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Electrogenerated Chemiluminescence. 83. Immunoassay of Human C-Reactive Protein by Using $Ru(bpy)_3^{2+}$-Encapsulated Liposomes as Labels

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Liposomes (∼100-nm diameter) containing $Ru(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine) were prepared as an electrogenerated chemiluminescent (ECL) tag for a sandwich-type immunoassay of human C-reactive protein (CRP). Polyclonal human CRP antibodies were introduced onto liposomes and magnetic beads through biotin−streptavidin interaction. The antigen−antibody conjugates formed on addition of a CRP-containing sample were separated from unreacted species magnetically. Addition of 0.1 M tri-*n*-propylamine and 0.1 M phosphate buffer (pH 7.6) containing 0.1 M NaCl and 1% (v/v) Triton X-100 caused liberation of the $Ru(bpy)_3^{2+}$ from the liposome. ECL obtained in this medium showed a detection limit of 100 ng/mL for human CRP with good linearity of ECL intensity versus antigen concentration over the range 100 ng/mL−10 μg/mL.

We describe here a new approach to electrogenerated chemiluminescent (ECL) labeling, with $Ru(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine)-encapsulated liposomes, and its application in a sandwich-type immunoassay of human C-reactive protein (CRP). A detection limit of 100 ng/mL for human CRP was obtained, and a good proportionality of ECL intensity versus antigen concentration was observed. Many biofunctional groups can be directly or indirectly attached to a liposome surface, and the capture of biologically complexed liposomes can be conveniently realized by magnetic separation. This study thus provides us a general route of detecting such biological interactions with ECL measurements.

Liposome-based immunosorbent assays (LISA) have been widely studied and practiced.[1] In contrast to a typical enzyme-linked immunosorbent assay, where antigen−antibody interactions are followed by monitoring enzymatic products, LISA is realized through detection of liposome-contained marker molecules.[2] While many marker systems, e.g., based on chemiluminescence,[3] electroactivity,[4] and fluorescence,[5] have been developed for LISA, this work represents the first liposome-based bioassay utilizing electrogenerated chemiluminescent[6] detection. The general operation of this system, which is based on a sandwich assay with antibody-tagged liposomes and magnetic beads, is shown in Figure 1.

We recently introduced an ECL method of adding multiple $Ru(bpy)_3^{2+}$ to a single antibody by encapsulating a hydrophobic $Ru(bpy)_3^{2+}$ compound in polymer (polystyrene) microspheres.[7,8] While this technique shows enormous signal amplification compared to the conventional, widely used ECL immunoassay with one label per antibody,[9] it requires the use of an organic solvent, acetonitrile, to release the $Ru(bpy)_3^{2+}$ and generate ECL. This approach is not generally compatible with current commercial ECL instrumentation. The liposome-based system described here, which is based on a similar idea of holding multiple labels in a larger container, allows the assay to be carried out in aqueous solution and has the potential to generate multiple labels after release.

EXPERIMENTAL SECTION

Materials and Methods. Chemicals and Materials. Several lipids, including 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DSPG), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)2000] (ammonium salt) (DSPE-PEG-(2000)biotin), all with purity above 99%, were obtained from Avanti Polar Lipids, Ltd. (Alabaster, AL) Cholesterol (purity >99%), $Ru(bpy)_3Cl_2 \cdot 6H_2O$ and bovine serum albumin (BSA) were from Aldrich. EZ-Link Sulfo-NHS-LC-biotin (sulfosuccinimidyl-6-(biotinamido)hexanoate), Neutravidin, and Immunopure D-biotin were obtained from Pierce Biotechnology, Inc. (Rockford, IL) Purified C-reactive protein (CRP, >95%) was from Alpha Diagnostic International (San Antonio, TX). Polyclonal affinity purified goat anti-human CRP was obtained from Immunology Consultants Laboratory (Newberg, OR). Tri-*n*-propylamine (>99%) was from

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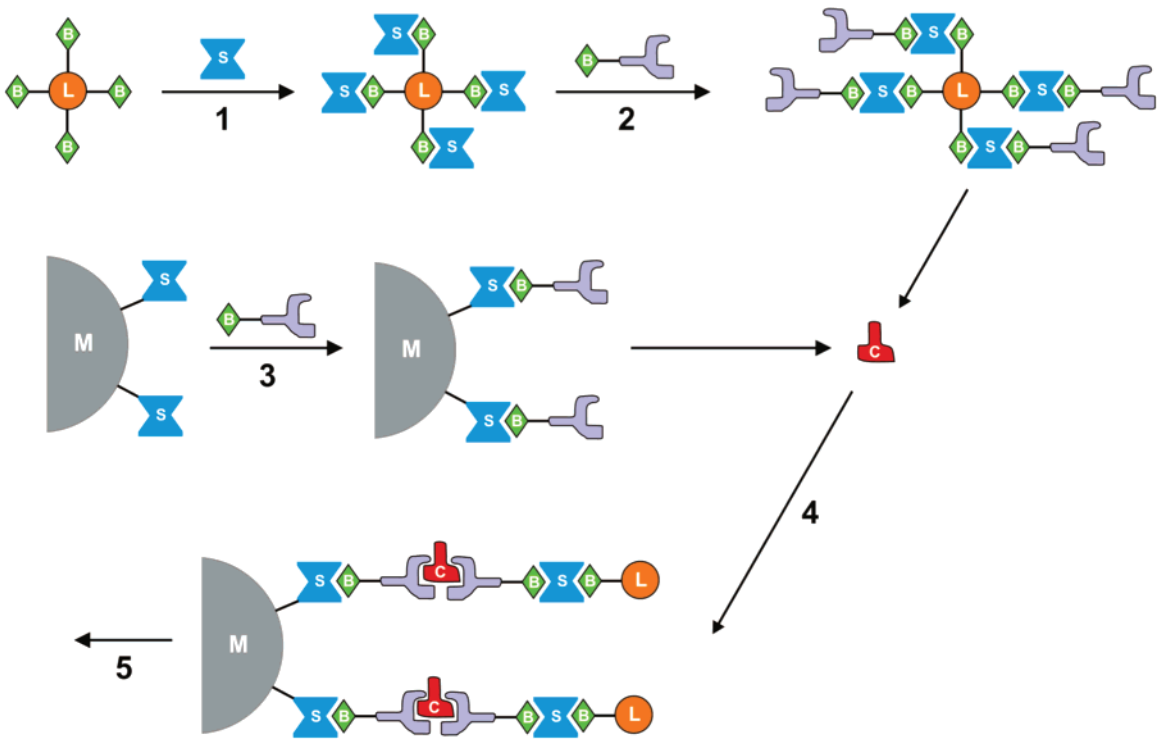

Figure 1. Immunoassay of human C-reactive protein. Species and materials involved: B, biotin; L, liposomes; S, streptavidin; C, C-reactive protein (analyte); and M, magnetic microbeads. Experimental steps: (1) coating of streptavidin on liposome surface; (2) coupling biotinylated anti-human CRP to liposomes; (3) decoration of streptavidin-coated magnetic beads with biotinylated anti-human CRP; (4) formation of anti CRP (liposomes)−CRP-anti CRP (magnetic beads) sandwich structure; and (5) quantification of CRP by detecting ECL signal based on Ru(bpy)$_3^{2+}$ and tri-*n*-propylamine.

Acros Organics. Tris(hydroxymethyl)aminomethane (molecular biology grade) was from FischerBiotech (Fair Lawn, NJ). Triton X-100 was purchased from MP Biomedicals, Inc. (Solon, OH). Streptavidin-coated magnetic polystyrene beads (6.5×10^8 beads/mL) with an average diameter of 2.8 μm (Dynabeads M-280) were a gift from BioVeris Corp. (Gaithersburg, MD). All samples were freshly prepared with 18 MΩ·cm deionized Milli-Q water (Millipore, Bedford, MA).

Preparation of Ru(bpy)$_3^{2+}$-Encapsulated Liposomes by Lipid Extrusion. Liposomes containing Ru(bpy)$_3^{2+}$ were prepared using a homemade miniextruder, which contains a stainless steel mesh screen (mesh opening size, ~250 μm) as a membrane holder connected to a 1-mL airtight syringe (Hamilton Co., Reno, NV) at each side for liquid pumping and lipid extrusion. To begin the procedure, 40 μmol of lipid mixture containing DSPC/cholesterol/DSPG/ DSPE-PEG(2000)biotin (molar ratio 50/40/10/2) was first dissolved in 4 mL of $CHCl_3$/ethyl ether (1:1, v/v). Into this lipid solution, 1 mL of 50 mM Ru(bpy)$_3^{2+}$ in Tris buffer (10 mM Tris and 15 mM NaCl, pH 7.2) was then added. After briefly vortexing the mixture, the organic solvent was removed by heating at 70 °C in a water bath and purging Ar into the flask, producing Ru(bpy)$_3^{2+}$-containing liposomes of micrometer size.[10] This aqueous liposome solution was further sonicated in a 55 °C bath for 3 min and ready for the lipid extrusion. Before each extrusion step, the syringes were warmed with Tris buffer and the lipid solution, both of which were preheated to 70 °C. In addition, the extruder was maintained at a high temperature by a heating tape wrap. Two sets of filter membranes (Nuclepore Track-Etched membranes, Whatman) were used, first of 1 μm and then of 0.2 μm. In each case, the lipid mixture was manually pumped back and forth through the filter membrane 10 times.

The liposomes were separated from untrapped materials and lipid debris by gel filtration (PD-10, GE Healthcare) followed by dialysis (Slide-A-Lyzer Dialysis Units (molecular weight cutoff, 10 000), Pierce Biotechnology, Inc.). The resulting liposomes were stored in Tris-buffered saline solution (TBS: 0.1 M Tris, 0.15 M NaCl, pH 7.2) at 4 °C until used.

Attachment of Anti-Human CRP to Liposomes and Magnetic Beads. Biotinylation of anti-human CRP was achieved by reacting 25 μL of 10 mM Sulfo-NHS-LC-biotin aqueous solution with 1 mL of 1.0 mg/mL anti-human CRP at room temperature for 1 h. The unreacted species were separated from the product by gel filtration (PD-10, GE Healthcare). This brought the final volume to 3.5 mL, and the concentration of biotin-conjugated antibodies was estimated to be ~1.9×10^{-6} M.

To conjugate anti-human CRP with streptavidin-coated magnetic beads, 2 mL of the above biotinylated anti-human CRP were reacted with 1 mL of magnetic beads at room temperature for 1 h. Based on the information provided by the manufacturer, the amount of antibodies used was at least 5 times more than the total binding capacity of the magnetic beads. After the reaction, the anti-human CRP conjugated beads were washed 3 times with 1 mL of TBS solution with the aid of a magnetic microcentrifuge tube holder/stand (MPC-S, Dynal Biotech Inc.). The unreacted streptavidin sites were then blocked by reacting the beads with 1 mL of 0.2 mM D-biotin at room temperature for 30 min, and the excess biotin was separated accordingly. Similarly, anti-human

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CRP was linked to biotin-containing liposomes by first reacting 0.2 mL of liposomes (6×10^{-11}–10^{-10} mol calculated biotin) with 1×10^{-10} mol of Neutravidin at room temperature for 1 h; this complex was further incubated with 1×10^{-10} mol of anti-human CRP for 1 h at room temperature. The resulting solution, with a total volume of 280 μL, was stored at 4 °C until use.

Sandwich-Type Immunoassay. Each assay contained a mixed solution of 100 μL of 2% (m/m) BSA in TBS solution, 25 μL of anti-human CRP conjugated magnetic beads, 25 μL of anti-human CRP conjugated liposomes (1:5 (v/v) diluted from the above as-prepared liposomes with TBS solution), and 50 μL of CRP samples of different concentrations in TBS solution. This mixture of total volume of 200 μL was incubated at room temperature for 1 h with constant gentle swirling. The anti CRP–CRP-anti CRP sandwich complex was then separated from other unreacted species magnetically. Successfully formed sandwich complexes are thus held on the magnetic beads and can be separated with an external magnet while other unreacted species are removed by washing the beads with 200 μL of TBS solution 3 times. After the final washing step, the liposomes carried by the magnetic beads were lysed and the encapsulated $Ru(bpy)_3^{2+}$ was released using 0.5 mL of ECL buffer (see below), and the magnetic beads were removed from the ECL cocktail solution with a magnet.

ECL Detection. An ECL generation and detection system similar to that reported previously[7] was used. A three-electrode cell containing a 2.2-mm-diameter Pt disk electrode (working), a 0.5-mm Pt wire (counter), and a 0.5-mm Ag/AgCl wire (reference) was used. Virgin glass vials (size, 19 × 48 mm) housing the three electrodes were used in each measurement. The cell was aligned with the photodetector (Hamamatsu H7421-40, Shizuoka-ken, Japan) such that the distance between the working electrode and the photocathode was held constant at ~2 cm. The electrochemical cell was connected to a potentiostat (model 900, CH Instruments, Austin, TX) controlled by a PC. The ECL intensity measured in counts per second was collected by a multichannel scaler (model 914P, EG&G) interfaced with another computer. To quantify the ECL signal, all of the photons collected during a single CV scan were integrated.

The ECL buffer was 0.1 M tri-*n*-propylamine, 0.1 M phosphate (sodium salt), 0.1 M NaCl, and 1% (v/v) Triton X-100, pH 7.6. Five cyclic voltammograms were taken for each sample, and between each measurement, the working electrode was swept between 0 to −1 V at 2 V/s for 10 cycles. Between samples, the working electrode was taken out of the ECL cell and rinsed with 2 M NaOH and then DI water. This procedure was used to improve the reproducibility of the ECL measurements.

The fluorescence images were recorded with a color CCD camera (Magnafire S99806, Olympus) connected to the front port of a microscope (Eclipse TE 300, Nikon).

RESULTS AND DISCUSSION

$Ru(bpy)_3^{2+}$-Encapsulated Liposome Formation and Characterization. We used the lipid extrusion method to generate submicrometer-size liposomes containing $Ru(bpy)_3^{2+}$. To prepare stable liposomes, lipids with a relatively high phase-transition temperature (T_c) such as DSPC and DSPG (both T_c = 55 °C) were used. For the same reason, the extrusion was performed above 55 °C because, otherwise, pumping gel-phase lipids through polycarbonate filter membrane resulted in membrane clogging and lipid damage.[11] Additionally, we included cholesterol to the preparation because it generally lowers the permeability of liposomes by enhancing the packing density of phospholipid acyl chains.[12,13] Two percent biotin-containing lipids were also included so that the liposomes could be further modified via biotin–avidin linkages.

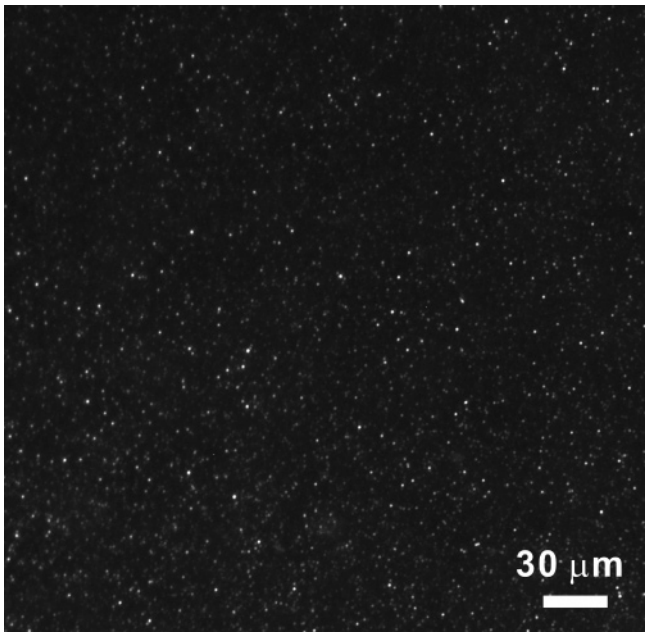

Figure 2. Fluorescence imaging of $Ru(bpy)_3^{2+}$-encapsulated liposomes dispersed in TBS solution (0.1 M Tris, 0.15 M NaCl, pH 7.2). Excitation, 490 nm; emission, 520 nm.

The fluorescence micrograph shown in Figure 2 indicates that $Ru(bpy)_3^{2+}$ was successfully encapsulated inside liposomes of submicrometer size with this method. Size characterization using a Coulter counter (MS3, Beckman Coulter) showed liposomes as large as a few micrometers were also formed by this preparation method, however, only at a very low abundance (data not shown). Additional measurements based on a light scattering technique (N5, Beckman Coulter) indicates most of the liposomes had a diameter of 180.3 ± 121.8 nm or an average volume of 2.8×10^{-18} L. When 50 μL of the liposome solution was diluted with 450 μL of ECL buffer and lysed, as described in the next section, the concentration of $Ru(bpy)_3^{2+}$ in this solution as determined by ECL was 6.7×10^{-6} M. Moreover, by taking the concentration of encapsulated $Ru(bpy)_3^{2+}$ to be 25–50 mM (assuming a maximum of 50% leakage), we find a biotin concentration in this solution that is in the range of 1.5–3 μM. If we further assume that the liposomes are unilamellar and take the area per molecule[14] for DSPC and DSPG as 0.71 nm^2 and cholesterol as 0.19 nm^2, respectively, we can estimate there are roughly 100 000 DSPC, 80 000 cholesterol, 20 000 DSPG, and 4000 DSPE-PEG(2000)biotin molecules per liposome.

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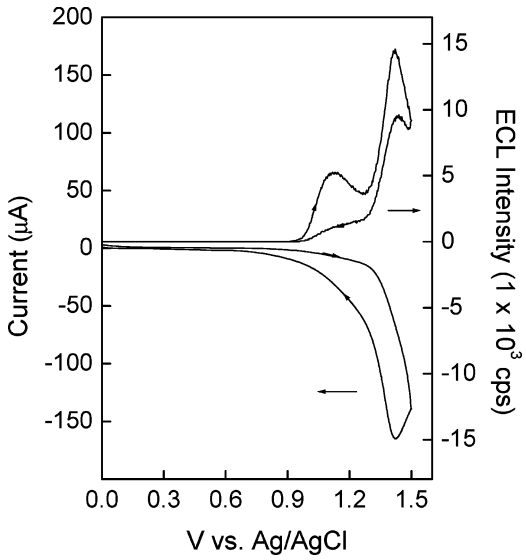

Figure 3. Luminescence (top) and electrochemical (bottom) responses of the ECL buffer containing 50 nM $Ru(bpy)_3^{2+}$ released from lysed liposomes. The ECL buffer contains 0.1 M tripropylamine, 0.1 M phosphate, 0.1 M NaCl, and 1% (v/v) Triton X-100, pH 7.6. Working electrode, 2.2-mm Pt disk. Potential scanning rate, 0.05 V/s.

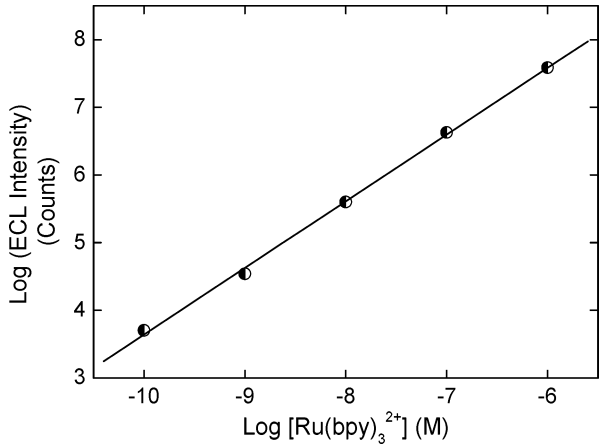

Figure 4. ECL intensity versus $Ru(bpy)_3^{2+}$ concentration. Experimental conditions are the same as in Figure 3.

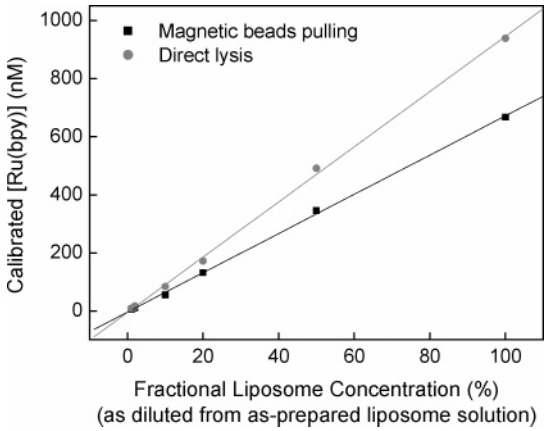

Figure 5. ECL monitoring of binding between streptavidin-coated magnetic beads and biotin-containing liposomes. Fifty microliters of magnetic beads and liposomes was incubated at room temperature for 1 h. After washing the aggregates with TBS solution for 3 times, the bead-captured liposomes were lysed by ECL buffer and ECL was measured. The ECL response resulted from biotin−avidin binding (black) is compared with that obtained from direct lysis (red).

To conjugate biotinylated antibodies onto biotinylated liposomes via avidin−biotin interaction (Figure 1), we sequentially added avidin and antibody to the above liposome solution in a mole ratio of 10:2:1 (biotin/avidin/antibody). This ensured most antibody molecules to be directly associated with liposomes.

Electrochemical and ECL Behavior of the Liposomes/Magnetic Beads Detection System. To lyse the captured liposomes and thus release the ECL label ($Ru(bpy)_3^{2+}$), 1% (v/v) Triton X-100, a nonionic surfactant, was added into the ECL detection buffer. Figure 3 shows a cyclic voltammogram (Figure 3, bottom) of the buffer containing $Ru(bpy)_3^{2+}$ released from surfactant-lysed liposomes, which features an irreversible oxidation wave for tripropylamine on Pt electrode similar to the response obtained from ECL buffer alone. The ECL intensity versus applied potential profile is shown by the top curve. The two ECL waves result from responses during the oxidation of TPrA and $Ru(bpy)_3^{2+}$.[15,16] The concentration in this solution can be found by preparing a calibration curve of ECL intensity versus $Ru(bpy)_3^{2+}$ concentration (Figure 4). From this plot, the actual $Ru(bpy)_3^{2+}$ concentration in the test solution was 50 nM, or considering the dilution factor, the concentration of $Ru(bpy)_3^{2+}$ in the as-prepared liposomes was 6.7×10^{-6} M.

To test whether the prepared liposomes can withstand the washing and magnetic separation processes, we first tested the interaction between biotin-linked liposomes and streptavidin-coated magnetic beads; i.e., the liposomes were attached to the magnetic beads via biotin−avidin interactions and detected by ECL after lysing. In this set of experiments, a series of liposome concentrations were prepared by serially diluting the as-prepared liposome sample. As indicated by Figure 5, the liposomes can be quantitatively captured by magnetic beads and reliably detected by ECL measurement. A comparison of the ECL response from magnetic bead-separated liposomes with that from direct lysis of the same amount of liposome demonstrates that there was some $Ru(bpy)_3^{2+}$ not collected by the beads. This could represent $Ru(bpy)_3^{2+}$ *outside* of the liposomes either because the liposome leak slightly or a fraction of the liposomes is not collected by the magnetic beads. As we discussed previously,[10] $Ru(bpy)_3^{2+}$ has a significant solubility in both organic and aqueous media, so some leakage is possible. Nevertheless, the issue of $Ru(bpy)_3^{2+}$ leakage does not affect the quantitative aspects of the assay because (1) this process is relatively slow,[10] and (2) any $Ru(bpy)_3^{2+}$ outside is effectively removed from the analysis by the magnetic separation step and thus does not contribute to final ECL quantification. This is also confirmed by the immunoassay of human CRP experiment discussed below.

ECL Detection of Human CRP. Of the three components in this sandwich assay, anti-human CRP conjugated liposomes and magnetic beads were always added in excess to a CRP test solution. Therefore, the number of liposomes that are conjugated to the magnetic beads was determined by the quantity of human CRP present in a sample, which thus led to different levels of ECL response after the $Ru(bpy)_3^{2+}$ complexes were released from the liposomes. To minimize nonspecific binding of the liposomes to

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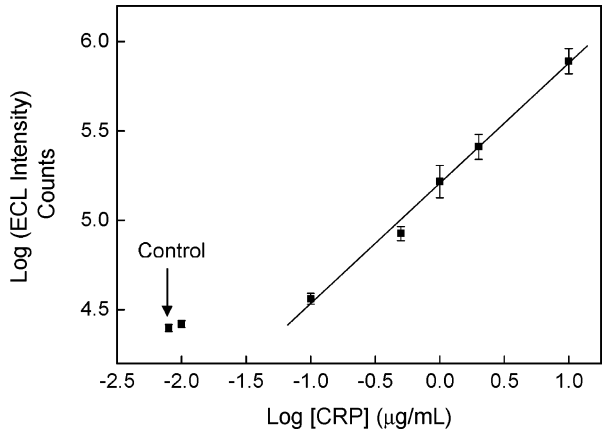

Figure 6. ECL detection of human CRP. Human CRP of different concentration was dissolved in TBS solution (0.1 M Tris, 0.15 M NaCl, pH 7.2). Error bars represent the standard deviation of three parallel measurements (samples) for each concentration. In the control experiments, CRP samples were replaced by TBS solution of same volume. See the Experimental Section for details.

the magnetic beads, 2% BSA was used. Shown in Figure 6 are the results of three replicate experiments, where human CRP concentrations as low as 100 ng/mL could be reliably detected with linearity of the ECL intensities with analyte concentration up to at least 10 μg/mL (the highest concentration tested). At a smaller CRP concentration, 10 ng/mL, the signal could not be discriminated from that of the control experiment where no human CRP was included.

The amplification effect of this immunoassay can be assessed by the number of $Ru(bpy)_3^{2+}$ complexes released per CRP protein (analyte) added, since we know the amount of CRP added and $Ru(bpy)_3^{2+}$ detected in each assay. This calculation yields an amplification factor of ~4 for 0.1 μg/mL CRP and ~1 for 10 μg/mL CRP. These low values imply that the number of liposomes captured by the magnetic beads is very low, since thousands of ECL labels are encapsulated in each liposome. Such effects have been discussed by others[17] and perhaps can be attributed to the large size of the liposomes, leading to steric effects, or blocking of the antigen−antibody interactions by BSA.

CONCLUSIONS

We have shown here that submicrometer-sized liposomes containing $Ru(bpy)_3^{2+}$ can be prepared by a lipid extrusion method, and by combining with streptavidin-coated, micrometer-sized magnetic beads, these liposomes can be used as a new ECL tag for immunoassay. The real attractiveness of this technique lies in that the surface moieties of the liposome can be tailored[13] by controlling the starting lipid molecules and that the analysis is compatible to a totally aqueous system. The low-level nonspecific binding achieved in the human CRP immunoassay can be attributed to the use of DSPE-PEG(2000)biotin, which contains a long-chain poly(ethylene glycol) that is known to be able to lessen nonspecific biological binding.[18] To further lower the nonspecific binding and thus the detection limit, other reported schemes are worth testing.

It may be possible to increase the sensitivity of this method by increasing the concentration of $Ru(bpy)_3^{2+}$ incorporated into the liposome and improving the collection efficiency by the magnetic beads. It would also be useful to suppress leakage of the liposome contents, e.g., by controlling the relative osmotic pressure of the solutions inside and outside the liposome and by the use of more hydrophilic $Ru(bpy)_3^{2+}$ derivatives. Finally, the density of anti-human CRP molecules on individual liposomes can be further increased by integrating a higher percentage of DSPE-PEG(2000)biotin into the liposome preparation, which could increase the binding events and therefore produce higher ECL signals.

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