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Versatile Apoferritin Nanoparticle Labels for Assay of Protein

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A versatile bioassay label based on marker-loaded apoferritin nanoparticles (MLANs) has been developed for sensitive protein detection. Dissociation and reconstitution characteristics at different pH as well as the special cavity structure of apoferritin provides a facile route to prepare nanoparticle labels and avoid the complicated and tedious synthesis process of conventional nanoparticle labels. The optical and electrochemical characteristics of the prepared nanoparticle labels are easily controlled by loading different optical or electrochemical markers. A fluorescence marker (fluorescein anion) and a redox marker [hexacyanoferrate(III)] were used as model markers to load into the cavity of apoferritin nanoparticles for microscopic fluorescence immunoassay and electrochemical immunoassay, respectively. Detection limits of 0.06 (0.39 pM) and 0.08 ng mL^{-1} (0.52 pM) IgG were obtained with fluorescein MLAN and hexacyanoferrate MLANs, respectively. The new nanoparticle labels hold great promise for multiplex protein detection (in connection with nanoparticles loaded with different markers) and for enhancing the sensitivity of other bioassays.

The unique optical, electronic, and mechanic properties of nanoscale materials offer excellent prospects for designing highly sensitive and selective biosensors for proteins and nucleic acids.[1] The creation of such designed nanomaterials for specific bioassays greatly benefits from the ability to vary the size, composition, and shape of the materials and hence to tailor their chemical and physical properties. Metal nanoparticles, such as gold,[2] silica nanoparticles,[3] quantum dots,[4,5] and carbon nanotubes,[6−8] have been widely used as labels for the sensitive electronic and optical transudation of different biomolecular recognition events in recent years. The synthesis of such nanomaterial labels requires a complicated and tedious process and is time-consuming. Additionally, a harsh releasing step (e.g., strong acid) is necessary in some cases.[5] Herein, we present a facile strategy to prepare versatile nanoparticle labels based on apoferritin for optical and electrochemical transudation of antibody−antigen recognition events.

Apoferritin is a native protein composed of 24 polypeptide subunits that interact to form a hollow cagelike structure 12.5 nm in diameter; the interior cavity of apoferritin is ~8 nm in diameter and has an interior volume that can store up to 4500 iron atoms as an iron oxide−hydroxide mineral.[9] Apoferritin has 14 channels, which are formed at subunit intersections with diameters of 3−4 Å and which connect the outside of the apoferritin molecule with its interior.[10] The protein cage of apoferritin can be disassociated into 24 subunits at low pH (2.0), and the subunits reconstitute in a high pH (8.5) environment.[11−13] Because of its unique cavity structure as well as its disassociation and reconstitution characteristics, apoferritin has been used widely as a protein cage to synthesize a size-restricted bioinorganic nanocomposite, e.g., cobalt,[14,15] manganese,[16] iron sulfide,[17] iron phosphate,[18] cadmium sulfide,[19] uranium,[20,21] Prussian blue,[13] cobalt and platinum,[22−24] nickel,[25] chromium,[26] and magnetite.[19] Small molecules, such as

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the pH indicator neutral red[11] and the gadolinium complex,[13] have been captured in the cavities of apoferritin. The applications of the synthesized bioinorganic nanocomposite include magnetic resonance imaging, uranium neutron-captured therapy, radiopharmaceuticals, quantum dots and nanobatteries, photocatalysts, and magnetic memory devices. To our best knowledge, there is no previous report on biosensing and bioassay application using this nanostructured protein as labels.

EXPERIMENTAL SECTION

Apparatus. Fluorescence microscopy images were taken from Nikon TE−300 inverted optical microscopes integrated with Cohu 4912 CCD cameras. A ScanArray Express HT microarray scanner from Packard BioScience BioChip Technologies (Billerica, MA) was used for fluorescence quantitative detection. Fluorescence spectra were taken from a SPEX, 1680 double spectrometer. A robotic printer MicroGrid from Apogent Discoveries (Wilmslow, Cheshire, UK) was used to prepare antibody microarrays. Atomic force microscopy (AFM) imaging was performed in air using intermittent contact on a Nanoscope IIIa Multimode system (Veeco Metrology). Square-wave voltammetric and cyclic voltammetric measurements were performed with an electrochemical analyzer CHI 660A (CH Instruments, Austin, TX) connected to a personal computer. A disposable screen-printed electrode (SPE) consisting of a carbon working electrode or streptavidin-modified carbon working electrode, a carbon counter electrode, and an Ag/AgCl reference electrode was bought from Alderon Biosciences, Inc. for electrochemical measurements. A sensor connector (Alderon Biosciences, Inc.) allows for connecting the SPE to the CHI electrochemical analyzer. Magnetic beads-based sandwich immunoassay was performed in a 1.5-mL centrifuge tube with an Orbital shaker (VWR Scientific Products). Magnetic separation was performed with a Multi-6 microcentrifuge tube separator (Bangs Laboratories, Inc.). Centrifugal filter devices (Amicon Ultra-15, 30000 MWCO, Millipore Corp.) were used to separate and concentrate the sample solution. Dialysis was performed with Float-A-Lyzer (MWCO 25 000, Spectrum Laboratories, Inc.). The Disposable PD-10 desalting column packed with Sephadex G-25 medium (exclusion limit 5000) was bought from Amersham Bioscience Corp. and used to purify the protein solution. Centrifuge was performed with a Sorvall RC 26 plus (Kendro Laboratory Product).

Reagents. All stock solutions were prepared using deionized or autoclaved water. Apoferritin from equine spleen, hydrochloride acid, potassium hexacyanoferrate [$K_3Fe(CN)_6$], 0.05 M phosphate buffer saline (PBS, pH 7.4), 1 × PBS/casein block solution, sodium dodecyl sulfate, fluorescein sodium salts, sodium borohydide, biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester (biotin-NHS), Tris-HCl buffer, bovine serum albumin (BSA), mouse IgG, anti-mouse IgG, anti-mouse IgG (whole molecule) biotin conjugate, and streptavidin were purchased from Sigma. BioMag@goat anti-mouse IgG microspheres (average diameter 1.5 μm, 1.1 mg mL^{-1}) were purchased from Bangs Laboratories (Fishers, IN). Aminosilanated glass slides were bought from Erie Scientific Co. (Portsmouth, NH).

Preparation of Marker-Loaded Apoferritin Nanoparticles. A fluorescence marker (fluorescein sodium salts) and a redox marker ($K_3Fe(CN)_6$) were used as model markers to load into the apoferritin nanoparticles for fluorescence microscopy immunoassay and electrochemical immunoassay, respectively. Briefly, 5 mg of apoferritin solution (equine spleen, Sigma) was prepurified on a gel-filtration column (Superose 12, Pharmacia-LKB) to remove aggregates. The collected eluent fractions (0.1 M ammonium acetate, pH 7.0) were mixed and concentrated to 0.1 mL by a centrifugal filter device (Amicon Ultra-15) and then washed with 3 mL of autoclaved water twice using the same filter. Then, 2.9 mL of autoclaved water was added. The purified apoferritin solution (3 mL, 1.1×10^{-5} M) was gradually adjusted to pH 2.0 by slowly adding HCl solution (0.1 M). This pH was maintained for 20 min. At this point, 0.1 mM fluorescein solution was very slowly added (final concentration 0.01 mM), and then the pH of the mixture was slowly raised to 8.5 by adding an NaOH solution (0.1 M). The resulting solution was stirred at room temperature for 2 h, concentrated to 0.1 mL by a centrifugal filter device (Amicon Ultra-15), and then washed with 3 mL of autoclaved water three times using the same filter. Next, 0.9 mL of 0.05 M phosphate buffer (pH 7.4) was added and exhaustively dialyzed for 24 h to remove free fluorescein against several changes of phosphate buffer using a Spectra/Por Float-A-lyzer with a molecular weight cutoff (MWCO) of 25 000. Finally, the fluorescein marker-loaded apoferritin nanoparticle (MLAN) solution was applied to a disposable PD-10 desalting column with an exclusion limit of 5000 for purification. The eluent was 0.05 M phosphate buffer, pH 7.4. The collected fractions were mixed together and concentrated to 1 mL with an Amicon Ultra-15 filter. Control experiments (the same as those described above), consisting of adding the same amount of fluorescein marker into the apoferritin solution, were carried out, except the pH was varied only between 4.0 and 5.0 so that the apoferritin never disassociated into its subunits.

Hexacyanoferrate MLANs were prepared by using the above procedure. In this case, a 0.5 M $K_3Fe(CN)_6$ solution was used, and the final concentration of hexacyanoferate in the mixture was 0.1 M.

Determination of Loading Amount of Markers in Apoferritin. The number of entrapped markers was estimated by calibrating the ratio of the concentrations of markers and apoferritin. The apoferritin concentration was measured by the bicinchoninic acid method (BCA). The fluorescein content in apoferritin was determined by comparing fluorescence intensities of the fluorescein MLANs with those of a standard fluorescein solution. A calibration curve for the standard fluorescein was developed by measuring fluorescence intensities of fluorescein at different concentrations. The hexacyanoferrate content in hexacyanoferrate MLAN was determined by comparing the square wave voltammetric (SWV) signal of the dissociated hexacyanoferrate MLANs with that of a standard hexacyanoferrate solution. Hexacyanoferrate MLANs were dissociated with 0.1 M HCl/KCl to release the captured hexacyanoferrate. The released hexacyanoferrate was electrochemically measured with a disposable SPE.

Preparation of Biotin-Functionalized MLAN Conjugate. Biotin was conjugated with the purified MLANs using biotin-NHS. Briefly, we mixed 800 μL of MLAN suspensions with 200 μL of 1

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mg mL^{-1} biotin-NHS, which was freshly prepared with 0.05 M phosphate buffer (pH 7.4). The reaction was allowed to continue for 3 h at room temperature. During the procedure, the solution was rotated or inverted continuously. After incubation, the mixture was dialyzed for 24 h against 0.05 M phosphate buffer to remove free biotin-NHS using a Spectra/Por Float-A-lyzer with an MWCO of 25 000. The resulting conjugate was concentrated to 0.1 mL with the above-mentioned procedure, and then 0.4 mL of 0.05 M phosphate buffer (pH 7.4) containing 0.1% BSA was added. The solution was stored at 4 ∀C.

Microscopy Fluorescence Immunoassay. A fluorescence microscopy immunoassay was performed with an aminosilanated glass slide. A robotic printer MicroGrid from Apogent Discoveries (Wilmslow, Cheshire, UK) was used to prepare antibody microarrays. Aminosilanated slides were modified with 200 μL of a fresh 0.3 mg/mL solution of the homobifunctional cross-linker bis-(sulfosuccinimidyl)suberate (BS3) in PBS (Dulbecco's phosphate-buffered saline) for 5 min. The slides were rinsed briefly in 70% ethanol and dried under a stream of N_2 gas. Anti-IgG antibody (0.5 mg/mL in PBS) was printed on the slides. Each spot was printed in 1-nL antibody solution.

The arrays were then circled with a hydrophobic pen to mark their location and to facilitate probing the array with small volumes. The hydrophobic barrier marked by the pen holds the small-volume samples over the spotted array. During this step, the arrays were allowed to dry for 5 min. Each array was blocked with 15 μL 1 × PBS/casein for 1 h. The excess was removed by aspiration. The slides were washed three times (5 min each time) with 0.1 M PBS, 0.05% Tween (PBST) buffer. Each array was incubated overnight with 20 μL of target antigen solution with a desired concentration, which was diluted in 0.1 × PBS/casein in a humidified chamber. The excess was removed by aspiration. The slides were again washed three times with PBST buffer. The arrays were probed with 20 μL of biotinylated secondary antibody, which was diluted in 0.1 × PBS/casein for 2 h. After aspiration and washing, the arrays were exposed to 20-μL streptavidin in 1:100 PBST buffer for 30 min. After eliminating the excess and standard washing step, the arrays were incubated with 20 μL of biotinylated fluorecein/apoferritin nanoparticles. The incubation proceeded for 30 min in a humidified chamber. After removing the excess and washing with PBST buffer three times, the array was further rinsed with deionized water twice and air-dried. Fluorescence microscopy images were taken from a Nikon TE-300 inverted optical microscope integrated with a Cohu 4912 CCD camera. A ScanArray Express HT microarray scanner from Packard BioScience BioChip Technologies (Billerica, MA) was used for fluorescence quantitative detection.

Electrochemical Immunoassay. Electrochemical sandwich immunoassay experiments were performed with a 1.5-mL centrifuge tube using a modified procedure recommended by Bangs Laboratories.[27] Briefly, 50 μL of the anti-IgG-coated magnetic beads was transferred into a 1.5-mL centrifuge tube. The beads were then washed three times with 150 μL of PBS, 0.05% BSA (PBSB) and suspended in 40 μL of PBSB buffer. Ten microliters of a desired concentration of IgG was added, and the immunoreaction was continued for 60 min under shaking. The resulting microspheres coated with antibody−antigen complex were then washed twice with 150 μL of PBSB and suspended again in 40 μL of PBSB. Ten microliters of biotin-modified secondary antibodies was then added followed by a 60-min incubation with gentle shaking, magnetic separation, and three washing steps with 150 μL of PBSB buffer. The magnetic beads were suspended in 40 μL of PBSB, and 10 μL of 1 mg mL^{-1} streptavidin was added. The streptavdin−biotin interaction was allowed to continue for 30 min. After the magnetic separation and washing steps, the beads were resuspended in 40 μL of PBSB, and 10 μL of the biotin-functionalized hexacyanoferrate MLAN was added. Following a 30-min incubation, magnetic separation, and washing steps, 50 μL of HCl−KCl (0.1 M) was added to release hexacyanoferrate from the captured apoferritin nanoparticles. After magnetic separation, the 50 μL of HCl/KCl solution containing the released hexacyanoferrate was transferred to an SPE surface for SWV measurement. SWV measurements were performed with an SPE consisting of a carbon working electrode, carbon counter electrode, and Ag/AgCl reference electrode. Before the electrochemical measurements, the SPE was pretreated 60 s in 0.05 M phosphate (pH 7.4) at the potential of 1.5 V to clean the electrode surface. After washing and drying with an air stream, a 50-μL sample solution was dropped in the area of the three electrodes to form a droplet. A sensor connector was used to connect the SPE and electrochemical analyzer (CHI 660). The potential was scanned from 0 to 0.45 V with a step of 4 mV, amplitude 25 mV.

(27) Technote 101, Bangs Laboratories Inc., Fishers, IN, 2002.

RESULTS AND DISCUSSION

In this study, a fluorescence marker (fluorescein) and a redox marker (hexacyanoferrate) were chosen as model markers to load in the cavity of apoferritin to develop versatile nanoparticle labels for fluorescence microscopy imaging and electrochemical bioassays of protein. Figure 1(A) schematically illustrates the procedure for the MLAN label, which involves a dissociation of apoferritin at pH 2.0 in the presence of markers and a reconstitution of subunits at pH 8.5 to entrap markers into the cavity of apoferritin. This was followed by separation and surface biotinylation with biotin-NHS. Figure 1B presents a typical AFM image of the fluorescein MLANs on a mica surface. Individual nanoparticles with a diameter of ~12.5 nm are clearly identifiable in the image, indicating that the protein shell of the apoferritin remained a substantially stable structure and sustained no major alteration during the dissociation and reconstruction process. A similar AFM image was also obtained with the hexacyanoferrate MLANs (not shown). To determine whether the markers were loaded into the cavity of apoferritin or just physically adsorbed at the external surface of apoferritin nanoparticles, control experiments were carried out consisting of adding the same amount of fluorescein markers to the apoferritin solution as described above (see details in Experimental Section). However, the pH was varied only between 4.0 and 5.0 so that the apoferritin would never disassociate into its subunits. Figure 2A shows (a) the photographs of the fluorescein MLAN solution and (b) the solution resulting from the control experiment. No color was observed in the control product; a brown-yellow color was clearly observed in the fluorescein MLAN solution, indicating that the fluorescein marker was successfully loaded into the cavity of apoferritin and that no physical adsorption occurred at the external surface of apoferritin nanoparticles. Figure 2B shows the emission spectra of the fluorescein MLAN solution at the excitation wavelength of 470

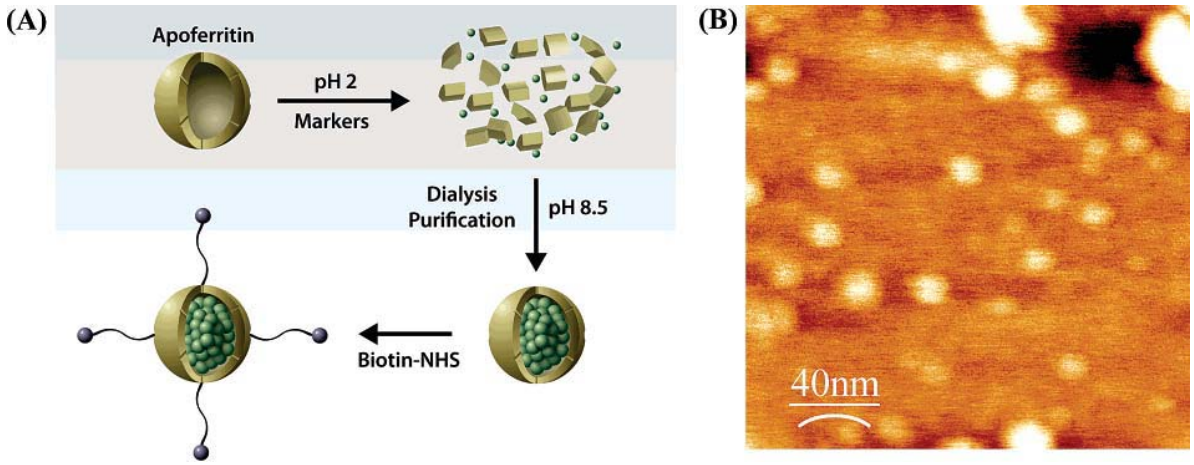

Figure 1. (A) Preparation of biotin-functionalized MLANs; (B) typical AFM image of fluorescein MLAN.

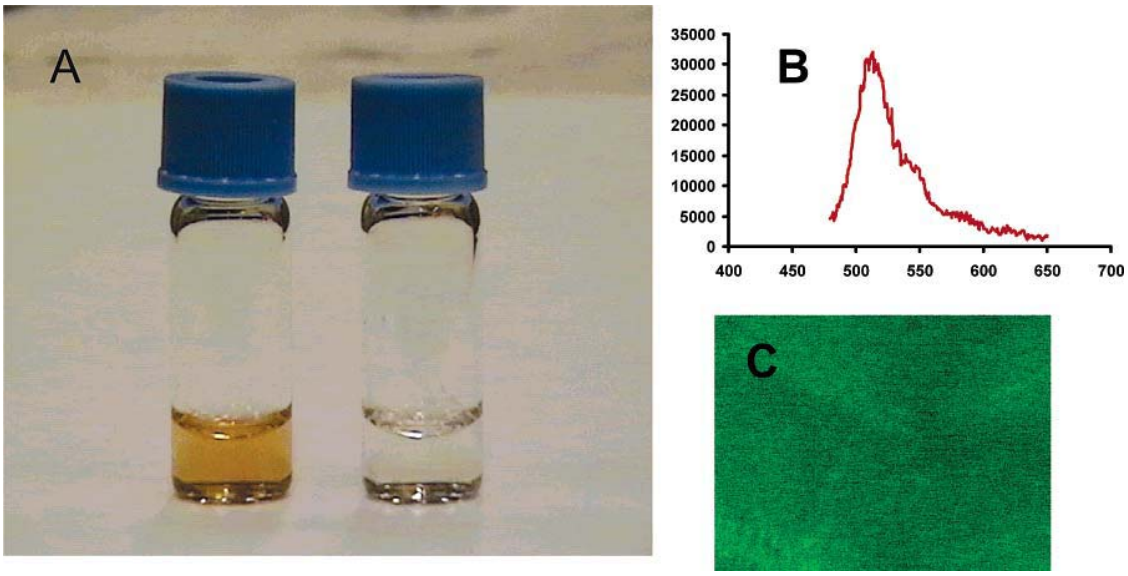

Figure 2. (A) Photographs of (a) fluorescein MLAN solution and (b) the resulting solution in control experiment. (B) Emission spectra of fluorescein MLAN (SPEX, 1680 double spectrometer). Excitation wavelength, 475 nm. (C) Fluorescence microscopy image of a glass slide coated with fluorescein MLANs. The sample was prepared by dropping 10 μL of the fluorescein MLAN solution on the cleaned microscope glass slide and dried at room temperature. The fluorescence microscopy image was taken from Nikon TE-300 inverted optical microscopes integrated with a Cohu 4912 CCD camera.

nm. A maximum emission wavelength was obtained at 520 nm, which is consistent with the standard emission spectra of fluorescein. It indicates that the protein cage does not change the spectral characteristic of the fluorescein, which was entrapped in the cavity of the apoferritin nanoparticles. The fluorescence microscopy image of the fluorescein MLANs (Figure 2C) further confirms this conclusion. Uniform green fluorescence was observed with a glass slide coated with the fluorescein MLANs.

SWV experiments were used to confirm that the loaded redox markers (hexacyanoferrate) were in the cavity of apoferritin. Figure 3 presents the typical SWV voltammograms of hexacyanoferrate MLAN in 0.1 M KCl (curve a) and 0.1 M KCl/HCl (curve b). As expected, no oxidation peak of hexacyanoferrate was observed in the voltammogram (curve a) before the dissociation step whereas a well-defined wave associated with the oxidation of hexacyanoferrate can be seen (Epa, 0.275,vs Ag/AgCl) after dissociation with 0.1 M HCl/KCl (curve b). No electrochemical signal was observed with the control samples in both 0.1 M KCl and 0.1 M HCl/KCl, which were prepared by mixing hexacyanoferrate and apoferritin solutions under a constant pH environment (5.0) and following extensive dialysis (results not shown). This indicates that the hexacyanoferrate markers are loaded into the cavity of apoferritin.

These favorable fluorescence and electrochemical signals provide a facile method to quantify the loading amount of markers in the cavity of apoferritin. The number of entrapped markers was then estimated by calibrating the ratio of concentrations of markers and protein. The protein concentration, measured by BCA, was 1.15×10^{-6} M. The concentration of fluorescein, determined by comparing fluorescence intensities of the fluorescein MLANs with that of a standard fluorescein solution, was 0.075 mM. The results show that ~65 fluorescein anions were trapped per apoferritin nanoparticle. The concentration of hexacyanoferrate, determined by comparing the current intensities of hexacyanoferrate MLANs with that of a standard hexacyanoferrate solution, was 0.18 mM. A loading of ~150 hexacyanoferrate anions

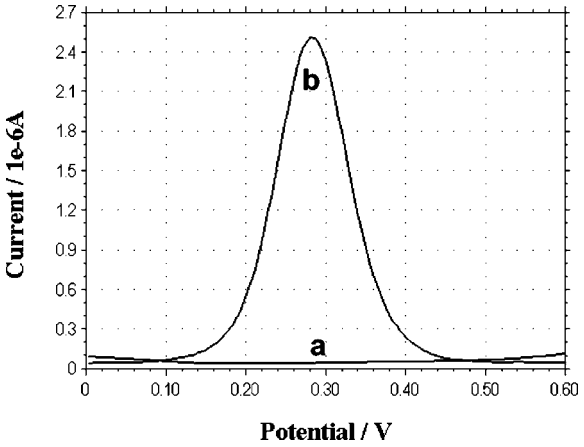

Figure 3. Square wave voltammograms of hexacyanoferrate-loaded apoferritin in 0.1 M KCl (a) and 0.1 M KCl−HCl (b). The measurements were performed with a 50-μL sample solution on a disposable SPE. The potential was scanned from 0 to 0.6 V with a step of 4 mV, amplitude 25 mV.

per apoferritin was obtained. Allen et al.[15] observed a similar loading amount. These values are much higher than the theoretical one proposed by Watt et al.[11] of 8.7 molecules per apoferritin, which was calculated by considering the size of the apoferritin cavity and the concentration of the molecule to be trapped and ignoring the latter's size or interaction with the apoferritin subunit. This result suggests the existence of a specific interaction between the subunits and markers (hexacyanoferrate(III) and fluorescein) in the apoferritin reassembly process. This interaction must have occurred at the internal surface of the subunits rather than at the external surface because no fluorescence or electrochemical signal was observed in control experiments (incubation of apoferritin with markers at fixed pH (5.0) followed by exhaustive dialysis). The interaction may be attributed to the binding between the positively charged subunits (at low pH) and negatively charged markers (anions). The difference of the loading numbers between fluorescein and hexacyanoferrate may be attributed to its different sizes and structure. The loading number of markers in the cavity decreases as the diameter of the loaded marker increases. The calculated cross diameters of fluorescein and hexacyanoferrate are 13 and 6.4 Å, respectively, based on geometry optimization from density functional theory. It is reasonable that the loading number of hexacyanoferrate is twice that of fluorescein.

The MLAN was then functionalized with biotin for bioassay application. The amino acid residues at the ends of the channels of apoferritin provide a facile route to conjugate with biotin. The conjugation was realized by incubating the MLAN solution with a biotin-NHS reagent for 3 h, followed by an extensive dialysis and purification process to remove the unconjugated biotin-NHS molecules. Avidin-modified glass slides and avidin-modified SPEs were used to attach the biotin-functionalized fluorescein MLANs and hexacyanoferrate MLANs, respectively. Fluorescence microscope imaging and the electrochemical method were used to examine the binding capability of biotin on the surface of fluorescein MLAN and hexacyanoferrate-MLAN labels. The binding reactions were allowed to perform 30 min at room temperature. Fluorescence microscopy and a CHI 660 electrochemical analyzer were used for fluorescence and electrochemical measurements

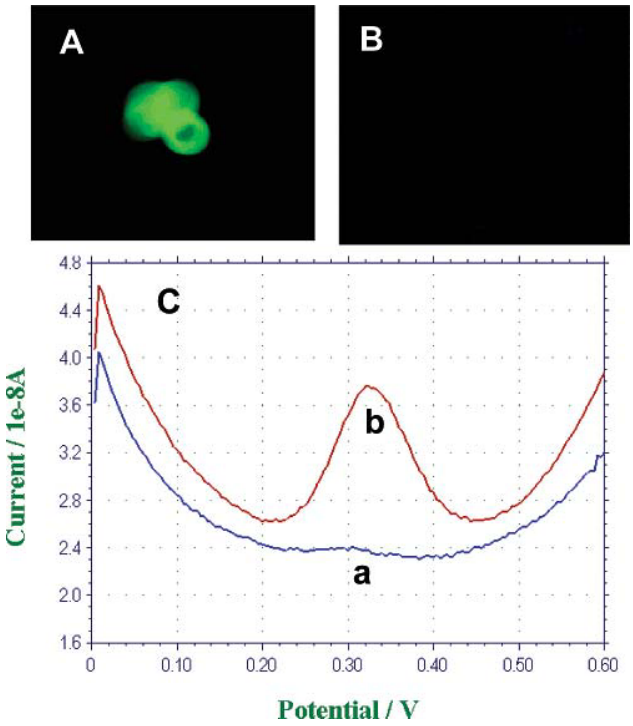

Figure 4. (A) Fluorescence microscopy image of the avidin-coated glass slide incubated with the biotin-functionalized fluorescein MLAN labels. (B) Fluorescence microscopy image of the avidin-coated glass slide incubated with fluorescein MLAN. (C) Square wave voltammograms of (a) an avidin-modified SPE incubated with hexacyanoferrate MLANs and (b) the biotin-functionalized hexacyanoferrate MLAN labels. The avidin-coated glass slide was prepared by the same procedure during the immobilization of antibodies (details in Experimental Section). The avidin-coated glass slide and avidin-modified SPE were incubated 30 min in the corresponding solution, followed by extensive washing steps.

after washing with 0.05 M phosphate buffer containing 0.1% BSA. Figure 4A shows a typical fluorescence microscopy image of the avidin-modified glass slide incubated with the biotin-functionalized fluorescein MLAN. A strong green fluorescence was observed at the avidin-coated area, indicating that the biotin-modified fluorescence MLAN labels were successfully attached to the glass slide through the biotin−avidin interaction. In contrast, no fluorescence was observed with a control avidin-modified glass slide, which was incubated with a fluorescein MLAN solution (Figure 4B). SWV scanning was used for electrochemical measurements of the binding biotin-functionalized hexacyanoferrate MLAN labels in the solution of released hexacyanoferrate by adding 50 μL of 0.1 M HCl/KCl to the incubated SPE surface. Figure 4C shows the typical voltammograms of the avidin-modified SPE incubated with (a) hexacyanoferrate MLAN and (b) biotin-functionalized hexacyanoferrate MLAN. A well-defined voltammetric peak (Epa 0.30 V) was observed at the avidin-modified SPE incubated with biotin-functionalized hexacyanoferrate MLAN; no signal was observed at the avidin-modified SPE incubated with hexacyanoferrate MLAN. The above results indicate that the prepared biotin-functionalized MLANs can be used as labels for bioassay application.

The use of biotin-functionalized MLANs as labels was further studied with fluorescence microscope and electrochemical im-

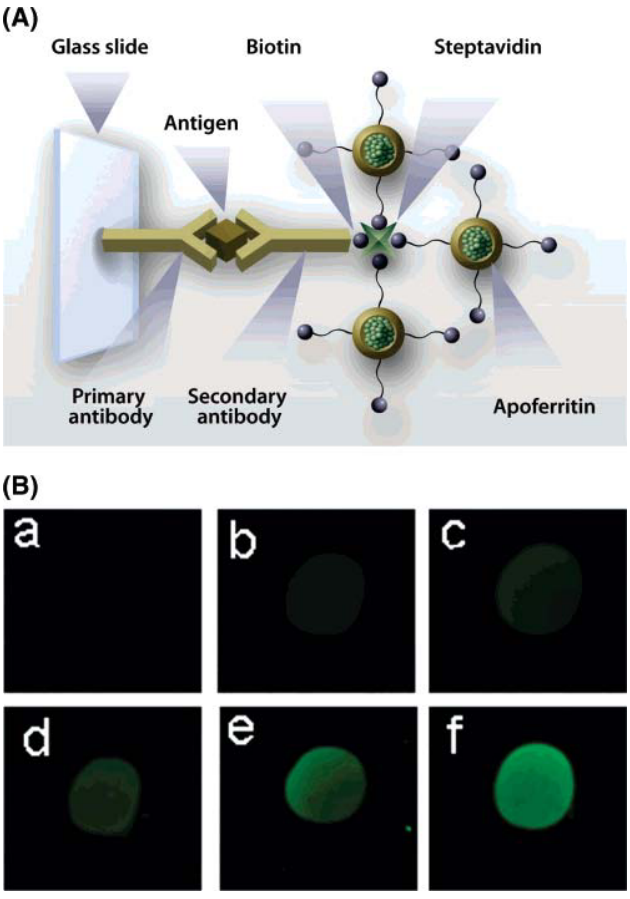

Figure 5. (A) Protocol of fluorescence microscopy immunoassay based on fluorescein MLAN label. (B) Fluorescence microscopy images of immunoassay with the increasing concentration of the IgG (from a to f, 0, 0.1, 1, 5, 10, 20 ng mL^{-1}, respectively). For details of the immunoassay procedure and fluorescence microscope imaging, see the Experimental Section.

munoassay. First, we investigated a fluorescence microscopy immunoassay based on the biotin-functionalized fluorescein MLAN label. The immunoassay was performed with an aminosilanated glass slide (Figure 5A). The primary antibody (anti-IgG) was first immobilized on an aminosilanated modified glassy slide, and then the mouse IgG was bound to the antibody, followed by interaction with a biotin-modified secondary antibody. Streptavidin was further incubated on the above modified slides, and this was followed by adding the biotin-functionalized fluorescein MLAN label. The procedure for this sandwich immunoassay is detailed in the Experimental Section. Figure 5B shows the fluorescence microscopy images of this sandwich immunoassay with increasing concentration of the IgG. We can see that the fluorescence intensity of the antibody-spotted area increased with the increase of IgG concentration ranging from 0.1 to 20 ng mL^{-1} (b−f). No fluorescence was observed at the spotted area in the absence of IgG control (Figure 4 Ba). Such a low background is attributed to a blocking step and extensive washing steps with the washing buffer containing 0.05% Tween (details in Experimental Section). A fluorescence was still observed with 0.06 ng mL^{-1} IgG (0.39 pM, see Figure S1 in Supporting Information). This detection limit is much lower than that of the colorimetric protein assay.[28] Comparable immunoassays were performed with a single fluorescein probe labeled anti-mouse IgG antibody (anti-mouse IgG−FITC antibody, Sigma, Ca. F4018) under similar conditions. Fluorescence microscopy images indicate that a minimum of 10 ng mL^{-1} IgG could be detected with this single fluorescence probe (not shown). Compared with that of the single fluorescence probe, the sensitivity of the biotin-functionalized fluorescein MLAN label was enhanced 125 times. The significant signal enhancement is attributed to two factors: first, apoferritin nanoparticles as a carrier to load ~65 fluorescence anions; second, streptavidin as a bridge to bind two or three biotin-functionalized MLAN labels (one streptavidin has four binding sites to biotin).

Application of the MLAN label in the electrochemical immunoassay was also demonstrated with the biotin-functionalized hexacyanoferrate MLAN label in connection with a magnetic bead-based sandwich immunoassay (Figure 6A). It involves two immunoreactions between the primary IgG antibody linked to magnetic beads and the biotin-modified secondary antibody in the absence or presence of antigen (IgG). This was followed by introducing streptavidin as a bridge to bind the biotin-functionalized hexacyanoferrate MLAN labels. The reaction mixture was subjected to a magnetic field to separate the excess and free reagents. This was then followed by the dissociation of captured apoferritin nanoparticle labels with 50 μL of 0.1 M HCl/KCl and SWV detection of the released hexacyanoferrate at a disposable SPE. Figure 6B shows the typical square wave voltammograms of this sandwich immunoassay with increasing concentration of the IgG. The voltammetric peaks are well defined and proportional to the concentration of the corresponding IgG. The resulting calibration plots are linear (left, top inset, correlation coefficients, 0.996). Also shown in Figure 6 (left, bottom inset) is the SWV signal for a 0.1 and 0 ng mL^{-1} (control) IgG solution. Such a response indicates a detection limit of 0.08 ng mL^{-1} (~0.52 pM; see Figure S2 in Supporting Information) in connection with the 60-min immunoreaction time. This detection limit corresponds to 26 amol in the 50-μL sample solution, which is comparable to that of the tagged liposome.[29] A series of six repetitive measurements of the 5 ng mL^{-1} IgG yielded reproducible SWV peaks with a relative standard deviation of 7.3% (not shown).

In conclusion, we have demonstrated a versatile bioassay label based on biotin-functionalized MLANs for sensitive protein detection. Dissociation and reconstitution characteristics at different pH as well as the special cavity structure of apoferritin provide a facile route to prepare nanoparticle labels. The optical and electrochemical characteristics of the prepared nanoparticle labels are easily controlled by loading different fluorescence or electrochemical markers. A fluorescence marker (fluorescein anion) and a redox marker [hexacyanoferrate(III)] were used as model markers to load into the cavities of apoferritin nanoparticles and developed for microscopic fluorescence immunoassay and electrochemical immunoassay, respectively. While the concept has been presented within the context of a fluorescein marker and hexacyanoferrate redox marker, it could be readily extended to other fluorescence and redox markers, multiplex immunoassays, drug delivery, and cell imaging. Simultaneously loading multiple markers makes it possible to build a marker molecular library.

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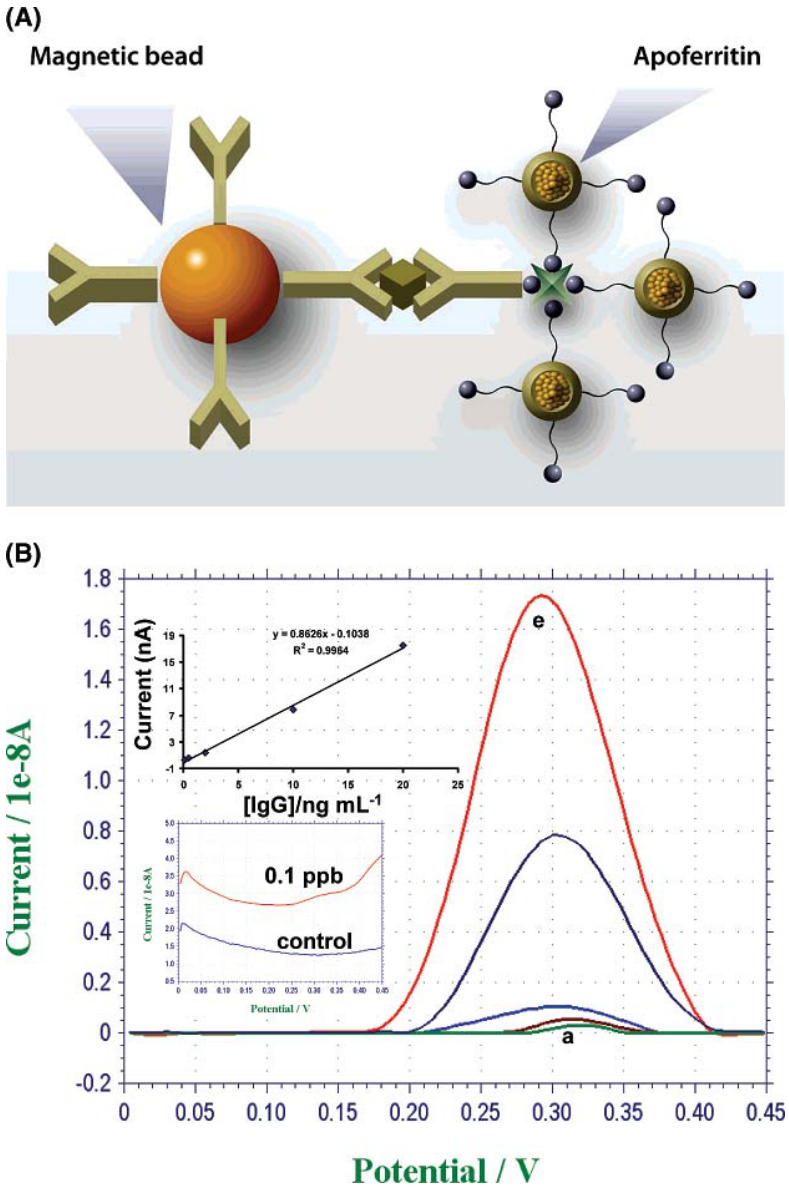

Figure 6. (A) Magnetic beads and electrochemical sandwich immunoassay protocol based on biotin-functionalized hexacyanoferrate MLAN labels. (B) Typical square wave voltammograms of electrochemical immunoassay with increasing concentration of the IgG (from a to e, 0.1, 0.5, 2, 10, and 20 ng mL^{-1} IgG, respectively). A baseline correction of the resulting voltammogram was performed using the "linear baseline correction" mode of the CHI 660 (CH Instruments) software. Also shown (insets), (top) the resulting calibration plot and (bottom) the square wave voltammograms (without baseline correction) of 0.1 and 0 ng mL^{-1} (control) IgG. After the sandwich hybridization assay, the magnetic bead−hexacyanoferrate loaded apoferritin hybrid was dispersed in 50 μL of 0.1 M HCl/KCl to release the captured hexacyanoferrate. Following a magnetic separation, the solution was transferred to a SPE surface for SWV scanning. For details of the immunoassay procedure and electrochemical detection, see the Experimental Section.

The new nanoparticle labels could be applicable to other biological assays, particularly DNA.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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