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Synthesis of Polystyrene Nanoparticles with Different Surface Modification by Emulsion Polymerization and Measurement of IgG Adsorption and Stability for the Application in Latex-Protein Complex Based Solid-Phase Immunoassay

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Summary

Unmodified and surface modified polystyrene (PS) nanoparticles with hydrophilic end group on surface were synthesized by emulsion polymerization for adsorption of a protein (immunoglobulin G, IgG) by passive adsorption and electrostatic attraction for the application on solid-phase immunoassay. The presence of formed IgG-PS particle complex was confirmed by dynamic light scattering (DLS) measurement which shows the increased size of particle (≈ 178nm and 220nm) after IgG immobilization. Increased Zeta (ζ) potential from -56.7mV to -3.84mV following the IgG immobilization by electrostatic attraction also provides the presence of the formed IgG-PS particle complex. Enzyme-linked immunosorbent assay (ELISA) results demonstrate that the formed IgG-PS complex was stable and the binding strength between IgG and PS was maintained after 1 month from initial IgG-PS particle complex formation.

Keywords

emulsion polymerization, surface modification, IgG adsorption, stability, Solid-phase immunoassay

Introduction

Monodispersed latex nanoparticles are widely used in many field of technology such as paint coatings, ceramics processing as well as recently many efforts have been devoted in biomedical applications such as diagnostic test, controlled drug release, bioaffinity chromatography, biomolecule adsorption, and phagocytosis researches [1-6]. Several methods for production of the monodispersed latex nanoparticles have been developed, such as emulsion polymerization, seeded polymerization, emulsifier-free polymerization, precipitation polymerization, and dispersion polymerization [7].

In biomedical application, especially, the protein adsorbed latex particle has been used for diagnostic test since Singer and Plots firstly reported the protein adsorbed on the latex particle as the diagnosis tool for rheumatoid arthritis in 1956 [8]. The latex-protein complex based solid-phase immunoassay system has been studied extensively since the rapidity, low cost, simplicity, and easy determination by using potable optical devices compared with conventional immunoassay [9]. PS latex particles-protein conjugate are widely used in biomedical field especially in the diagnosis of chagas disease and cancer, the application in white blood cell counting, and the attempt in the field of biosensor [10-14]. The solid-phase immunoassay system is based on the antigen-antibody interaction as detection system following conjugation of particular antibody of interest on the surface of PS particles [15]. For the application to solid-phase immunodiagnostic assay, protein-PS latex particle must be formed and stable for desired period until the immunological reaction is complete as well as conjugated proteins on the surface must have homogeneous distribution [16].
Protein adsorption to the PS surface is known to be formed in several ways such as Van der Waals forces, hydrogen bonding, and electrostatic and hydrophobic forces [17]. Interaction between particular protein and solid support interface is greatly influenced by many variables such as pH, ionic strength, temperature, properties of surface of solid support, and the nature of solvent [18-19]. Passive adsorption is one of the classical methods which allow the physical interaction between protein and surface of PS particle in aqueous solution by hydrophobic forces [9,17,20]. Electrostatic attraction allows the conjugation of protein to PS latex particle via surface charge modification by changing pH condition of proteins greater/smaller than their isoelectric point (pI) [10,20]. Protein adsorption on its surface is processed by functionally modified reactive surface groups such as aldehyde, amino, carboxyl, hydroxyl, and sulfate [3]. Surface modification with functionalized reactive group is achieved by hydrophilic copolymerization which processed in the presence of hydrophilic reagents such as acrylic acid [21], sodium tetraborate [22], 2-hydroxyethly methacrlyate [1] or by introducing a cationic initiator [23].
In this study, we aimed to determine the stability of IgG-PS complex and binding strength of IgG adsorbed on PS nanoparticles between hydrophobic and electrostatic attraction for the establishment of solid-phase immunoassay detection system. To achieve this goal, we established the emulsion polymerization system for the preparation of PS nanoparticles by combined emulsion polymerization. In addition, functional surface modification of PS nanoparticles was process in the presence of hydrophilic co-monomers which generate the negatively charged functional end group. ELISA data show that formed IgG-PS complex by the mechanism of electrostatic attraction showed slightly higher binding strength between IgG and PS, and the stability of IgG-PS complex than those of hydrophobic interaction. These results suggest that the IgG-PS complex formed by electrostatic attraction has the potential for the clinical application as solid-phase immunoassay.

Experimental

Materials

Styrene, acrylic acid, divinylbenzene (DVB), sodium tetraborate, sodium dodecyl sulfate (SDS), and potassium peroxosulfate(KPS) were purchased from Sigma Aldrich (St. Louis MO, USA). All other chemicals of sodium acetate, citric acid, and

chromium bromide used for making acid buffer were purchased from Sigma Aldrich. All chemical reagents were used as received without any purification.

Synthesis of Seed PS nanoparticles

Monodispersed crosslinked PS nanoparticles were synthesized through an emulsion polymerization process. For a typical polymerization procedure, polymerization reactions of crosslinked PS seed nanoparticles were carried out in glass vessel under 200rpm at 70°C for 24 hr. Styrene (20g), DVB, SDS (360mg) were added to 250ml D.W in the 500 ml round bottom three neck flask. The polymerization processes were started by adding KPS (50 mg) with nitrogen purging. The polymerization process was performed overnight in the presence of potassium peroxosulfate (50mg) with nitrogen purging. Used fractions of crosslinker, monomer, emulsifier, and initiator are listed in Table 1. The negatively surface charged PS nanoparticles were produced via emulsion polymerization with sodium tetraborate (0.85, %w/v) and acrylic acid (2.52, %w/v).

Polystyrene Latex Particle Characterization

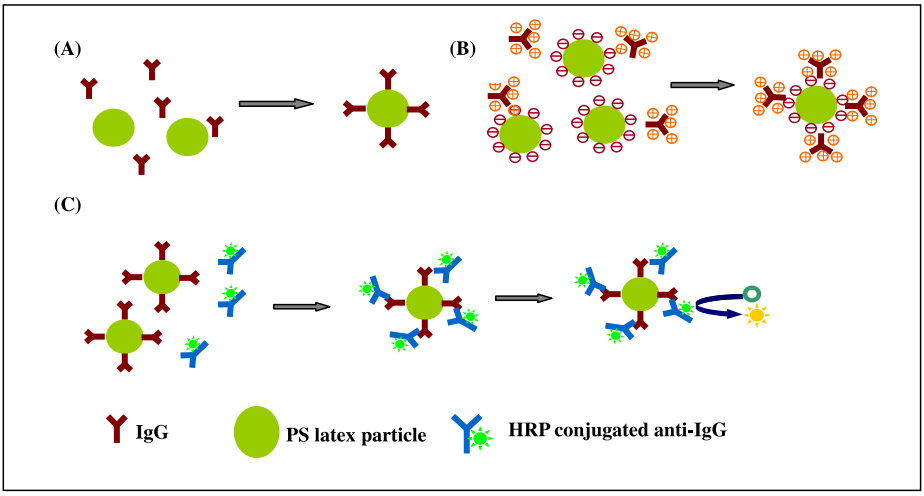
The size of PS latex particle was measured by dynamic light scattering (DLS) (DLS-700, Otsuka Electronics Co. Ltd., Kyoto, Japan) which generates the hydrodynamic diameters (D_h) of the PS particles with polydispersity. Zeta(ζ) potential of surface charge from negatively charged PS latex particle also determined by DLS-700. The morphology of synthesized PS latex particles was observed with Sirion scanning electron microscope (SEM, FEI Company, Oregon, USA). For SEM examination, emulsion was placed onto silicon wafer and allowed to air dry at room temperature. In addition, the unreacted monomers and water was evaporated at 55°C under vacuum drying to measure the weight of polymer.

IgG Adsorption on the PS Latex Particle

IgG adsorption mechanisms on the PS nanoparticle surface are illustrated in Schematic 1. IgG adsorption on the surface of PS latex particles (d= 111nm and 93nm) were performed by passive adsorption and electrostatic attraction. For passive adsorption, 400 µl of murine IgG (1.0mg/ml) was added to 4.24×10^{13} PS latex particles in 5ml of PBS(phosphate buffered saline, pH 7.2) and acidic buffer (0.1M CH_3CO_2Na, 0.1M $C_6H_7O_8$, 0.1M KBr, pH 5.0). For electrostatic attraction, IgG with isoelectric point at pH7.2 was treated in acidic buffer (pH 5.0) to induce positive charge. 4.81×10^{13} PS latex particles were introduced to 5 ml reaction buffer containing 500 µl IgG (1.0 mg/ml). The mixture was incubated for 5hrs at room temperature by gentle shaking and processed to washing for buffer exchange to PBST (PBS with 0.05 % of Tween 20) and further blocking with 1% casein. Formed IgG-PS complex was confirmed by ELISA, Zeta-potential (electrostatic attraction), and DLS measurement.

ELISA Test for the Measurement of Adsorbed IgG

The adsorbed murine IgG on PS latex particles was measured by enzymatic activities of HRP (horseradish peroxidase) which conjugated to anti-IgG antibody, specifically bind to IgG on the PS latex surface (Schematic 1). In brief, IgG-PS latex particles complex were rinsed in PBST and centrifugation at 28,500 *g* for 1 hr at 4°C. This



Schematic 1. IgG-PS Latex Particle Complex Formation. IgG Adsorption on PS Latex Particle by Passive Adsorption (A). IgG Adsorption on PS Latex Particle by Electrostatic Attraction (B). ELISA Test with HRP-conjugate anti-IgG for the Measurement of Adsorbed IgG on PS Latex Particle (C), Scale is not fit.

centrifugation step was repeated for 3 times to remove free IgG in buffer. Then, the pellet fraction of IgG-PS particle complexes were incubated with HRP-conjugated goat anti-mouse IgG (Sigma) in 1% casein (Pierce) for 1 hr at room temperature with gentle agitation. The unbound HRP-conjugated anti-IgG was removed by centrifugation as previously mentioned and pellet was resuspended in PBST. Finally, the rinsed pellet fraction was step-diluted in PBS then process to enzyme reaction in the presence of HRP substrate of TMB (3,3',5,5'-tetramethylbenzidine, Pierce). The enzyme activity was measured by ELISA reader (Molecular Devices, Union City, CA) at 450 nm upon completion of enzymatic reaction. Additional ELISA was performed after 1 month from the initial binding to determine the stability of formed IgG-PS particle complex. Desorbed fraction of IgG proteins from pre-structured IgG-PS particle complex were removed by rinsing and centrifugation in prior to enzymatic activity measurement.

Results and discussion

Synthesized PS latex particles by emulsion polymerization under different reaction conditions were characterized by DLS measurement and SEM image detection and showed monodisperse distribution and slight different sizes from each reaction condition as seen in Table 1.

SEM images showed that synthesized PS latex particles from each reaction condition were spherical in shape with uniform distribution. In the case of high concentration of divinylbenzene (30, %v/v), size in diameter of synthesized PS latex particle was smaller than 100 nm measured by SEM image detection. The size of PS latex particles was ranging between 110 to 120 nm. We did not observe significant size increment by altering the concentration of divinylbenzene. SDS concentrations did not have an effect on the size distribution and surface morphology of particles as confirmed from

SEM measurement. In addition, as described in Table 1, micelle formation time did not induce size differences. Our results indicate that the size of PS nanoparticles was not influenced by altered reaction conditions in terms of cross-linker concentration, stirring rate and micelle formation period.

Table 1. Variations in PS nanoparticle size as function of cross-linker concentration (v/v)

Experiment No.	Styrene (ml)	SDS (mg)	DVB (%, v/v)	Micelle Formation (min)	Diameter (nm)	Polydispersity
1	20	360	10	120	117	1.10
2	20	360	1	120	115	1.14
3*	20	360	1	60	111	1.16
4	10	180	1	60	93	1.13

*: Denotes the particles that were used for IgG adsorption.

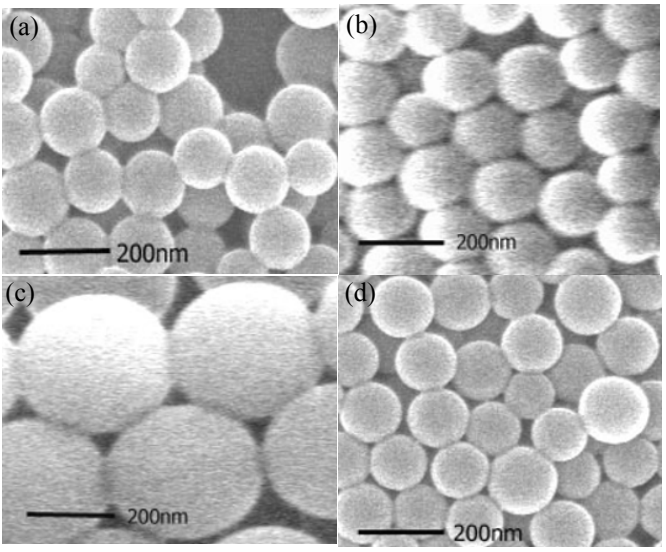


Figure 1. SEM Image of PS latex particles in various diameters synthesized by emulsion polymerization. PS latex particles from different reaction conditions. (a) Experiment #3, (b) Experiment #4, (c) Experiment #6 in Table 2, (d) Experiment #9 in Table 3. All reactions were conducted at 200 rpm and further overnight polymerization.

Cross-linked PS latex particle has been known to have high degree of surface hydrophobicity [4], in this study the surface modification of PS latex was conducted by using two different types of hydrophilic functional groups allowing the negatively charged sulfate and carboxyl group on PS surface. First, the emulsion polymerization was conducted in the presence of hydrophilic co-monomer, sodium tetraborate by which the synthesized the PS nanoparticles surface is arranged with sulfate end group ($-SO_4$). The surface distribution of sulfate group is oriented toward to the water phase as consequence the surface charge of PS latex becomes negative. Secondly, we synthesized another types of PS latex particles in the presence of co-monomer of acrylic acid which functionalize the surface of PS latex particle with carboxyl group (-COOH) upon completion of polymerization. Change in hydrophobicity of PS latex

surface by introducing hydrophilic groups was confirmed by Zeta (ζ) potential measurement and showed moderate and dramatic changes of values depending on the different functional group. The Zeta potential by functionally modified by sulfate group was -51mV and that of carboxyl group was -2.1mV respectively as seen in Table 2.

Table 2. Size distribution and functional surface modification of polystyrene latex particles

Experiment No	Functional Group	Charge on the Surface		Zeta Potential (ζ)	Diameter (nm)
		Co-momoner	Conc.(w/v%)		
1	-	-	-	0.37 mV	117
5	$-SO_4$	Na_2B_4O7	0.85	-56.7 mV	93*
6	-COOH	$C_3H_4O_8$	2.52	-2.1 mV	258

*: Denotes that the IgG-PS latex complex was formed based on electrostatic attraction.

The Zeta potential value of the unmodified PS latex was 0.37 mV. The result supports the notion that the concentration of sodium tetraborate (0.85, %w/v) was optimal in this experimental condition to make the synthesized particles be in stable homogeneous dispersion through repulsion force from negative charge on the surface. In the case of carboxyl group, the hydrophilicity of surface was less strong compared to that of sulfate group. The PS latex particle which has functionally modified carboxyl group on the surface was not used for the IgG adsorption since the size in diameter was twice as large as the particle which is functionalized by sulfate group and unmodified. We intended to observed surface charge effect on IgG adsorption within the particles in similar size having almost same surface area. The total number of PS latex particle was calculated as previously described by Yang et al [21].
The total number of PS latex particle =

$$\frac{W_{ps,p} + W_{pdvb,p}}{\overline{\rho_p}} \frac{1}{4\pi R^3 / 3} \qquad \text{[Eq. 1]}$$

Where the $W_{ps,p}$ and the $W_{pdvb,p}$ are the weights of styrene and DVB in polymer particle and the measured weight of total polymer weight in 1ml of was 0.033g (bare PS latex) and 0.022g (sulfated PS latex) respectively. Where $\overline{\rho}$ is the density of polymer particles, and the measured value was 1.0868 g cm^{-3}. The volume of one polymer particle is $4\pi R^3 / 3$. Therefore the total number of particles used in IgG adsorption for each different PS latex particle could be calculated. The total numbers of PS latex particle for IgG adsorption are shown in Table 3. The total number of IgG used for the adsorption also calculated as previously described by Sell [24] as follows:

$$\text{Total number of molecules of IgG} = \frac{\text{Weight in grams} \times \text{Avogadro's number}}{\text{Molecular weight of IgG}} \qquad \text{[Eq.2]}$$

The total numbers of IgG molecules introduced for adsorption on the surface of two different types of PS latex particles are shown in Table 3. We fixed the molecular

weight of IgG as 150,000 Da. Finally, the formed IgG-PS latex particle complex was confirmed by immunoassay and DLS measurement as described in Experimental.

Table 3. Reaction conditions for PS latex particle/IgG complex and altered size in diameter of PS-IgG complex after IgG adsorption determined by DLS measurement

Experiment Number	Diameter (nm)	Number of PS latex particles	pH of reaction buffer	IgG		Diameter of IgG-PS latex complex
				Number of IgG molecules	Conc. (mg/ml)	
7	111	4.24×10^{13}	7.2	1.6×10^{15}	1.0	178nm
8	111	4.24×10^{13}	5.0	1.6×10^{15}	1.0	207nm
9	93*	4.81×10^{13}	5.0	2.0×10^{15}	1.0	220nm

*: Denotes that the IgG-PS latex complex was formed based on electrostatic attraction.

DLS measurement was performed to observe the characteristic size increase of PS latex particle by adsorbed IgG on the surface. DLS results provided the hydrodynamic diameter(D_h) of measured particle and results support that IgG-PS latex particle complex has been formed since the increased diameter of IgG-PS complex was in agreement with the size of IgG ($\approx 21.9 \times 15.5 \times 1.5$nm) adsorbed on the surface of particle [17]. In the case of acidic condition of reaction buffer (pH 5.0, Experiment #9 in Table 3) the size increase was slight higher than that of neutral condition. It would be explained that the abundance of H^+ ions in reaction buffer resulted in the formation of extended electrical double layer.

Our immunoassay results showed that the signal increase of HRP support the notion that the surface of PS latex particle is covered by adsorbed IgG in phosphate buffer-based reaction (pH7.2). It is expected that the surface distribution of IgG would be homogeneous and each single particle made complex with IgG since the signal generated by HRP showed linear increase as function of the number of particles added in each reaction as shown in Fig. 2. If the surface distribution of IgG would not be homogeneous, no linear pattern of signal increase could be determined. According to different pH condition of acidic buffer (pH5.0), IgG-PS latex particle complex formation also has been confirmed by immunoassay results of linear increase in HRP

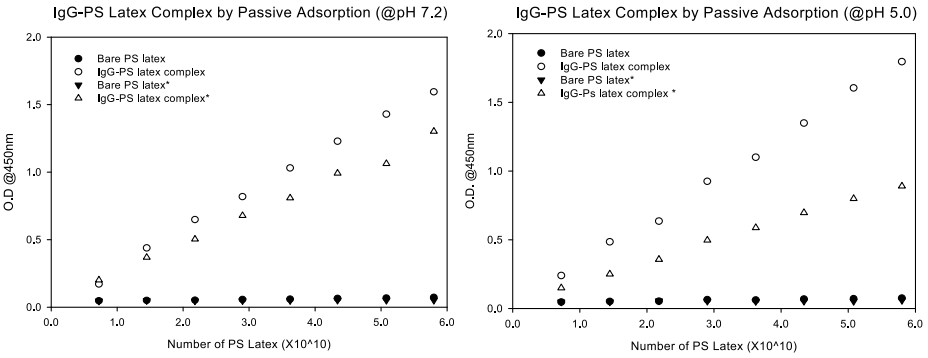


Figure 2. IgG adsorption on polystyrene nanoparticles by passive adsorption
*: Denotes the HRP signal measurement after 1 month from the initial complex formation

signal as function of different number of particles. HRP signal increase between different pH conditions did not reveal pH preference for IgG adsorption.
IgG conjugation on the functionally modified surface of PS latex particle was performed based on the electrostatic attraction. IgG, the i.e.p(isoelectric point) of pH 7.2, becomes positively charged when it treated with acidic buffer (pH 5.0) and bind to negatively charged sulfate group on the surface of PS latex particle by charge interaction. Upon completion of conjugation, the formation of IgG-PS latex particle complex was confirmed by immunoassay and change of Zeta potential (ζ) respectively. Immunoassay result of IgG conjugation by electrostatic attraction similarly with that of passive adsorption method showed the linear increase of HRP signal as function of different number of latex particles whose surface is covered by IgG proteins. The magnitude of the measured intensity values of HRP signal was higher than that of passive adsorption suggesting high binding affinity of electrostatic attraction under same experimental condition. It would be explained that negatively charged surface of latex particles is covered by positive charge of IgG and induced the dramatic increase of Zeta potential (from -56.1mV to -3.84mV). It is also support the evidence that surface distribution of IgG is very homogeneous and most of particles were formed the complex with IgG proteins.

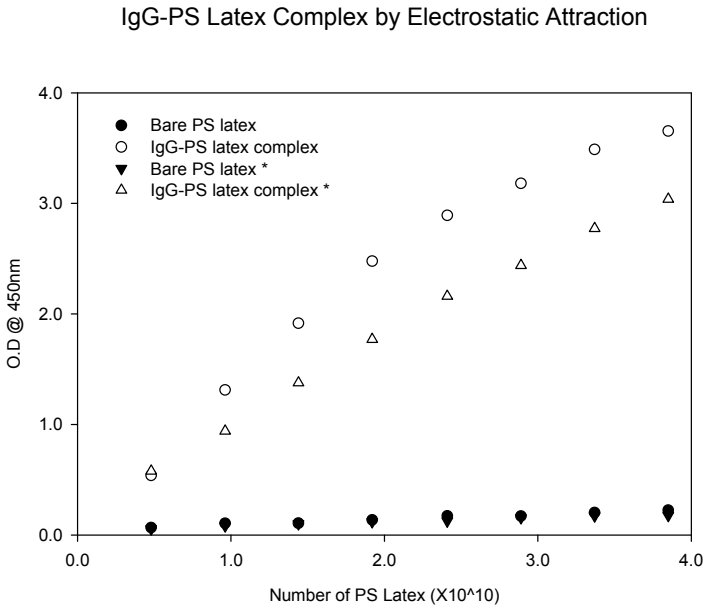


Figure 3. IgG adsorption on polystyrene nanoparticles by electrostatic attraction
*: Denotes the HRP signal measurement after 1month from the initial complex formation

After 1 month upon initial IgG-PS latex particle complex formation, the activity of HRP was determined and showed similar pattern in linear increase as function of number of particles both from passive adsorption and electrostatic attraction. Desorbed fractions of anti-IgG-HRP from previously formed IgG-PS complex stored at room were removed by centrifugation. Therefore, the HRP signal could be generated only by the complexes that maintain the structured of PS latex-IgG/anti-

IgG-HRP. In the case of passive adsorption, the HRP signal showed same pattern with that of initial curve, however, reduced intensity values were determined both from different pH conditions. In the case of acidic buffer condition, the decrease was more significant than that of neutral condition. Decreased HRP signal intensity also observed with the IgG-PS complex in electrostatic attraction, however, the decreased HRP signal intensity was moderate than that of passive adsorption. According to the decrease of HRP signal intensity, one another possible expectation would be the decrease in signal intensity which may be resulted from the decay of fluorescence activity of HRP rather than the dissociation of anti-IgG from IgG-PS complex.

Conclusions

In this study, we synthesized surface unmodified and modified with negatively charged poly (styrene/divinylbenzene) latex particles and processed further IgG immobilization. DLS and Zeta potential measurement showed the presence of IgG-PS particle complex which formed by passive adsorption and electrostatic attraction. Importantly, our immunoassay results showed the linear increase in HRP signal which provide the evidence that the surface distribution of IgG is homogeneous. In addition, immunoassay results support the notion that the number of IgG for the reaction condition was sufficient enough to optimal binding point. Additional immunoassay conducted on 1 month after initial IgG-PS complex formation demonstrated that the formed IgG-PS latex complex was stable up to 1month from initial complex formation both from passive adsorption and electrostatic attraction. However, the electrostatic attraction method revealed higher binding affinity and strength than that of passive adsorption as previously reported [16]. Thus, we could propose that IgG-PS latex particle complex formed by electrostatic attraction as suitable candidate for solid-phase immunoassay requires long-term stability for the application in clinical application.

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