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High-Performance Immunolatex Possessing a Mixed-PEG/Antibody Coimmobilized Surface: Highly Sensitive Ferritin Immunodiagnostics

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To create a high-performance immunoassay system based on a nanosphere/antibody complex, pentaethylenehexamine-ended poly(ethylene glycol), N6-PEG comprising N6-PEG-5k (M_n = 6000 g/mol) and N6-PEG-2k (M_n = 2000 g/mol) was employed as a novel blocking agent to modify the surface of nanospheres. Both the antibody (antiferritin) and the N6-PEG were covalently bonded onto the nanospheres by the linkage of their amino groups with the activated carboxyl groups of those particles. The quantification of antiferritin and tethered N6-PEG polymer was carried out using the copper reduction/bicinchoninic acid reaction (the Micro BCA method). Dynamic-light-scattering (DLS) and electrophoretic mobility (μ_e) measurements were performed to characterize the nanosphere/antiferritin/N6-PEG complex, which was prepared under various conditions. Simultaneously, the immune response of the complex obtained in this manner was measured by the turbidimetric monitoring method in phosphate buffer (10 mM, pH = 7.4). On the basis of all the results, the optimum conditions for preparing an acceptable nanosphere/antiferritin/N6-PEG complex were determined. Interestingly, compared to the blocking treatment with bovine serum albumin (BSA), which is a well-known blocking agent, surface modification with N6-PEG, especially that using a mixture of N6-PEG-5k and N6-PEG-2k, improved the performance (increased immune response yield and decreased detection limit) of the nanosphere/antiferritin complex to a remarkable degree in both phosphate buffer and 100% fetal bovine serum (FBS), thus significantly demonstrating the potential of the nanosphere/antibody/mixed-PEG complex as central to a high-performance immunoassay system.

As an exceptional carrier of antibodies or antigens, surface-functionalized polystyrene colloidal particles (nanospheres) are of great interest in many types of serological diagnosis. This is not only because they are low-cost, chemically and colloidally stable, and available in homogeneous sizes, but they also have a conveniently varied functional surface, which favors the covalent attachment of antibody or antigen.[1−7] Compared to physical adsorption, this covalent binding of antibody or antigen may improve the immunoassay performance of these particles, since they are more stable over time and can orientate appropriately, rendering them more accessible to complementary analytes.[8]

In principle, serological diagnosis using a nanosphere/antibody complex is based on the phenomenon of aggregation of the nanospheres, which is triggered by the agglutination of a specific antigen in the serum with several complementary polyclonal antibodies on different nanospheres. Hence, the nonspecific adsorption of antigen and/or other contaminants in the serum onto the nanosphere/antibody complex, the low dispersion stability of the nanosphere/antibody complex caused by antibody immobilization onto the nanospheres, and the electrical repulsion between the nanospheres and the antigen, which prevents the approach of the antigen to the located antibody, are major issues related to the diagnostic efficiency of this immune diagnosis method.[7,9−11] These problems occur depending on the surface

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characteristics of nanospheres (e.g., electric charge and hydrophobicity) and the protein feature of the antigen (e.g., isoelectric point and structural stability),[12] which cause different obstacles in different systems. To solve these complex problems, effective surface modification of the nanospheres after antibody attachment is necessary to remove any nonspecific interaction between the surface of the nanospheres and the antigen.

Though bovine serum albumin (BSA) is generally used as a blocking agent, its efficiency is very limited because BSA is an acidic protein, as is negatively charged under physiological conditions. This feature enhances the dispersion stability of the nanosphere/antibody complex, whereas it is unfavorable for the dense application of BSA molecules onto a negatively charged surface, for example, the carboxylated surface of nanospheres, because of the electrical repulsion between BSA molecules and that between the surface and BSA. Similarly, a negatively charged BSA-covered surface tends to halt the approach of a negatively charged antigen[13] and preclude its interaction with an immobilized antibody. Moreover, BSA may also influence the formation of immune complexes by occupying the epitopes of the prelocated antibody if excess BSA is loaded onto the nanosphere surface.

Poly(ethylene glycol) (PEG) is a well-known hydrophilic, flexible, and neutral polymer of low toxicity. It has been widely used to stabilize various nano- and microscale particles. Additionally, since a PEG chain possesses low affinity for various biomacromolecules, a PEGylated surface improves the performance of biodiagnosis, especially in immunodiagnostic systems.[9,14−16] In this case, the long chain length and high chain density of the PEG layer were both required, but increased polymer chain length is always accompanied by decreased polymer chain density, owing to the steric exclusion of the tethered PEG chains.[17,18] Many approaches have been tried to improve the polymer chain density of the PEG layer by suppressing this kind of steric extension. One of these approaches is to reduce the size of the PEG molecules in a poor solvent of PEG during the course of the PEGylation reaction, which may suppress the steric extension and result in increased amounts of tethered PEG chains under these conditions. For example, aqueous media with high salt concentrations and/or at high temperatures are employed to improve the PEG chain density.[19] Unfortunately, these conditions were not suitable in the present study, since these methods may damage the prelocated antibody and decrease its reactivity. According to our previous investigations of PEGylated surfaces, a mixed-PEG chain layer with both long-chain (5k) PEGs and short-chain (2k) ones resolved this trade-off relation, in that the long PEG chains retained the chain length of the layer, while the short ones easily occupied the interspace between two long chains to remarkably increase the total chain density of the layer.[9,20−22] Besides this efficient suppression of various nonspecific interactions, a PEG layer may also create a suitable environment for specific types of bio-recognition. For example, gold nanoparticles with coimmobilized enzyme and PEG polymer (nanozymes) were found to increase the enzymatic reactivity of the enzyme as compared to that of the free enzyme, while boosting the dispersion stability considerably.[23,24] On the basis of these facts, it is considered to be an ideal strategy to replace BSA with PEG polymer, especially a mixture of PEGs, which then would serve as a novel blocking agent. The objective of this study was to create a stably dispersed and highly responsive nanosphere/antibody/PEG complex by the construction of a compact and neutral PEG layer around the located antibody on the surface of nanospheres.

In this paper, rabbit antihuman ferritin polyclonal antibody (antiferritin) was used as the model antibody. It was covalently linked to nanospheres through the reaction of its amino groups with the activated carboxyl groups (active ester) of the particles, followed by the similar linkage of α-methoxy-poly(ethylene glycol)-pentaethylenehexamine (N6-PEG), which possesses six amino groups at one end. Compared to monofunctional amino groups, this positively charged six-amino-group structure is more electrically attracted to the negatively charged surface of nanospheres at pH = 7.4, which accelerates the approach and reaction of N6-PEG with the active ester on the nanosphere surface. The excess amino groups in N6-PEG may electrically interact with the carboxyl groups due to the possible hydration of the active ester on the surface. This is crucial for the construction of a densely packed PEG layer because the active ester is unstable in aqueous solutions and susceptible to hydrolysis and thereby to the loss of its reactivity.[25] The preparation conditions for the nanosphere/antibody/PEG (LAP) complex were investigated in detail to obtain an LAP complex possessing both high immune performance and high dispersion stability. Although the obtained LAP and nanosphere/antiferritin/BSA (LAB, as a control) complexes possessed similar sizes and distributions (260−270 nm; polydispersity index (μ/Γ^2) < 0.1), the LAP complex showed evidently improved immune response yields and detection limits in both phosphate buffer (pH = 7.4) and 100% fetal bovine serum (FBS) as compared to those of the LAB complex, clearly suggesting its potentiality to displace the nanosphere/antiferritin/BSA complex in clinical serum diagnosis applications.

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EXPERIMENTAL SECTION

Materials. Carboxylated polystyrene nanospheres (AJ26COOH-Clean, 10% w/v, Ikerlat, Spain), bovine serum albumin (BSA; lot 651, Serologicals, U.S.), rabbit antihuman ferritin polyclonal antibody (antiferritin, 9.7 mg/mL), and human ferritin (31.284 μg/mL) were a kind gift from Biokit S.A. (Barcelona, Spain). All of these products were used as received. According to the manufacturer's data, the surface charge density of the nanospheres is 25.28 μC cm^{-2}, and the area occupied by each carboxyl functional group is 63 Å^2/group. The particle size (251 ± 3 nm) and electrophoretic mobility (μ_e = −5.7 ± 0.1 μm cm/Vs) of the nanospheres in phosphate buffer solution (buffer A; 10 mM, pH = 7.4) were measured on a Malvern Zetasizer Nano ZS instrument (He−Ne laser, U.K.). The antiferritin was diluted with buffer A to 0.516 mg/mL, which corresponds to a fairly low amount of antiferritin added to the particles (0.45 mg/m^2), to confirm the efficiency of PEG-modification in detail. Ferritin was also diluted with buffer A to 1 μg/mL just before use.

Two kinds of α-methoxy-poly(ethylene glycol)-pentaethylene-hexamine (N6-PEG) were used in this study. One was N6-PEG-5k (CE510, lot 070719AR, 2.1% w/v), which was a kind gift from JSR Co. (Japan). The M_n value of the N6-PEG-5k (6000 g/mol; M_w/M_n= 1.13) and the N6 end-functionality (81%) were determined by ^{1}H NMR spectrum analysis (270 MHz). Malmsten, et al.[17b] have reported that densely tethered PEG polymers (molecular weight: 6650) on the substrate were about 8−14 nm long in aqueous solution. The hydrodynamic diameter of antiferritin in solution was 13 ± 4 nm based on DLS measurements. N6-PEG-5k polymer with a molecular weight of 5k−6k is suitable for modifying the surface of nanospheres. Another one was N6-PEG-2k (M_n = 2000 g/mol; M_w/M_n= 1.05; N6 end-functionality: 87%), which was synthesized as described in our previous papers and similarly characterized as above.[9] This polymer was used to make an under-brushed N6-PEG-2k layer to increase the total tethered-chain density of the PEG layer. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, special grade) and fetal bovine serum (FBS) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) and Invitrogen Co. (lot 1412447, Tokyo, Japan), respectively. All chemicals were used without further purification.

Activation of the Carboxyl Functional Groups of the Nanospheres. A nanosphere suspension (30 μL) was poured into a 1.5 mL plastic tube and diluted with 167 μL of NaH_2PO_4 solution (buffer B; 10 mM, pH = 4.8). Then, 3 μL of an EDC aqueous solution were poured in, followed by shaking for 20 min at 25 °C (shaking velocity = 1000 rpm; shaking incubator SI-300, As One Co., Japan). The EDC concentrations used were 6.25, 12.5, and 25 mg/mL, corresponding to the molar ratios of EDC to the carboxyl groups of the nanospheres (*ECR*) of 1:2, 1:1, and 2:1, respectively. The obtained activated nanospheres, which possessed active ester groups on the surface, were further diluted with 100 μL of buffer A and used immediately. The final nanosphere concentration was 1% w/v. In this paper, the temperature was 25 °C, and the shaking velocity was 1000 rpm in all experiments unless otherwise stated.

It is notable that the activation of the nanospheres by EDC only was similar to that achieved by a mixture of EDC and *N*-hydroxysuccinidmide (NHS), which is a well-known method using NHS to stabilize active esters.[25] The similarity is probably due to the speedy antibody immobilization treatment after the activation of the nanospheres, as described in the following sections. Since NHS strongly interfered with the quantification of the immobilized antibody because of the copper reduction/bicinchoninic acid reaction (the Micro BCA method), it is preferable to employ EDC only for the activation reaction. The optimal *ECR* value, namely, the amount of EDC required to activate the nanospheres, was determined to be 1:1, as described in detail in section S2 of the Supporting Information. This *ECR* value was the main one used to prepare various forms of the nanosphere complex in the ensuing experiments.

Construction of the Nanosphere/Antibody/PEG (LAP) Complex. *Estimation of the Reaction Time of the Antibody to the Nanospheres.* Activated nanospheres (*ECR* = 1:1, 50 μL) were mixed with 275 μL of buffer A and 10 μL of antiferritin solution (0.516 mg/mL, the molar ratio of the carboxyl group of the nanospheres to antiferritin equal to 879.). This mixture was continuously vibrated for the prescribed periods (15, 30, 45, 60, and 90 min) to enable the coupling of antiferritin with the activated nanospheres. The obtained nanosphere/antibody (LA) complex (nanosphere conc. = 0.15% w/v) was in a microaggregation state, and was mono-dispersed again by ultrasonication treatment (35 W, 50−60 Hz, room temperature, 2−5 s several times). Then, 300 μL of the LA complex were added in 150 μL of N6-PEG-5k polymer solution (buffer A, 0.3% w/v), followed by shaking for another 30 min, as determined by the detailed investigations presented in section S1 of the Supporting Information. The final nanosphere concentration was 0.1% w/v.

Evaluation of the Proper N6-PEG-5k Concentration. For the N6-PEG modification reaction, the suitable N6-PEG concentrations were investigated. After the LA complex (300 μL, nanosphere conc. = 0.15% w/v), which was obtained by 1 h of vibration of a mixture of antiferritin and activated nanospheres (*ECR* = 0, 2:1, 1:1 and 2:1), was re-mono-dispersed as described above, 150 μL of N6-PEG-5k solution of various concentrations (0.03, 0.06, 0.12, 0.3, and 0.6% w/v, corresponding to the molar ratio of N6-PEG-5k to the carboxyl group of the nanospheres of 0.3, 0.7, 1.3, 3.3, and 5.8, respectively.) were added and vibrated for another 1 h. The obtained LAP complex was slightly suspended by ultrasonication (35 W, 50−60 Hz, room temperature) for 5 s and maintained at 4 °C for further studies.

Construction of the Nanosphere/Antibody/Mixed-PEG (LAmP) Complex. As with the preparation of the LAP complex, the obtained LA complex (200 μL, *ECR* = 1:1) was first reacted with 50 μL of N6-PEG-5k solution by shaking for 30 min. Then, 50 μL of N6-PEG-2k solution of various concentrations were added to the mixture, followed by vibration for another 30 min. The final N6-PEG-5k concentration in the solution was 0.1% w/v. The mass ratios of N6-PEG-5k to N6-PEG-2k (5k/2k) were 1:0 (i.e., N6-PEG-5k only), 1:0.25, 1:0.43, 1:0.67, and 1:1. As a control, the same experiment using N6-PEG-5k was carried out; namely, after the preparation of the LAP complex with N6-PEG-5k, N6-PEG-5k was added again and vibrated for further 30 min. The final concentration of N6-PEG-5k in the solution was 0.2% w/v (sample code: 5k/5k = 1:1).

Immune Response Measurements. The immune response of the test samples was measured by turbidimetric monitoring

on a PL-2500 spectrophotometer (Shimadzu, Japan). Briefly, 50 μL of the test sample suspension (nanosphere conc. = 0.1% w/v) were mixed well with a given amount of human ferritin solution, which was obtained by the dilution of ferritin solution (1 μg/mL) using buffer A or FBS solution of various concentrations from 0 to 100% v/v (0% v/v and 100% v/v denotes buffer A and original FBS, respectively.). The total final volume was 500 μL. Then, turbidimetric monitoring was performed immediately at a wavelength of 550 nm for 10 min at room temperature, and the slope of the initial part of the obtained curve was identified as the immune response in this study. The turbidity change caused by the immuno-coagglutination of the nanosphere complex was much more significant at 550 nm than that at 750 nm, which is consistent with light scattering theory[26] according to which size-dependent variation in light scattering may be amplified when suspended particles are monitored at short wavelength.[27] But the initial O.D. value (light scattering signal) at time = 0 was over 0.5 when using a monitoring wavelength of 500 nm and 50 μL of test sample. To obtain correct and accurate immune response experimental values, 550 nm was selected thenceforth as the monitoring wavelength in this study. The volume of the test sample (50 μL) was determined in detail and is described in section S2 of the Supporting Information. It should be noted that no appreciable aggregation of various forms of the nanosphere complex occurred without ferritin in buffer A and FBS in this measurement.

RESULTS AND DISCUSSION

Since Singer, et al.[28] first reported the preparation of immunolatex particles in 1956, various preparation approaches have been described using different nanospheres and antibodies. Thus, it is necessary to investigate the suitable conditions in detail for our system, such as the immobilization time, ratio, concentration of antibody and PEG polymer, and so forth.

Reaction Time of Antiferritin. The adsorption of antibody onto nanospheres, which is similar to that of protein onto a solid surface, is influenced by various factors, for example, antibody concentration, adsorption time, surface characteristics, and so on. The antiferritin concentration (0.516 mg/mL) and other factors were fixed in this study to optimize the reaction time of antiferritin to the activated nanospheres. The rationalization of this parameter is important, since antiferritin may physically adsorb onto the particles, which means that the antibody is unstably immobilized and easily desorbed during diagnostic treatment. Conversely, an excessively long reaction time may lead to the denaturation of antiferritin or change its orientation on the surface to decrease its immune response.

Figure 1 shows the immune response of nanospheres modified with antiferritin under different antibody-immobilization conditions. N6-PEG at a concentration of 0.1% w/v was used as a blocking agent in this study. When the reaction time of antiferritin was 15 min, the LAP complex yielded a slightly low immune response compared to the others. The response became almost constant when the immobilization reaction was allowed to continue for more than 30 min, indicating that a period of more than 30 min was required for a complete immobilization reaction. Actually, the amount of immobilized antibody, which was quantified by the Micro BCA method, after 15 min was slightly lower than that for the other complexes (Table S-1 in section S2 of the Supporting Information). About 95% of antiferritin was immobilized onto the nanospheres when its reaction time was longer than 30 min. Since there was no significant difference in both the immune response value and the loaded antiferritin amount for the other forms of LAP complex within the experimental error margin, a 1 h reaction time of antiferritin was employed in the subsequent experiments.

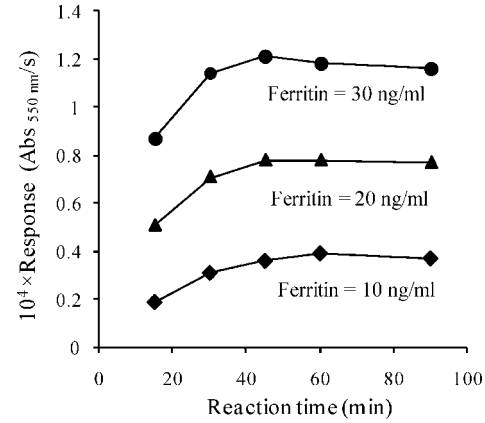

Figure 1. Immune response of the LAP complex (*ECR* = 1:1) at various ferritin concentrations in the solution as a function of the reaction time of antiferritin with the nanospheres.

Prior to the evaluation of the PEGylation efficiency of antibody-immobilized nanosphere (LA complex), the conventional blocking treatment using BSA was examined.

BSA Concentration. Most of the immunolatexes produced so far have been prepared by BSA blocking treatment after antibody immobilization. Although various BSA concentrations have been used in different systems, little explanation has been given on how to establish the right concentration. It is imperative to identify the proper BSA concentration, in order to prepare an acceptable nanosphere/antibody/BSA (LAB) complex.

The proper concentration of BSA was determined by two methods, that is, according to the variations in the electrophoretic mobility value (μ_e) and according to the immune response of the LAB complex with increasing BSA concentration. As described in section S2 of the Supporting Information, the suitable ratio of EDC to the carboxyl groups on the nanosphere surface (*ECR*) was determined to be 1:1. The LAB complex (*ECR* = 1:1) was prepared under the same conditions. BSA concentrations ranging from 6.7×10^{-3} to 3.3% w/v in the solution (i.e., from 0.01 to 5.13 μM, the molar ratio of the carboxyl group of the nanospheres to BSA changed from 5876 to 12) were employed for this modification. Figure 2a shows the μ_e and immune response of the LAB complex as a function of the BSA concentration. As shown in this figure, the μ_e value of the LAB complex changed from -3.8 to -1.9 with increasing BSA concentration, but notable variations from -3.8 to -2.7 occurred below BSA = 2.7×10^{-2} % w/v. This fact indicates that the negatively charged activated nanosphere was effectively covered by BSA when the BSA concentration was above 2.7×10^{-2}% w/v. Moreover, the immune response of the LAB complex significantly increased to the maximum value at a BSA

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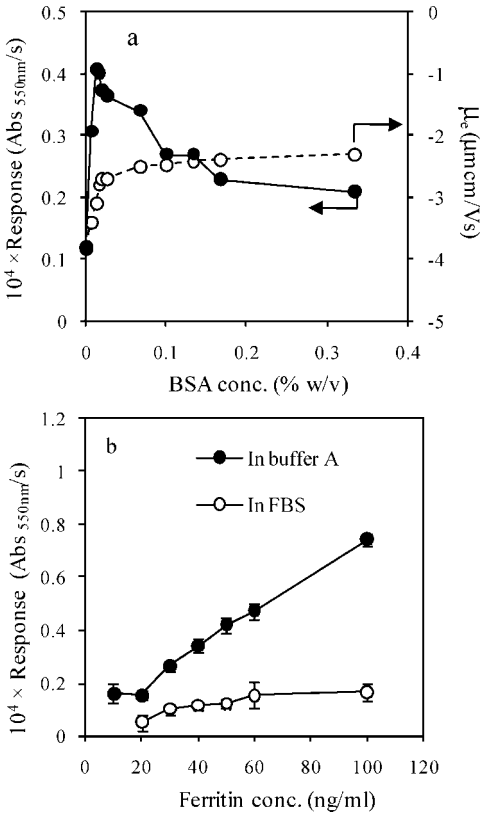

Figure 2. (a) Immune response and μ_e value of the LAB complex as a function of the BSA concentration in the solution. (b) Immune response of the LAB complex (BSA conc. = 1.3×10^{-2} % w/v) measured in buffer A and FBS.

concentration in the solution of about 1.3×10^{-2} % w/v, and then progressively decreased with increasing BSA concentration. At low BSA concentrations (<2.7×10^{-2} % w/v), the change in the μ_e value of the LAB complex is consistent with that of the immune response. This consistence indicates that the addition of BSA as a blocking agent might weaken the electrical repulsion between ferritin (isoelectric point <6) and the nanospheres, which promotes the approach of ferritin to the nanospheres to interact with the immobilized antibody, resulting in an increased response of the LAB complex. But too much BSA may have a tendency to occupy the epitopes of antiferritin and interfere with its agglutination with ferritin, leading to decreased immunoreactivity of the LAB complex. It is interesting to note that the BSA concentration of 1.3×10^{-2} % w/v (0.02 μM) is twice higher than that needed to completely cover a nanosphere, assuming that the nanosphere and the BSA molecule are spheres with diameters of 250 and 8 nm (DLS experimental results), respectively. Consequently, it is plausible to assume that there is a critical BSA concentration, 1.3×10^{-2} % w/v (0.02 μM), for preparing a high-performance LAB complex, and this was used in this study.

Figure 2b shows the immune response yields of the LAB complex. In buffer A, the yields were proportional to the ferritin concentration when it was higher than 30 ng/mL, but this tendency completely disappeared in 100% fetal bovine serum (FBS) within the experimental range, clearly indicating the limited efficiency of BSA as a blocking agent under the present conditions.

We then investigated the suitable conditions for the PEGylation of antibody-immobilized nanosphere (LA complex).

N6-PEG-5k Concentration. The proper N6-PEG-5k concentration should meet two requirements. One is the capability of N6-PEG-5k to completely cover the residual unmodified surface of each nanosphere after antibody immobilization, to avoid any nonspecific recognition. The other is to create a favorable environment for antibody−antigen agglutination. To obtain suitable PEGylation conditions, the μ_e value of PEGylated nanospheres without antibody on the surface (LP complex) was evaluated simply to understand the PEGylation effect. In addition, the immune response of the LAP complex was then estimated as a function of the N6-PEG-5k concentration in the solution.

The preparation of the LP complex was carried out by mixing different concentrations of N6-EPG solution with activated nanospheres (*ECR* = 2:1). As shown in Figure 3a, the μ_e value of the nanospheres changed from −5.7 to −4.2 after activation. When the activated nanosphere was treated with N6-PEG-5k, the μ_e value of the LP complex increased and reached zero with increasing N6-PEG-5k concentration in the solution up to 0.01% w/v. This phenomenon reflects the fact that the conjugation of the neutral and hydrophilic PEG polymer to nanospheres shifted the hydrodynamic plane of shear away from the particle surface, which resulted in a reduced μ_e value.[17b,18b] It also points to the generation of a N6-PEG-5k polymer layer around the nanosphere. The proper concentration of N6-PEG-5k in the solution, thus, should be higher than 0.01% w/v to completely cover the surface of each nanosphere.

The immune response of the LAP complex as a function of the N6-PEG-5k concentration in the solution gave a profile similar to that of the μ_e value; that is, the response increased with increasing N6-PEG-5k concentration in the solution from 0.01 to 0.04% w/v before it became constant (Figure 3b, the representative result of *ECR* = 1:2). In other words, the N6-PEG-5k concentration in the solution should be higher than 0.04% w/v to obtain a high and constant immune response value, even though the nanosphere surface is fully covered as a result of the treatment with 0.01% w/v of N6-PEG-5k. These facts indicate that the excess free N6-PEG-5k polymer present in the LAP complex suspension improved its immune response. Actually, if centrifugation is performed once to exchange the supernatant for buffer A, the immune response of the LAP complex decreases. It should be noted that the immune response of the LAB complex decreased when it was exposed to excess BSA blocking, as stated above. In the case of the LAP complex, no decrease in the immune response was observed even upon treatment with N6-PEG-5k at a high concentration. This fact means that N6-PEG-5k does not interact with any epitope of the prelocated antibody on the nanosphere surface. On the basis of these results, 0.1% w/v was selected as the suitable N6-PEG-5k concentration in the solution for preparing a desirable LAP complex in the subsequent experiments.

As mentioned above, the N6-PEG-5k polymer may chemically (covalent bond) and/or physically (electrical attraction) modify carboxylated nanospheres, since the oligoamine segment of the N6-PEG-5k polymer is positively charged at pH = 7.4. Thus, covalent PEGylation via an active ester and N6-PEG-5k might not be required for the modification. For example, the electrically PEGylated nanosphere (n-LP complexes), which was prepared with no activation agent (*ECR* = 0), showed a μ_e profile similar

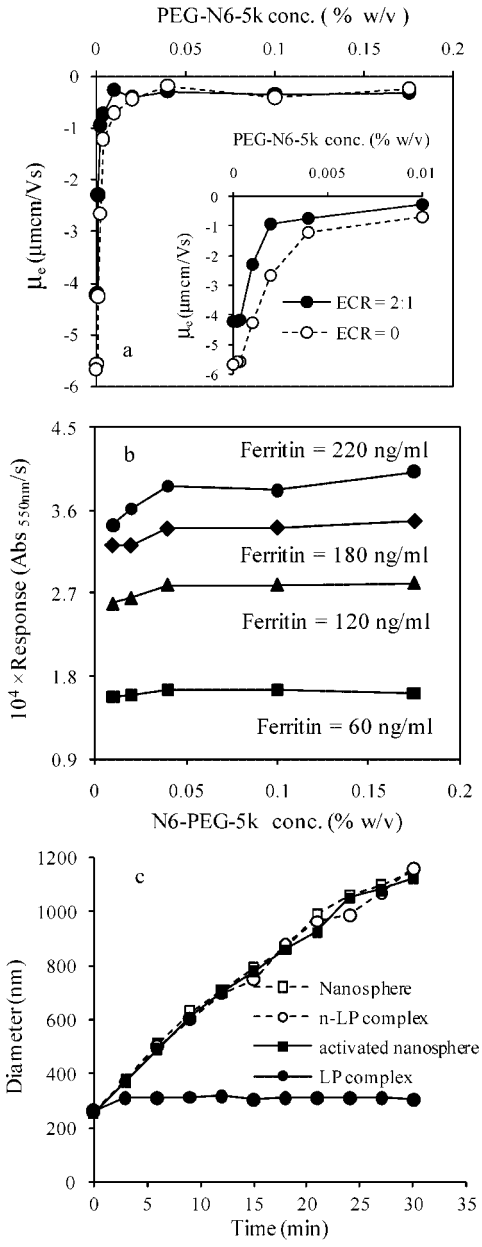

Figure 3. (a) Variations in the μ_e value of the LP complex (*ECR* = 2:1; reaction time of N6-PEG-5k with the nanospheres: 60 min) and the n-LP complex (*ECR* = 0; reaction time of N6-PEG-5k with the nanospheres: 60 min) with increasing N6-PEG-5k concentration in the solution. (b) Immunoreactivity of the LAP complex (*ECR* = 1:2) corresponding to various ferritin amounts as a function of the N6-PEG-5k concentration in the solution. (c) Time-dependent variation in the size of nanosphere and activated nanospheres and the LP (*ECR* = 2:1) and n-LP (*ECR* = 0) complexes in 2 M NaCl solution.

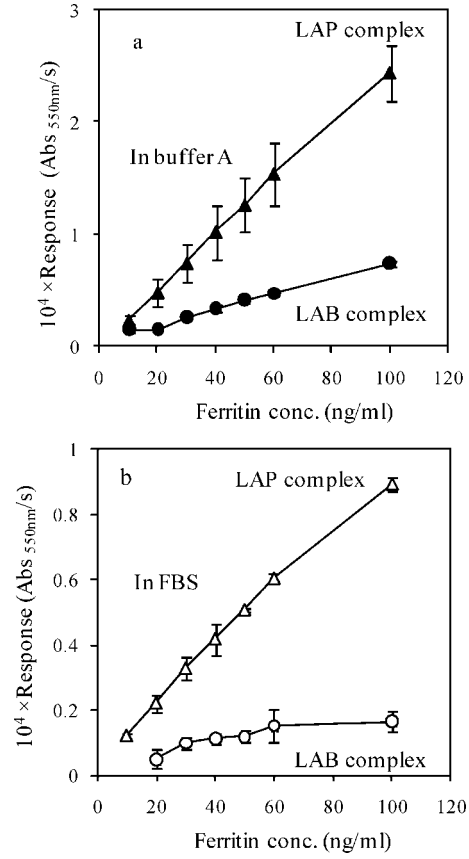

Figure 4. Immune response of the LAP and LAB complexes in (a) buffer A and (b) FBS solution.

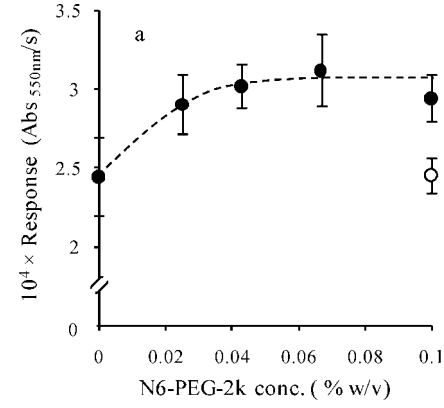

Figure 5. N6-PEG-2k-concentration-dependent immune response of the LAmP complex (N6-PEG-5k = 0.1% w/v) measured at 100 ng/mL ferritin. ○ denotes the immune response of the LAP complex (5k/5k = 1: 1, N6-PEG-5k = 0.2% w/v) for comparison.

to that of covalently PEGylated nanosphere (LP complexes) regardless of the N6-PEG-5k concentration in the solution. (Figure 3a) To determine whether or not covalent linkage (activation step) is required, the dispersion stability of the LP (*ECR* = 2:1) and n-LP (*ECR* = 0) complexes (N6-PEG-5k = 0.1% w/v) and nanospheres was investigated at a high salt concentration (2 M NaCl). Figure 3c shows the time course of the particle sizes within 30 min of salt addition on a Malvern Zetasizer Nano ZS instrument. The size of the nanosphere itself, regardless of the activation process, significantly increased after salt addition, apparently indicating that the nanospheres were unstable without PEG-modification and rapidly aggregated in the 2 M NaCl solution. This is because the salt ions thinned the electrical double layer of the nanosphere and weakened the electrical interparticle repulsion, thus promoting their aggregation. The n-LP complexes also aggregated in exactly the same way as the nanosphere without PEG. When N6-PEG-5k was covalently immobilized on the nanospheres, the LP complex almost retained its size even after 1 day at 4 °C. From these data, it is obvious that the electrical interaction between nanosphere and N6-PEG-5k is not sufficiently strong to stably disperse the nanospheres at high salt concentrations because of the electrostatic shielding effect; that is, the added

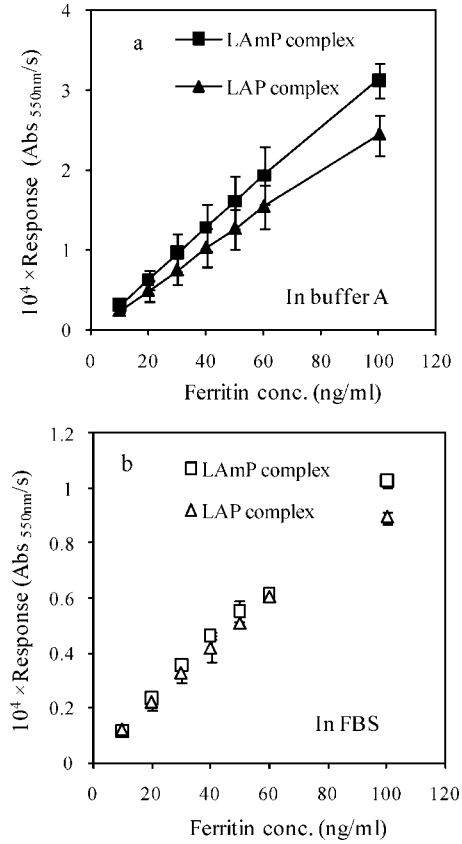

Figure 6. Immune response of the LAmP (5k/2k = 1:0.67) and LAP complexes in (a) buffer A and (b) FBS solution.

salt ions displaced the electrically coupled N6-PEG-5k molecules and released them from the n-LP complex, whereas they had no effect on the covalently coupled N6-PEG-5k of the LP complex. Covalent PEGylation is thus crucial for the preparation of a stable complex.

Comparison between the LAP and LAB Complexes. Since PEGylated immunolatex (LAP complex) was successfully prepared under suitable conditions, it was compared to conventional BSA-treated immunolatex (LAB complex). After DLS and μ_e measurements to characterize these two types of nanosphere complex, the immune responses of the LAP and LAB complexes were compared in both buffer A and 100% FBS. The LAP and LAB complexes were mono-dispersed and possessed a similar size (260−270 nm), but the LAP complex ($\mu_e = -0.4$ μm cm/Vs) was more neutral than the LAB complex ($\mu_e = -3.1$ μm cm/Vs), suggesting that LAP complex may efficiently suppress the electrical repulsion between the nanospheres and ferritin, as described above. Two parameters were used to characterize the performance of these nanosphere complexes. One is the directly measured immune response value, and the other is the analytical sensitivity, which is determined by the minimum value of antigen concentration that can be accurately measured and distinguished from background noise. Here, the minimum antigen concentration, which corresponds to the minimum response value proportional to the ferritin concentration, was defined as the detection limit of the nanosphere complexes. The responses of the LAB complex shown in Figure 1b are presented in Figure 4 again for comparison.

In buffer A, the immune response of the LAP complex was about 3 times higher than that of the LAB complex, and its detection limit was less than 6 ng/mL, which was 5 times lower than that of the LAB complex (30 ng/mL) (Figure 4a). Since the LAP and LAB complexes should possess almost the same antiferritin load, this high performance and low detection limit of the LAP complex might be due to the suitable environment for antiferritin on the PEGylated surface. The following synergetic effects can be considered: (i) densely packed PEG chains prevent nonspecific interactions of antigen with the surface of the nanospheres; (ii) a densely packed PEG-chain layer may retain the conformation of the antibody on the neighbor; (iii) N6-PEG does not influence the epitopes of the immobilized antibody. As shown in Figure 4b, the performance of the immune response in 100% FBS was remarkable. Actually, almost no immune response was observed in the LAB complex. In contrast, the LAP complexes showed a proportional response against the ferritin concentration, although the absolute value in 100% FBS was lower than that in buffer A. The detection limit of the LAP complex was 20 ng/mL in 100% FBS, while it was higher than 100 ng/mL in the LAB complex. For the conventional and sensitive analysis of crude samples, it is concluded that N6-PEG-5k-modified immunolatex (LAP complex) has an extremely high performance compared to BSA-modified immunolatex (LAB complex).

N6-PEG-5k/N6-PEG-2k (5k/2k) Ratio. Compared with the LAB complex, the obtained LAP complex was a stably mono-dispersed and highly performing immunolatex, demonstrating its potentiality in clinical diagnosis. As described in the introduction, we found that surface modification using both long and short PEG chains creates a denser PEG layer than that using only long PEG chains. If this treatment is effective for the present immunolatex system, its performance will be improved. To further increase the reactivity of the LAP complex based on this concept, the generation of a nanosphere/antibody/mix-ed N6-PEG polymer (LAmP) complex at *ECR* = 1:1 was examined.

The proper ratio of long-chain PEG (N6-PEG-5k) to short-chain PEG (N6-PEG-2k), 5k/2k, was determined by immune response measurements at a ferritin concentration of 100 ng/mL. Figure 5 (●) shows the immune response of the LAmP complex prepared under several conditions. The LAmP complex was prepared by the PEGylation of the LA complex with N6-PEG-5k, followed by a similar treatment with N6-PEG-2k of a certain concentration. No meaningful variations in size and μ_e occurred between the LAP and LAmP complexes (data not shown). Interestingly, the immune response of the LAmP complex increased after the addition of N6-PEG-2k, especially when its concentration was 0.07% w/v (5k/2k = 1:0.67). In contrast, the response of the LAP complex did not increase at all, even though a consecutive treatment with 0.1% w/v of N6-PEG-5k in the solution was performed (total polymer conc. = 0.2% w/v, Figure 5 ○; 5k/5k = 1:1); that is, the polymer concentration was equivalent to 5k/2k = 1:1. These inconsistent immune responses for 5k/2k = 1:1 with 5k/5k = 1:1 clearly indicate that the improved performance yield of the LAmP complex was not due to increased polymer concentration in the system but probably to the increased chain density of the PEG layer around the nanospheres, as described above. The LAmP complex prepared at 5k/2k = 1:0.67 showed a definitely high immune response regardless of the

antigen concentration, and this ratio was used as the proper one to prepare the LAmP complex thereafter.

Figure 6a shows the immune responses of the LAmP and LAP complexes corresponding to various ferritin concentrations in buffer A. The LAmP complex gave a higher performance than the LAP complex regardless of the ferritin concentration examined in this study (10−100 ng/mL). Figure 6b shows the results in 100% FBS solution of the experiment, like Figure 6a. Similar results were obtained in FBS solution, although the absolute values of the immune response decreased compared to those in buffer A, because FBS solution contains various contaminants, which strongly interfere with the antiferritin-ferritin interaction to reduce the response. All of the results represented in Figure 6 agree well with previous reports, suggesting that increasing the chain density of the PEG layer around the nanospheres effectively improved the antiferritin−ferritin interaction in this study.

CONCLUSIONS

To obtain a stably mono-dispersed and highly immune-performing nanosphere/antiferritin/PEG complex, various preparation conditions were evaluated in detail. As a result, one LAmP complex (5k/2k = 1:0.67) of about 270 nm ($\mu_e = -0.4$ μm cm/Vs; polydispersity index (μ/Γ^2) < 0.1) was constructed under optimum conditions. Its preparation required no purification and took only about 2 h, which is advantageous for practical utilization. This complex showed a significantly higher immune response yield and a lower detection limit than the LAB complex in 100% FBS solution, clearly demonstrating its usefulness in the direct analysis of samples without dilution and its potential as the basis of a novel serological diagnosis system. Further investigations of the experimental conditions are now in progress, and the results will be published elsewhere.

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SUPPORTING INFORMATION AVAILABLE

Further details are given in Tables S-1 and S-2 and Figures S-1 and S-2. This material is available free of charge via the Internet at http://pubs.acs.org.

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