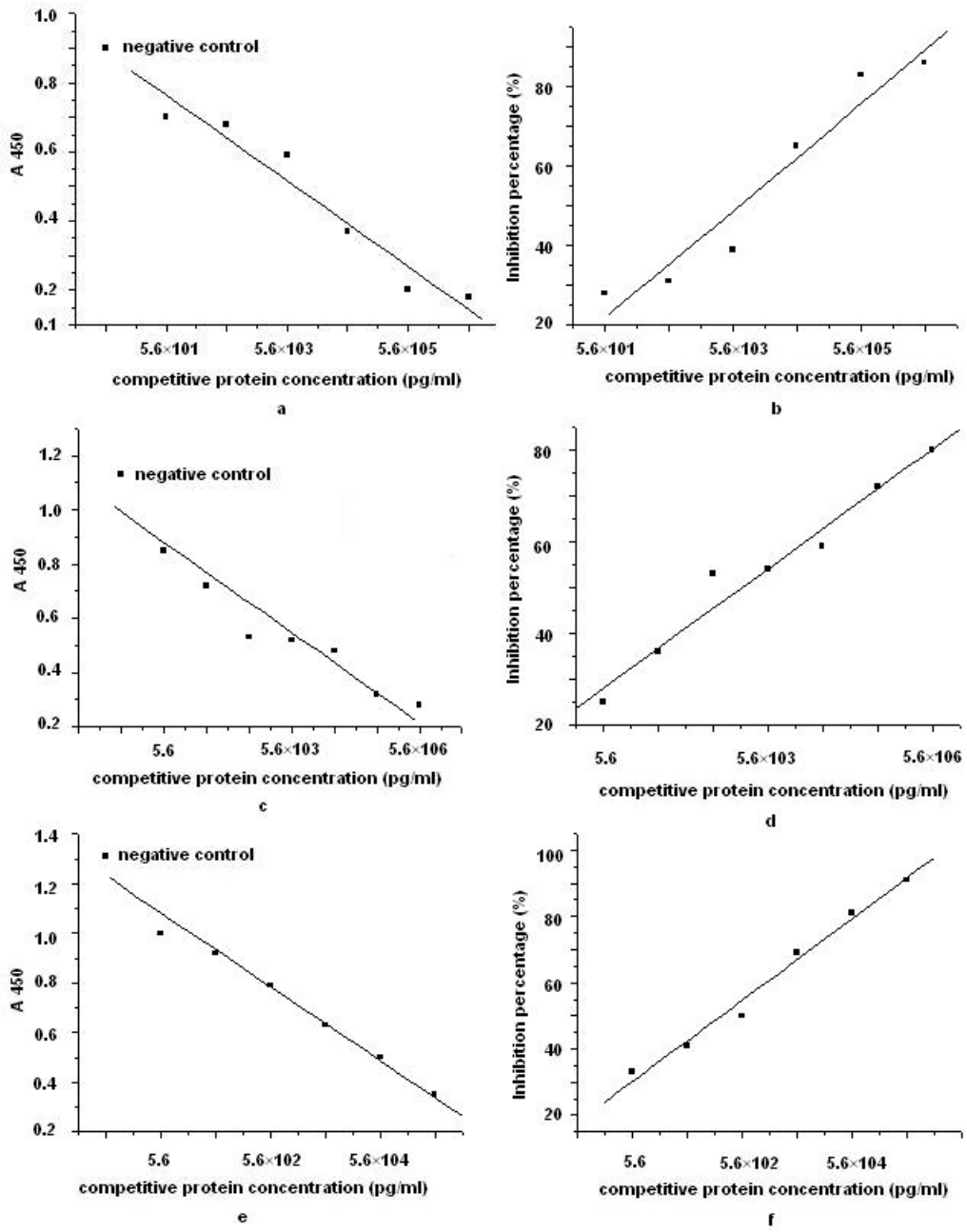

Figure 4. Inhibition competitive ELISA results obtained from protein immobilized PDMS: (a, b). Rabbit IgG passively adsorbed onto APTES-PDMS surface; 4(c, d). Rabbit IgG covalently immobilized by GA on PDMS; 3(e, f). SAA/EDC cross-linking PDMS surface, where the coating protein concentration, 1.12 μg/ml is 10 fold lower than GA immobilization.

observed, indicating poor protein immobilization capability in the direct passive protein adsorption on APTES-PDMS substrate. APTES grafting generated a hydrophilic thin layer on the PDMS surface. This not only changed the surface property of the PDMS, but also provided function group, amino groups, for future chemical modification. In Figure 3 (c) and (d), the morphology characteristics are obviously different from 3(a) and (b). More protein immobilization could be observed on the solid surface in both 3 (c) and (d), in which the protein were covalently immobilized on the APTES-PDMS surface. In 3(c), where GA served as the cross-linker, the image roughness was at the medial level showing that the protein immobilization was not uniform, and was stacked at some places. This could be caused by the internal protein-protein crosslink generated by GA, which has two identical reaction sites as a homobifunctional cross-linker for reacting with the amino group, resulting in internal protein-protein cross-linking for leading protein conglomeration. By comparing 3(c) with 3(d), it shows that the immobilized protein uniformly

distributed on the APTES-PDMS solid surface. The AFM morphology demonstrates that SAA generated sufficient carboxyl groups to react with the heterobifunctional cross-linker EDC, which could react with the amino group of the protein and carboxyl groups on the solid surface for higher surface concentration and more uniform immobilization. Apparently, protein internal cross-linking lost its binding sites for enzyme-labeled antibody or indicator protein interactions, resulting in lower sensitivity. AFM based experimental results show that SAA/EDC immobilization method is more efficient than GA method to tether protein while avoiding the protein internal cross-linking. This also demonstrates that AFM is a powerful tool to directly investigate biomolecule interactions at a molecular scale.

4.4. Immunoassay results

With the optimized reaction condition of immunoassay, the immobilized rabbit IgG, rat IgG, goat IgG and human IgA on PDMS solid-surfaces were studied. Figure 4 shows typical absorbance curves obtained from the competitive immunoassay. In Figure 4, the curves represent the absorbance changes measured with rabbit IgG immobilized by different methods. Similar experiments were also conducted with Rat IgG, goat IgG and human IgA, and similar results were obtained. The absorbance changes correspond to captured quantity of the enzyme-labeled protein by the immobilized protein on the patch surface, while **t**he inhibition percentage was directly derived from the data of absorbance. Figure 4(a) shows the results obtained from the immobilization by the passive adsorption method and the absorbance value is lower than that obtained from the covalent immobilization method; 4(b) shows that the lowest limit concentration detected was 56 pg/ml with 30% inhibition. Figure 4(c, d) show the results from the rabbit IgG immobilized by GA. In the test, the protein immobilization was conducted with 11.2 μg/ml rabbit IgG solution. There is a linear relationship observed in the absorbance curve (Figure 4(c)). 4(d) clearly shows that the lowest protein concentration detected was 5.6pg/ml with 27% inhibition. Figure 4(e, f) show the results obtained from EDC based immobilization method. The immobilization protein concentration employed in the experiments was 1.12 μg/ml, which was 10 fold lower than GA immobilization. The absorbance value obtained was higher than that with the GA based method. The lowest protein that was distinguished from the test was 5.6 pg/ml, which is not inferior to the GA based immobilization method.

The absorbance of the enzymatic converted substrate is proportional to the concentration of the enzyme-labeled antibody. Due to the specific reaction between the antibody and antigen, we can deduce the protein immobilization concentration by the concentration of the captured enzyme-labeled antibody. Therefore, the immobilization capability of different methods can be evaluated by ELISA. The results described above show covalent immobilization methods with GA and SAA/EDC on the PDMS-APTES surface are superior to the passive adsorption method. The results obtained with the immunoassay well agree with those observed using AFM topographies on different PDMS solid surfaces for protein immobilization.

5. CONCLUSION

Through investigation of different immobilization methods, SAA/EDC based protein immobilization method was demonstrated to be the simplest and most effective approach to attach probe proteins on PDMS solid surface for sensitive protein detection. In this method, fewer chemicals were used and less experimental steps were required in comparison to existing PDMS modification methods. Both AFM and competitive immunoassay proved that the SAA/EDC immobilization method is more efficient than GA method. AFM studies further showed that the poorer protein immobilization efficiency of the GA based method was caused by protein-protein conglomeration or self cross-linking. This is possibly due to the homobifunctional cross-linker of GA, which has two identical functional groups to react with the primary amine residues of proteins, readily leading denature of immobilized proteins. However, EDC is a heterobifunctional cross-linker with two different reactive groups that allow both sequential (two-step) and one-step conjugations for higher immobilization efficiency.

Experimental results showed that the detection limit with the simple and efficient protein immobilization method was reached at pg/ml level. This detection limit is sensitive and could have wide applications in clinical diagnostics.

PDMS is an important substrate to construct plastic microfluidic devices for lab-on-chip systems. ELISA is the most sensitive and reliable diagnostic method for different disease recognition. The surface immobilization method reported here could bridge PDMS and ELISA, and provides a very promising approach to build a PDMS based ELISA lab-on-chip system for clinical diagnostic applications.

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