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Some Key Factors in a Bead-based Fluorescence Immunoassay

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Abstract

In an effort to understand some key factors in a bead-based fluorescence immunoassay, a series of sandwich-type immunoassays were carried out on a glass plate using a latex bead embedded with dye molecules. The fluorescence bead functionalized with an amine functionality was conjugated with an anti-prostate specific antigen (anti-PSA) using glutaraldehyde. The glass surface was functionalized with the amine group using 3-aminopropltriethoxysilane (3-APT). The amine functionality on the glass surface was converted to aldehyde using glutaraldehyde followed by conjugation with the anti-PSA. A series of sandwich-type immunoassays were carried out by incubating the anti-PSA bead with PSA followed by incubating the PSA/bead complex on the anti-PSA glass surface. The dependency of the fluorescence intensity in the immunoassay was observed to increase when the fluorescence bead concentration was increased from 10^{-3} mg/mL to 10^{1} mg/mL with some signs of saturation around the range of 10^{0} mg/mL to 10^{1} mg/mL. Also, the fluorescence intensity was observed to increase when the incubation time of the PSA /anti-PSA bead complex on the anti-PSA glass was increased from 5 minutes to 60 minutes. The shear force generated during the washing step was observed to affect the bead-based immunoassay.

Keywords: Bead-based immunoassay, Dye-embedded particle, Fluorescence bead, Shear force

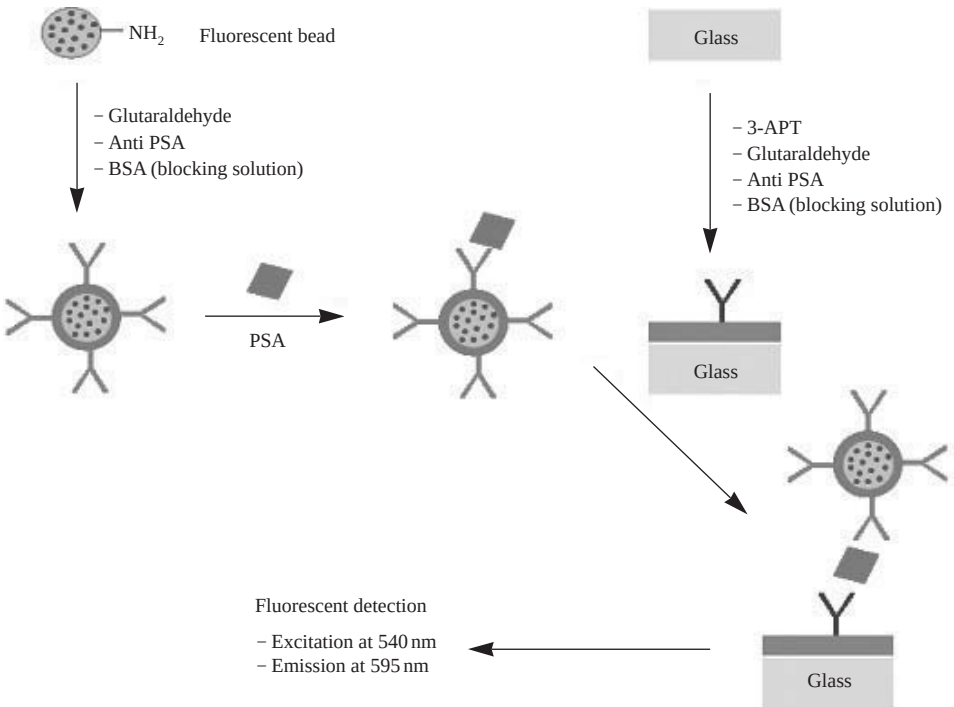
Introduction

Successful incorporation of an immunoassay system on a fluidic chip may provide a facile alternative to the complicated diagnoses now practiced in hospitals. Much research has been concentrated not only on developing specific part of fluidic diagnosis system but also on the total integration of each part[1-3]. A bead-based fluorescence immunoassay is one of the major interests due to such advantages as high fluorescence intensity and resistance to photobleaching[4-6]. Recent developments in the fields of nanoparticle synthesis[7] and surface chemistry[8] have also contributed to the interest and development of the particle-based immunoassay.

The use of a fluorescent dye-embedded bead in the immunoassay is reported to be superior especially with regard to the controllability, stability, and intensity of the fluorescence signal, providing the possibility of embodying a nano-gram level detection capability in a fluidic chip system. Although a bead-based system has the advantages mentioned above, diverse intricate factors have to be understood before a successful application of a bead system in a fluidic system is embodied. In this report, we used a functionalized latex bead embedded with fluorescent dye molecules for immunoassaying PSA on a glass slide. We did a series of immunoassays in order to observe the dependency of the immunoassay upon the amount of the beads used in the assay, the incubation time, and the washing conditions.

Results and Discussion

Scheme 1 shows all the reaction steps involved with the sandwich-type bead-based immunoassay toward the prostate specific antigen (PSA) carried out in this report. The commercial amine-functionalized bead used in this report was reported to fluorescence at 610 nm when excited at 575 nm. We chose the antibody pair as recommended by the manufacturer. One antibody was conjugated on the bead and the other on a glass surface using the well-known amine-aldehyde reaction followed by blocking with a bovine serum albumin (BSA) solution[9]. The amine functionality on the bead surface was converted to aldehyde using glutaraldehyde. Next, the bead aldehyde was conjugated with amine functionality on the antibody. When conjugating the antibody on a glass substrate, 3-aminopropyltrimethoxysilane (3-APT) was used to prepare the amine group. The antibody was immobilized



Scheme 1. A flow diagrammic view of all the reactions involved with the sandwich-type bead-based immunoassay. The antibody immobilization steps on a bead and a glass surface, and the incubation steps for the PSA on the anti-PSA bead and the PSA/anti-PSA bead complex on the anti-PSA conjugated glass, are described.

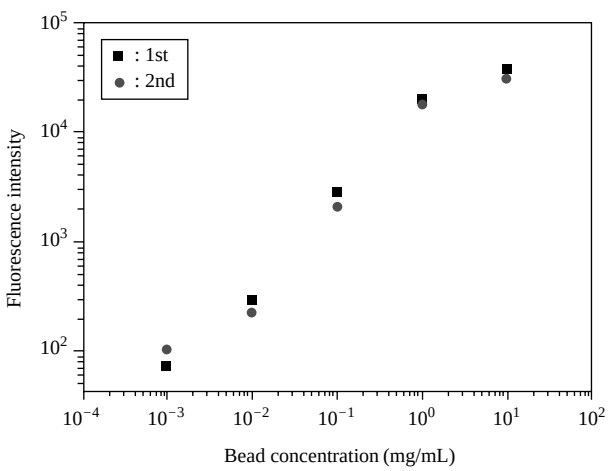


Figure 1. The relationship between the fluorescence intensity and bead concentration for the bead-based immunoassay. The fluorescence immunoassay was repeated twice by varying the bead concentration from 10^{-3} mg/mL to 10^{1} mg/mL. All of the immunoassays were carried out using 100 ng/mL of PSA.

using the same procedure carried out for preparing the antibodies on the bead.

In order to successfully carry out the bead-based immunoassay, some factors have to be considered. For example, the signal intensity of the immunoassay under a given amount of analytes depends on the number of fluorescent dye molecules in the bead, the number of antibodies on the bead surface, the concentration of the fluorescent bead conjugated with antibodies, etc. Since we used a commercially available fluorescent amine bead as the starting material for preparing the antibody bead, the number of dye molecules and antibodies in a single bead was considered to be fixed. Now, we have to understand the dependency of the bead concentration used in the immunoassay.

In an effort to understand the dependency of the bead concentration, we carried out a series of immunoassays by varying the bead concentration from 10^{-3} mg/mL to 10^{1} mg/mL. We used a PSA concentration of 100 ng/mL all throughout the experiment, as this amount is large enough to produce a strong fluorescence intensity in the bead-based immunoassay. Figure 1 shows the relationship between the bead concentration used in the immunoassay and the fluorescence intensity. A near linear relationship was observed in the bead concentration ranging from 10^{-3} mg/mL to 10^{0} mg/mL. The fluorescence intensity seems to reach saturation around the range of 10^{-3} mg/mL to 10^{1} mg/mL. For the purpose of confirmation, we did the same experiment twice. The two series of data points show the same pattern. Figure 2 shows scanned images of the immunoassays. As can be seen from (a) in Figure 2, a very large difference in brightness between the spots corresponding to 10^{0} and 10^{-1} mg/mL was ob-

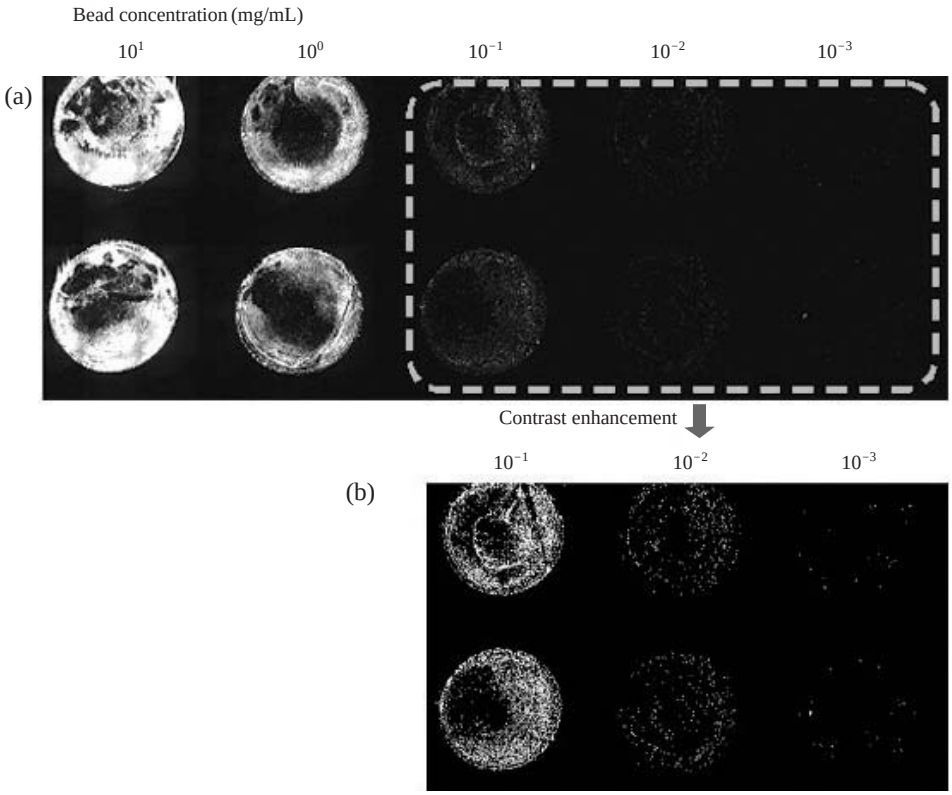


Figure 2. (a) A scanned image produced from the bead-based immunoassay toward 100 ng/mL of PSA as the bead concentration was varied from 10^{-3} mg/mL to 10^{1} mg/mL, and (b) a processed image for contrast enhancement in a range from 10^{-3} mg/mL to 10^{1} mg/mL.

served when compared with those between 10^{1} mg/mL and 10^{0} mg/mL, which is in good agreement with the plotted results in Figure 1. When the contrast was enhanced by image modification, we were able to recognize the spot at 10^{-2} mg/mL. Such large differences in brightness between spots in the bead concentration range of 10^{-3} mg/mL to 10^{0} mg/mL are also well matched with the digitized relationship between the bead concentration and fluorescence intensity in Figure 1.

It is interesting to take note of the donut-type pattern of the fluorescence spots. The donut-shaped fluorescence image has been reported to be a typical phenomenon in spot-related analysis[10,11]. The shear forces involved in the washing steps are thought to be one of the major causes of such patterns when considering the use of a pipette in the small volume of a micro-well (3.0 mm diameter and 1.0 mm thickness). The flow rate generated during the washing step is considered to be at maximum at the center of the well. It may be a reasonable guess that the relatively large volume of the bead with a diameter of 1 μm interrupts the stability of the complexation between PSA and anti-PSA, especially in the middle of the well, resulting in the hollow pattern.

We have studied the effect of incubation time for the complexation between PSA and anti-PSA. Two steps are involved with the complexation in the bead-based sandwich-type immunoassay: one for the complexation between the PSA and the anti-PSA conjugated on the fluorescence bead, and the other between the PSA complexed with the anti-PSA on the bead and the anti-PSA on the glass plate (Figure 1). In this experiment, the incubation time on the glass plate was varied from 5 minutes to 60 minutes, while the incubation time between the PSA and bead anti-PSA was fixed at 60 minutes. The effect of the incubation time was carried out using three different concentrations of PSA, 10 ng/mL, 100 ng/mL, and 1,000 ng/mL. As expected, the fluorescence intensity measured for the immunoassays generally increased when the incubation time was increased for the three different PSA concentrations (Figure 3). In the cases of the immunoassays using the 100 ng/mL and 1,000 ng/mL concentrations, the fluorescence intensity did not seem to reach saturation. In contrast, 20 minutes seems to be enough to bring the system to saturation for the 10 ng/mL concentration.

One of the other factors we studied was the effect of the severity of the washing procedure. As has been briefly mentioned in the fluorescence spot images (Figure 2), the washing conditions, which can be directly related with the shear force exerted on the complexed bead on the glass plate, may affect the

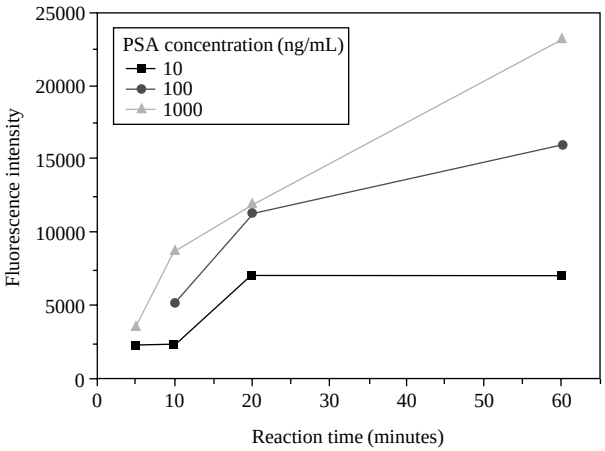


Figure 3. The effect of incubation time toward fluorescence intensity. The incubation step for the complexation of the PSA/anti-PSA bead complex with the anti-PSA on glass was considered as the reaction time was varied from 5 minutes to 60 minutes. The time dependency experiment was carried out using three different PSA concentrations, 10 ng/mL, 100 ng/mL and 1,000 ng/mL.

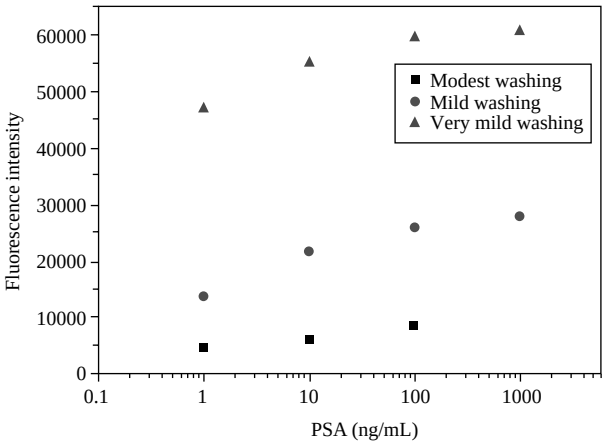


Figure 4. The effect of washing condition on the bead-based immunoassay. Three different degrees of washing condition were compared, very mild, mild, and modest. In all of the PSA concentrations concerned in this experiment, the effects of shear force exerted during the washing step are well described.

image patterns. Another reason for studying this effect was due to some difficulties in obtaining reproducible results in the bead-based sandwich-type fluorescence immunoassay. We came to think that the fluorescence bead immobilized on the glass plate by the immuno-complexation of one PSA with two anti-PSAs, one conjugated on the bead and the other on the glass (Scheme 1), may not hold fast enough to stay attached on the plate when the shear force exerted during the washing step is large enough. Although not quantitative, we preliminarily studied the shear force effect. Based on long periods of hands-on experiments with the bead-based immunoassay, we conducted the study qualitatively by controlling the severity of the squeezing and suctioning during the washing steps. Depending on the severity of the squeezing and suctioning, we categorized the degrees into three levels: very mild, mild, and modest washing. Figure 4 shows the effect of washing conditions based on fluorescence intensity. Also, the experiments were carried out using different concentrations of PSA, 1 ng/mL, 10 ng/mL, 100 ng/mL, and 1,000 ng/mL. Generally, with the three different washing conditions the fluorescence intensity increased as the PSA increased. It is interesting to take note that the increase rate in intensity slowed as the PSA concentration increased, while showing some signs of saturation in the concentration range of around 100 ng/mL to 1,000 ng/mL. As expected, the effect of the shear force was evident in the PSA concentration ranges concerned in this experiment. When the washing condition was more severe, the fluorescence became less intense in all the PSA concentration ranges concerned. When considering the 1 μm diameter of the bead staying on a glass slide fastened only with the interaction forces between antibody and antigen, of which the sizes are around only a few nanometers, it may be reasonable to think that the shear force exerted on the bead during the washing process should affect the amount of the fluorescence of the bead based immunoassay. We are planning to extend the shear force study for a quantitative understanding. The study with regard to the dependency of bead-based immunoassay on the shear force generated by fluid is thought to be one of the key factors when considering applying the bead system in a microfluidic chip.

Conclusions

We have studied three types of factors that may affect the bead-based fluorescence immunoassay on a glass slide: the effect of the number of beads, incubation time, and the severity of the washing condition. We observed that the bead concentration has to be in the proper range to produce a reasonable result in the bead-based immunoassay. The intensity of the immunofluorescence seemed to reach a saturation state at around a bead concentration of 10^0 mg/mL to 10^1 mg/mL. When it comes to analyzing a low concentration target, it may be reasonable to recommend using a high bead concentration. As for the incubation time, a prolonged incubation generally increases the immuno-

fluorescence intensity, while for lower PSA concentrations (for example, 10 ng/mL) the incubation time reached a plateau earlier than for higher PSA amounts. The shear force exerted on the fluorescence bead during the washing process is thought to affect the results of the bead-based immunoassay to a significant degree. We are generally working to further understand the diverse factors involved in the bead-based immunoassay. Elucidation of these factors may contribute to the application of a bead-based immunoassay in fluidic systems.

Materials and Methods

Materials and Instrumentation

The amine-modified fluorescence latex bead (size: 1 μm and solid content: 2.5%), bovine serum albumin (BSA), (3-aminopropyl)triethoxysilane (3-APT) and chambered coverslip (50-wells, diameter: 3 mm, depth: 1 mm) were purchased from Sigma-Aldrich. A pair of monoclonal antibodies to free the prostate specific antigen (PSA) was purchased from Biodesign. The fluorescence intensity was measured by scanning the spotted slide in the arrayWoRx® Biochip Reader (Applied Precision) with the excitation maximum at 540 nm and the emission at 595 nm.

Antibody Conjugation on Amine-functionalized Fluorescence Particle

A glutaraldehyde solution (2%) was prepared using a 10 mM PBS buffer at pH 8.4. The glutaraldehyde buffer solution (750 μL) was mixed with an amine bead (250 μL). After vortexing for 3 hours, the solution mixture was centrifuged for 10 minutes at 18˚C with an 8,000 g force. The precipitated particles were washed two times using a PBST buffer (0.05% tween, pH 7.4). The aldehyde particle was dispersed using 0.5 mL of PBS buffer (pH 8.4). The anti-PSA in the 0.5 mL of PBS buffer solution (120 μg/mL, pH 8.4) was incubated with the aldehyde-bead solution. After 3 hours of gentle shaking, the antibody bead was again separated by centrifuging with an 8,000 g-force for 10 minutes. The precipitated particle was washed twice with the PBST buffer. The PBS solution containing 1% BSA and 0.05% PBST was used as a blocking solution and stored for later use at 4˚C.

Antibody Immobilization on a Glass Plate

A slide glass was immersed in a mixture of hydrochloric acid and methanol (HCl : MeOH 1 : 1 v/v) for 30 minutes followed by washing with DI and methanol a few times. Next, the slide was immersed in H_2SO_4. After being washed with DI and acetone, the slide glass was immersed in 2% 3-APT in acetone for 3 hours and baked at 120˚C for 30 minutes. The amine functionalized slide glass was treated with 2% glutaraldehyde for 3 hours. The aldehyde slide glass was attached with a chambered coverslip. In each well, 10 μL of antibody solution (200 ng/mL) was placed for 3 hours. The PBS solution containing 1% BSA and 0.05% PBST was used as a blocking solution.

Complexation of a PSA with an Anti-PSA Conjugated on a Fluorescence Bead, and Fluorescence Immunoassay on a Slide Glass

The required amount of PSA was incubated with the anti-PSA conjugated fluorescence bead for 60 minutes. The antigen-particle complex solution was incubated on the anti-PSA immobilized glass substrate for an hour followed by three washings with PBST.

Acknowledgements

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