Efficient Inhibition of Interfacial Nonspecific Interaction to Create Practically Utilizable High Ferritin-Response Immunolatex

ARTICLE *in* ANALYTICAL CHEMISTRY · NOVEMBER 2009

Impact Factor: 5.64 · DOI: 10.1021/ac902055w · Source: PubMed

4 AUTHORS, INCLUDING:

Xiaofei Yuan
University of Glasgow
18 PUBLICATIONS **255** CITATIONS
SEE PROFILE

Keitaro Yoshimoto
The University of Tokyo, Komaba 3-8-1, Me…
44 PUBLICATIONS **844** CITATIONS
SEE PROFILE

Anal. Chem. **2009,** *81,* 10097–10105

Efficient Inhibition of Interfacial Nonspecific Interaction to Create Practically Utilizable High Ferritin-Response Immunolatex

Xiaofei Yuan,[†,‡,§] Dolça Fabregat,[◆] Keitaro Yoshimoto,[†,‡,§] and Yukio Nagasaki*[,†,‡,§,⊥,▽]

Graduate School of Pure and Applied Sciences, University of Tsukuba, Ten-noudai 1-1-1, Tsukuba, Ibaraki 305-8577, Japan, Tsukuba Research Center for Interdisciplinary Materials Science (TIMS), Tsukuba Advanced Research Alliance (TARA), Master School of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ten-noudai 1-1-1, Tsukuba, Ibaraki 305-8577, Japan, Satellite Laboratory of International Center for Materials Nanoarchitectonics (MANA) in National Institute for Materials Science (NIMS), Ten-noudai 1-1-1, Tsukuba, Ibaraki 305-8573, Japan, and Biokit Latex R&D Department, 08186 Lliçà d'Amunt, Barcelona, Spain

Immunolatex particles (LAmP-s), which were prepared by covalently co-immobilizing antiferritin and a mixture of pentaethylenehexamine-ended poly(ethylene glycol) (N6-PEG) of two different chain lengths onto the surface of polystyrene submicroparticles, were formulated with various antiferritin loads. Following the quantification of the bound antiferritin, as well as the differentiation of the physically adsorbed antiferritin from chemically bound antiferritin, 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 size, homogeneity, and surface charge of the complex. Compared to the control immunolatex particles, which were prepared similarly but traditionally using bovine serum albumin (BSA) as a blocking agent (LAB-s), the LAmP-s complex showed a difference only in the surface charge property, because of the altered surface treatment in the case of the LAmP-s (PEGylation) and LAB-s complexes (BSA covering). However, the LAmP-s complex was much more reactive than the LAB-s complex, not only in phosphate buffer (PB, 10 mM, pH 7.4) but also in 100% fetal bovine serum (FBS), as measured by the turbidimetric monitoring method. The electrical repulsion between the negatively charged LAB-s complex and the antigen was the primary obstacle in the former case, and the overwhelming nonspecific deposition of contaminants from FBS onto the LAB-s complex was the main reason in the latter case. Moreover, the PEGylation procedure allowed the LAmP-s complex to possess invariable size and reactivity for at least one month at 4 °C without salt, and colloidal stability at high salt concentrations up to 2.0 M for at least one week, obviously demonstrating that the PEG surface modification technique described in this paper is a promising method of constructing efficient immunoassay systems.

Immunoassay is a biochemical analysis method that takes advantage of the specific binding of an antibody to its corresponding antigen. It has been routinely used in hospitals to determine the concentration of a substance in a biological liquid, typically serum or urine, to ascertain the disease and/or disorder of patients. Also, it is a well-known basic assay method widely used in multidisciplinary research, because of its high specificity and sensitivity. Although there are many types of immunoassay (e.g., enzyme-linked immunosorbent assay (ELISA), fluorescence immunoassay (FIA), and radioimmunoassay (RIA)), the disadvantages of toxicity, limited shelf life, high cost, high time consumption, and so forth limit their extensive use. However, the immunolatex agglutination test, of which the result can be rapidly evaluated with the naked eye, is inexpensive and user-friendly, needs no special equipment or skilled personnel, yields a result within 2 min,[1−3] and is claimed to be the quickest and easiest method. It is not only particularly suitable for use under field conditions, but also for automatic diagnosis on a machine, as has been realized in so many countries in the quest for efficiency improvement. Consequently, prompt, accurate, convenient (not time-consuming or labor-intensive), and low-cost immunolatex diagnosis is the key to effective disease management, with regard to both endemic spread and the problem of drug resistance.

The formulation of immunolatex usually includes bovine serum albumin (BSA) to cover the residual hydrophobic particle surface after the sensitization of antibodies or antigens,[4−6] with the purpose of stabilizing the particle and protecting it from any interfacial disturbance during assay. However, the inherent protein nature of BSA is an obstacle to this function, and a novel surface

* To whom correspondence should be addressed. Tel.: +81-29-853-5749. Fax: +81-29-853-5749. E-mail: yukio@nagalabo.jp.

† Graduate School of Pure and Applied Sciences, University of Tsukuba.

‡ Tsukuba Research Center for Interdisciplinary Materials Science (TIMS).

§ Tsukuba Advanced Research Alliance (TARA).

◆ Biokit Latex R&D Department.

⊥ Master School of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba.

▽ Satellite Laboratory of International Center for Materials Nanoarchitectonics (MANA) in National Institute for Materials Science (NIMS).

(1) Polpanich, D.; Tangboriboonrat, P.; Elaissari, A.; Udomsangpetch, R. *Anal. Chem.* **2007**, *79*, 4690–4695.

(2) Chen, J.; Jin, M.; Yu, Z.; Dan, H.; Zhang, A.; Song, Y.; Chen, H. *J. Vet. Diagn. Invest.* **2007**, *19*, 155–160.

(3) Bhaskar, S.; Banavaliker, J. N.; Hanif, M. *FEMS Immunol. Med. Microbiol.* **2003**, *39*, 235–239.

10.1021/ac902055w CCC: $40.75
Published on Web 11/12/2009

modification technique is required to solve the problem.[7] In view of this fact, we successfully designed and formulated a novel high-performance antiferritin-immunolatex particle using a special PEGylation technique, instead of the traditional BSA surface treatment, as described in our previous paper.[7] Ferritin is an iron storage protein, and its blood level is in equilibrium with the body's depot iron. Determination of ferritin in blood using antiferritin-immunolatex is, hence, profoundly meaningful in clinical applications, above all in iron metabolism diagnosis (e.g., prelatent and latent iron deficiency, as well as iron overloading), monitoring iron therapy, ascertaining the iron reserves in groups at risk (cancer and infectious disease) and in the differential diagnosis of anemia (such as hypoferric anemia and hypochromic anemia). Currently, determination of the ferritin level in hospitals is mainly done using the approach of antiferritin-immunolatex, because of its charming merits (previously mentioned).

In the previous research, a very low amount of antiferritin was used to accentuate the effects of PEGylation. To construct an acceptable and applicable antiferritin-immunolatex, valid antiferritin loads and formulation conditions are necessary. Generally, high antibody load corresponds to high reactivity of the immunolatex particles, accompanied by high risk of nonspecific aggregation during the sensitization procedure triggered by its decreased colloidal stability.[8,9] In this study, the increased prelocalized antiferritin may also occupy the space for N6-PEG polymer linkage, interfering in the subsequent PEGylation process, which plays an important role in suspending the particles.[10−13] That is, a good balance between the antiferritin load and the surface-tethered PEG chains is required.

In this study, immunolatex particles with four different antiferritin loads were prepared in similar ways, and they are characterized here in detail, in terms of particle size, size distribution, electrophoretic mobility, colloidal stability, and immunoreactivity. The purpose of our study was to determine the most suitable immunolatex particle, endowed with both high performance and colloidal stability. In addition, the nonspecific adsorption phenomenon and the reactivity at various salt concentrations of the immunolatex were explored to obtain further insights into its high performance, in comparison to immunolatex prepared similarly but using BSA as a blocking agent.

EXPERIMENTAL SECTION

Materials. Carboxylated polystyrene submicrosphere (AJ26-COOH-Clean, 10% w/v, without surfactant, Ikerlat, Spain), bovine serum albumin (BSA) (Lot No. 651, Serologicals, Norcross, GA), rabbit antihuman ferritin polyclonal antibodies (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 submicroparticle (sMP) 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 (−5.7 ± 0.1 μm cm/(V s)) were measured in phosphate buffer solution (buffer A; 10 mM, pH 7.4) on a Zetasizer Nano ZS instrument (He-Ne laser, Malvern, Worcestershire, U.K.). Ferritin was diluted with buffer A to 1 μg/mL just before use.

Two types of α-methoxy-poly(ethylene glycol)-pentaethylene-hexamine (N6-PEG) were used in this study: N6-PEG-5k and N6-PEG-2k (molecular weights of M_n = 6000 and 2000 g/mol, respectively, with M_w/M_n = 1.13 and 1.05; N6 end-functionality = 81% and 87%, respectively; obtained from JSR Co. (Japan) and synthesized as described in our previous papers[14]). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, special grade), fetal bovine serum (FBS, Lot No. 1412447), and fluorescein isothiocyanate conjugate bovine albumin (BSA-FITC) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), Invitrogen Co. (Tokyo, Japan), and Sigma (St. Louis, MO), respectively. All chemicals were used without further purification.

In this paper, the temperature (25 °C) was identical in all experiments unless otherwise stated.

Quantification of Antiferritin. The antiferritin surface concentration was quantified using the copper reduction/bicinchoninic acid reaction (the Micro BCA method). This quantification method has been reported elsewhere.[7] Briefly, a freshly prepared sMP/antiferritin (LA) complex (300 μL) of a given amount of antiferritin was divided for the supernatant (250 μL) and the particles (50 μL, another 250 μL of buffer A were added later to suspend the particles again) through a centrifuge procedure (15000 rpm, 20 min, 25 °C), followed by a separate antiferritin quantification using the Micro BCA method. Moreover, physically adsorbed antiferritin within the total immobilized antibody was also similarly quantified after incubation of a freshly prepared LA complex in 0.1% w/v of Tween 20 solution for 24 h at room temperature under slow continuous shaking, because the physically adsorbed antiferritin is reported to be removed from the antiferritin chemically bonded onto the particles by the surfactant.[15−17] Note that even 0.1% w/v of Tween 20 positively interfered with the Micro BCA method and a new calibration of antiferritin in 0.1% w/v of Tween 20 solution was required.

Formulation of a sMP/Antibody/Mixed-PEG (LAmP-s) Complex. Preparation of a LAmP-s complex (where "s" stands for the antibody load (in milligrams) per area of the particle surface (expressed in units of m^2); e.g., LAmP-1.0 was the LAmP complex at s = 1.0 mg/m^2) was conducted under the conditions described previously.[7] (see Scheme S1 in the Supporting Information). Briefly, a sMP suspension (30 μL) was poured into

(4) Senthilkumar, T.; Subathra, M.; Phil, M.; Ramadass, P.; Ramaswamy, V. *Ind. J. Med. Microbiol.* **2008**, *26*, 45–49.
(5) Hamano, A.; Umeda, M.; Ueno, Y.; Tanaka, S.; Mimuro, J.; Sakata, Y. *Clin. Chem.* **2005**, *51*, 183–188.
(6) Benecky, M.; Post, D. R.; Schmitt, S. M.; Kochar, M. S. *Clin. Chem.* **1997**, *43*, 1764–1770.
(7) Yuan, X.; Yoshimoto, K.; Nagasaki, Y. *Anal. Chem.* **2009**, *81*, 1549–1556.
(8) Price, C. P.; Newman, D. J. *Principles and Practice of Immunoassay*, 2nd Edition; Macmillan Reference, Ltd.: London, U.K.; 1997; Chapter 18.
(9) Nakamura, M.; Ohshima, H.; Kondo, T. *J. Colloid Interface Sci.* **1992**, *154*, 393–399.
(10) Miyamoto, D.; Oishi, M.; Kojima, K.; Yoshimoto, K.; Nagasaki, Y. *Langmuir* **2008**, *24*, 5010–5017.
(11) Nagasaki, Y.; Yoshinaga, K.; Kurokawa, K.; Iijima, M. *Colloid Polym. Sci.* **2007**, *285*, 563–567.
(12) Kamimura, M.; Miyamoto, D.; Saito, Y.; Soga, K.; Nagasaki, Y. *Langmuir* **2008**, *24*, 8864–8870.
(13) Nagasaki, Y. *Chem. Lett.* **2008**, *37*, 564–569.
(14) Nagasaki, Y.; Kobayashi, H.; Katsytana, Y.; Jomura, T.; Sakura, T. *J. Colloid Interface Sci.* **2007**, *309*, 524–530.
(15) Musyanovych, A.; Adler, H. J. P. *Langmuir* **2005**, *21*, 2209–2217.
(16) Rapoza, R. J.; Horbett, T. A. *J. Colloid Interface Sci.* **1990**, *136*, 480–493.
(17) Ortega-Vinuesa, J. L.; Hidalgo-Alvarez, R.; de las Nieves, F. J.; Davey, C. L.; Newman, D. J.; Price, C. P. *J. Colloid Interface Sci.* **1998**, *204*, 300–311.

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 (12.5 mg/mL) were poured into the mixture, followed by shaking for 20 min at 25 °C (shaking velocity = 1000 rpm, using a Model SI-300 shaking incubator, from AS ONE Co., Japan) to activate the surface carboxyl groups of the particles. The thus-obtained activated sMPs were further diluted by 100 μL of buffer A before mixing them (50 μL) with buffer A (275 μL) and 10 μL of antiferritin solution in buffer A (0.514, 1.14, 1.71, and 2.28 mg/mL, which corresponds to s = 0.45, 1.0, 1.5, and 2.0 mg/m^2, respectively), followed by 1 h of shaking incubation to bind the antiferritin onto the particles. Then, 300 μL of the obtained sMP/antibody (LA) complex, which was monodispersed again by ultrasonication treatment (35 W, 50−60 Hz, room temperature, 2−5 s for several rounds), were successively reacted with N6-PEG-5k (75 μL, 0.6% w/v) and N6-PEG-2k (75 μL, 0.4% w/v) solutions by separate shaking incubation for 30 min. The final concentrations of the sMP, N6-PEG-5k, and N6-PEG-2k were 0.1%, 0.1%, and 0.067% w/v, respectively. The thus-obtained sMP/antiferritin/mixed-PEG complex (abbreviated as "LAmP-s") was kept at 4 °C for 1 day before measurement.

The proper reaction times of antiferritin with the activated sMP at s = 1.0, 1.5, and 2.0 mg/m^2 were separately estimated to be similar to that at s = 0.45 mg/m^2.[7] The LAP-s complex, which was obtained by reacting the monodispersed LA complex (300 μL) with N6-PEG-5k (150 μL, 0.3% w/v) for 30 min under shaking incubation, showed a maximum and constant immune response when the time was in the range of 30−60 min for s = 1.0 and 1.5 mg/m^2, which was completely consistent with the case of s = 0.45 mg/m^2. The dissimilar result at s = 2.0 mg/m^2 was possibly due to the colloidal instability of the complex (see the Results and Discussion sections). The mass ratio of N6-PEG-5k to N6-PEG-2k (5k/2k) was fixed to 1:0.67, which is the proper ratio of the LAmP-0.45 complex, for comparison of the LAmP-s complex at various values of s. It is worth mentioning that the LAmP-s complex at this ratio was eventually more reactive than the LAP-s complex (see Figure S1 in the Supporting Information), in good agreement with the conclusions of the previous paper that the PEGylation procedure, using both the long and short PEG chains, was more suitable than that using only long PEG chains. Therefore, the LAmP-s complex, but not the LAP-s complex, was mainly characterized in this study.

Construction of the sMP/Antibody/BSA (LAB-s) Complex. As a control, a sMP/antiferritin/BSA (LAB-s) complex was prepared similarly to that of the LAP-s complex, except that BSA was used instead of N6-PEG polymer. As described previously, the selection of the proper BSA concentration was critical for preparing an acceptable LAB-s complex, because both low and high BSA concentration resulted in low reactivity.[7] After the s value was altered, validation of the BSA concentration had to be performed by monitoring the size, electrophoretic mobility (μ_e), and immune response of the LAB-s complex formulated using various BSA concentrations in solution from 0% to 0.027% w/v (data not shown). The LAB-s complexes described in this paper as a control were all prepared using the corresponding proper BSA concentration; i.e., BSA concentrations (in solution) of 0.012%, 0.01%, and 0.008% w/v were separately used in the formulation of the LAB-1.0, LAB-1.5, and LAB-2.0 complexes.

Dynamic Light Scattering (DLS) and Electrophoretic Mobility (μ_e) Measurements. Both dynamic light scattering (DLS), to give the diameter and polydispersity index (PDI) values of particles, and electrophoretic mobility (μ_e) measurements were performed on a Zetasizer Nano ZS instrument (He−Ne laser, Malvern, Worcestershire, U.K.). The sMP concentration of each test sample was 1.25×10^{-3}% w/v, obtained by the dilution of the sample suspension (5 μL, sMP concentration = 0.1% w/v) with a corresponding solvent (395 μL). The sample for the μ_e measurements, with respect to pH, was separately diluted with various solutions, including acetate buffer (pH 4 and pH 5), phosphate buffer (pH 6 and pH 7), and borate buffer (pH 8 and pH 9) of identical ionic strength.

Immune Response Measurements. The immune response of the test samples was measured by turbidimetric monitoring, using a PL-2500 spectrophotometer (Shimadzu, Japan). Briefly, 20 μL of the test sample suspension (latex concentration = 0.1% w/v) were added into a mixture of a given amount of human ferritin solution (1 μg/mL) with buffer A or FBS solution to a total final volume of 500 μL, followed by turbidimetric monitoring at a wavelength of 550 nm for 5 min at room temperature. The initial slope of the turbidimetric progress curve was identified as the immune response here.

The antibody load was determined to significantly influence the time dependency of the immune response of the LAP-s complex. At s <1.5 mg/m^2, the immune response slowly increased with time until ~24 h, because of the possible readjustment of the structure and/or orientation of the localized antibody at 4 °C. Several authors[18−21] have reported this type of behavior of the adsorbed proteins in a slow dynamic state, and they attributed it to minimization of the free energy of the system. Conversely, no change of this type was observed at s ≥1.5 mg/m^2, which may be explained by the densely immobilized antibody on the sMP surface with no requirement for orientation modulation (i.e., they were initially more homogeneously orientated) and/or with insufficient space for this readjustment. Based on this phenomenon, all the samples were used after at least 24 h at 4 °C.

Nonspecific Adsorption of BSA-FITC. The LAB-1.0 and LAmP-1.0 complexes (sMP concentration = 0.1% w/v, 0.2 mL) were separately mixed with BSA-FITC solution (0.2 mL, 10 μg/mL) in buffer A with or without NaCl, to final salt concentrations of 0, 0.05, 0.15, and 0.5 M. After 1 h of mild vibration of these mixtures under cover at 25 °C, a separation procedure (15 000 rpm, 20 min, 25 °C) was performed, followed by fluorescence measurements (emission wavelength (λ_{ex}) = 492 nm, 25 °C, F-7000, Hitachi High-Technologies Co., Japan) of the supernatant (0.35 mL) to identify its fluorescence intensity at 520 nm (Int_{520}). Because coexistence with BSA or N6-PEG polymer alternated the Int_{520} of BSA-FITC, compared to that in buffer A, BSA (0.012% w/v) and a mixed N6-PEG polymer solution at

(18) Molina-Bolivar, J. A.; Ortega-Vinuesa, J. L. *Langmuir* **1999**, *15*, 2644–2653.
(19) Andrade, J. D.; Hlady, V. *Ann. N.Y. Acad. Sci.* **1987**, *516*, 158–172.
(20) Van Oss, C. J. *Interfacial Forces in Aqueous Media*; Marcel Dekker: New York, 1994.
(21) Brash, J. L., Horbett, T. A., Eds. *Advances in Chemistry Series, Vol. 343*; Proteins at Interfaces: Physicochemical and Biochemical Studies; American Chemical Society: Washington, DC, 1987; pp 324−338.

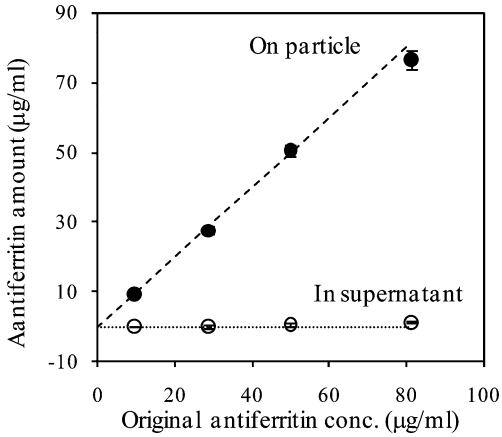

Figure 1. Relationship of (●) the antiferritin immobilized onto the sMP and (○) the free antiferritin in the supernatant with the original antiferritin concentration in solution.

a 5k/2k ratio of 1:0.67 in buffer A were separately used as controls for the supernatant of the LAB-1.0 and LAmP-1.0 complexes, and they were named "BSA-control" and "PEG-control" solutions, respectively. The amount of BSA-FITC nonspecifically adsorbed onto the complex was then derived from the difference in the Int_{520} of the supernatants in the control and test samples, based on the calibration curve of the BSA-FITC in the BSA-control and PEG-control solutions.

In the presence of salt, the BSA-control (or PEG-control) solution with salt was used as the control, because the fluorescence intensity of BSA-FITC changed with the addition of salt. To avoid having to construct calibration curves for BSA-FITC at each salt concentration, the Int_{520} value with salt was changed to that without salt, based on the ratio obtained from the BSA-control (or PEG-control) solution with and without salt under the same conditions. The amount of BSA-FITC nonspecifically adsorbed onto the complex with salt was therefore similarly obtained as described previously.

RESULTS

1. Binding Efficiency of Antiferritin. Figure 1 reveals the amount of antiferritin adsorbed onto the sMP and dissolved in the supernatant as a function of the total/original antiferritin concentration in solution, as measured by the Micro BCA method. The broken line denotes the case of complete antibody binding, and the dotted line shows the case without antibody. Although there are slight deviations at the points corresponding to 80 μg/mL (i.e., below the broken line and above the dotted line, respectively), the measured amount of antibody on the sMPs and that in the supernatant separately overlapped with these two lines, clearly suggesting that a total added antibody amount of <80 μg/mL resulted in complete binding onto the particles. On the other hand, physically adsorbed antiferritin (in the supernatant) increased slightly as the total antiferritin load increased; i.e., the antibody load of the LA complex before surfactant addition, along with the correspondingly decreased chemically bonded antiferritin (on the particles), implies that a small amount of physically adsorbed antiferritin existed, especially at a high antiferritin load (see Table S1 in the Supporting Information).

2. Size and PDI. Table 1 summarizes the results obtained from a Malvern Zetasizer Nano ZS instrument. It can be observed that the size of the LAmP-s complex was almost constant, regardless of the value of *s*, and that the PDI was <0.1, except for the case of the LAmP-2.0 complex. Considering the average size of a sMP particle (251 ± 3 nm) and that of an antiferritin molecule (13 ± 3 nm), these results suggest that the LAmP-0.45, LAmP-1.0 and LAmP-1.5 were monodispersed immunolatex particles. In comparison, the LAmP-2.0 complex became large with a PDI >0.1, which indicates that it was heterogeneous. On the other hand, the LAB-s complex showed results very similar to those for the LAmP-s complex, despite the LAB-2.0 complex, which was slightly larger than the other LAB-s complexes, with PDI <0.1.

Table 1. Size, Polydispersity Index (PDI), and Electrophoretic Mobility (μ_e) Values of Versatile Latex Complex

latex complex	diameter (nm)	PDI	μ_e (μm cm/(V s))
LAmP-0.45	268 ± 2	<0.1	−0.23 ± 0.17
LAmP-1.0	269 ± 1	<0.1	−0.31 ± 0.09
LAmP-1.5	275 ± 6	<0.1	−0.39 ± 0.03
LAmP-2.0	359 ± 41	0.26 ± 0.07	−0.52 ± 0.04
LAB-0.45	264 ± 4	<0.1	−3.12 ± 0.09
LAB-1.0	272 ± 4	<0.1	−2.36 ± 0.08
LAB-1.5	273 ± 6	<0.1	−1.60 ± 0.12
LAB-2.0	284 ± 6	<0.1	−1.37 ± 0.06

To explain these discrepancies in size and PDI between s = 2.0 and other values, the colloidal stability of the complex was monitored in terms of size distribution. (See Figure S2 in the Supporting Information, which shows the results for several forms of the complex at s = 2.0 mg/m^2 after 1 day and 3 days at 4 °C, using the complex at s = 1.5 mg/m^2 as a control.) Compared to the invariable size distribution of the LAB-1.5 and LAmP-1.5 complexes, that of the complex at s = 2.0 mg/m^2 became broad after 3 days because some large particles appeared in the system with time, leading to an increase in average size to ~9 nm, ~47 nm, and ~37 nm for the LAB-2.0, LAmP-2.0, and LAP-2.0 complexes, respectively. These remarkably dissimilar sizes and size distributions among the forms of the complex at s = 2.0 mg/m^2 undoubtedly indicate the aggregation of the particles as a result of their colloidal instability.

3. μ_e Values versus pH. With the purpose of distinguishing the effects of PEGylation from those of BSA surface treatment, and to understand the interface structure and explain the colloidal stability of the LAmP-s and LAB-s complexes, electrophoretic mobility (μ_e) measurements at various pH values were performed, the results of which are presented in Figure 2. As a control, the same experiment was also performed using polystyrene sMPs and analogues of the LAB-0.45, LAB-1.0, and LAB-1.5 complexes, called "LAB-0-1", "LAB-0-2", and "LAB-0-3" complex, respectively, which were prepared without antiferritin but using the same BSA treatment as that for the LAB-0.45, LAB-1.0, and LAB-1.5 complexes, respectively.

As shown in Figure 2a, the polystyrene sMP gave a large negative μ_e value across the entire the pH range, which suggests the presence of many negative charges on its surface. These coincide with its surface carboxyl groups. The BSA surface treatment significantly diminished the μ_e value of the sMPs by BSA coverage. The higher the BSA surface concentration, the lower the absolute μ_e value, shown as the variation in the μ_e values of the LAB-0-1, LAB-0-2, and LAB-0-3 com-

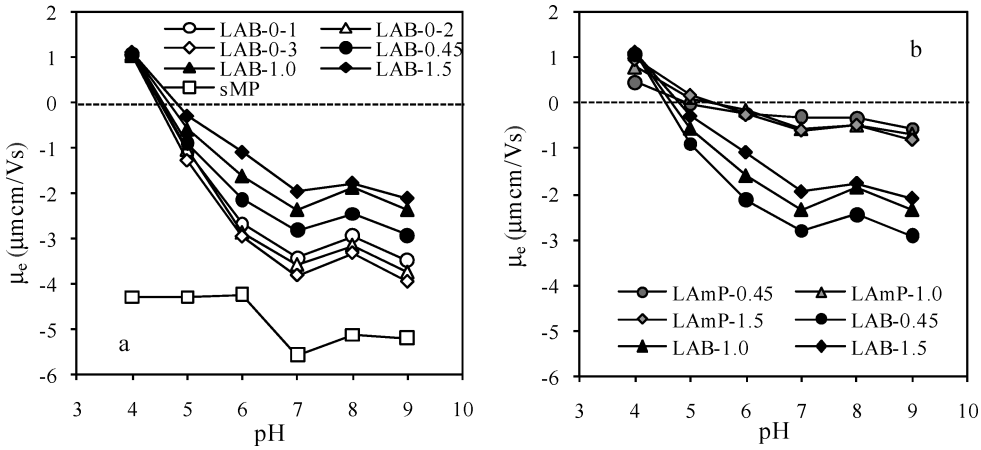

Figure 2. Effect of pH on the electrophoretic mobility (μ_e) of the sMP and various forms of (a) LAB-s complex with or without antiferritin and (b) the LAmP-s and LAB-s complexes.

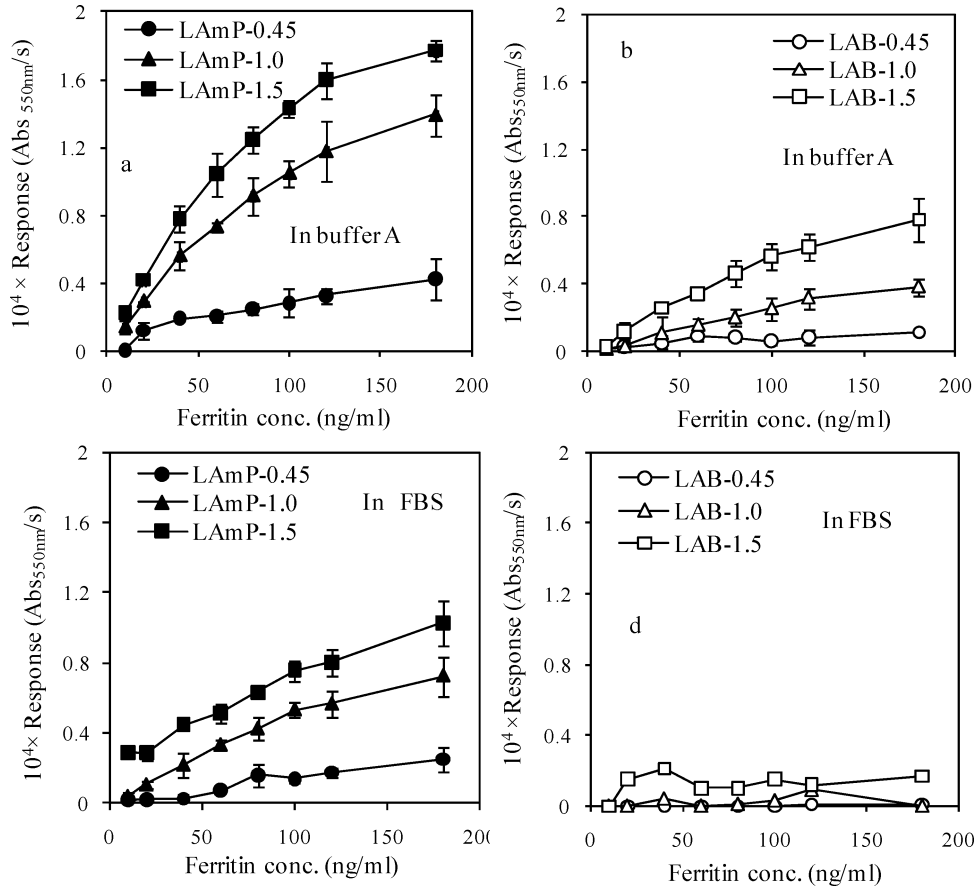

Figure 3. (a and c) Immune response of the LAmP-s complex and (b and d) the LAB-s complex in buffer A (panels a and b) and in fetal bovine serum (panels c and d).

plexes. The localization of both antiferritin and BSA onto the particles further underlined this phenomenon, because of the compensation of the surface charges of the sMPs by the net charge of these proteins.[17,22] In consideration of the isoelectric point (iep) of BSA (4.8−5)[18,23] and that of the antiferritin (6−7), the gradually decreasing μ_e of the LAB-s complex with increasing *s* excellently supports the upward tendency of antiferritin coverage in the same order. The negative μ_e values of the LAB-s complexes obviously indicate that all of them were dispersed by interparticle electrostatic repulsion at pH 7.4. Figure 2b shows that the LAmP-s complexes possessed zero closed mobility at any pH, regardless of the antiferritin load, confirming that all of them were dispersed by steric exclusion from the neutral and hydrophilic PEG chains, which were considered to cover the residual bare surface of the particles compactly after the sensitization.

(22) Ortega-Vinuesa, J. L.; Bastos-Gonzalez, D.; Hidalgo-Alvarez, R. J. *J. Colloid Interface Sci.* **1995**, *176*, 240–247.

(23) Zhang, S.; Sun, Y. *Biotechnol. Bioeng.* **2001**, *75*, 710–717.

4. Immune Response. Figure 3 illustrates the immune response of the LAmP-s and LAB-s complexes measured in buffer A and in 100% fetal bovine serum (FBS). The LAmP-s complex (Figures 3a and 3c) responded strongly to the ferritin antigen in both buffer A and FBS, although it was more sensitive in buffer A, possibly because of the nonspecific adsorption of the contaminants from the FBS. The response of the LAmP-s complex was notably dependent on the parameter *s*, because the functionality of the LAmP-s complex was proportional to the antiferritin load. According to the definition given in the previous paper,[7] the detection limits of the LAmP-0.45, LAmP-1.0, and LAmP-1.5 complexes were equal to 40, 10, and 10 ng/mL ferritin in buffer A, and to 100, 20, and 20 ng/mL ferritin in FBS, respectively. Figure 3b represents the immune response of the LAB-s complex in buffer A, which also increased with the antiferritin load, but the absolute value was lower than that of the LAmP-s complex, particularly in the case of $s = 0.45$ mg/m^2. The detection limits of the LAB-1.0 and LAB-1.5 complexes in buffer A (ca. 40 ng/mL) were 4 times higher than those of the corresponding LAmP-s complexes. The LAB-0.45 complex did not show this value, because no response proportional to the antigen concentration was available within the experimental range. A similar phenomenon also occurred for the measurements in FBS, leading to the absence of a detection limit for all three LAB-s complexes (see Figure 3d). The low functionality, in combination with the extremely high detection limit, of the LAB-s complex evidently demonstrates that the LAmP-s complex is a more sensitive and efficient form of immunolatex.

5. Colloidal Stability. Long-time colloidal stability is one of the primary requirements for the practical utilization of immunolatex particles, because of the diagnosis mechanism involved. Estimation of the colloidal stability of the LAmP-s complex (containing 0.1% w/v of NaN_3 at 4 °C), in terms of the time-dependent size (DLS) and reactivity for one month in buffer A, was performed to show its potential in clinical diagnosis. A freshly prepared ferritin solution (1 μg/mL) was used each time for the reactivity measurements. Figure 4 shows the diameter and immune response of the LAmP-s complex over the course of a one-month period at 4 °C. No significant variation in these two parameters was observed for the LAmP-s complex, although LAmP-1.5 showed a slight decreasing tendency in its reactivity with time.

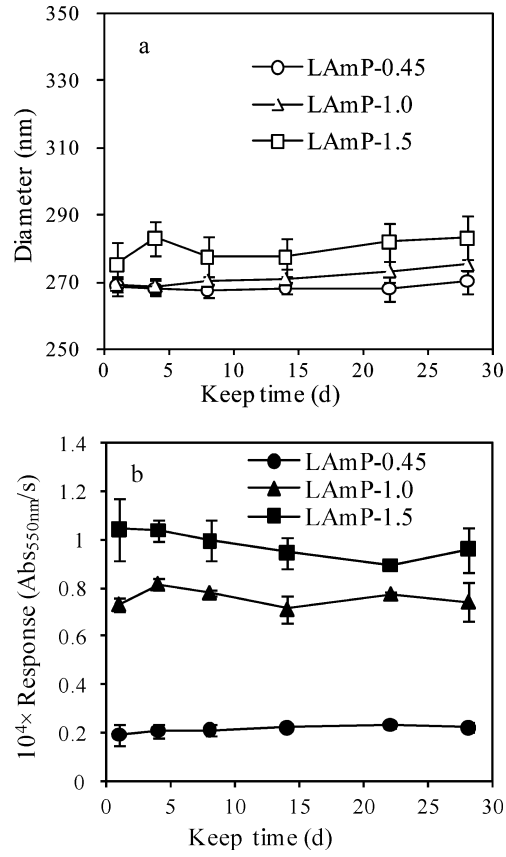

Figure 4. Time-dependent (a) size and (b) immune response of the LAmP-s complex over the course of a one month at 4 °C.

DISCUSSION

1. Characterization of Antiferritin Immobilization. The immobilization efficiency of antiferritin was estimated, first of all, to determine the appropriate range of antiferritin concentration for LAmP-s complex formulation. Figure 1 implies that efficient antibody immobilization occurred when using a total/original antiferritin concentration of <80 μg/mL. Although the antiferritin was chemically (covalently) immobilized onto the sMPs in this study, physical (passive) adsorption was also considered, along with chemical adsorption, because the passive adsorption of proteins on hydrophobic sorbents (such as polystyrene sMPs) usually occurs very rapidly and irreversibly, although it is susceptible to various experimental conditions (e.g., the ionic strength and pH).[24,25] Moreover, it has been demonstrated that physical adsorption occurs significantly in the chemical binding of anti-C reactive protein (a-CRP) antibody to polystyrene sMPs, using the same EDC method.[22] Because the physically adsorbed antibody, compared to the chemical bound one, was more randomly orientated and possibly desorbed from the sorbent to disfavor antibody−antigen recognition,[22] it was necessary to ascertain the amount of the physically adsorbed antiferritin. Fortunately, at most, 10% of the total antiferritin load was comprised of this type of antiferritin, at the original antiferritin concentration of 72.6 ng/mL (see Table S1 in the Supporting Information), implying that the possible influence of the physically adsorbed antiferritin on the antibody−antigen interaction may eventually become negligible.

2. Characteristics of the Various Forms of the Complex. To maintain a high antiferritin immobilization efficiency and avoid time-consuming purification processes during complex preparation, the antiferritin concentration in solution was limited to within 80 μg/mL, which corresponds to an *s* value of <2.0 mg/m^2 (68 μg/mL). Accordingly, two forms of the complex—LAmP-s and LAB-s—at $s \leq 2.0$ mg/m^2 were prepared as described in the Experimental Section. Comparison between these two complexes, in terms of size, electrophoretic mobility (μ_e), and immune reactivity, was performed after the samples were kept at 4 °C for ~24 h.

(24) Serra, J.; Puig, J.; Martin, A.; Galisteo, F.; Galvez, M.; Alvarez-Hidalgo, R. *Colloid Polym. Sci.* **1992**, *270*, 574–583.
(25) Van Dulm, P.; Norde, W. *J. Colloid Interface Sci.* **1983**, *91*, 248–254.

Table 1 shows that all the forms of the complex processed a similar size and were monodispersed, except for those at $s = 2.0$. The fact that free antiferritin began to appear in the supernatant at an antiferritin loading of ~80 μg/mL (Figure 1) suggests that the antiferritin should adsorb compactly on the particle surface at $s = 2.0$ mg/m^2 (68 μg/mL). In agreement with this assumption, Figure S2 in the Supporting Information gives the colloidal instabilities of all forms of the complex at $s = 2.0$ mg/m^2, in comparison with the stabilities at $s = 1.5$ mg/m^2. Therefore, the altered size and PDI at $s = 2.0$ mg/m^2 might be attributed to the relatively excessive antiferritin coverage of the particles, which occupied the space required for PEG-chain linkage or BSA adsorption to stabilize the LA complex. Interestingly, the LAB-2.0 complex seems to have been slightly more stable than the LAP-2.0 and LAmP-2.0 complexes, in that it had a relative small size and a low PDI value. This result is consistent with the phenomenon that one optimum BSA concentration exists for LAB-s complex preparation, possibly because of the epitope coverage of the prelocalized antiferritin by nonspecifically adsorbed BSA molecules in excess; this result was not obtained for PEGylation.[7] The passive deposition of BSA molecules may play a central role in suspending the complex, to some extent. The unstable complex at $s = 2.0$ mg/m^2 was excluded from subsequent experiments.

3. Immune Response. Based on the structural properties of the LAmP-s and LAB-s complexes, several possible facts relative to interfacial interactions may explain the results shown in Figures 3a and 3b. For example, the charged LAB-s complexes hardly coalesced with each other; also, the negatively charged LAB-s complex (see Figure 2) may have electrically repelled identically charged ferritin ($\mu_e = -0.6$ μm cm/(V s) in buffer A), whereas this type of interference did not exist in the case of the LAmP-s complex, because of its hydrophilic, flexible, and almost net-neutral PEG surface layer, which is well-known to be capable of inhibiting various types of nonspecific interfacial adsorption of biomaterials.[14,26−30] On this assumption, the discrepancy between the LAmP-s and LAB-s complexes in the reactivity change from $s = 0.45$ mg/m^2 to $s = 1.0$ mg/m^2 in buffer A is also easily understood. The striking reactivity increment of the former may result from its increased antiferritin load, but the slightly improved functionality of the latter seems to result primarily from the progressively decreased total surface charge of the complex, as depicted in Figure 2, because this decrement favors the contact of the complex particles and/or the complex with the ferritin, as mentioned previously.

To obtain experimental evidence, immune response measurements were also performed in buffer A that contained salt. If the electric repulsion between particles and/or between the particles and ferritin is the reason for the low response of the LAB-s complex, salt addition should result in the response recovery of the complex, because salt weakens the interparticle electric repulsion (screening effect) of the LAB-s complex and thins the PEG layer of the LAmP-s complex (see the following explanation) to facilitate particle coalescence,[17,31] while also quenching the electric repulsion between the LAB-s complex and ferritin if it is governed mainly by the electric interaction. Figures 5a and 5b give the salt-induced variations in the immune response of the LAmP-s and LAB-s complexes at 60 ng/mL of ferritin. The LAmP-s and LAB-s complexes showed a similar tendency, in that the immune response increased to a maximum value and then decreased again with increasing salt concentration. This behavior implies that two factors, in fact, control the reactivity of the complex as the salt concentration varies. However, the salt-induced nonspecific aggregation of the LAmP-s and LAB-s complexes under the same measurement condition but without ferritin (Figures 5c and 5d), which reflects particle coalescence without antibody−antigen recognition, reveals a salt dependence dissimilar to those in Figures 5a and 5b, indicating that the salt-induced easy assembly of the particles was not the reason, or at least not the main reason, for this salt-induced reactivity recovery of the LAmP-s and LAB-s complexes.

To explain the response measured in FBS (Figures 3c and 3d), it is helpful to compare them with the results measured in buffer A that contained 0.15 M NaCl (Figures 5a and 5b). Although the only difference between them is the presence (from FBS) or absence (in buffer A) of contaminants in the system, the responses in FBS were very low, implying that the nonspecific adsorption of the contaminants (comprising abundant BSA) from FBS may relate to this phenomenon. This hypothesis is supported by the results shown in Figure 6, which illustrates the average number of passively adsorbed BSA-FITC molecules per LAB-1.0 particle and per LAmP-1.0 particle, as a function of the salt concentration. The deposition of BSA-FITC for both the LAB-1.0 complex and the LAmP-1.0 complex increased with the salt concentration; however, the number of attached BSA-FITC molecules per particle for the LAmP-1.0 complex was apparently lower than that for the LAB-1.0 complex, especially at NaCl = 0 M, confirming that BSA-FITC molecules were easily deposited onto the latter. This salt-boosted BSA-FITC adsorption phenomenon is consistent with the literature.[32,33] Generally, the adsorption of proteins is a complex process involving various driving forces such as hydrophobic interaction, electrostatic interaction, and hydrogen bonding. Therefore, the effects of salt on the adsorption event of BSA are multiple and dependent on what driving force predominantly results in adsorption. For example, if electric attraction is the dominant force, a high salt concentration disfavors the adsorption of BSA.[34−36] Conversely, salt promotes BSA adsorption onto the sorbent when electric repulsion is the dominant force.[23] Thus, salt is considered to accelerate the attachment of BSA-FITC molecules onto the identically charged LAB-s complex. Similarly, it is plausible that the protein contaminants from FBS completely

(26) Yoshimoto, K.; Hirase, T.; Nemoto, S.; Hatta, T.; Nagasaki, Y. *Langmuir* **2008**, *24*, 9623–9629.
(27) Uchida, K.; Hoshino, Y.; Tamura, A.; Yoshimoto, K.; Kojima, S.; Yamashita, K.; Yamanaka, I.; Otsuka, H.; Kataoka, K.; Nagasaki, Y. *Biointerphases* **2007**, *2*, 126–130.
(28) Satomi, T.; Nagasaki, Y.; Kobayashi, H.; Tateishi, T.; Kataoka, K.; Otsuka, H. *J. Nanosci. Nanotechnol.* **2007**, *7*, 2394–2399.
(29) Yoshimoto, K.; Matsumoto, S.; Asakawa, R.; Uchida, K.; Ishii, T.; Nagasaki, Y. *Chem. Lett.* **2007**, *36*, 1444–1445.
(30) Takae, S.; Akiyama, Y.; Yamasaki, Y.; Nagasaki, Y.; Kataoka, K. *Bioconjugate Chem.* **2007**, *18*, 1241–1245.
(31) Perez-Amodio, S.; Holownia, P.; Davey, C. L.; Price, C. P. *Anal. Chem.* **2001**, *73*, 3417–3425.
(32) Tamai, H.; Hasegawa, M.; Suzawa, T. *Colloids Surf.* **1990**, *51*, 271–280.
(33) Tamai, H.; Fujii, A.; Suzawa, T. *J. Colloid Interface Sci.* **1987**, *1*, 176–181.
(34) Wittemann, A.; Haupt, B.; Ballauff, M. *Phys. Chem. Chem. Phys.* **2003**, *5*, 1671–1677.
(35) Rabe, M.; Verdes, D.; Zimmermann, J.; Seeger, S. *J. Phys. Chem. B* **2008**, *112*, 13971–13980.
(36) Gao, D.; Lin, D.; Yao, S. *J. Chem. Eng. Data* **2006**, *51*, 1205–1211.

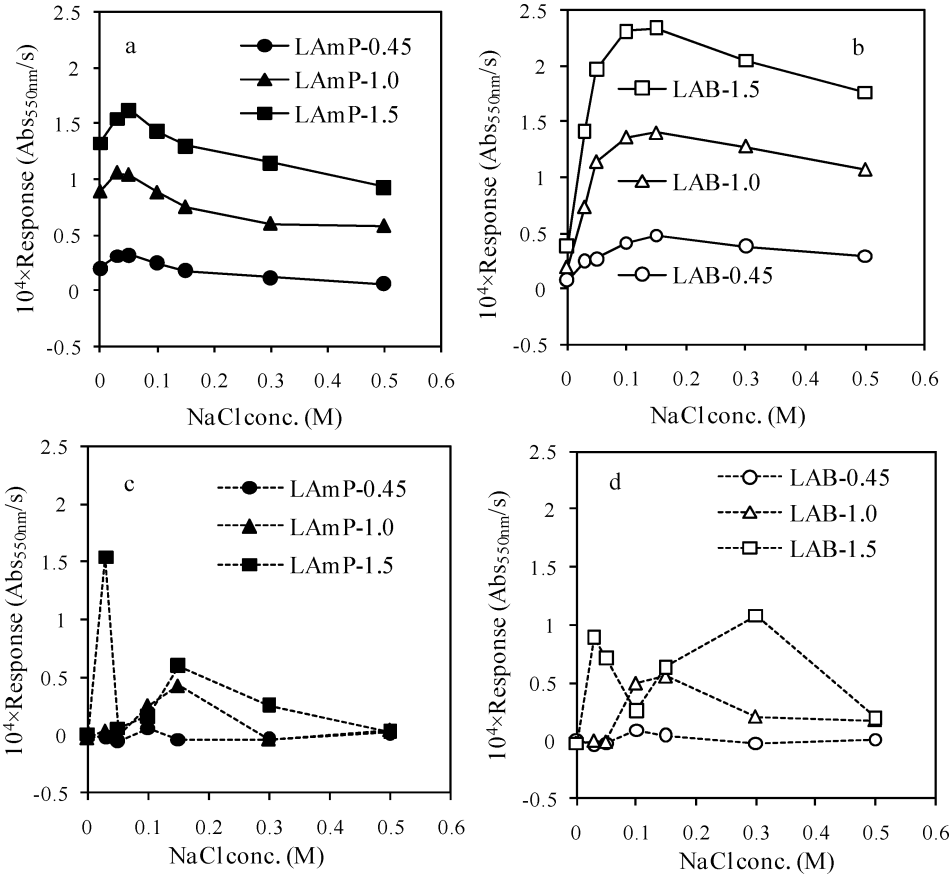

Figure 5. Immune response of the (a) LAmP-s and (b) LAB-s complex at 60 ng/mL ferritin in buffer A, and nonspecific aggregation of the (c) LAmP-s and (d) LAB-s complex without ferritin, as a function of the NaCl concentration.

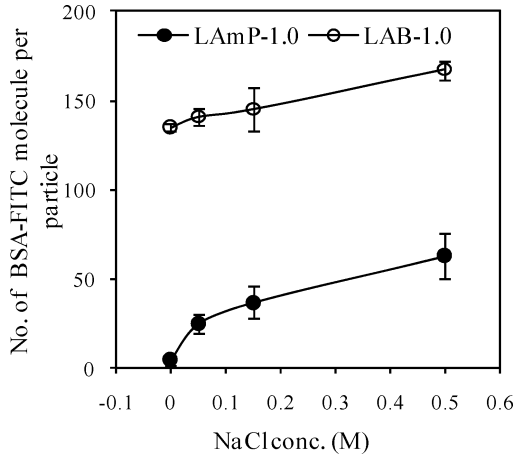

Figure 6. NaCl dependency of the number of nonspecifically adsorbed BSA-FITC molecules per particle of LAB-1.0 and LAmP-1.0.

turned off the reactivity of the LAB-s complex in FBS by epitope coverage of the antiferritin, to inhibit antiferritin−ferritin recognition, and the colloidal stabilization of the complex might also have depressed the interparticle agglutination simultaneously.

Combined with the results shown in Figures 3, 5, and 6, the salt-induced high immune response of the LAB-s complex at low salt concentration (0−0.15 M) experimentally agrees with the hypothesis that the addition of salt weakened the electric repulsion between the LAB-s complex and ferritin to allow rapid antiferritin−ferritin recognition. At high salt concentration (0.15−0.5 M), the salt-reduced repellent force conversely promoted the passive adsorption of the ferritin onto the LAB-s complex, similarly to the salt-promoted nonspecific deposition of BSA-FITC molecules onto the LAB-1.0 complex (see Figure 6), to decrease the valid ferritin concentration in the solution, showing a reverse effect. Note that the antiferritin concentration was ~1000 times higher than that of ferritin under the measurement conditions, and that subtle changes in ferritin concentration were sensitively reflected by the reactivity of the complex. For the LAmP-s complex, the salt-induced response recovery was insignificant, in comparison with that of the LAB-s complex, because of its hydrophilic and almost-neutral interface structure. However, at high NaCl concentration, the immune response of the LAmP-s complex decreased as the salt concentration increased; the salt possibly disrupted the PEG-bound water and contracted the PEG chain layer,[37,38] to render the PEG chain layer partially incapable of inhibiting nonspecific adsorption, as illustrated in Figure 6. Consequently, the PEGylation technique is more suitable than the traditional BSA surface treatment method in the preparation of highly functional antiferritin-immunolatex particles, because of its effective inhibition of various interfacial interactions, in correlation with the interfacial structure of the particles.

4. Colloidal Stability. The long-term size stability and immune response of the LAmP-s complex, particularly at s <1.5, evidently indicates that PEGylation surface treatment may not only

(37) Molina-Bolvar, J. A.; Aguiar, J.; Carnero Ruiz, C. *J. Phys. Chem. B* **2002**, *106*, 870–877.
(38) Charlton, I. D.; Doherty, A. P. *J. Phys. Chem. B* **2000**, *104*, 8327–8332.

colloidally stabilize the LA complex, but also favor the maintenance of the configuration of the attached antibody for a long period (see Figure 4). The aforesaid possible interference of desorption of the physically adsorbed antiferritin was clearly negligible, as expected at least for the complex at s <1.5. Moreover, the LAmP-s complex was so stable that only a subtle variation in its size was observed even after being kept for one week at 2.0 M NaCl (4 °C), which is the salt concentration at which the sMPs immediately aggregated[7] (see Figure S3 in the Supporting Information). However, the LAB-s complex aggregated at 0.15 M NaCl, because of the screening effect of salt on the coagulation of the LAB-s complex, especially that with a high antiferritin load.

CONCLUSION

As a result of the thorough characterization and comparison of LAmP-s complexes with different antibody loads, the LAmP-1.0 complex was concluded to be the most suitable or applicable, because of its high immune response per localized antiferritin in both buffer A and FBS, and high colloidal stability for a long period. All of these advantages of the LAmP-1.0 complex were attributed to the PEG surface treatment using both long-chain and short-chain N6-PEG polymer, which not only stabilized the complex and maintained the antiferritin configuration on the surface, but also markedly depressed the disturbance caused by various nonspecific interfacial interactions. The LAmP-1.0 complex has the potential for practical utilization as the basis of a novel serological diagnosis system.

ACKNOWLEDGMENT

This research was supported by Biokit S. A. (Barcelona, Spain), a Grant-in-Aid for Scientific Research (A), No. 18200033, and a Grant-in-Aid for Scientific Research on Innovative Areas, No. 20106011 (Soft Interface), from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. The authors would like to express their gratitude to Dr. Miquel Sales (Biokit S.A.) for supplying various laboratory materials and for his helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Further details are given in Figures S1, S2, and S3, as well as Scheme S1 and Table S1. (PDF) This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 26, 2009. Accepted October 21, 2009.

AC902055W