Surface Enhancements of Polymer-Based Microfluidic Enzyme-Linked Immunosorbent Assay

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Introduction

Due to the high selective molecular recognition and high sensitivity of the immunoassay system, assays via antibody-antigen interaction have drawn a great deal of interest. Enzyme-linked immunosorbent assay (ELISA) is the most commonly used method among various immunoassays. It has been developed for detection and quantification of a wide variety of materials in the biotechnology and clinical medicine, including diagnostics assays, environmental monitoring, and food safety testing. However, the tedious and laborious protocol involving a series of mixing (reaction) and washing steps used in the conventional (manual) ELISA, which is typically carried out in a 96-well microtiter plate, often results in large errors and inconsistent results. A better approach is to use novel microfabrication technologies to develop low-cost microfluidic ELISA devices with automatic and reliable (precise) liquid handling functions.

The integration of immunoassays on microchips have been shown to have great advantages of higher specific volume, lower sample and reagent consumption, reduced assay time[1-7]. In designing a biosensor, controlling surface properties is one of the most important issues. From an operational perspective, the surface activity of immobilized antibodies determines the performance of an immunoassay microdevice. To achieve high sensitivity and reliable result, not only a high number of active protein (enzyme or antibody) molecules needs to be immobilized on the microchip, non-specific protein binding that interferes with the reaction must be minimized as well. Various approaches have been developed for binding biomolecules to the surface of the microfluidic biosensors, which are made of silicon, metal or glass. The polymer-based microdevice is an attractive alternative because of its biocompatibility, optical clarity, high impact strength and feasibility of mass production. However, compared to the well-established surface modification methods for glass substrate, the development of surface treatment for polymeric materials is still in its infancy. Sato et al. developed an immunoassay system with antigen passive adsorbed onto polystyrene microbeads embedded inside lithographically patterned mcirochannels[3]. Yang et al. presented a microfluidic poly(dimethylsiloxane) (PDMS) immunoassay system with lipid layers modified surface and dinitrophenyl (DNP)-conjugated lipid providing the binding sites for the bivalent anti-DNP

antibodies[8]. They also used biotinylated phospholipids bilayers to provide binding site for streptavidin-conjugated alkaline phosphatase[1]. Some other researcher adsorbed protein A onto solid surface and bind antibody molecules to the surface via specific recognition of these two molecules[6, 9].

In our previous work, we have demonstrated the feasibility of integrating CD microfluidic biosensor with ELISA [10]. However, the direct adsorption of antibodies to the polymer surface without surface modification yielded low sensitivity and high non-specific adsorption[9]. Our studies have shown that antibodies bind poorly to the poly(methyl methacrylate) (PMMA) surface. In order to address this issue, we have developed a novel method to functionalize the PMMA surface with amine group via poly-ethyleneimine (PEI), and antibodies can be readily immobilized on this modified surface via a crosslinker, glutaraldehyde. Our results showed that the modified surface can possess the binding capacity of antibodies about 4 times more than untreated surface. Furthermore, the surface modification was through the covalent binding, which is more stable than the physical adsorption and, hence, substantially improved the performance consistence of the CD-ELISA device. It showed that the prototype CD-ELISA microfluidic device greatly reduced the reagent use, shortened the assay time, improved the assay range, while maintained similar sensitivity as those from the conventional 96-well plate.

Materials and Methods

Regents. Rat IgG was the 'analyte' used in these studies. The first antibody used is a polyclonal affinipure goat anti-rat IgG (H+L). The second antibody used for detection is horseradish peroxidase-conjugated Affinipure Goat anti-rat IgG(H+L). The first antibody and second antibody were purchased from Jackson immno labtorary. Dulbecco's phosphate buffered saline with pH 7 was from Invitrogen Life Technologies (Carlsbad, CA), Polyoxyethylenesorbitan monolaurate (Tween-20) from Bio-Rad, Bovine serium albmine from , Sodium meta-periodate from Pierce, NAP-10 column from Pharmacia, and the other chemicals were from Sigma Aldrich.

Microchannel fabrication:

Microchannels with 140 μm in width, 125 μm in depth, and 1.5 cm in length were microfabricated on polymethyl methacrylate (PMMA) substrate using computer numerical control (CNC) machine. Polydimethylsiloxane (PDMS), which was prepared by thoroughly mixing 10:1 wt% of base and curing agent and degassed under vacuum for 30 min, was poured over the microchannels and cured in an oven at 70°C for 1.5-2 hours. The cured PDMS was peeled off from the mcirochannels and used as the mold to emboss PMMA substrate, yielding PMMA microchannels. .

PMMA surface treatment:

Poly-ethyleneimine method: PMMA chips with microchannels were first treated in 1M NaOH solution at 55°C for 30mins. Then, they were immersed in 0.2% PEI at room temperature for 1 hour, followed by 1% glutaraldehyde treatment at room temperature for 30mins. The chips were thoroughly rinsed with distilled water between the steps. Finally, the treated microchannels were rinsed, air blow dried, and ready for antibody binding.

Hexamethyl diamine method: This method has been described by Fixe et al. [11] Briefly, the PMMA chips with microchannels were incubated with 10% hexamethyl diamine in 100mM borate buffer, pH 11.5, for 2 hours. Then, they were immersed into 1% glutaraldehyde solution for 30 mins. The chips were thoroughly rinsed with distilled water between the steps. Finally, the microchannels were rinsed, air blow dried, and ready for antibody binding.

Sandwich type Immunoassay system setup:

The buffer solution: PBW washing buffer (PBS, 0.1% Tween 20, 0.5% BSA), blocking buffer (PBS, 0.1% Tween 20, 1% BSA, sodium azide), Tris-HCl buffer solution were prepared in our laboratory. The substrate HPPA (3 mg/mL) was prepared in a 0.15 M Tris-HCl buffer solutin (pH8.5). One microliter of 30% hydrogen peroxide was added to every 7.5 mL of HPPA solution and mixed thoroughly prior to use. The concentration of first and second antibody used was 10 μg/ml without further notification. The time response of the enzymatic reaction of HPPA was recorded. The fluorescence intensity of the enzymatic reaction product was used as an indicator of the extent of the enzymatic reaction and monitored as a function of time. The Unit of RFU/min was utilized to indicate the enzyme amount and related to the amount of antigen bound to the surface.

ELISA procedure in 96-well plate: 100 μL solution was added into wells for the first antibody, blocking solution, antigen solution, second antibody solution, and substrate solution. Three times of 200 μL of washing solution were added into well and washed the individual wells. First antibody was incubated in the well at 4°C overnight. The other incubation steps were done at room temperature for 3 hours or at 4°C overnight. The reaction was detected with a CyFluor 96-well plate fluorescence reader.

ELISA procedure in microchannel: A single microchannel was utilized to study the ELISA assay. 1 μL of the same concentration of first antibody, antigen, and second antibody as those used in the 96-well plate assay was applied. Three times of 10 μL of washing solution was added between the steps to ensure complete washing. The detection was carried out using an inverted fluorescence microscope (Nikon Ecllipse TE2000-U). A 100-W mercury light source with a 335/20 nm filter was used as the excitation source. The fluorescence emission signal was obtained through a dichrooic mirror and a 405/40 nm filter. Images were recorded with a 12-bit high-resolution monochrome digital camera system (CoolSnap HQ) and the intensity of fluorescence was analyzed using the Fryer Metamorph Image Analysis System.

AFM image: All AFM experiments were performed in tapping mode. AFM images were obtained using the 'height mode'. To obtain the best imaging conditions, the applied force was minimized and stabilized by adjusting the height of cantilever during scanning of the sample surface.

Antigen binding Kinetics study: Antigen binding kinetics was studied with 100 ng/mL antigen added to microchannel and incubated at various time periods. Excess second antibody (horseradish peroxidase conjugated antibody) was then added and incubated for 1 hour. The final enzyme activity expressed as RFU/min was measured to quantify the amount of antigen binding to surface at various incubation times.

Antibody modification with sodium meta-periodate: Weighed out 2.5 mg of sodium meta-periodate in a 1 ml of amber microcentrifuge tube. Added 2.4 mg/ml first antibody solution 0.5 ml into the amber tube containing sodium met-periodate and gently swirl the tube till the powder is dissolved. Incubate the sample for 30 minutes at room temperature. Then, apply the sample to a pH7.4 PBS equilibrated NAP-10 column and elute with this pH 7.4 PBS buffer. Collect ten 0.25 ml fractions and analyze the protein concentration of these fractions. Collect the fractions with protein in it, aliquot, and stored at -84 °C. The protein concentration of the treated and untreated antibody was analyzed by the method of Bradford[12]. The concentration of the treated antibody was then calculated using the untreated antibody as the standard. 10μg/mL of the treated antibody was applied to the PEI modified microchannel directly. Same concentration of the untreated antibody was applied to the microchannel after glutaraldehyde treatment. The following steps were the same as we described in the microchannel ELISA procedure. 0.1 μg/mL and 10 μg/mL of antigen were applied to both untreated antibody binding microchannels and treated antibody binding ones.

Results and Discussion

Effect of surface treatment on the ELISA performance:

The enzyme reaction signal obtained from different kinds of surfaces was examined. According to our results, low reaction signal was obtained with passive adsorption under both low and high antigen concentrations. The reaction rate was about 4 times faster in the chemically modified microchannels. Because the enzyme reaction signal is directly related to the antigen bound to the surface, this result showed the efficiency of first antibody binding. Protein passive adsorption is caused by the hydrophobic and hydrophilic interaction. Since the hydrophobic domains are buried inside the protein at physiological conditions, when exposed to hydrophobic surface, protein molecules undergo conformational change and expose its hydrophobic core, with which the protein molecule attaches to the surface. During this process, some of the molecules will lose their native activity.

Two chemically modification methods were studied and compared. Our results have shown

that PEI method gave slightly higher reading than the HMD method. The HMD method presented by Fixe et al. was reported to have a surface amine density of 0.28 ± 0.03 nmol NH_2/cm^2. Overall diameter of IgG antibodies has been estimated from AFM image to be between 7 and 10 nm[11]. The molecular weight of the IgG antibody is 160kD. Therefore, a densely packed antibody layer has the surface density of 0.00432 nmol antibody/cm^2 and the primary surface amine obtained with this aminolysis method is in excess of the amine needed for the antibody binding. With PEI modification method, the layer of PEI molecules increased the distance between the antibody and surface, thus providing more freedom and availability of antibody on the surface.

We also studied the antibody binding with AFM. AFM images of the plain PMMA surface and PMMA surface with antibody binding are quite different from each other. We also investigated the effect of the orientation of antibody on the performance of immunoassay. Antibodies are glycoproteins with polysaccharides residules on the Fc fragment. Sodium meta-periodate cleaves adjacent hydroxyl groups in sugar residues to create highly reactive aldehyde functionalities. Because the sugar residues are located on the Fc fragment, the aldehyde functionalities are far from the binding site and can be directed onto surface with amine group. The results showed that at lower concentration of antigen, untreated and treated antibodies yielded similar final signal. However, at high antigen concentration, treated antibody showed higher signal, implying that the modified antibody has better orientation and thus has higher surface activity for the antigen bindind.

Comparison of traditional 96-well assay and microchip assay:

According to our experiment, the 96-well assay has a linear range of 2 ng/ml to 100 ng/ml, whilethe microchannel assay from 5 ng/ml to 1000 ng/ml. Hence, the microchannel has a wider detection range of the immunoassay.

Antigen binding kinetics:

The antigen binding kinetics at different temperature was further investigated. Rapid adsorption occurred and reached saturation after 20mins' incubation at 30°C. The adsorption is slower at 22°C, with more than 30mins to reach the saturation.

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