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Research Article

Improvement of Accuracy in Flow Immunosensor System by Introduction of *Poly*-2-[3-(methacryloylamino)propylammonio]ethyl 3-aminopropyl Phosphate

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In order to improve the accuracy of immunosensor systems, *poly*-2-[3-(methacryloylamino)propylammonio]ethyl 3-aminopropyl phosphate (*poly*-3MAm3AP), which includes both phosphorylcholine and amino groups, was synthesized and applied to the preparation of antibody-immobilized beads. Acting as an antibody-immobilizing material, *poly*-3MAm3AP is expected to significantly lower nonspecific adsorption due to the presence of the phosphorylcholine group and recognize large numbers of analytes due to the increase in antibody-immobilizing sites. The elimination of nonspecific adsorption was compared between the formation of a blocking layer on antibody-immobilized beads and the introduction of a material to combine antibody with beads. Determination with specific and nonspecific antibodies was then investigated for the estimation of signal-to-noise ratio. Signal intensities with superior signal-to-noise ratios were obtained when *poly*-3MAm3AP was introduced. This may be due to the increase in antibody-immobilizing sites and the extended space for antigen-antibody interaction resulting from the electrostatic repulsion of *poly*-3MAm3AP. Thus, the application of *poly*-3MAm3AP coatings to immunoassay beads was able to improve the accuracy of flow immunosensor systems.

1. Introduction

Immunosensors quantify analytes based on binding reactions between antibody and antigen, and various immunosensing methods have been reported for the quantitative determination of a broad range of analytes of clinical, medical, biotechnological, and environmental significance [1–4]. However, nonspecific adsorption can lead to a decline in sensor accuracy and sensitivity, and this has limited the further development of immunosensors toward practical application. Nonspecific adsorption decreases the signal-to-noise (S/N) ratio, preventing the improvement of immunosensor systems. Therefore, eliminating nonspecific adsorption is crucial in realizing a practical immunosensor system.

Blocking reagents such as nonspecific sera, detergents, milk proteins, bovine serum albumin (BSA), and ovalbumin are typically used to reduce nonspecific adsorption [5–9]. On the other hand, blocking reagents also block the binding sites for specific interaction between antibodies and antigens [10]; thus, blocking methods interfere with sensitive and accurate determination, despite their efficacy in reducing nonspecific adsorption. Moreover, the composition of nonspecific adsorption reducing layers with blocking reagents typically requires additional technical know-how for incubation in sensing. Such incubation processes prevent application to rapid and continuous measurements. Therefore, alternative methods for reducing nonspecific adsorption have been investigated.

In recent years, hydrophilic polymers such as *poly*-2-methacryloyloxyethyl phosphorylcholine (*poly*-MPC) have been utilized as materials for reducing protein sorption [11–14]. It has been reported that the adsorption of protein onto a film of phosphorylcholine derivatives is limited due to the presence of free water at the polymer-liquid interface [13, 14]. However, the introduction of phosphorylcholine-containing polymer to the antigen-antibody interface did not increase the signal intensity caused by the specific reaction, while nonspecific adsorption was eliminated. Therefore, analyte selectivity is improved by its introduction, but the sensitivity and accuracy are unsatisfactory. To overcome this issue, the construction of an interface that not only reduces nonspecific adsorption but also increases the specific reaction-based determination is of intrinsic importance.

Poly-2-[3-(methacryloylamino)propylammonio]ethyl 3-aminopropyl phosphate (*poly*-3MAm3AP), which has a phosphorylcholine moiety for nonspecific adsorption removal and an amino group for covalent bonding with antibody, was synthesized and applied to antigen-antibody interfaces. As the phosphorylcholine group has both hydrophilic and electrolytic properties, *poly*-3MAm3AP would be effective in preventing entanglement with other molecules. Therefore, the introduction of antibody attached to *poly*-3MAm3AP would be effective for trapping larger numbers of antigens due to the introduction of large numbers of antibodies.

The reaction area (reactor) for antigen (analyte) recognition and the detection area (detector) for signal translation were separated with the construction of an immunosensor system. Otherwise, recognizing an antigen on the surface of the detector as in our previous reports would cause an increase in background noise due to adsorption of high molecular weight protein [4, 10]. By separating the reactor and detector, antigen recognition and signal translation should not affect one another in the detection process.

The effects of nonspecific adsorption removal were evaluated as follows: (i) formation of a nonspecific adsorption preventing layer on antibody-immobilized substrate by incubation with blocking reagents; (ii) immobilization of antibodies on a nonspecific adsorption-preventing polymer formed on the substrate.

Poly-MPC and *poly*-3MAm3AP were used as blocking reagents and antibody-immobilizing material, respectively. BSA, which is known to be useful as both a blocking reagent and an antibody-immobilizing material, was used for comparison. In addition, an acrylamide polymer containing positively charged choline and amino groups, *poly*-4-[3-(acryloylamino)propylammonio]-1-butylamine (*poly*-3AAm1BA), which differs from *poly*-3MAm3AP in that *poly*-3AAm1BA does not contain a phosphoric ester, was also employed as the antibody-immobilizing material for comparison of the polymer electrolyte effect.

Luteinizing hormone (LH), which is useful for sterility treatment, was employed as a model antigen and was labeled with fluorescein (F-LH) for sensitive fluorescence detection [15, 16]. Two monoclonal IgG class antibodies, antiluteinizing hormone antibody (anti-LH) and antihemoglobin anti body (anti-hemo), were employed for the estimation of nonspecific adsorption levels, as the difference obtained between F-LH and anti-hemo will be only nonspecific adsorption. Antibodies were directly immobilized onto agarose gel beads or by the mediation of antibody-immobilizing material and packed into a reactor column. The other conditions for fabricating the flow system were as described previously [4, 17, 18].

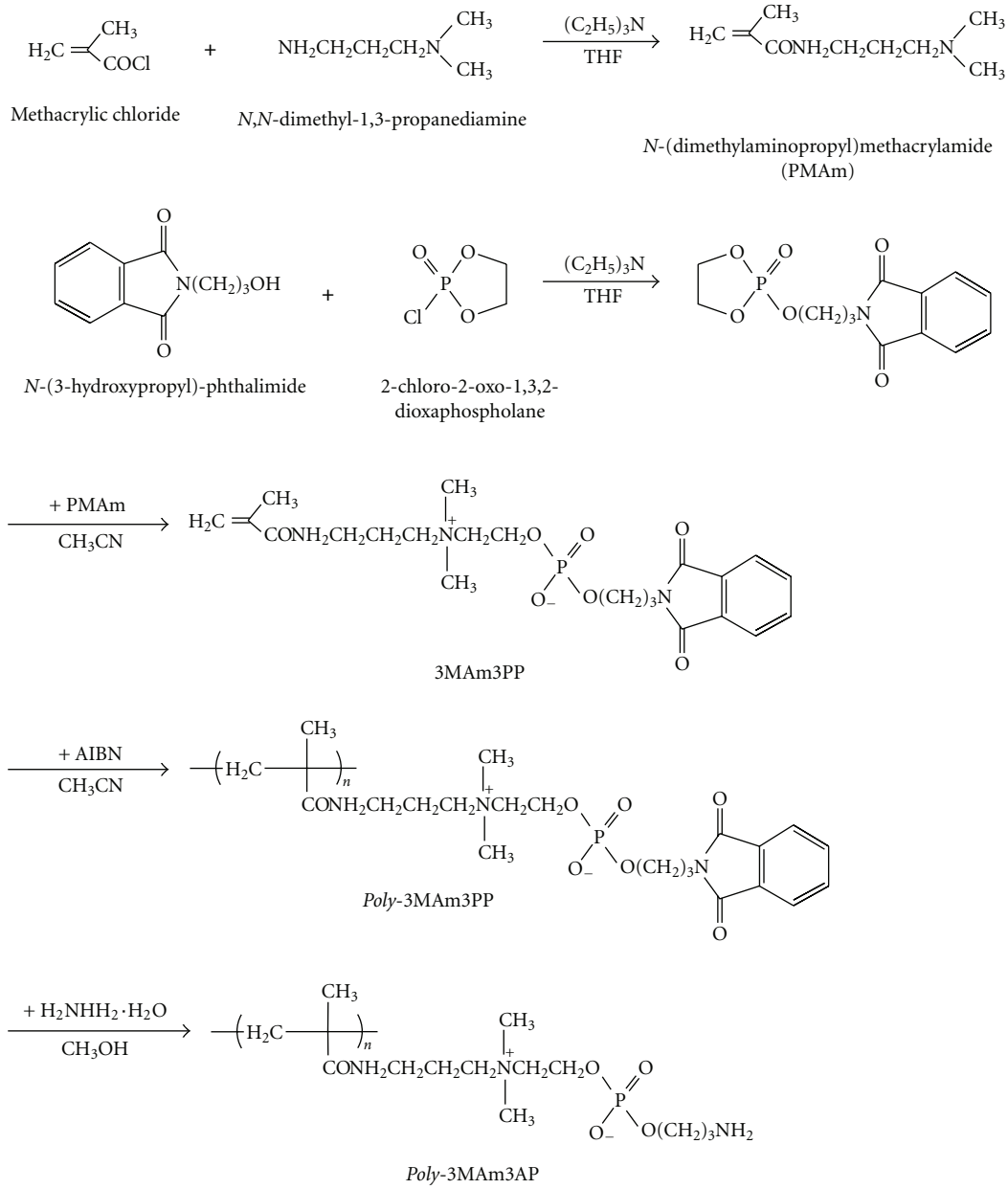
Compared with recent technologies that allow the accuracy and sensitivity to be increased, this paper describes an approach for enhancing immunosensor accuracy and sensitivity by introducing antibodies immobilized on beads coated with *poly*-3MAm3AP [19–23]. Furthermore, *poly*-3MAm3AP beads do not require incubation with blocking reagents or bound/free separation.

We truly believe that this system will give a significant impact on immunosensor technologies in future.

2. Experimental

2.1. Reagents and Materials. Luteinizing hormone (LH), monoclonal IgG class anti-LH, and antihemoglobin antibodies were kindly provided by ROHTO Pharmaceutical Co. Bovine serum albumin (BSA, 96%–99%) was obtained from Sigma Chemical. Commercial 2-chloro-1,3,2-dioxaphospholane was purified by vacuum distillation. Commercial 2,2′-azobisisobutyronitrile (AIBN) was recrystallized from methanol. All other solvents were purchased as the best commercial grade and dried over molecular sieves (4 Å) prior to use. All other chemicals and solvents were of commercial grade and used without further purification. Standard stock solutions containing antigens were prepared with phosphate buffer solution (PBS, pH 7.0) and were stored at 4°C. Antibodies were stored in a frozen state, and standard solutions were prepared daily with PBS solution as needed. All aqueous solutions were prepared in deionized distilled water. Affi-Gel Hz hydrazide gel beads were purchased from Bio-Rad Laboratories and were used to immobilize antibodies. LH was modified with fluorescein isothiocyanate to prepare fluorescein-labeled LH (F-LH) for use as an antigen in the flow immunosensor system.

2.2. Synthesis of Poly-3MAm3AP. Hydrophilic phosphorylcholine-containing polymer, *poly*-2-[3-(methacryloylamino) propylammonio] ethyl 3-aminopropyl phosphate (*poly*-3MAm3AP), was synthesized as shown in Scheme 1. To a stirred dry THF solution of *N*-(3-hydroxypropyl) phthalimide and triethylamine, 2-chloro-2-oxo-1,3,2-dioxophosphorane was added at −20°C and the reaction mixture was maintained at 0°C for 2 h. After filtration of triethylamine hydrochloride, the filtrate and THF solutions were evaporated in vacuo in a stream of nitrogen. The resulting product was reacted with *N,N*-(dimethylamino propyl)methacrylamide in acetonitrile at 80°C for 20 h, yielding 2-[3-(methacryloylamino)propylammonio]ethyl 3-phthalimidopropyl phosphate (3MAm3PP). 3MAm3PP was

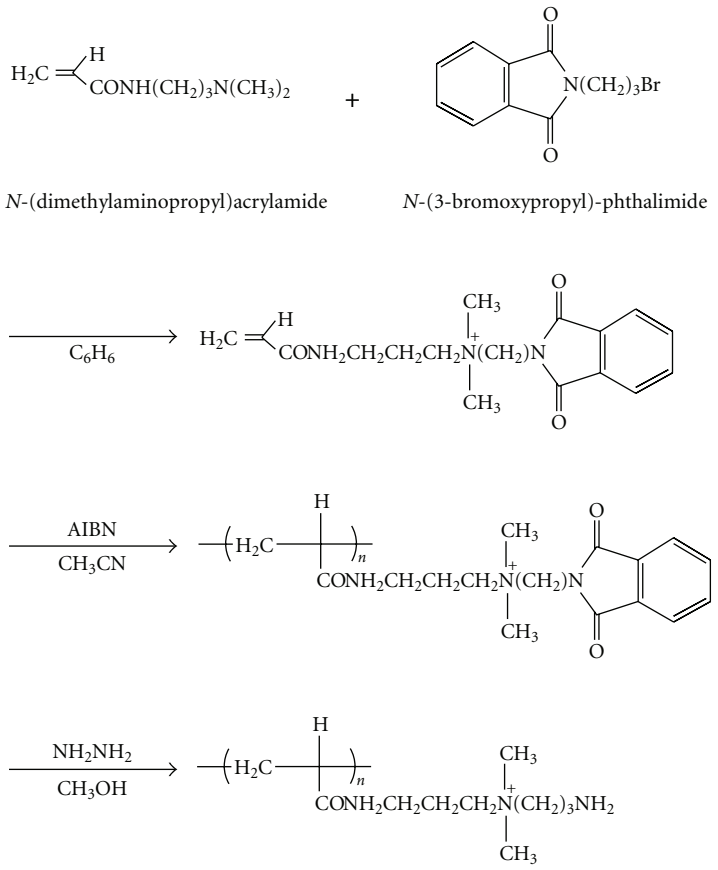


Scheme 1: Reaction route for synthesis of *poly*-3MAm3AP.

then polymerized at 80°C for 20 h in acetonitrile using AIBN as an initiator, and *poly*-3MAm3PP was obtained. Finally, *poly*-3MAm3PP was subjected to a hydrolysis reaction with hydrazine hydrate in methanol, and the phthalimide protecting groups were removed to afford *poly*-3MAm3AP.

2.3. Synthesis of Poly-3AAm1BA. Synthesis of acrylamide polymer containing both positive amino groups and positively charged choline groups and lacking phosphate groups (*poly*-3AAm1BA) was performed according to Scheme 2. *N*-(Dimethylaminopropyl) acrylamide and *N*-(3-bromoxypropyl) phthalimide were reacted in benzene and were then polymerized in acetonitrile. The phthalimide protecting groups were removed to afford the acrylamide polymer of *poly*-4-[3-(acryloylamino) propylammonio]-1-butylamine, *poly*-3AAm1BA.

2.4. Preparation of Antibody-Immobilized Beads. Antibody-immobilized beads for blocking reagent treatment were prepared according to instruction manual provided by Bio-Rad Laboratories [24], and the beads were packed into the reactor column (50 × 3.0 mm I.D. EYELA). Antibody-immobilized beads attached through polymers were prepared according to the procedure in Scheme 3. Carboxylic groups were introduced to the surface of the beads by succinic anhydride, and the activated ester was prepared using *N*-ethyl-*N*-(3-



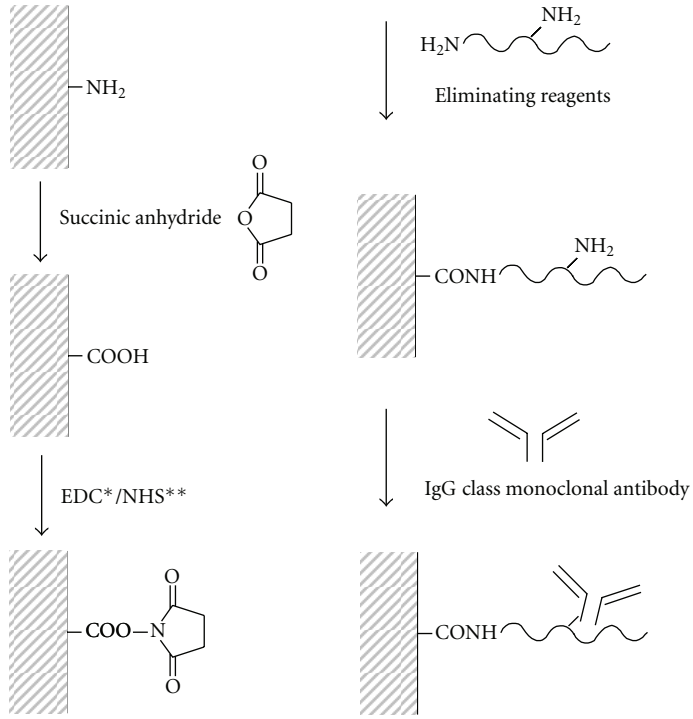
Scheme 2: Reaction route for synthesis of *poly*-3AAm1BA.

dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy succinimide (NHS). Beads were reacted with the polymers to form amide bonds. Prior to IgG antibody binding to the polymer, the antibody was partially purified for maximizing the binding capacity and oxidized the carbohydrate moieties on the Fc region to form aldehydes [24]. Finally, the antibodies were attached to the polymers, and the prepared antibody-immobilized beads were attached through polymers.

2.5. Principle and Apparatus of Reactor/Detector Separated Flow System. Measurements using the reactor/detector separated flow system were performed as follows. First, F-LH dissolved in PBS was injected onto the reactor column with peristaltic pumps (EYELA MP-3 microtube pump) and was trapped by the antibody-immobilized beads. Second, acetic acid solution was injected to cleave the antigen-antibody binding. Third, free F-LH antigen in acetic acid solution was mixed with 0.02 M sodium hydrate solution through a flowing process before loading onto the optical detector, as the fluorescence of F-LH was only minimally detected in solutions of less than pH 6.0. Finally, the F-LH solution flowed into the detector and fluorescence intensity was measured. All of the measurements were automatically performed with the fabricated flow system at room temperature, as shown in Figure 1. BSA blocking procedure was performed as reported previously [17, 18, 25]. Tween 20 solution (0.5%) was employed for cleaning both the quartz crystal cell used in the optical detector and the flow path in the system. Position valve C was set as the flow channel in order to avoid loading the Tween 20 solution onto the reactor column. Thus, the adsorption of F-LH onto the quartz cell could be washed between measurements by loading the Tween 20 solution from position valve B.

3. Results and Discussion

3.1. Study of F-LH Detection in Reactor and Detector Separated Immunosensor System. A calibration curve was produced using various concentrations of F-LH with anti-LH antibody-immobilized bead (anti-LH) column and anti-hemoglobin (anti-hemo) columns (Figures 2(a)–2(c)). Measurement was performed three times against each concentration. To reduce nonspecific adsorption, BSA and poly-MPC were used as blocking reagents. As a result, the reactor and detector separated immunosensor system was able to detect different concentrations of F-LH, and their fluorescence res-



EDC*: *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide,
NHS**: *N*-hydroxysuccinimide

Scheme 3: Preparation procedure for antibody-immobilized beads including nonspecific adsorption-reducing reagents.

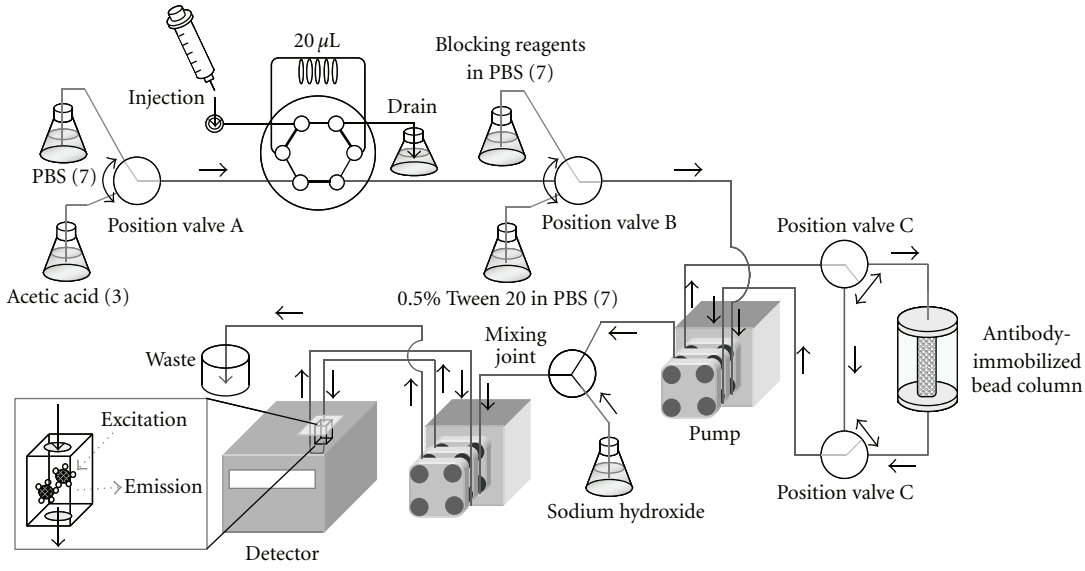


Figure 1: Flow immunosensor system.

ponses depended on the linear increase of F-LH concentration up to 3.5 μg cm^{-3}(Figure 2). Although the anti-hemo columns contained no specific binding sites for F-LH, they all exhibited linear increases with increasing F-LH concentration, even when blocking reagents were used. Moreover, the *poly*-MPC blocking layer had no effect on eliminating nonspecific adsorption, while BSA had some effect observed. This indicates that nonspecific adsorption occurred on the anti-hemo-immobilized beads.

3.2. Effects of Poly-3MAm3AP Beads on Nonspecific Adsorption. We planned to immobilize antibodies covalently on

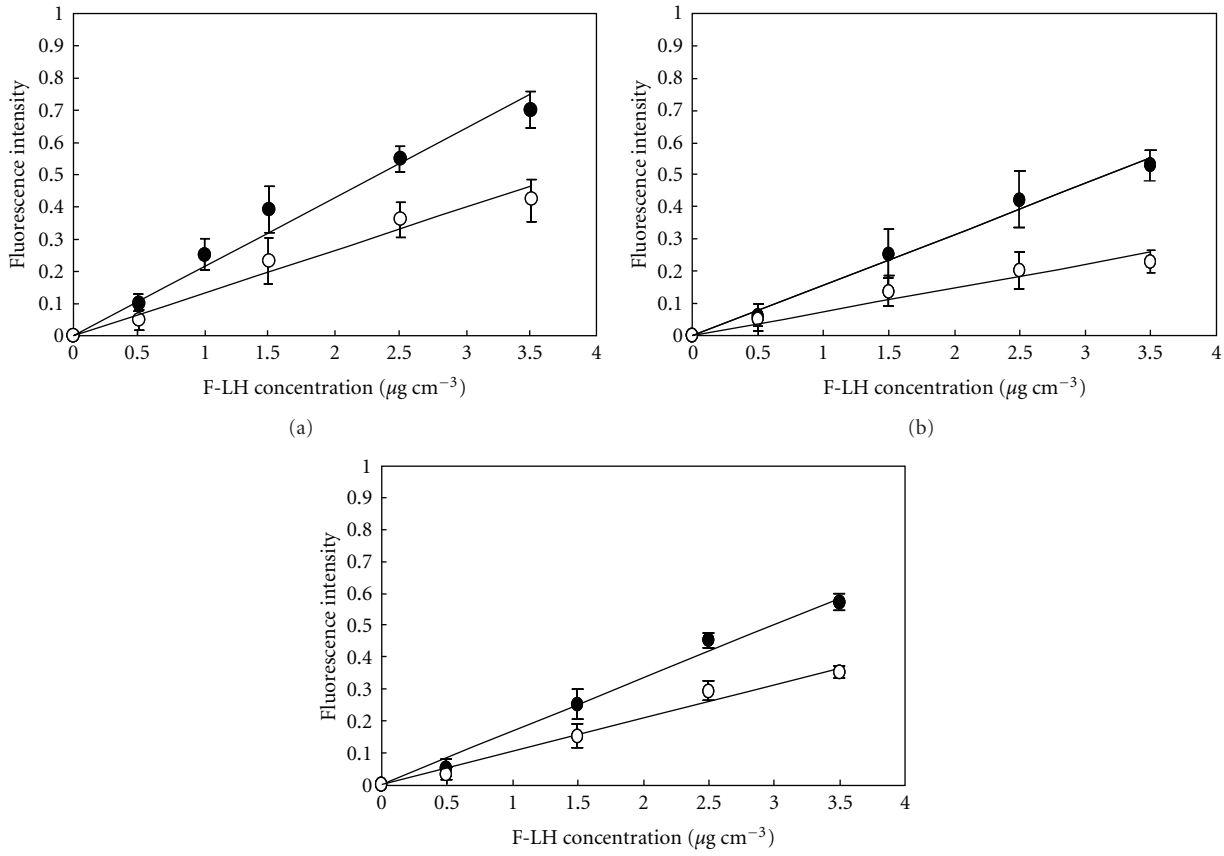


Figure 2: Relationship between different concentrations of F-LH and fluorescence intensity, under (a) nonblocking conditions, (b) with BSA, and (c) with *poly*-MPC. Measurements were performed with anti-LH-(closed symbols) and anti-hemoglobin-(open symbols) immobilized beads, respectively.

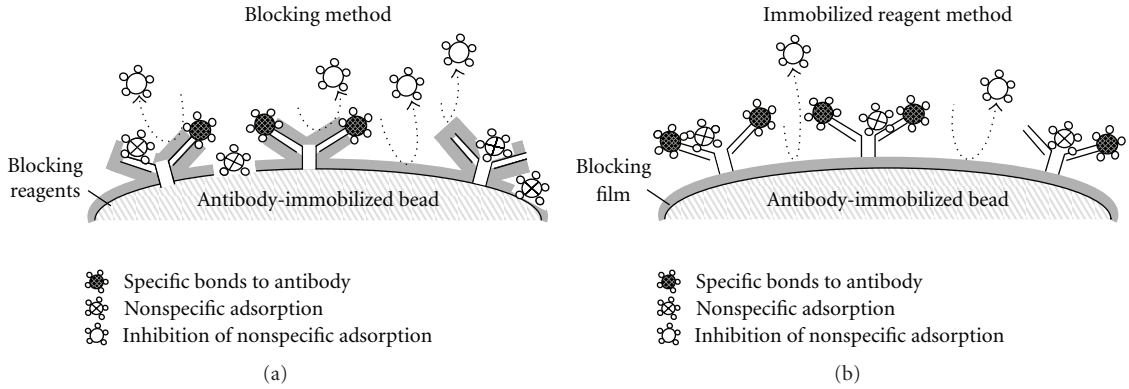


Figure 3: Removal of nonspecific adsorption with (a) blocking method and (b) immobilized reagent method.

the beads via poly-3MAm3AP, which reduces nonspecific adsorption (Figure 3). Such a method would not require complex procedures or technical knowledge, blocking of anti body-antigen interaction sites, or release of film for nonspecific adsorption removal.

Poly-3MAm3AP, which had amino groups for covalent attachment with antibodies and beads, was synthesized and used as a phosphorylcholine-containing polymer. Acrylamide polymer (*poly*-3AAm1BA), which contains both amino and positively charged choline groups, but no negatively charged phosphoric ester, and the known blocking reagent BSA were employed for comparison. Calibration curves were fabricated as shown in Figure 4, and the data were obtained three times for each concentration. The slopes of

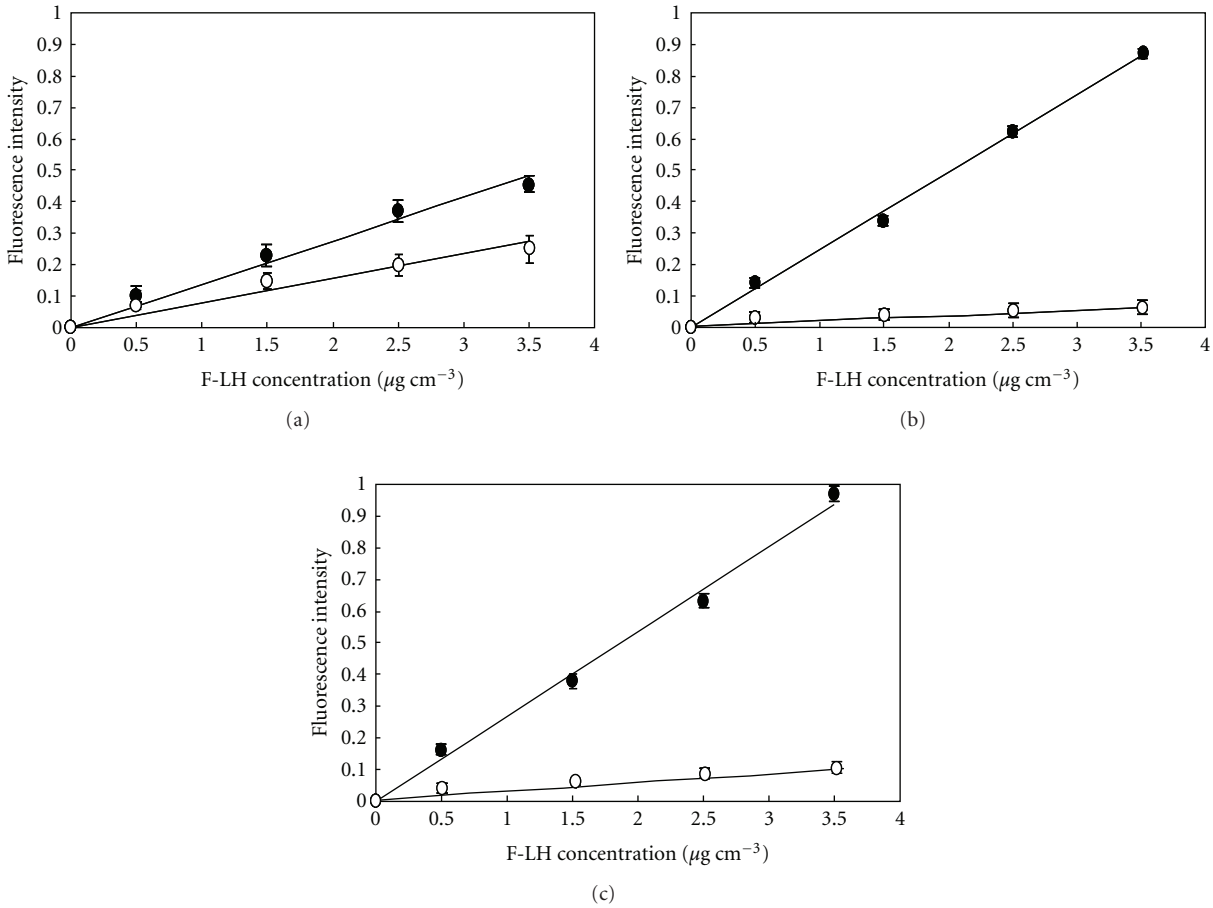


Figure 4: Relationship between concentration of F-LH and fluorescence intensity. (a) BSA membrane, (b) *poly*-3MAm3AP, and (c) *poly*-3AAm1BA were, respectively, immobilized on the surface of antibody-immobilized beads before the antibody-binding process.

calibration curves obtained from anti-hemo-immobilized beads were significantly lower when *poly*-3MAm3AP and *poly*-3AAm1BA were employed (Figure 4). This indicates that nonspecific adsorption was successfully reduced (Figures 4(b) and 4(c)). The effectiveness of *poly*-3MAm3AP was attributed to the phosphorylcholine derivative which removed nonspecific adsorption due to the presence of free water. The most likely explanation for the successful reduction of nonspecific adsorption by *poly*-3AAm1BA is that positively charged *poly*-3AAm1BA produced a highly hydrophilic surface [10, 16]. However, the strongly positively charged *poly*-3AAm1BA may limit the reduction of nonspecific adsorption when compared with nearly neutral *poly*-3MAm3AP, as negatively charged antigens will adsorb *poly*-3AAm1BA by electrostatic attraction. The isoelectric point of the model sample LH was in the range of 6.51–6.77 [26], and the antigen-antibody reaction was performed in pH 7.0 solution; thus, the measurement conditions were suitable for *poly*-3AAm1BA to act as an effective antibody-immobilized material. On the other hand, the BSA-immobilized beads scarcely reduced the nonspecific adsorption.

For the slopes of anti-LH, notable results were obtained. The slopes of *poly*-3MAm3AP and *poly*-3AAm1BA were higher than those of the BSA-immobilized beads (Figure 4). This is attributable to the fact that the number of the antibodies immobilized to the beads increased by modifying those polymers around the beads (Figure 5(a)). Although the antibodies were directly immobilized on the surface of the beads, a substantial number of antibodies could be introduced to the small area on the beads by using these polymers, as shown in Figure 5(a). Generally, the densely immobilized polymer chains would become entangled, making it difficult for the host molecule attached to the polymer to bind the guest molecule. On the other hand, phosphatidylcholine analogues are reported to have an overall positive charge due to the stronger positive charge of the choline groups, producing hydrophilic properties [27–29]. As *poly*-3AAm1BA possessed only the positively charged choline groups, the strong repulsion between the polymer chains and the antigen F-LH readily allowed access to the antibodies on the polymers. The anti-LH slope of *poly*-3MAm3AP was therefore similar to that of *poly*-3AAm1BA. This indicates that *poly*-3MAm3AP was able to capture a larger quantity of antigen F-LH due to the repulsion between polymers preventing entanglement (Figure 5(b)). In addition, acrylamide polymers not containing choline groups did not show such increases in anti-LH slope. Therefore, sufficient space for the antigen-antibody reaction

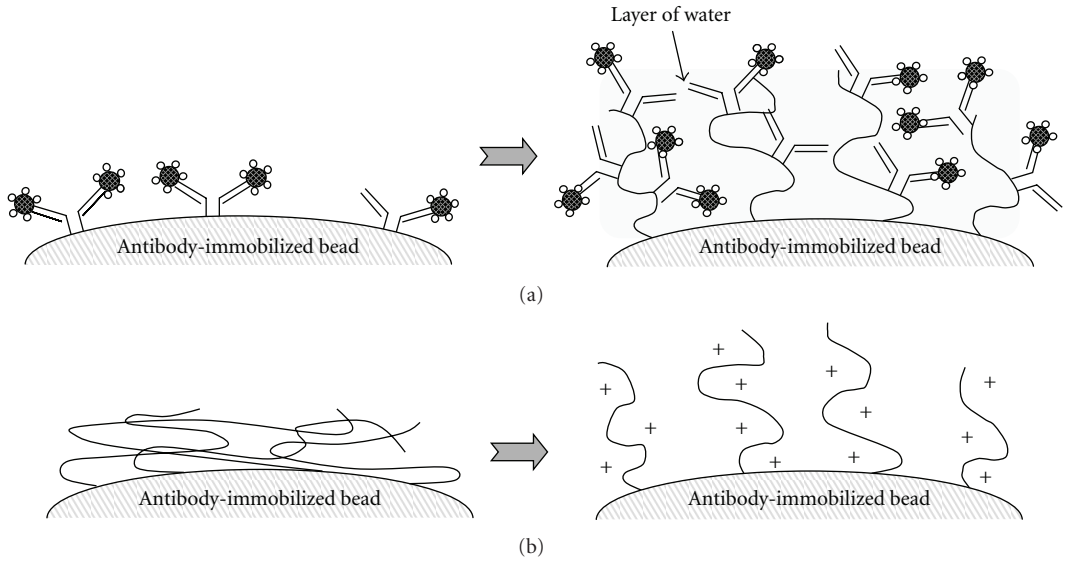


Figure 5: Image on the surface of *poly*-3MAm3AP-immobilized beads. (a) Increase in immobilized antibodies by introducing *poly*-3MAm3AP to eliminate nonspecific adsorption. (b) Swelling effect of electrolytic properties of *poly*-3MAm3AP.

Table 1: Comparison of the slopes and variations obtained from calibration curves of the sensor system.

Method	Blocking			Immobilization		
	None	BSA	Poly-MPC	BSA	*Poly*-3MAm3AP	*Poly*-3AAm1BA
Anti-LH	0.214	0.158	0.168	0.138	0.246	0.298
Variation*	±0.112	±0.121	±0.087	±0.080	±0.032	±0.054
Anti-hemo	0.133	0.074	0.104	0.078	0.014	0.021
Ratio**	0.622	0.468	0.619	0.565	0.057	0.070

* Average relative deviation obtained from calibration plot of anti-LH.
** Anti-hemo/Anti-LH.

may remain on the surface of *poly*-3MAm3AP-immobilized beads, and F-LH antigens could easily access the anti-LH antibodies attached to the polymer for specific binding.

3.3. Evaluation of Accuracy of Immunosensor System. In order to evaluate sensor accuracy, the variations and slopes obtained from the calibration curves were analyzed and compared, as shown in Table 1. Variation was calculated from 12 measurements. The effects of specific bonds were estimated based on the ratio of the slope of the anti-hemo curve to that of the anti-LH curve (Table 1). Among the blocking methods, BSA was effective in reducing nonspecific adsorption, as shown by a lower ratio than the nonblocking case; however, the variation became wider and the slope of anti-LH decreased. The ratios of poly-3AAm1BA and poly-3MAm3AP were nearer to zero when compared with the others due to almost all nonspecific adsorption removal. Moreover, the variations with poly-3AAm1BA and poly-3MAm3AP were also smaller than the others due to their allowing specific interactions between antibodies and antigens, while reducing nonspecific adsorption. This indicates that immunosensors using these hydrophilic polymer-immobilized beads would be able to more accurately detect antigen F-LH. Generally, nonspecific adsorption causes both a decrease in the S/N ratio and a decrease in sensor accuracy. However, blocking methods also decrease these factors, in addition to requiring complex technical knowledge to fully function, thus making them unsuitable for use in an accurate flow-sensor with automatic determination and various sensing applications.

The results obtained for poly-3MAm3AP beads confirmed satisfying effects for both the reduction of nonspecific adsorption and the increase in signal intensity caused by specific reactions.

4. Conclusion

Poly-3MAm3AP was introduced to the antigen-antibody interface in an effort to improve the accuracy of the immunosensor system. Anti-LH and anti-hemo were employed for the estimation of nonspecific adsorption levels. *Poly*-3MAm3AP-immobilized beads exhibited a high anti-LH slope and a low anti-hemo slope, resulting in a considerably lower ratio of anti-LH to anti-hemo. This indicates that *poly*-3MAm3AP markedly reduces nonspecific adsorption and specifically trapped large amounts of F-LH. The introduction

of *poly*-3MAm3AP to the interface significantly reduced nonspecific adsorption with high accuracy in the fabricated immunosensor system. This immunosensor device may assist in the development for other biosensing devices by substituting other biological receptors for antibodies.

Acknowledgments

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