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Nanocrystal Biolabels with Releasable Fluorophores for Immunoassays

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A novel signal amplification technology based on a new class of biofunctional fluorescent nanocrystals holds promise to improve the sensitivity and the limits of detection of immunoassays. A two-step approach without layer-by-layer techniques is described to encapsulate the fluorogenic precursor fluorescein diacetate (FDA) nanocrystals (107-nm average size) followed by conjugation of the antibody. Distearoylphosphatidylethanolamine (DSPE) modified with amino(poly(ethylene glycol)) (PEG(2000)-Amine) is coated on the surface of the FDA nanocrystals to provide a interface for the antibody coupling. Anti-mouse antibodies are attached to the nanocrystalline FDA biolabels by adsorption. A high molar ratio of fluorescent molecules to biomolecules (2.8×10^4) is achieved in this nanocrystal biolabel system. The analytical performance of the nanocrystal-based label system is evaluated in a model sandwich immunoassay for the detection of mouse IgG. After separation of the nonbound antibody nanocrystal labels, fluorophores are released by hydrolysis and dissolution of the nanocrystalline FDA. Due to the release of the fluorophores (fluoresceins) into a large volume of organic solvent/sodium hydroxide mixture, self-quenching is suppressed. The FDA[DSPE-PEG(2000)Amine]-modified biolabels have a highly stable colloidal suspension with minimized nonspecific interactions. The limit of detection was lowered by a factor of 5−28, and the sensitivity was 400−2700-fold higher compared with a state-of-the-art immunoassay using directly fluorescent-labeled antibodies. Our approach provides high sensitivity and low limits of detection without the need for long incubation times, making it an interesting alternative in biolabel technology.

Achieving very high sensitivity is a major goal in assay development. As the trend toward assay miniaturization continues in high-throughput screening, the need for highly sensitive detection reagents/systems is of increasing importance. Enzymatic amplification systems have challenged the assay gold standard, radioisotopes. They are advantageous due to continuous enzymatic generation of detectable product molecules. Sensitivity and limits of detection can be improved by extending incubation times in these systems. However, enzymatic methods require the addition of enzymes, substrates, and sometimes stopping or developing solutions to maintain detectable products. Such additions have tradeoffs in ease of use and analysis time. The present trend toward utilizing nonradioactive labeling strategies with powerful fluorescent conjugates to provide sensitivity equal to or even better than the enzymatic methods bypasses the disadvantages of the enzymatic methods without sacrificing sensitivity.

The sensitivity of a fluorescence assay is determined by the number of light quanta emitted per analyte molecule. Increasing the ratio of fluorescent dyes to proteins (i.e., the F/P ratio) while minimizing dye self-quenching effects to achieve signal amplification is highly desirable. However, conventional fluorescence systems have self-quenching problems: If more than 10−15 fluorophores are attached to 1 antibody, their distance is close to or within their Foerster radius, resulting in significant loss of fluorescence emission intensity due to energy transfer. There are several methods based on fluorescence detection that permit signal amplification and exhibit high versatility. Long-lived fluorescence lanthanide chelates,[1] liposome-encapsulated fluorophores,[2,3] chemiluminescent microemulsions,[4] fluorescent-conjugated dendrimers,[5] and other related systems[6−8] all display a high F/P ratio and are suitable for use in immunoassays. However, time-resolved fluorescence immunoassays using lanthanide chelates as fluorophores require sensitive and expensive instrumentation. Also, this detection method is prone to lifetime quenching

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by some impurities.[1] The preparation of microcapsules with liposomes is rather elaborate, and the stability of such a system is critical.[9,10] The precipitation method to prepare perylene microparticles for chemiluminescent microemulsions[4] or fluorescence intensity[8] requires a large amount of antigen or antibody, which is not economical for most biological analyses. Given the limitations of existing fluorescence-based biochemical assays, the development of new strategies and systems to perform fluorescent immunoassays is desirable.

In our previous study,[11] fluorescent labels were constructed by encapsulating microcrystalline fluorescein diacetate (FDA) with an average size of 500 nm within ultrathin polyelectrolyte layers of poly(allylamine hydrochloride) and poly(sodium 4-styrenesulfonate) via layer-by-layer (LbL) techniques.[12] The polyelectrolyte coating was subsequently used as an interface for the attachment of antibodies through adsorption. The fluorescein equivalent (FE) value ($\sim 1.5 \times 10^8$) and the F/P ratio (ranging from 5×10^4 to 2×10^5) of our labels have been exceptionally high. We reported amplification rates of 70−2000-fold for our microcrystal label-based assay compared with the corresponding immunoassay performed with directly fluorescent-labeled antibodies.

To simplify the preparation of the fluorescent conjugates and enhance the sensitivity of the immunoassay, we developed a two-step approach without LbL techniques. FDA nanocrystals are first encapsulated by distearoylphosphatidylethanolamine (DSPE) lipids coupled to amino(poly(ethylene glycol)) (PEG-Amine) and subsequently adsorbed to biorecognition molecules (Figure 1).[11] The aim of the two-step approach is to optimize novel nanocrystalline biolabels based on fluorogenic precursor FDA encapsulated with PEG-Amine-modified phospholipids and conjugated to biomolecules in order to (a) achieve a highly stable colloidal suspension, (b) enhance the sensitivity of the immunoassay, and (c) minimize nonspecific binding.

EXPERIMENTAL SECTION

Materials. FDA and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Steinheim, Germany). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(poly(ethylene glycol))-2000] (ammonium salt) [DSPE-PEG(2000)Amine] with a molecular weight of 2,790.52 and the molecular formula $C_{132}H_{266}N_3O_{54}P$ (Figure 1)[11] was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Affinity-purified polyclonal goat anti-mouse IgG (Gt α M IgG, whole molecule), mouse IgG (M IgG), rabbit IgG (Rb IgG), and goat IgG (Gt IgG) were supplied by Arista Biologicals Inc. (Allentown, PA). Goat anti-mouse IgG−FITC conjugates were purchased from Molecular Probes (Eugene, OR; protein concentration 2 mg/mL, F/P ratio 6.3) and from Sigma-Aldrich (protein concentration 1.1 mg/mL, F/P ratio 4.2). Bovine serum albumin (BSA, fraction V) was from Roth (Karlsruhe, Germany). Fluorescein 5-isothiocyanate (FITC) and all other chemicals were obtained from Sigma-Aldrich and were of analytical grade.

Step 1. Encapsulation by Treatment of Nanocrystals with DSPE-PEG(2000)Amine. A suspension of 5% (w/w) FDA in 1.25% (w/w) DSPE-PEG(2000)Amine was milled for 48−72 h by using a process developed at Elan Drug Delivery Inc. (King of Prussia, PA). The temperature was maintained at 20 °C to prevent the material from hydrolyzing. The morphology of milled FDA nanocrystals was examined with a JEOL 6300F ultrahigh-resolution scanning electron microscope (SEM), operating at 10 kV. Particle size distribution was measured based on both the Fraunhofer and Mie theories of light scattering using a Coulter LS230 laser diffraction particle size analyzer (Beckman Coulter Inc.) by polarization intensity differential scattering technology.

Step 2. Conjugation of Encapsulated Nanocrystals with Antibodies. The particle suspension from step 1 was diluted to 0.0626% (w/v) and incubated with 200 μg/mL Gt α M IgG in 10 mM phosphate-buffered saline (PBS, pH 7.4) at 20 °C for 1 h. After centrifugation at 16000*g* for 10 min, the supernatant was removed and its UV absorption was measured at 280 nm (Cary 50 Conc UV−visible spectrophotometer). The antibody surface coverage of the nanocrystals was determined by the difference in absorption at 280 nm between the supernatant and the original protein solution. The IgG-coated particles were then separated from the soluble IgG by three centrifugation/washing cycles.

Preparation of Direct FITC-Labeled Antibody. A solution of Gt α M IgG (1 mg/mL, whole molecule, Arista Biologicals Inc.) in 500 mM carbonate buffer, pH 9.5, was incubated with FITC (320 μg/mg of antibody in the same buffer) for 1 h at room temperature. Free unconjugated FITC was removed by dialyzing the mixture in a Pierce Slide-A-Lyzer Dialysis Cassette (Pierce Biotechnology Inc.) against 10 mM PBS. The number of FITC molecules bound per protein molecule and the protein concentration were estimated by measuring the optical density of the solution at 280 and 495 nm.

Microelectrophoresis. The surface charges of the FDA nanocrystals and nanocrystals adsorbed with IgG were examined at different pH by microelectrophoresis using a ZetaPlus potential analyzer (Brookhaven Instrument Corp., Holtsville, NY) by taking the average of five measurements at the stationary level. The mobility, u, was converted to the zeta potential (ξ-potential) using the Helmholtz−Smoluchowski relation, $\xi = 4\pi u\eta/\epsilon$, where η is the viscosity of the solution and ϵ is the permittivity.[13]

Solid-Phase Sandwich Fluorescence Immunoassay. The applicability of the nanocrystal-based label system was demonstrated in a model sandwich immunoassay for mouse IgG detection (Figure 2).[11] A 1 μg/mL solution of Gt α M IgG (100 μL/well) was coated on Nunc Maxisorp 96-well microplates (Nunc International, Rochester, NY) in 0.1 mol/L carbonate buffer (pH 9.6) at 4 °C overnight. After rinsing three times with a washing buffer [10 mM PBS, 0.1% (w/v) BSA, 0.5% (w/v) Tween-20], the wells were each blocked with 300 μL of 1.0% BSA solution for 0.5 h at 37 °C. The plates were then washed four times and incubated with dilutions (100 μL/well) of M IgG as the analyte at 37 °C for 1 h. After washing five times, the anti-mouse-coated nanocrystal suspension (0.0125%, w/v) was dispensed into the wells (100 μL/well), and the microplates were incubated again at 37 °C for 1 h. Soluble direct-label Gt α M IgG-FITC dilutions (100 μL/well) of

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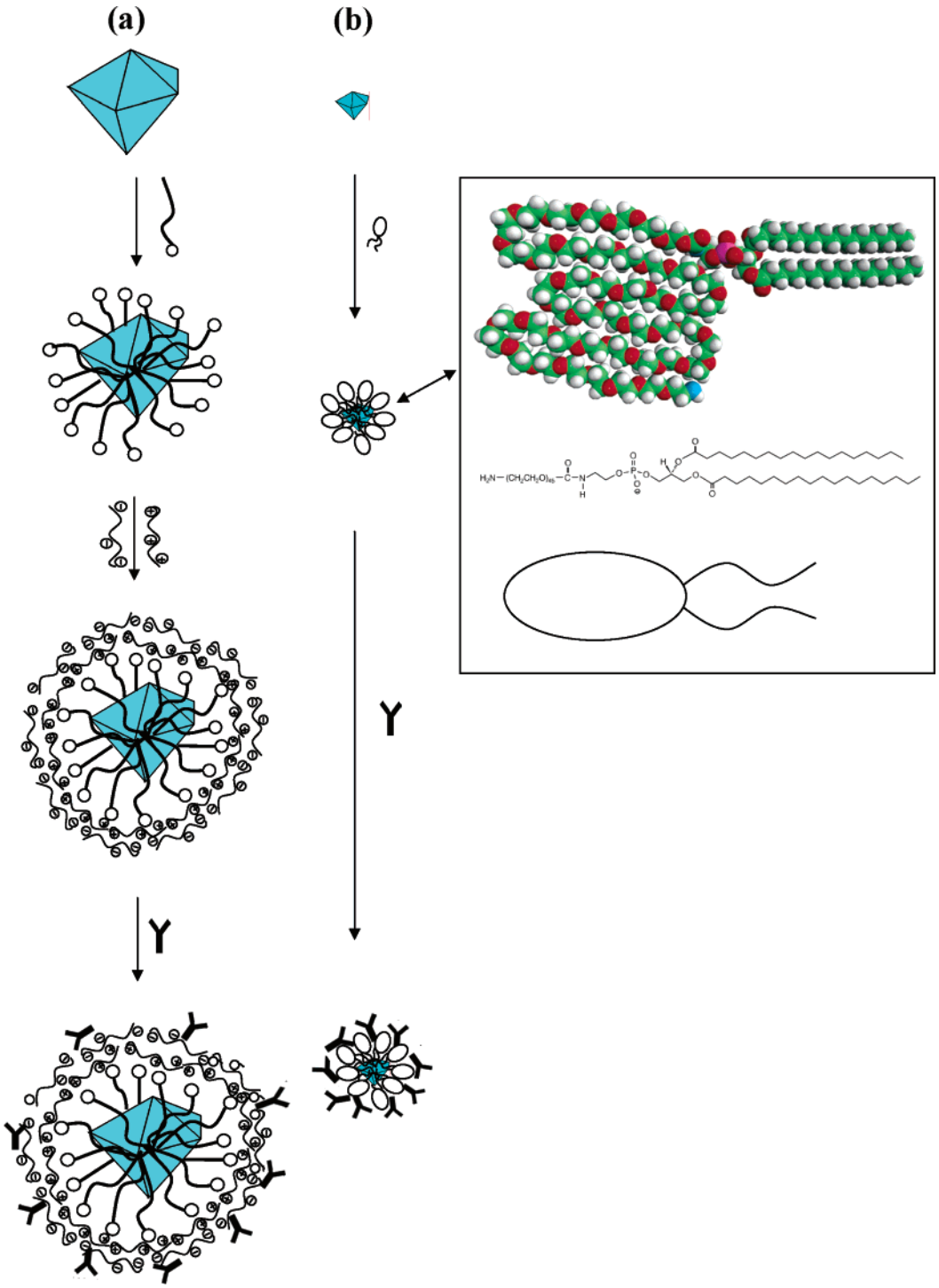

Figure 1. Schematic illustration of the preparation of biofunctional fluorescent labels. In a previous study,[11] FDA was milled into microcrystals with an average size of 500 nm, stabilized with SDS surfactant, followed by encapsulation of polyelectrolyte multilayers, and the attachment of biorecognition molecules, e.g., antibodies (a). A simple two-step approach is described in the current study: FDA was milled into nanometer-sized (107 nm on average) crystals in an aqueous surfactant (DSPE-PEG(2000)Amine) medium and subsequently biorecognition molecules, e.g., antibodies were deposited (b). The structure of DSPE-PEG(2000)Amine is shown in the inset.

1:200 (Molecular Probes), 1:128 (Arista, protein concentration 1.1 mg/mL, F/P ratio 4.4), and 1:64 (Sigma-Aldrich) were used for comparison. After incubation, excess antibody conjugates were washed off in five washing cycles with the buffer. An aliquot of 100 μL of releasing reagent per well (DMSO and 1 M NaOH in a 1:1 ratio) was added. The fluorescence intensity was measured using a MFX Microplate Fluorometer (Dynex Technologies Inc., Chantilly, VA) with an excitation/emission wavelength of 485/538 nm. However, this was not sensitive enough to measure the fluorescence signal of the FITC conjugates, which was measured using a FLUOstarOPTIMA multifunctional microplate reader (BMG Labtechnologies GmbH) with an excitation/emission wavelength of 485/520 nm and compared with those of the FDA-labeled antibodies. A fluorescence reader gain setting of 1600 was

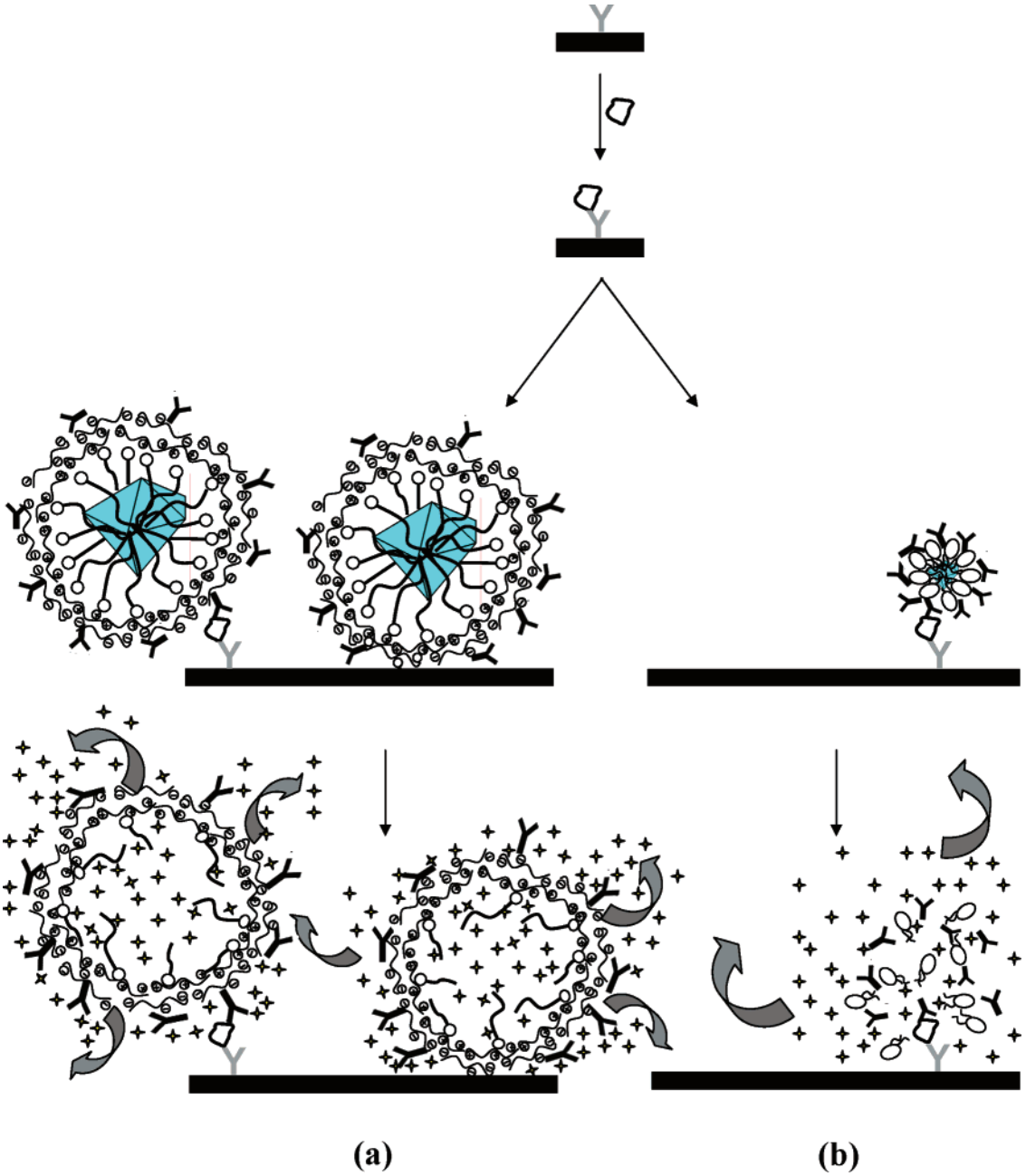

Figure 2. Principle of a sandwich fluorescent immunoassay using nanocrystalline FDA conjugates. The analyte is first incubated with the capture antibody preadsorbed on the microtiter plate and then exposed to nanocrystalline FDA conjugates. High signal amplification is achieved after solubilization, release, and conversion of the precursor FDA into fluorescein molecules by the addition of DMSO and NaOH. The previous microcrystal (500 nm in average size) contained $\sim 10^8$ FDA molecules, and a limit of detection of 4 μg/L was achieved (a).[11] A much lower limit of detection of 0.057 μg/L and minimized nonspecific interactions are demonstrated using the DSPE-PEG(2000)Amine-modified FDA as labels although each nanocrystal contains 2 orders of magnitude fewer fluorescein molecules (b).

used for measurements of the FDA-labeled and the FITC-labeled antibodies.

Negative controls were prepared by omitting the Gt α M IgG coating in the microtiter plate, by omitting the M IgG as the analyte, or by exchanging it with Rb IgG or Gt IgG and by omitting the conjugate.

Samples were run in replicates of 8 except that 16 wells were run without added M IgG as the blank to establish the limit of detection (LOD) of the assay, which is defined as the concentration of M IgG corresponding to the mean fluorescence of the zero dose response plus two times the standard deviation of this measurement. This LOD value determined the sensitivity of the assay at a 95% confidence interval ($P < 0.05$).

RESULTS AND DISCUSSION

Size Distribution of the FDA Nanocrystals. The particle size distribution of the FDA nanocrystals, determined by light-scattering measurements, is shown in Figure 3. Approximately 90% of the particles were found to be smaller than 236 nm, and 100% were smaller than 1.0 μm. This result is in agreement with SEM analysis of the particles as shown in Figure 3. The SEM image shows that the particles have a range of shapes.

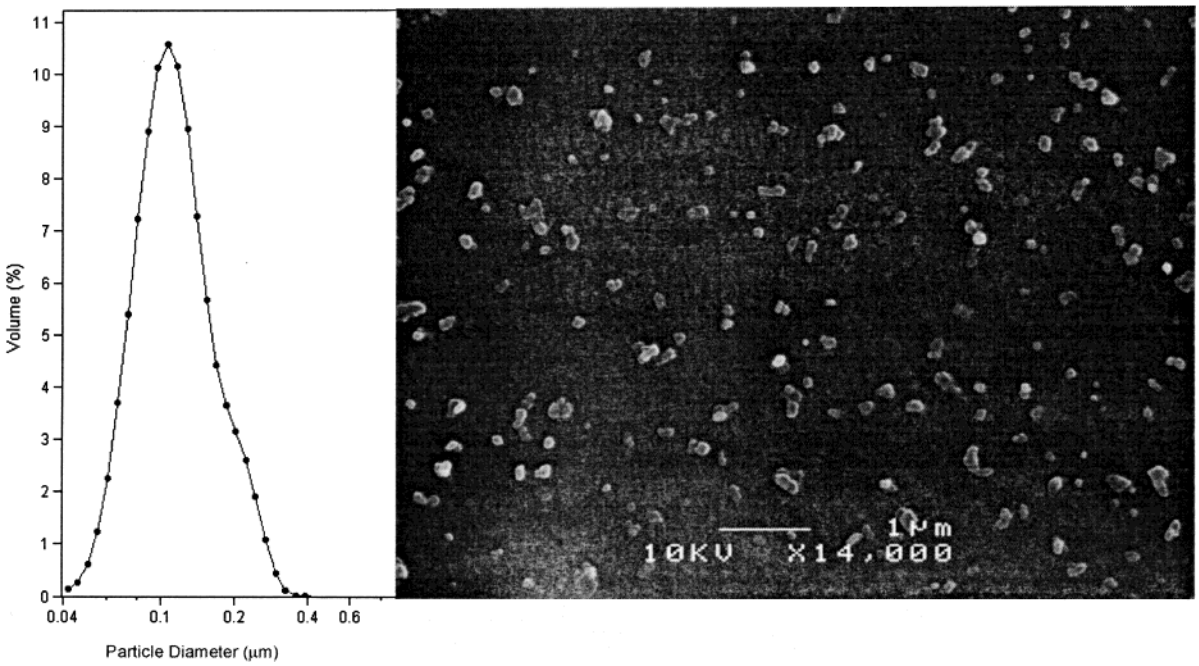

Figure 3. Particle size distribution and SEM micrograph of milled DSPE-PEG(2000)Amine-stabilized FDA nanocrystals. The image shows the shapes of the particles.

During the milling process in the presence of the DSPE-PEG(2000)Amine solution, newly generated FDA/water interfaces were immediately coated with DSPE-PEG(2000)Amine molecules. The hydrophobic surface of the stabilizer is most likely associated with the hydrophobic surface of the nanocrystals, and the hydrophilic portion of the DSPE-PEG(2000)Amine is oriented toward the aqueous phase of the suspension.[14] The adsorbed DSPE-PEG(2000)Amine layer causes the FDA nanocrystals to disperse in water and prevents their aggregation, hence conferring colloidal stability.

Biolabeled FDA Nanocrystals. The DSPE-PEG(2000)Amine contains one amino functional group. Antibodies could be assembled onto the DSPE-PEG(2000)Amine-encapsulated nanocrystals via a covalent binding protocol.[15−18] However, we attached the antibodies by simply adsorbing them onto the FDA nanocrystals. Adsorptive immobilization of antibodies onto polyelectrolyte-coated colloid particles has been extensively studied and has been shown to be a successful means for the stable attachment of biomolecules by retaining their specific immunorecognition abilities.[19−22]

Adsorption was confirmed by our microelectrophoresis study of the IgG assembled particles under different pH conditions. Figure 4 shows the ζ-potentials of the nanocrystals without and

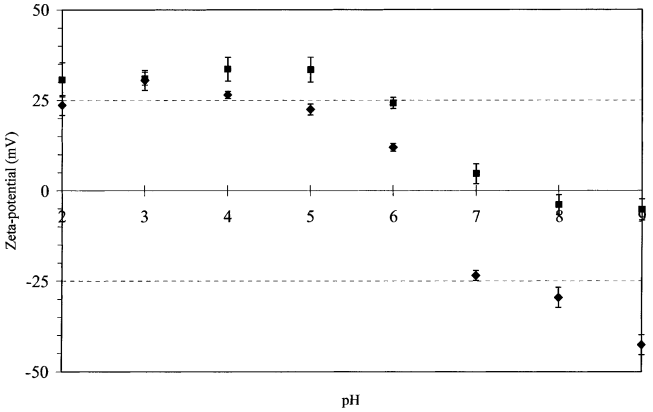

Figure 4. Microelectrophoresis measurements of the FDA nanocrystals (■) and nanocrystals adsorbed with IgG (◆) under different pH conditions by taking the average of five measurements at the stationary level.

with adsorbed antibodies at different pH. FDA nanocrystals coated with DSPE-PEG(2000)Amine exhibited positive ζ-potentials at pH less than or equal to 7. This clearly indicates that the amino groups in the DSPE-PEG(2000)Amine chains were protonated. When the pH values increased, the ζ-potentials decreased to nearly neutral values. The bulky PEG chain may "mask" the negatively charged phosphate groups in the DSPE chain. After incubation of DSPE-PEG(2000)Amine-coated nanocrystals with IgG, a clear change in the ζ-potential of the nanocrystals was observed. The ζ-potential of the IgG-coated nanocrystals changed steadily from −42.6 mV at pH 9 to +23.6 mV at pH 2, with an isoelectric point (IEP) between pH 6 and 7, which is close to the IEP of native IgG. It is expected that the IEP of the protein-coated colloids is similar to that of the native protein when the protein surface coverage is relatively high.[23,24] The microelectrophoresis data of the

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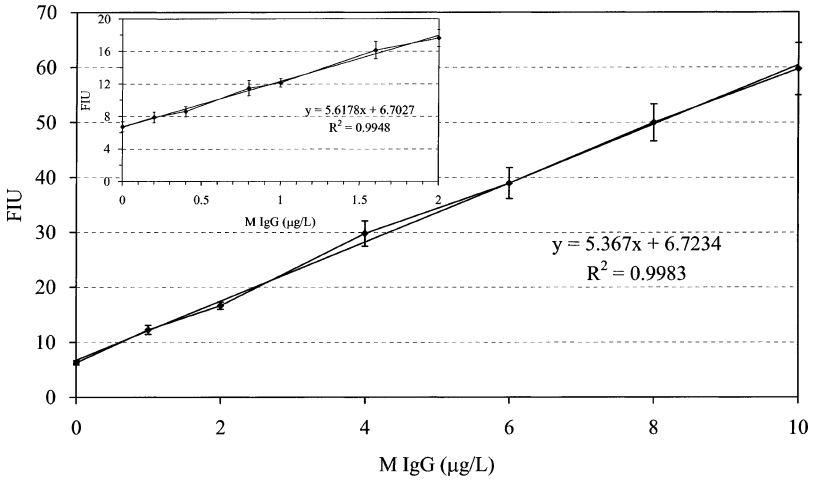

Figure 5. Sandwich fluorescence immunoassay of M IgG using Gt α M IgG-FDA nanocrystals (0.0125%, w/v) as labels. Good linearity is observed at analyte concentrations from 0 to 10 μg/L ($R^2 = 0.9983$). An ultrasensitive sandwich fluorescence immunoassay with analyte range from 0 to 2 μg/L ($R^2 = 0.9948$) is shown in the inset. Error bars correspond to standard deviations (±SD, $n = 8$).

nanocrystals assembled with IgG suggested the successful adsorption of IgG on the particle surface. The mechanism for adsorption is not yet fully understood. Given that IgG was readily adsorbed, the electrostatic attraction between the protonated PEG-Amine chains of the nanocrystal surface and the carboxyl groups of the antibodies does have influence on the adsorption of antibodies, especially on the orientation of the adsorbed protein molecules. Under some circumstances, additional forces, such as hydrophobic interaction, the van der Waals force, H-binding, etc., are also at work.

The adsorption of antibodies on the nanocrystal surface was further confirmed by adsorption spectroscopy. The amount of Gt α M IgG adsorbed onto the FDA nanocrystals was determined spectrophotometrically. The protein surface coverage was calculated by assuming an average size of 107 nm for the FDA nanocrystals. Incubation of the FDA nanocrystals with Gt α M IgG (200 μg/mL) in 10 mM PBS buffer for 1 h at 20 °C resulted in the adsorption of 39.6% of the added protein, correlating with a surface coverage of 3.35 mg/m^2. The theoretically calculated surface coverage value for a close-packed IgG monolayer is in the range of 2.0−5.5 mg/m^2, depending on the different orientations of the adsorbed IgG molecules.[24,25]

To calculate the number of fluorescein molecules released by a single DSPE-PEG(2000)Amine-coated FDA nanocrystal label, a cubic crystal morphology with dimensions of 107 × 107 × 107 nm was assumed. As each FDA molecule can be converted into one fluorescein molecule, the number of fluorescein molecules per nanocrystal is calculated to be ∼2.6×10^6, which is 2 orders of magnitude less than the previously reported FE value (∼1.5×10^8) due to the smaller size of the FDA nanocrystals (Figure 2).[11] The FDA nanocrystal is totally composed of precursor fluorescein molecules in a crystalline state, providing the highest possible packing density. From the calculated number of fluorescein molecules per FDA nanocrystal and the measured values of the protein surface coverage, the F/P ratio can be calculated. The calculated F/P value for nanocrystal labels is 2.8×10^4, which is 1 order of magnitude lower than the previously reported F/P value[11] (5×10^4−2×10^5). However, it is still much higher (by a factor ∼10^3) than the ratios of directly covalent labeled antibodies (which normally carry 4−8 fluorophores). The F/P ratio of an immune detection system reflects its potential amplification rate.

Application of Nanocrystalline FDA Biolabels in Immunoassays. Figure 5 shows calibration curves of assays performed with the Gt α M IgG−FDA nanocrystal labels. The fluorescence signal is directly proportional to the M IgG concentration in the range of 0−10 μg/L. It shows a further increase until a concentration of 25 μg/L (data not shown) is reached. A limit of detection of 0.057 μg/L (57 pg/mL) was achieved, which was much lower than that of 4 μg/L obtained in the previous study.[11] An ultrasensitive calibration curve from 0 to 2 μg/L with a linearity of $r^2 = 0.9948$ is shown in the inset of Figure 5. To minimize nonspecific binding, low concentrations of the fluorescent-labeled antibody conjugate are typically favorable. Nonspecific binding is defined as the signal that is observed in the negative controls. By omitting the nanocrystalline label system, the negative controls gave extremely low fluorescence signals (0.33 ± 0.09 FIU). Without immobilization of the capture antibodies or without addition of the analytes, signals of 4.27 ± 0.46 and 6.27 ± 0.38 FIU were registered, respectively. No increase in the signal intensity was registered for increasing concentrations of Gt IgG and Rb IgG as analytes up to 100 μg/L (Figure 6). Only minor cross-reactivity was observed using the less-specific Gt α M IgG coated FDA-[DSPE-PEG(2000)Amine] as the conjugate in the immunoassay, confirming that the conjugate is immunoactive and recognizes its specific antigen.

Comparison of Nanocrystalline FDA-Labeled Antibodies with Commercial Direct-Labeled Antibodies. Figure 7a shows the calibration curves of Gt α M IgG-FDA nanocrystal labels in comparison with a direct FITC-labeled antibody conjugate. A limit of detection of 0.060 μg/L was achieved with the FDA-labeled antibodies while the limits of detection of 0.27, 0.38, and 1.66 μg/L were achieved with the directly fluorescent-labeled antibodies from Arista, Molecular Probes, and Sigma-Aldrich, respectively. The

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Table 1. Signal-to-Noise Ratios (S/N) and Sensitivity-to-Sensitivity Ratios (S/S) of FDA-Labeled and FITC-Labeled Antibodies[a]

analyte (μg/L)	S/N of Gt α M IgG detector antibody				S/S of Gt α M IgG-FDA/Gt α M IgG-FITC		
	FDA	FITC (Arista)	FITC (Sigma-Aldrich)	FITC (Molecular Probes)	FDA/FITC (Arista)	FDA/FITC (Sigma-Aldrich)	FDA/FITC (Molecular Probes)
1	2.27	1.47	1.09	1.19	843.01	2276.13	1138.06
2	3.56	1.74	1.16	1.47	1065.63	2672.95	916.44
4	5.39	2.28	1.39	1.81	1061.92	1896.81	901.76
6	6.85	2.83	1.53	2.08	988.20	1880.11	905.24
8	8.77	3.34	1.80	2.49	1022.81	1650.37	869.39
10	10.96	3.90	1.89	2.79	1061.44	1891.30	931.53
100	31.87	12.93	9.11	13.95	798.84	644.93	398.52

[a] Raw data were extracted from the measurements presented in Figure 7.

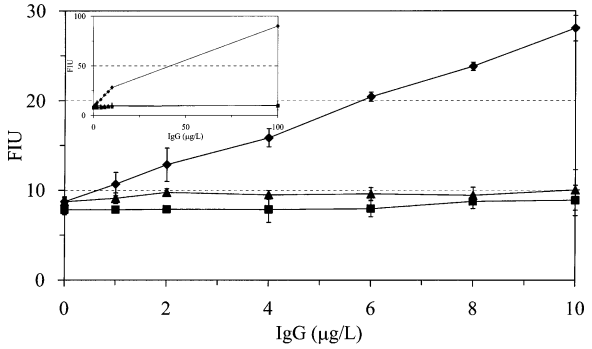

Figure 6. Sandwich fluorescence immunoassay using FDA nanocrystal-labeled detector antibody. Different concentrations of M IgG (◆) are added as the analyte and the fluorescent signals are compared with those of Gt IgG (■) and Rb IgG (▲) as negative controls. Error bars correspond to standard deviations (±SD, $n = 8$).

detection limit of the FDA conjugate is a factor of 5−28 lower than those of the direct FITC conjugates. The signal-to-noise ratio of the assays using nanocrystalline FDA-labeled antibodies was higher than the ratios of assays using the direct FITC-labeled antibodies at all analyte concentrations and was also higher than the ratios of assays using microcrystalline FDA conjugates in the previous study (Table 1).[11] The greatest increase in the signal-to-noise ratio was observed in the low concentration range (Figure 7b). Therefore, the FDA conjugates appear to be very useful in maximizing the sensitivity of a fluorescent assay. Using nanocrystalline FDA antibody conjugates makes a low detection limit possible.

The assay sensitivity observed using FDA-labeled antibody nanocrystals was compared with the sensitivity observed using direct FITC-labeled antibodies. The comparisons are summarized in Table 1. The table shows a 400−2700-fold higher sensitivity with the assays using the FDA nanocrystal labels, depending on the analyte concentration. Compared with the previous study,[11] the current amplification rate is close to the theoretical value of ∼4000−7000-fold, calculated from the F/P ratios of the two systems.

Immunoassay sensitivity and performance were observed to be higher than with standard FITC conjugates in which the FITC is directly conjugated to the protein. The high sensitivity of the nanocrystalline FDA antibody conjugates may be explained by the boosting effect of the higher ratio of dye molecules to binding molecules, but the minimized quenching could also have contributed to the improved signal.

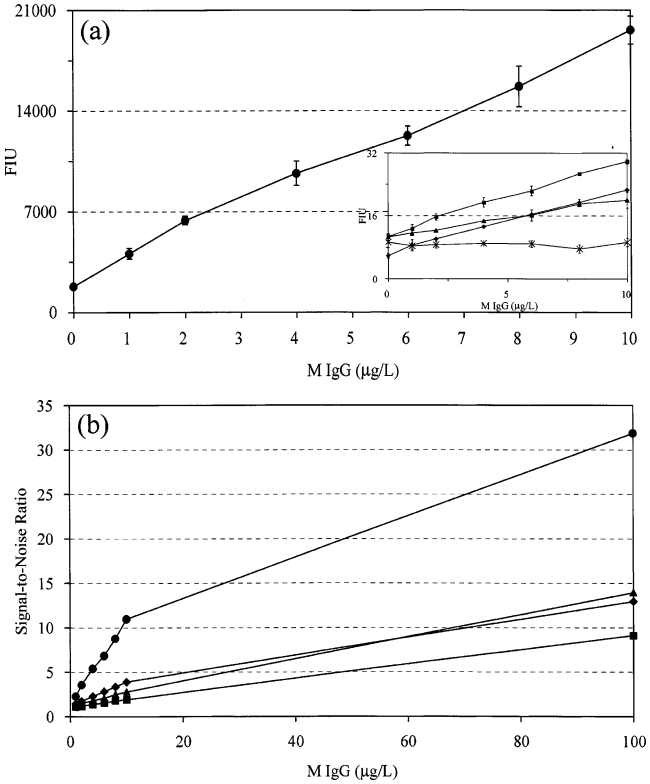

Figure 7. (a) Sandwich fluorescence immunoassay using Gt α M IgG-FDA nanocrystals (●) and using Gt α M IgG-FITC from Arista (◆), Sigma-Aldrich (▲), and Molecular Probes (■) as labels. The inset graph shows the amplification of the fluorescent intensity (*). Good linearity is observed in all cases using FDA nanocrystals as the label ($R^2 = 0.9954$) and direct FITC labels from Arista ($R^2 = 0.9962$), Sigma-Aldrich ($R^2 = 0.9915$), and Molecular Probes ($R^2 = 0.9947$). There is no increase in fluorescent intensity by increasing the M IgG concentrations using FDA-nanocrystal labels without addition of DMSO/NaOH as the releasing agent (*). (b) Alternatively, use of the signal-to-noise ratio instead of the fluorescence intensity unit to compare the sensitivity of the assay using FDA-nanocrystals and Gt α M IgG-FITC as detector antibodies. Error bars correspond to standard deviations (±SD, $n = 3$).

The core of the nanocrystals is composed of a fluorogenic precursor, FDA, which occupies the majority of the volume of the crystal and is densely packed. There is also a layer of biomolecules on the encapsulated crystal surface, leading to high FE values and high F/P ratios. The nanocrystals are dissolved in a large volume of organic solvent to release the fluorescein molecules into the surrounding medium. The quenching problem

normally arising from high F/P ratios is thereby prevented with our approach. A highly stable colloidal suspension is achieved by encapsulating the nanocrystalline particles with DSPE-PEG(2000)-Amine, which possesses groups for bioconjugation and minimizes nonspecific interactions due to a smaller charge density on the surface compared with the polyelectrolyte-coated microcrystals in the previous study.[11] Although the microcrystals have lower surface-to-volume ratios, causing the amount of FDA molecules per particle to be higher, every additional handling step increases the risk of aggregation. Bigger particles tend to form much bigger aggregates and consequently sediments and they interact with surfaces, often resulting in higher background signals. Nanocrystalline labels are advantageous over previously reported microcrystalline labels and other systems.

CONCLUSION

A novel signal amplification technology without the use of LbL techniques for immunoassays based on FDA nanocrystals encapsulated within PEG-Amine-modified phospholipids (DSPE-PEG-(2000)Amine), which provide an interface for the conjugation with antibodies, is described. The surface modification of the nanocrystalline biolabel system prevents nonspecific binding and particle aggregation. The FDA nanocrystals are nearly water-insoluble precursors of fluorescein, which is released by treatment with an organic solvent/hydroxide mixture. Nanocrystals with an average size of 107 nm were chosen as labels for utilization in a sandwich immunoassay for M IgG. Gt α M IgG was adsorbed onto FDA[DSPE-PEG(2000)Amine] nanocrystals and was confirmed to be immunologically active. The protein surface coverage was determined to be 3.35 mg/m^2, leading to a calculated F/P value of $\sim 10^4$. Utilization of nanocrystal labels in sandwich fluorescence immunoassays was observed to lower the limit of detection by 5−28 times and to produce up to a 400−2700-fold increase in signal intensity compared with conventional FITC conjugates. Numerous clinical immunoassays would benefit from better detection limits and higher sensitivity to detect diseases at an earlier stage than is possible with the present technology. Our system has the potential for further improvement of the signal-to-noise ratio and sensitivity by keeping the analysis time short and to make extremely sensitive and instant immunoassays for early disease detection a reality.

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