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Development of an Aggregation-Based Immunoassay for Anti-Protein A Using Gold Nanoparticles

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A unique, sensitive, and highly specific immunoassay system for antibodies using gold nanoparticles has been developed. The assay is based on the aggregation of gold nanoparticles that are coated with protein antigens in the presence of their corresponding antibodies. The aggregation of the gold nanoparticles results in an absorption change at 620 nm that is monitored using an absorption plate reader. To demonstrate the analytical capabilities of the new technique, monodispersed protein A-coated gold particles, averaging 10 nm in diameter, were used to determine the level of anti-protein A in serum samples. The effects of the pH, the temperature, and the concentration of protein A-coated gold nanoparticles on the sensitivity of the assay were investigated using transmission electron microscopy (TEM) and UV/vis absorption spectroscopy. A dynamic range of 2 orders of magnitude and a limit of detection of 1 μg/mL of anti-protein A were observed. The new technique could be used for fast, high-throughput screening of antibodies in clinical diagnostic applications.

Aggregation-based immunoassays were first developed in 1956 by Singer and Plotz.[1] They utilized latex microparticles that were chemically derivatized with antibody molecules to bind antigens. The reaction of the antibody-coated particles with the corresponding antigens resulted in cross-linking of the antibody-coated particles that led to their aggregation. In the original test, a drop of a homogeneous suspension of milky-white antibody-coated particles was applied to a glass slide and mixed with a drop of an antigen solution. The aggregation of the particles into what looked like curdled milk was observed.[2] Quantitative aggregation assays were later developed using absorption spectrometry and nephelometry.[2] For example, Medcalf et al. developed an immunoturbidimetric assay for urine albumin, an indicator of kidney problems.[3] In their assay, poly(vinylnaphthalene) particles, averaging 40 nm in diameter, were coated with an outer layer of a chloromethylstyrene polymer, which was used to immobilize anti-human serum albumin. Aggregation in the presence of urine albumin was detected by measuring the change in light scattering intensity at 340 nm. The same approach was later used by Thakkar et al. to detect urinary retinol-binding protein.[4] In their assay, rabbit anti-human retinol-binding proteins were covalently coupled to 40-nm latex particles. Rosenzweig and Yeung also developed a particle-counting immunoassay for the analysis of single human erythrocytes.[5] The assay is based on the aggregation of antibody-coated particles in the presence of an antigen. The particles were electrophoretically migrated in a capillary through the light-scattering detector.

Using nanoparticles in aggregation-based immunoassays offers several advantages: Suspensions of nanoparticles do not appreciably scatter visible light, which reduces the background signal in turbidimetric assays and lowers the detection limit. In addition, nanoparticles form more stable suspensions and are, therefore, less susceptible to nonspecific aggregation.[6] Gold nanoparticles have been previously used in aggregation-based immunoassays in conjunction with micrometer-sized latex particles to develop a home pregnancy test.[2] In this assay, the colorless micro- and nanoparticles are derivatized using antibodies to human chorionic gonadotropin, β-hCG, a hormone released by pregnant women. When mixed with a urine sample containing this hormone, the micro- and nanoparticles are coagglutinated to form noticeably pink aggregates.

Gold nanoparticles attract great scientific and technological interest because of their physical and chemical characteristics. Because of their high electron density, colloidal gold particles are commonly used as tracers in electron microscopic studies of cellular biological samples.[7−12] Gold nanoparticles are red in color because of the Mie absorption by their surface-plasmon oscillation that peaks at 520 nm.[13] The aggregation of gold nanoparticles leads to the formation of a new absorption band at longer wavelengths as a result of electric dipole−dipole interaction and coupling between the plasmons of neighboring particles in the formed aggregates. Nanoparticle aggregates with interparticle distances

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substantially greater than the average particle diameter appear red, but as the interparticle distance in these aggregates decreases to less than approximately the average particle diameter, the color of the aggregates turns blue.[14] Mirkin et al. recently developed a colorimetric DNA hybridization assay using the spectral properties of gold nanoparticles.[15−18] In their experiments, gold nanoparticles averaging 13 nm in diameter were coated with single-stranded DNA oligonucleotides. The gold nanoparticles agglutinated in the presence of target oligonucleotides with a matching sequence as indicated by a color change of the sample from red to purple. They were able to detect femtomole levels of matching oligonucleotides using this technique. Recently, Otsuka et al. used gold nanoparticles modified with α-lactosyl-ω-mercaptopoly(ethylene glycol) to induce selective aggregation of the particles in the presence of lectin.[19] The intensity of the plasmon resonance absorption band at 520 nm was used to monitor the aggregation of the particles. This led to a long assay time of about 8 h, since the intensity of the plasmon band decreases only when large aggregates are formed. In a similar study, Hupp et al. used funtionalized gold nanoparticles to sense spectroscopically silent heavy metal ions via an ion-chelation-induced aggregation process.[20]

Our study shows for the first time that Mirkin's approach to DNA hybridization assays could be used to quantify the level of antibodies in aqueous and serum samples on the basis of the aggregation of gold nanoparticles. Gold nanoparticles coated with protein A were used to determine the level of anti-protein A in aqueous and serum solutions. The rate of aggregation of the protein A-coated gold nanoparticles in the presence of anti-protein A was monitored by measuring the absorption of the gold colloid suspension at 620 nm. The aggregation process was also monitored by transmission electron microscopy (TEM). The analytical capabilities of the new assay are discussed.

EXPERIMENTAL SECTION

UV/Vis Spectroscopic Measurements. UV/vis absorption spectra were obtained using a Cary 500 UV/vis/NIR spectrophotometer, which is run by Varian's Cary Win UV software, version 2.0.

Transmission Electron Microscopic (TEM) Measurements. A 10-μL portion of sample solution was dropped on a copper grid (Catalog No. 01801, Ted Pella Inc, CA). The grid was then drained dry with a tissue paper and air-dried. The samples were examined using a high-resolution transmission electron microscope (TEM) (JEOL EM 2010, Tokyo, Japan).

pH-, Temperature-, and Gold Nanoparticle Concentration-Dependence Measurements. The rate of aggregation of the protein A-coated gold nanoparticles was measured as a function of pH, temperature, and gold nanoparticle concentration. pH dependence measurements of the aggregation rate of gold nanoparticles were carried out using phosphate-buffered solutions at pH 4.0−9.0 at room temperature. The temperature-dependence experiments were conducted at various temperatures ranging from 20 to 37 °C. Solutions containing protein A-coated gold nanoparticles (7.8×10^{11} particles/mL) and anti-protein A (0.32 mg/mL) were used in these experiments. Different concentrations of protein A-coated gold nanoparticles (7.8×10^{11} to 2.34×10^{12} particles/mL) were used to determine the optimal concentration for the immunoaggregation assays. All experiments were carried out in triplicate measurements using the Genios absorption microplate reader (Tecan U.S. Inc.) at 620 nm. The plate reader was equipped with a temperature controller that regulated the temperature of the plate with variability of 0.1 °C.

Control Experiments. Several control experiments were carried out to prove that nonspecific aggregation does not occur under our experimental conditions. Solutions containing 300 μL of (a) phosphate buffer solution at pH 7.0, (b) 0.32 mg/mL anti-protein A, (c) 7.8×10^{11} protein A-coated gold nanoparticles/mL, (d) 7.8×10^{11} protein A-coated gold nanoparticles/mL and 0.6 mg/mL monoclonal anti-protein A, and (e) 7.8×10^{11} protein A-coated gold nanoparticles/mL and 0.72 mg/mL anti-albumin were used as negative controls. The positive control solution contained 7.8×10^{11} protein A-coated gold nanoparticles/mL and 0.32 mg/mL anti-protein A. The aggregation assays were monitored for 2 h at room temperature.

Materials and Reagents. Colloidal gold nanoparticles (10-nm) labeled with protein A; monoclonal and polyclonal antibodies to protein A; anti-human albumin; bovine serum; and phosphate-buffered saline, pH 7.4, were purchased from Sigma Ltd. Protein A-coated gold nanoparticles from Sigma, lot no. 048H9220, were used throughout the study to eliminate possible errors resulting from variation in protein A coverage on the gold nanoparticles between lots.

Polyclonal anti-protein A was reconstituted using 2 mL of deionized water. The solution was stored frozen in working aliquots. Protein A-coated gold nanoparticle suspensions were kept under light-tight conditions at −20 °C to protect the gold nanoparticles from oxidation.[21]

RESULTS AND DISCUSSION

The Aggregation Process. The aggregation of protein A-coated gold nanoparticles in the presence of anti-protein A takes several hours to complete. However, a color change from red to purple could be observed within 30 min. To maximize the absorption change at 620 nm, we used an assay time of 2 h. At longer times, precipitation of large aggregates and a clear supernatant are observed. As a result, the absorption intensity is no longer concentration-dependent. The UV/vis absorption spectra of solutions containing protein A-coated gold nanoparticles and anti-protein A at different times during the assay are shown in Figure 1. Spectrum a, measured at $t = 0$, is a typical spectrum of a gold nanoparticles solution showing a plasmon resonance peak at 520 nm. The aggregation of gold nanoparticles leads to an increase in absorbance at longer wavelength (600−750 nm) as seen in spectrum b (t = 30 min), and c (t = 60 min). The aggregation splits the energy levels of gold nanoparticles. The low-energy flank of the absorption spectrum is shifted strongly

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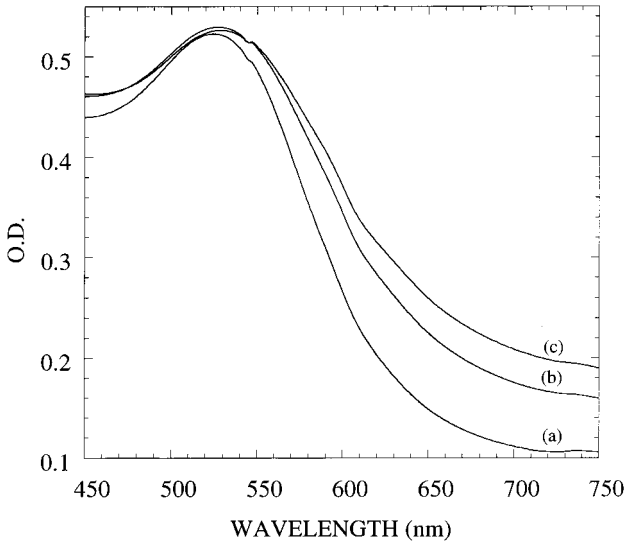

Figure 1. Spectra of the aggregation process were measured at 620 nm at (a) $t = 0$; (b) $t = 30$; and (c) $t = 60$ min. The solution contained of 7.8×10^{11} gold nanoparticles/mL and 0.32 mg/mL anti-protein A in phosphate-buffered solution at pH 7.0.

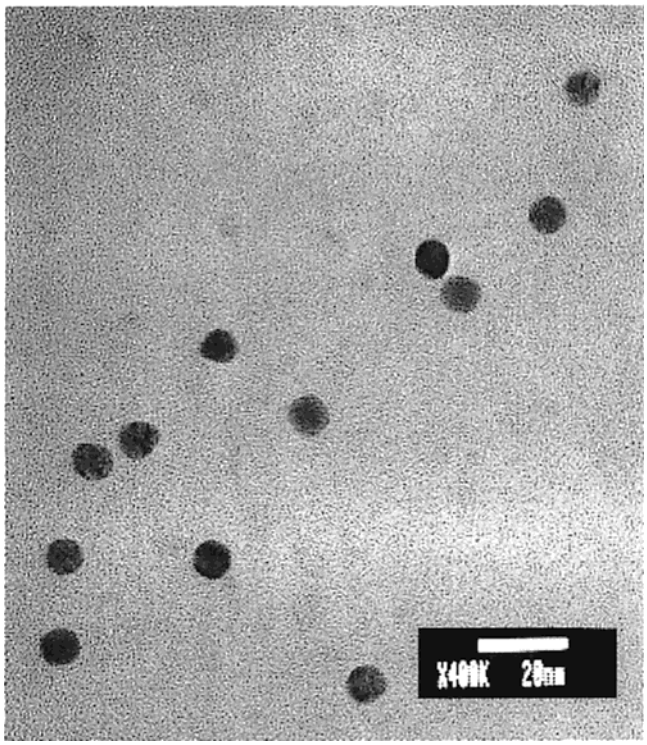

Figure 2. TEM image (400 000×) of monodispersed protein A-coated gold nanoparticles prior to immunoaggregation.

toward lower energies and high absorption is obtained even in the IR with samples containing some large particle aggregates.[14] In our experiments, we found that the largest change in absorption during the aggregation of the protein A-coated gold nanoparticles is at 620 nm. A TEM image (400 000×) of protein A-coated nanoparticles prior to aggregation is shown in Figure 2. The monodispersed particles average 10 nm in diameter. The progress of the aggregation process is shown in Figure 3. The TEM image taken at $t = 30$ min showed that the density of the particles in the field of view increased (Figure 3a). At $t = 1$ h, the presence of islands of small aggregates was seen (Figure 3b). Finally, at $t = 2$ h, large micrometric aggregates were observed (Figure 3c). The number of particles seen in Figure 3c increased as a result of movement of particles into the observed field of view during the aggregate formation.

Effect of the Density of Protein A-Coated Gold Nanoparticles on the Rate of Aggregation. Two major factors affect the rate of aggregation of protein A-coated gold nanoparticles in the presence of anti-protein A molecules. The first is the frequency of efficient collisions, which depends on the density of active sites. The second is the affinity between the antigen and antibody molecules. In our experiments, we assume that the binding constant between the antigen and antibody molecules is very high. Therefore, the aggregation rate depends mostly on the collision frequency of the particles, which is directly related to the density of gold nanoparticles in the analyte solution. As expected, the absorption change is larger at a higher concentration of gold nanoparticles. However, increasing the concentration of the gold nanoparticles over 1.5×10^{12} protein A-coated gold nanoparticles/mL does not improve the sensitivity of the assay, because there is also an increase in the viscosity of the solution, which inhibits aggregation. We determined that for the lot of gold nanoparticles used in our experiments, the optimum concentration of protein A-coated gold nanoparticles was 7.8×10^{11} particles/mL. Since the aggregation rate depends on the coverage of protein A on the gold nanoparticles as well as on the density of the particles in the solution, the assay would require recalibration should the lot of anti-protein A-coated gold nanoparticles be changed. As mentioned previously, we conducted our study using the same lot of anti-protein A-coated gold nanopraticles to prevent variation in aggregation rates that could result from the difference in protein A coverage on the surface of the particles.

pH Dependence. The immunoaggregation process involves several stages. The first stage is a primary reaction involving binding of the antigen-coated particles to antibody molecules. The second stage involves reaction between particles to form aggregates.[22] Since both reactions involve charged species, it is expected that the pH in the analyte solution would affect the aggregation rate. The effect of the pH on the rate of aggregation of the protein A-coated gold nanoparticles in the presence of anti-protein A is shown in Figure 4. The aggregation rate peaks at ~pH 7, which may be attributed to repulsive electrostatic forces at lower or higher pH.[15] It is also expected that the optimum pH would be system-dependent and should, therefore, be determined for each aggregation assay.

Temperature Dependence. The effect of temperature on the aggregation rate is shown in Figure 5. The aggregation rate increases with decreasing temperature. The rate of aggregation at 20 °C is 2.5 times higher than the rate of aggregation at 37 °C. Although the rate of chemical reactions usually increases with temperature, the aggregation reaction involves a negative change in entropy $\Delta S < 0$. Therefore, increasing the temperature results in an increase in the free energy ΔG, and a decrease in the stability of the formed aggregate. We also found that decreasing the temperature to 4 °C increases the rate of nonspecific aggregation.

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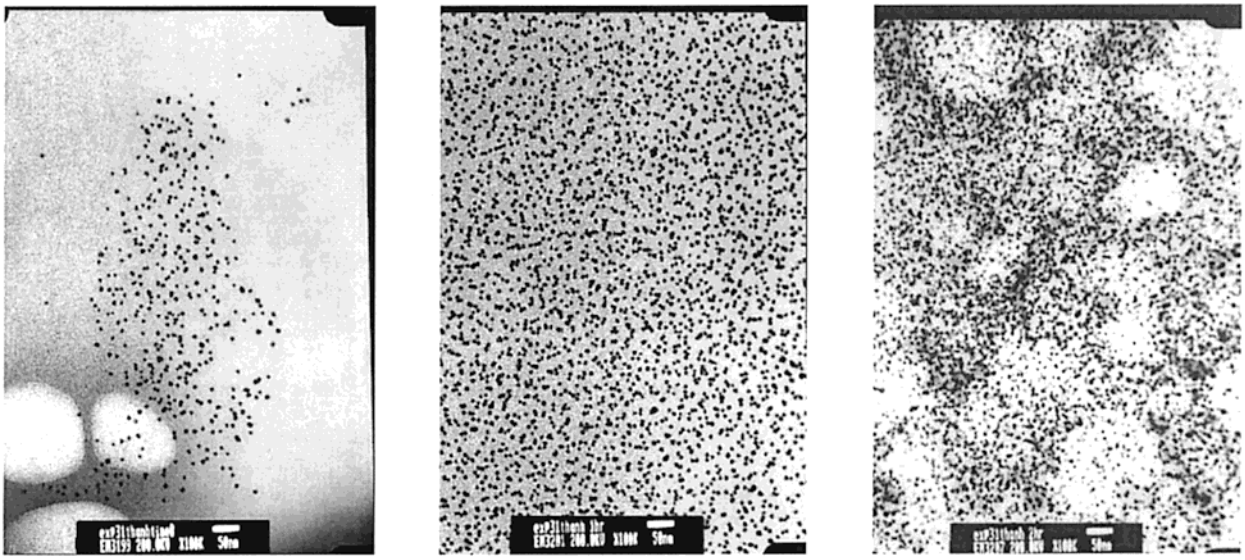

Figure 3. TEM images (100 000×) during the aggregation process at (a) $t = 30$ min, (b) $t = 1$ h, and (c) $t = 2$ h.

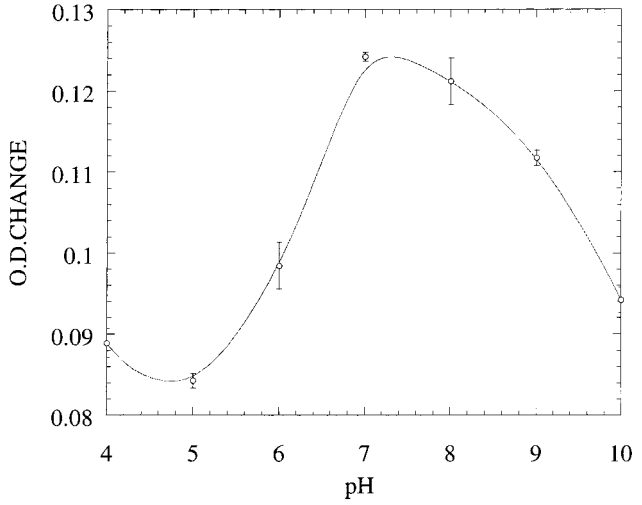

Figure 4. pH dependence of the aggregation rate measured at pH 4.0−9.0. The absorption change of samples containing protein A-coated nanoparticles (7.8×10^{11} particles/mL) and anti-protein A (0.32 mg/mL), incubated for 2 h, was measured at 620 nm. The optimal aggregation rate was found at pH 7.0.

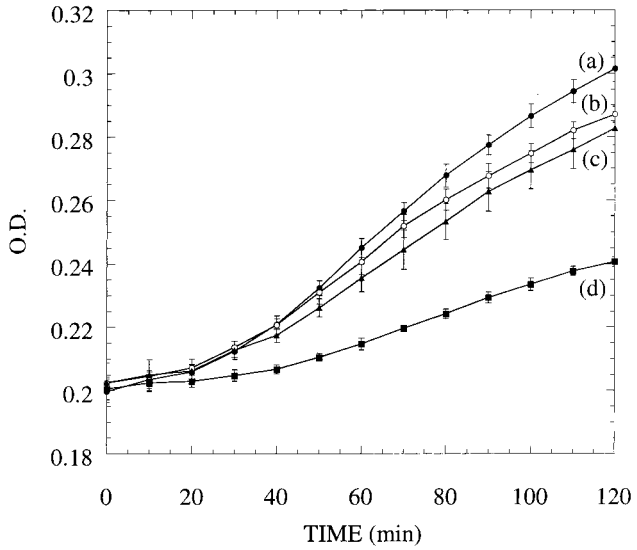

Figure 5. Temperature dependence of the immunoaggregation process. The assays were carried out in phosphate-buffered solutions at pH 7.0 and monitored for 2 h. The absorption change at 620 nm was measured at (a) 20, (b) 25, (c) 30, and (d) 37 °C.

This temperature also presents instrumental complications, because temperature control is needed throughout the assay. Although it is relatively simple to carry out the experiments at 4 °C under laboratory conditions, this requirement would preclude the use of the technique in field applications. We, therefore, decided to characterize the assay performance at ambient temperature. As seen in Figure 5, variations of temperature clearly affect the aggregation rate. Therefore, the reproducibility of the assay is greatly improved when the assay is conducted in a thermostated environment. Nevertheless, the assay could be conducted in an unregulated temperature environment. Fluctuations of ±1 °C could be tolerated, since the variation in aggregation rate would be smaller than 5%.

Control Experiments. Several control experiments were carried out to validate the selectivity of the aggregation assay. The results of these experiments are shown in Figure 6. Curve a shows the absorption intensity at 620 nm of a phosphate buffer solution at pH 7.4. Curve b shows the absorption intensity at 620 nm of a solution containing polyclonal anti-protein A in the absence of protein A-coated gold nanoparticles. Curve c shows the absorption intensity at 620 nm of a solution containing protein A-coated nanoparticles in the absence of anti-protein A. The absorption intensity at 620 nm remained constant for 2 h in these control experiments. Curve d shows the absorption intensity at 620 nm of a solution containing protein A-coated nanoparticles and a monoclonal anti-protein A. No absorption change was observed for 2 h, indicating that aggregation did not take place. This is attributed to the lack of multiple binding sites and the inability of monoclonal antibodies to form aggregates among a large numbers of particles. Curve e shows another control experiment in which protein A-coated nanoparticles are mixed with anti-albumin. No absorption change was observed, indicating that

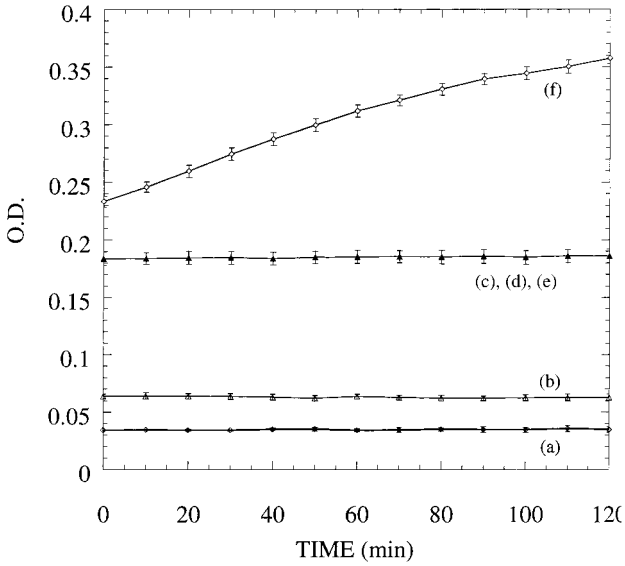

Figure 6. Control experiments to validate the selectivity of the aggregation process: (a) phosphate buffer solution at pH 7.0, (b) 0.32 mg/mL polyclonal anti-protein A, (c) 7.8×10^{11} protein A-coated gold nanoparticles/mL, (d) 7.8×10^{11} protein A-coated gold nanoparticles/mL and 0.6 mg/mL monoclonal anti-protein A, (e) 7.8×10^{11} protein A-coated gold nanoparticles/mL and 0.72 mg/mL anti-albumin, and (f) 7.8×10^{11} protein A-coated gold nanoparticles/mL and 0.32 mg/mL polyclonal anti-protein A. All experiments were carried out in phosphate-buffered solution at pH 7.0 for 2 h.

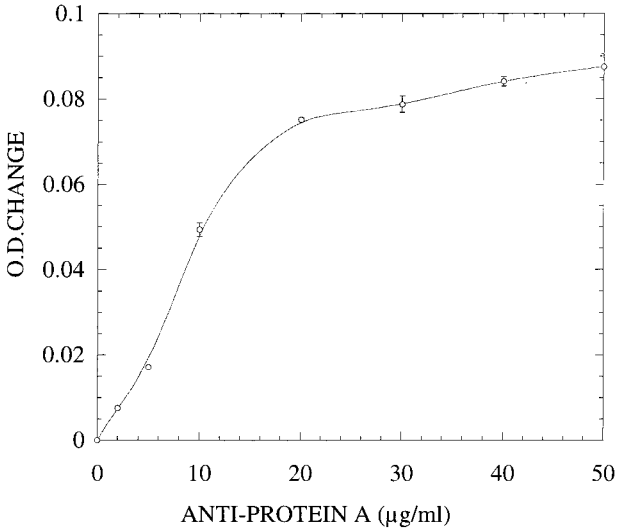

Figure 7. Calibration curve describing the absorption change at 620 nm against anti-protein A concentration in a 10% bovine serum solution. A dynamic range of 2 orders of magnitude and a limit of detection of 1 μg/mL anti-protein A were observed.

no nonspecific aggregation and no cross-reactivity of the antigen occurred. Curve f shows a positive response of the assay when protein A-coated gold nanoparticles and anti-protein A were present in the analyte sample. As expected, a noticeable absorption change at 620 nm occurred, indicating aggregation of the protein A-coated gold nanoparticles.

Calibration Curve of the Gold Nanoparticle-Based Assay. An absorption change at 620 nm vs concentration calibration curve for anti-protein A in 10% bovine serum is shown in Figure 7. The anti-protein A solution concentrations ranged from 0 to 50 μg/mL. A dynamic range of 2 orders of magnitude in concentration was observed. At higher concentration, the aggregation process was inhibited, which may be due to the blocking of active sites. Each data point represents the average of triplicate measurements. The relative standard deviation within the plate of 3−5 samples was 0.3−2.6%. The relative standard deviation among 3 runs was 1−2%. The limit of detection of the assay in serum samples was 1 μg/mL of anti-protein A, which is comparable with other immunological methods such as ELISA.[23]

SUMMARY AND CONCLUSIONS

A new aggregation immunoassay technique based on the use of gold nanoparticles was developed. The rate of aggregation of antigen coated gold nanoparticles in the presence of antibodies toward the antigen was measured by monitoring the absorption change of the gold nanoparticles upon aggregation. In our experiments, the aggregation process was monitored at 620 nm. The use of red light decreases the effect of light scattering and absorbance of analyte solutions at lower wavelengths on the precision of the results. In general, aggregation reactions are faster at low temperatures. Our assays were conducted at ambient temperature where a significant absorption signal change occurred in 1−2 h. This observation was also verified by TEM measurements. Although decreasing the temperature may increase the aggregation rate, it would also complicate the experimental system as a result of the required temperature control. It would also preclude the possibility of using this assay in field applications. As is, the new gold nanoparticles-based assay is sensitive to concentrations of anti-protein A as low as 1 μg/mL, which is comparable with the sensitivity of ELISA. However, the new assay is much simpler to perform, because it involves only one step, as compared to ELISA assays, which require multiple washing steps and separation between bound and unbound labeled macromolecules. Both ELISA and our new gold nanoparticles-based aggregation assay are capable of analyzing a large number of samples simultaneously using a microplate reader format; however, the gold nanoparticles-based assay would be easier to automate because of the small number of steps involved in the aggregation assay. Although gold is an expensive metal, the cost of the assay is kept low as a result of the use of small amounts of gold nanoparticles. To further reduce cost and increase simplicity, we are currently developing a simple diode-laser-based absorption spectrometer that will eventually replace the full-frame absorption spectrometer used in these studies. The new system could be suitable for field applications for aggregation-based immunoassays.

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