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Electrogenerated Chemiluminescence. 80. C-Reactive Protein Determination at High Amplification with $[Ru(bpy)_3]^{2+}$-Containing Microspheres

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Biotinylated anti-C-reactive protein (CRP) species were attached to the surface of streptavidin-coated magnetic beads (MB) and avidin-coated polystyrene microspheres/beads (PSB) entrapping a large number of electrogenerated chemiluminescence (ECL) labels (~10^9 $Ru(bpy)_3[B(C_6F_5)_4]_2$/bead) to form anti-CRP↔MB and Ru(II)⊂PSB/avidin↔anti-CRP conjugates, respectively. Sandwich-type Ru(II)⊂PSB/avidin↔anti-CRP ⟨CRP⟩ anti-CRP↔MB aggregates were formed when Ru(II)⊂PSB/avidin↔anti-CRP was mixed with anti-CRP↔MB conjugates in the presence of analyte CRP. The newly formed aggregates were magnetically separated from the reaction media and dissolved in MeCN containing tri-*n*-propylamine as an ECL coreactant. ECL was carried out with a potential scan from 0 to 2.8 V vs Ag/Ag^+, and the ECL intensity was found to be proportional to the analyte CRP concentration over the range of 0.010−10 μg/mL. The CRP concentration of an unknown human plasma specimen was measured by the standard addition method based on this technique. Elimination of the nonspecific adsorption of the CRP system with several different blocking agents was also studied, and 2.0% bovine serum albumin was found to be best.

An ultrasensitive DNA hybridization detection methodology that utilizes polystyrene microspheres/beads (PSB) as the carrier of a large number of the electrogenerated chemiluminescence (ECL) labels, namely, tris(2,2′-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) species, was recently reported.[1] With this technique, a detection limit of 1.0 fM for the target single-stranded DNA was achieved. The principle described in that paper is now extended to the development of a very sensitive sandwich-type immunoassay for the analyte C-reactive protein (CRP).[2] Here a ~100-fold improvement in the sensitivity in CRP determination compared to a recently reported immobilized CRP ECL detection on Au(111) electrodes using $Ru(bpy)_3^{2+}$ labels[3] is demonstrated. Unlike the usual immunoassays based on, for example, fluorescence[4] or ECL,[5] where only one or few fluorophore molecules are attached directly to an antibody, so that self-quenching of the fluorescence and nonspecific adsorption can be avoided and the bioactivity of the antibody remains, the current technique does not need the direct attachment of the ECL labels to the antibody, so that the loading capacity of the ECL labels per PSB can be as high as ~10^9 molecules.[1] Thus, there is a very large amplification factor of $Ru(bpy)_3^{2+}$ label molecules for each antibody, i.e., for each molecule of analyte, given that a limited number of antibody species are attached to one PSB via the immobilization layer (of avidin). A number of approaches have been developed in which a target species (i.e., DNA, antibody, antigen) can be tagged with a larger number of labels, including using liposome-encapsulated fluorophores,[6] chemiluminescent microemulsions,[7] nanocrystal or nanoparticle biolabels,[8,9] and microspheres entrapped with electroactive species,[10] so that signal amplification can be achieved. Figure 1 schematically displays the general principle of the technique currently employed. A magnetic bead (MB) immobilized with an antibody is mixed and reacts with an antibody-coated PSB preloaded with a large number of ECL labels in the presence of the antigen (analyte) species to form a sandwich-type MB<antigen>PSB aggregate that is separated magnetically from the reaction medium and transferred into an acetonitrile solution where the PSB is dissolved and the ECL label released. This is followed by an anodic ECL detection of the released ECL label using tri-*n*-propylamine (TPrA) as the coreactant, and the ECL signal produced within in a certain potential range is detected with a photomultiplier tube or CCD camera.

CRP is an acute-phase protein found in human serum.[2,11−13] Concentrations of CRP can be elevated up to 1000-fold (200 μg/

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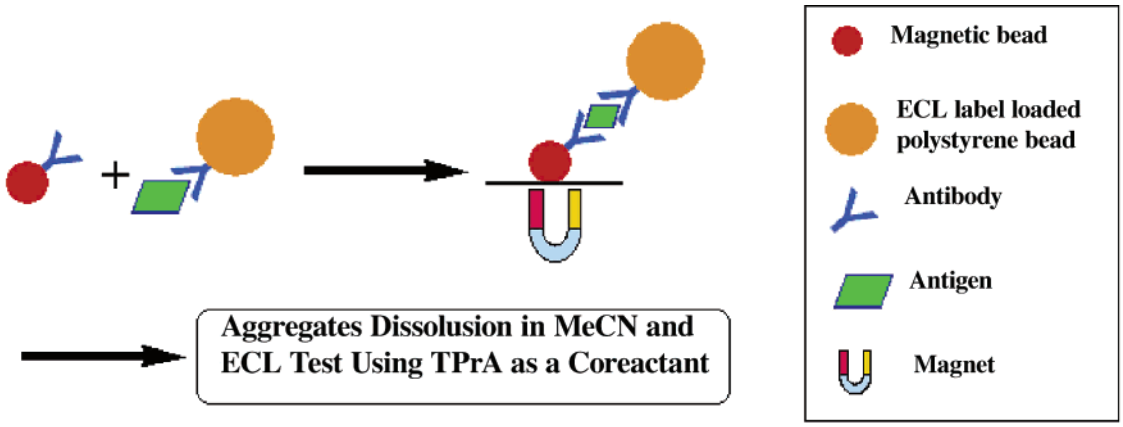

Figure 1. Schematic diagram showing the formation of a sandwich-type aggregate between an antibody-coated MB and an antibody-coated PSB containing entrapped ECL labels in the presence of the antigen species, and the separation of the newly formed aggregate with a magnet as well as the subsequent dissolution and ECL detection in MeCN using TPrA as the coreactant.

mL) in response to inflammation, injury, or infection.[13,14] A number of recent studies have demonstrated that CRP can be used to help predict the risk of acute events in patients with atherosclerosis.[11,13,15−21] CRP has also been shown to predict risk of future events in patients with acute coronary syndromes and in patients with stable angina and coronary artery stents.[11−25] As a result, CRP determination could play an important role as an assessor of risk factors for future coronary events, and numerous investigations toward high-sensitivity CRP detection techniques based on fluorescent nanoparticle,[26] thin-layer immunoaffinity chromatography,[27] surfactant- enhanced latex particle immunoassay,[28,29] polymeric nanoparticles,[30] and micro-"taste chips"[31] have recently been reported.

In this paper, the electrochemical and ECL behavior of the sandwich-type PSB⟨CRP⟩MB aggregates studied in MeCN with TPrA as the coreactant is described, followed by the examination of several different blocking agents for the suppression of the nonspecific adsorption between PSB and MB that interferes with the analysis. Finally, the ECL detection of pure CRP and the CRP in a human plasma sample is demonstrated.

EXPERIMENTAL SECTION

Chemicals and Materials. Tris(2,2′-bipyridyl)ruthenium(II) dichloride hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$), trifluoroacetic acid (TFAA, 99%), silver tetrafluoroborate (98%), Triton X-100, and tri-*n*-propylamine (99+%) from Aldrich (Milwaukee, WI); lithium tetrakis(pentafluorophenyl)borate ($Li[B(C_6F_5)_4] \cdot nEt_2O$, $n = 2-3$) from Boulder Scientific Co. (Mead, CO); tetrabutylammonium tetrafluoroborate ($(TBA)BF_4$, electrochemical grade) from Fluka (Milwaukee, WI); tris(hydroxymethyl)aminomethane (Tris, ultrapure) from Life Technologies (Rockville, MD); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (SigmaUltra), *N*-hydroxysuccinimide (NHS), 1-methylimidazole, C-reactive protein (from human plasma), human plasma, anti-human C-reactive protein (anti-CRP, developed in rabbit, ~90 mg/mL), and bovine serum albumin (BSA, 98−99%) from Sigma (St. Louis, MO); sodium hydroxide (GR), hydrochloric acid (GR), sodium chloride (GR), acetonitrile (HPLC), tetrahydrofuran (GR), and ethylenedinitrilotetraacetic acid (EDTA) from EM (Gibbstown, NJ); avidin (NeutrAvidin), BupH phosphate-buffered saline (PBS buffer, 0.10 M sodium phosphate−0.15 M sodium chloride, pH 7.2), sulfo-NHS-LC-biotin, and SuperBlock blocking buffer in PBS from Pierce (Rockford, IL); and methanol (spectroanalyzed grade) from Fisher (Fairlawn, NJ) were used as received. Carboxylate polystyrene microspheres/beads (10 μm in diameter, 2.6% (w/w) aqueous suspension with ~6.5×10^4 beads/μL) and streptavidin-coated superparamagnetic polystyrene beads (referred to as magnetic beads or MB, 1.0 μm in diameter, 10 mg/mL aqueous suspension with ~9.5×10^6 beads/μL) were purchased from PolySciences Inc. (Warington, PA) and Dynal Biotech Inc. (Lake Success, NY), respectively. Unless otherwise stated, all solutions were freshly prepared with 18 MΩ·cm deionized Milli-Q water (Millipore Corp., Bedford, MA).

Synthesis of Hydrophobic $Ru(bpy)_3^{2+}$ ECL Labels, Loading of the ECL Labels into Polystyrene Beads, and Immobilization of Avidin on the Surface of the Polystyrene Beads Entrapped with the ECL Labels. The relevant procedures associated with the above processes have been described in detail

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elsewhere,[1] and a brief description of the procedures can be also found in the Supporting Information.

Biotinylation of Anti-CRP. Biotinylated anti-CRP was prepared according to the procedure described previously[3] and is summarized in the Supporting Information.

Attachment of Biotinylated Anti-CRP to the Surface of MB and Ru(II)⊂PSB/Avidin Beads. Based on the manufacturer's instructions, before the attachment of biotinylated anti-CRP, the streptavidin-coated magnetic beads were thoroughly washed with a "binding and washing buffer" (B&W buffer) which consists of 5 mM Tris-HCl (pH 7.5)−0.5 mM EDTA−1.0 M NaCl. *[Caution: Care must be taken during the washing process, because the received MB contains 0.02% sodium azide as a preservative, which is highly toxic if ingested.]* Freshly washed 1-μm solid MB corresponding to 200 μL of the original MB suspension was immersed in 1.5 mL of ∼2.0 mg/mL biotinylated anti-CRP solution, and the mixture was then rotated with a Dynal sample mixer (Dynal Biotech Inc., Lake Success, NY) at ∼40 rpm for 1 h. With this treatment, a saturated layer of anti-CRP is expected to be formed on the surface of MB, since streptavidin and biotin interactions are very strong and specific,[32] and a large excess of biotinylated anti-CRP was initially used compared with the overall loading capacity of MB for biotin on the basis of our previous data.[1] Newly formed conjugates, designated as anti-CRP↔MB, were separated from the solution mixture with a magnet (Dynal MPC-S), followed by washing with 1.0 mL of 1× B&W buffer three times, transferred to a new 2-mL microcentrifuge tube to avoid possible biotinylated anti-CRP adsorption on the wall of the previous tube, and resuspended in 200 μL of PBS buffer (pH 7.2). Similarly, a saturated layer of anti-CRP immobilized on the surface of Ru(II)⊂PSB/avidin was obtained by mixing 400 μL of Ru(II)⊂PSB/avidin with 1.5 mL of ∼2.0 mg/mL biotinylated anti-CRP and rotating the mixture at ∼40 rpm for ∼1 h. Unreacted antibodies were then removed from the newly formed conjugates, referred to as Ru(II)⊂PSB/avidin↔anti-CRP, by centrifugation and washing with 1.0 mL of 1× B&W buffer three times, transferred to a new 2-mL microcentrifuge tube, and resuspended in 400 μL of PBS buffer (pH 7.2). Both anti-CRP↔MB (∼9.5×10^6 beads/μL) and Ru(II)⊂PSB/Avidin↔anti-CRP (∼6.5×10^4 beads/μL) conjugates were stored at ∼4 °C until use.

Sandwich-Type Ru(II)⊂PSB/Avidin↔Anti-CRP ⟨CRP⟩ Anti-CRP↔MB Aggregates Formation. A 20-μL aliquot of Ru(II)⊂PSB/avidin↔anti-CRP, 130 μL of a blocking agent, e.g., 2.0% BSA in 0.10 M PBS (pH 7.2) buffer, and 5 μL of anti-CRP↔MB conjugates (i.e., MB/PSB ≈ 36), together with either 20 μL of an appropriate concentration of pure CRP or 20 μL of human plasma sample with the addition of the desired volume (0−20 μL) of a standard concentration of pure CRP were used to produce the sandwich-type Ru(II)⊂PSB/avidin↔anti-CRP ⟨CRP⟩ anti-CRP↔MB aggregates. This sandwich-type aggregate will be referred to as PSB⟨CRP⟩MB in the following discussions for simplicity. Up to 25 μL of 0.10 M PBS (pH 7.2) buffer was also added to the above mixture to make an overall volume of 200 μL whenever necessary. The antibody−antigen reactions were carried out at room temperature for ∼3 h with frequent gentle shaking. Poor reproducibility in ECL intensities was often observed when mixing the suspension by rotating it rather than by gentle shaking, suggesting that the binding between PSB and MB beads is not strong enough to overcome the separation force induced by vigorous rotation. Alternatively, not all of the suspension adsorbed on the wall and cap of the microcentrifuge tube was fully collected during the following magnetic separation and washing processes. Newly produced sandwich-type aggregates, along with any unbound anti-CRP↔MB conjugates, were magnetically separated from the solution medium containing free unbound Ru(II)⊂PSB/avidin↔anti-CRP, washed gently with 200 μL of 1× B&W buffer thee times, and carefully transferred to a new centrifuge tube to minimize the possible adsorption of free Ru(II)⊂PSB/avidin↔anti-CRP beads on the wall of the tube, hence to reduce the background light emission signals during the process of subsequent ECL experiments.[1] The aggregates were finally dissolved in a 0.50-mL solution of 0.10 M TPrA−0.055 M TFAA−0.10 M (TBA)BF_4 in MeCN for the later ECL measurements. Previously, we reported that a combination of 0.10 M TPrA with 0.055 M TFAA produced the largest ECL response from Ru(bpy)$_2^{3+}$ in MeCN.[1] Note that, before the aggregate dissolution in MeCN, any noticeable aqueous droplets on the wall of the centrifuge tube should be removed, e.g., via centrifugation, because more than trace amounts of water in MeCN (e.g. >2% (v/v)) can cause a distortion of the anodic voltammetric and ECL response for the Ru(bpy)$_3^{2+}$−TPrA system, resulting in inaccuracy of CRP quantification.

Standard CRP solutions were prepared from a known concentration of a pure human CRP sample by diluting it with 0.10 M PBS (pH 7.2) prior to use, while human plasma samples containing CRP were reconstituted with deionized water according to the manufacturer's instructions. CRP-removed human plasma, which was used to replace the original plasma sample and to obtain the nonspecific binding data between Ru(II)⊂PSB/avidin↔anti-CRP and anti-CRP↔MB in the absence of CRP, was prepared by mixing 1.0 mL of human plasma sample with 50 μL of 1-μm-diameter anti-CRP↔MB and rotating at ∼20 rpm for ∼2 h. After removal of the anti-CRP↔MB beads coupled with CRP species from the plasma solution with a magnet, CRP-free plasma should be obtained, since, on the basis of the MB beads binding capacity for biotin[3,33] and the general concentration of CRP in human plasma,[3] a ∼10-fold excess of anti-CRP↔MB was used.

ECL and Electrochemical Measurements. A three-electrode, ∼5-mL disposable glass vial cell was used, with a 2.0-mm-diameter Pt disk as the working electrode, a Pt wire as the counter electrode, and a Ag/Ag^+ (10 mM $AgBF_4$ and 50 mM (TBA)BF_4 in MeCN) as the reference electrode. All electrodes were carefully cleaned before each experiment, including immersing all electrodes into MeCN, polishing the Pt working electrode with a 0.3-μm alumina slurry (Buehler Ltd., Lake Bluff, IL), washing with copious amounts of water, and rinsing with MeCN. The ECL intensities, along with the cyclic voltammograms (CV), were measured simultaneously with a home-built potentiostat combined with a photomultiplier tube (PMT, Hamamatsu R4220p) installed under the electrochemical cell. A voltage of −750 V was supplied to the PMT with a high-voltage power supply (Bertan High Voltage Corp., Series 225, Hicksville, NY).

All measurements were conducted at a temperature of 20 ± 2 °C, unless otherwise stated.

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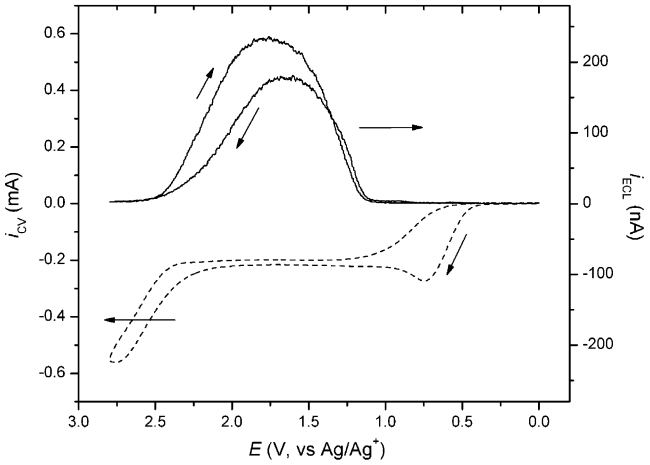

Figure 2. (a, dotted line) Cyclic voltammetric response obtained from PSB⟨CRP⟩MB aggregates dissolved in MeCN containing 0.10 M $(TBA)BF_4$ electrolyte−0.10 M TPrA coreactant at a 2.0-mm-diameter Pt electrode with a scan rate of 50 mV/s. (b, solid line) ECL response during CV as in (a). A CRP concentration of 1.0 μg/mL and 2.0% BSA as blocking agent was used for the experiment.

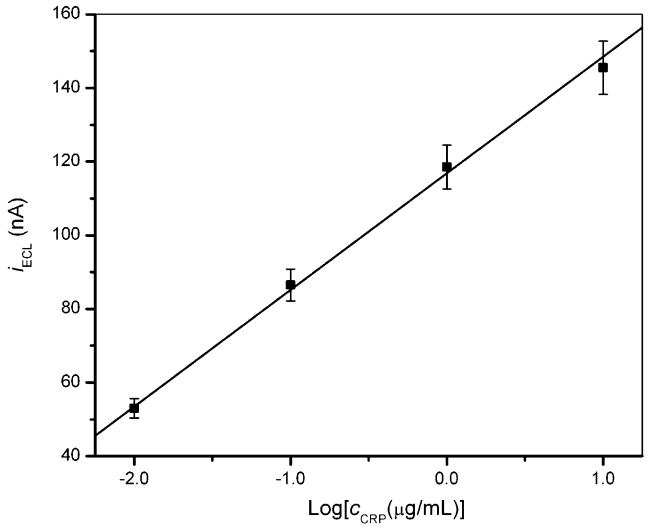

Figure 3. ECL intensity as a function pure CRP concentration. The experiments were carried out in 0.50 mL of 0.10 M TPrA−0.055 M TFAA−0.10 $(TBA)BF_4$ MeCN at a 2.0-mm-diameter Pt electrode by applying CV potential sweeps between 0 and 2.8 V vs Ag/Ag^+ at a scan rate of 50 mV/s. A subtraction of background ECL signal of 88 nA obtained in the absence of CRP was applied to all data.

RESULTS AND DISCUSSION

Electrochemical and ECL Behavior of PSB⟨CRP⟩MB Aggregates Dissolved in MeCN Using TPrA as a Coreactant. As in the case of DNA detection reported previously[1] using the same PSB−MB procedures described in this paper, the anodic electrochemical and ECL behavior of PSB⟨CRP⟩MB aggregates dissolved in MeCN using TPrA as a coreactant is essentially the same as that obtained from the $Ru(bpy)_3^{2+}$−TPrA system. PSB and MB beads are not electroactive, and trace amounts of DNA and proteins dissolved in the electrolyte solution do not have any noticeable effect on the overall oxidation behavior of the system, which is predominantly governed by the oxidation of the relatively high concentration of TPrA at the electrode. As discussed previously,[1,34−36] ECL generated from the present system is mainly produced by reaction of $Ru(bpy)_3^{3+}$ with the reducing TPrA radical, regardless of the existence of DNA or proteins. Figure 2 shows typical CV and ECL responses of PSB⟨CRP⟩MB aggregates dissolved in MeCN containing 0.10 M $(TBA)BF_4$ electrolyte−0.10 M TPrA coreactant when the Pt electrode was swept between 0 and 2.8 V versus Ag/Ag^+ at a scan rate of 50 mV/s. In this case, 1.0 μg/mL CRP analyte and 2.0% BSA blocking agent were used.

Nonspecific Adsorption Reduction. Nonspecific adsorption is a common phenomenon in immunoassays, and numerous blocking agents have been used to suppress the background signals.[37−41] In the present study, significant background ECL signals were observed in the absence of CRP when no blocking agent was used. Several blocking agents were examined, including 0.5% Triton X-100, 10% SuperBlock blocking buffer in PBS, 2.0% BSA, and combinations of these. Only 2.0% BSA gave good nonspecific adsorption suppression without disturbing the normal interactions of anti-CRP with CRP, and all others showed over-blocking effects. That is, except for 2.0% BSA, all ECL signals were suppressed to the background ECL level, even in the presence of relatively high concentrations of CRP (0.010−10.0 μg/mL). A 10-fold reduction of nonspecific adsorption was generally obtained using 2.0% BSA as a blocking agent in the current investigation.

Additionally, nonspecific adsorption can also be reduced by mixing PSB with the blocking agent and the analyte solution first and allowing the reactions to proceed for a few minutes before adding MB into the solution suspension. In this way, the direct contact of PSB with MB was minimized.

ECL Determination of CRP. The ECL intensity varied linearly with CRP concentration over the range 0.010−10.0 μg/mL in 0.10 M TPrA−0.055 M TFAA−0.10 $(TBA)BF_4$ MeCN after the dissolution of PSB⟨CRP⟩MB aggregates in the solution (Figure 3). A 2.0% solution of BSA was used to curb the nonspecific adsorption between Ru(II)⊂PSB/avidin↔anti-CRP and anti-CRP↔MB during the formation of sandwich-type PSB⟨CRP⟩MB aggregates. The ECL intensity (i_{ECL}) shown in Figure 3 was an average value of the forward and the reverse ECL peak currents (see Figure 2). Compared with our recently reported ECL determination of the CRP method,[3] in which Au(111) served as the substrate for the immobilization of anti-CRP⟨CRP⟩anti-CRP−$Ru(bpy)_3^{2+}$ on the working electrode, and only a few $Ru(bpy)_3^{2+}$ molecules were attached to each anti-CRP, the present technique shows at least a 100-fold lower in CRP detection limit (i.e., 0.010 μg/mL), which is comparable with the data obtained from several commercially available automated CRP systems such as the Hitachi 911 (Iatron), BN II (Dade Behring), Immulite 2000 (Diagnostic Products Corp.), Hitachi 911 (Denka Seiken), Hitachi 917 (Waco), and Olympus AU640 (Olympus) with quoted limits

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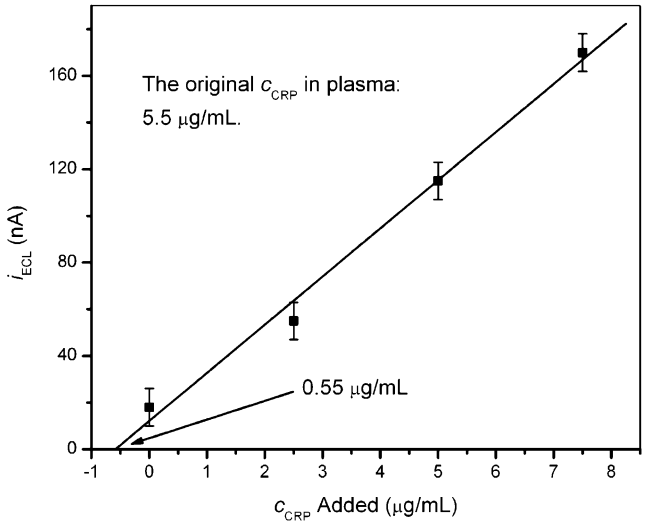

Figure 4. ECL determination of CRP in human plasma. The experiments were carried out using the same conditions as in Figure 3. All data shown in the figure were subtracted with a background ECL value of 101 nA, which was obtained from the experiment in which CRP-containing human plasma was replaced by human plasma from which CRP had been removed.

of detection of 0.005, 0.02, 0.02, 0.03, 0.06, and 0.08 μg/mL, respectively.[23]

Another set of experiments were designed to determine the CRP concentration in a human plasma sample, in which a different batch of Ru(II)⊂PSB/avidin↔anti-CRP beads, from the above, for the pure CRP assays was used, and a standard addition method was adopted to avoid any plasma matrix effect on the binding of anti-CRP with CRP. Figure 4 demonstrates the results of such a set experiments. Note that, in this figure, an average background ECL signal of 101 nA, which was obtained by using human plasma from which the CRP was removed (instead of a CRP-containing one in the absence of added CRP), was subtracted from all of the data. An original CRP concentration of 5.5 μg/mL in human plasma is found from this figure, given that in each case, 20 μL of plasma sample was added to an overall 200 μL of reaction media. This measured CRP value is consistent with the data reported previously.[3,20,23,24]

CONCLUSIONS

A very sensitive sandwich-type immunoassay method based on ECL using $Ru(bpy)_3^{2+}$ labels loaded into polystyrene beads has been carried out. ECL detection of CRP was accomplished in 0.10 M TPrA−0.055 TFAA−0.10 M $(TBA)BF_4$ MeCN at a Pt electrode after the PSB⟨CRP⟩MB aggregates were dissolved in the above solution. With this technique, the CRP detection limit is as low as 0.010 μg/mL, which is lower than those obtained from most of the presently available automated high-sensitivity CRP assay systems.[23] CRP detection for human plasma was conducted with the traditional standard addition method to avoid possible sample matrix effects, and the CRP value obtained was consistent with the literature data. Nonspecific adsorption in CRP immunoassay was also studied using several different blocking agents, of which 2.0% BSA was the best one for the CRP system.

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SUPPORTING INFORMATION AVAILABLE

A description of the synthesis of hydrophobic $Ru(bpy)_3^{2+}$ ECL labels, loading of the ECL labels into polystyrene beads, immobilization of avidin on the surface of the polystyrene beads entrapped with the ECL labels, and preparation of biotinylated anti-CRP. This material is available free of charge via the Internet at http://pubs.acs.org.

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