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Electrogenerated Chemiluminescence. 77. DNA Hybridization Detection at High Amplification with $[Ru(bpy)_3]^{2+}$-Containing Microspheres

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An ultrasensitive DNA hybridization detection method based on electrogenerated chemiluminescence (ECL) using polystyrene microspheres/beads (PSB) as the carrier of the ECL labels, namely, tris(2,2′-bipyridyl)ruthenium(II) tetrakis(pentafluorophenyl)borate ($Ru(bpy)_3[B(C_6F_5)_4]_2$), is reported. Probe single-stranded DNA (p-ssDNA) was attached to the surface of magnetic beads (MB) and hybridized with target-ssDNA (t-ssDNA) with immobilized PSB containing a large number of water insoluble $Ru(bpy)_3[B(C_6F_5)_4]_2$ species ($\sim 7.5 \times 10^9$ molecules/bead). With this approach a large amplification factor of $Ru(bpy)_3[B(C_6F_5)_4]_2$ molecules for each t-ssDNA can be achieved, when each PSB is attached to a limited number of t-ssDNA. The p-ssDNA−MB ↔ t-ssDNA−PSB/$Ru(bpy)_3^{2+}$ conjugates formed were magnetically separated from the reaction media and dissolved in MeCN containing tri-*n*-propylamine (TPrA) as an ECL coreactant. ECL was produced with a potential scan from 0 to 3.0 V versus Ag/Ag^+, and the integrated ECL intensity was found to be linearly proportional to the t-ssDNA concentration in a range of 1.0 fM to 10 nM under optimized conditions. ECL signals associated with two base pair mismatched ssDNA and noncomplementary ssDNA can be distinguished well from the ECL signal related to the complementary DNA hybridization. A Poisson distribution is followed when a large number of MB reacts with PSB, and the minimum number of 1.0- and 2.8-μm diameter MB required to bind and magnetically separate a single 10-μm diameter PSB from the reaction solution was estimated to be three and one, respectively. The principle described in this paper could be also applied to many other ECL analyses, such as immunoassays.

We report here 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. The loading capacity of the ECL labels per PSB can be as high as $\sim 7.5 \times 10^9$ molecules; thus, a very large amplification factor of $Ru(bpy)_3^{2+}$ label molecules for each target molecule of single-stranded DNA (t-ssDNA) compared to the usual single label is expected, if each PSB is only immobilized with a limited number of t-ssDNA. As a result, a very low concentration of t-ssDNA, of the order of the femtomolar level, can be detected. Figure 1 schematically shows the general principle of this technique. A known ssDNA (probe ssDNA) is first attached to the surface of a magnetic bead, and the complementary ssDNA (t-ssDNA), labeled with a coated polystyrene bead preloaded with a large number of ECL labels, hybridizes with the probe ssDNA. The t-ssDNA−polystyrene beads containing $Ru(bpy)_3^{2+}$ are magnetically separated and transferred into an acetonitrile solution where the polystyrene beads dissolve and the ECL label is released. This is followed by ECL detection of the released ECL label mixed with a coreactant such as tri-*n*-propylamine (TPrA) solution at an electrode. When the appropriate positive potential is applied to the electrode or it is scanned over the positive potential range, an ECL signal is generated and detected with a photomultiplier tube or CCD camera.

Sensitivity in DNA hybridization and other bioassays is important in clinical diagnostics,[1−3] forensic chemistry,[4,5] environmental[6,7] investigations, pharmaceutical studies,[4,8] and biological warfare agent detection.[9,10] ECL methods have been widely used in such studies because of their high sensitivity, wide dynamic range, and selectivity.[11] A variety of techniques are available for the detection of DNA, of which electrochemical, fluorescent, and ECL active labels attached to the t-ssDNA produce the measurable signal in the analysis process.[1,12−15] Since the intensity of the measured signal is generally proportional to the amount of

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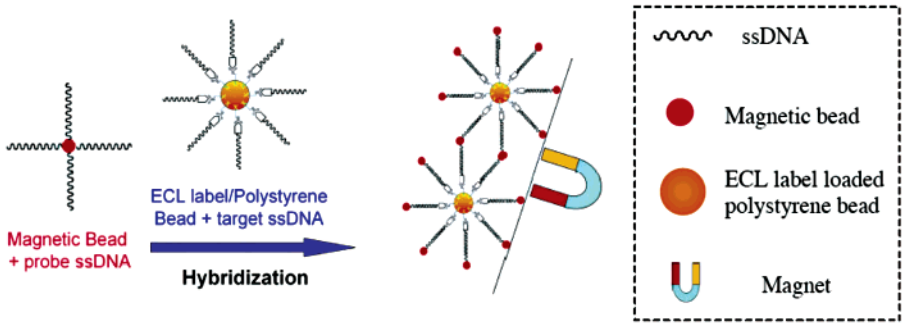

Figure 1. Schematic diagram of DNA hybridization on a polystyrene bead as the ECL label carrier and a magnetic bead for the separation of analyte-contained ECL label/polystyrene beads.

t-ssDNA, and traditionally only one or a few labels can be attached to one t-ssDNA, a number of approaches have been developed in which one DNA can be labeled with a large number of labels so that a higher sensitivity can be achieved.[16,17]

Compared with the recently reported ECL determination of immobilized DNA,[10] our present study has a number of advantages: (a) high sensitivity/low detection limit—in the present study 1.0 fM t-ssDNA can be detected; (b) high selectivity/low nonspecific adsorption—complementary, two base pair mismatched and noncomplementary DNA hybridizations can be distinguished and no complicated treatment to eliminate nonspecific adsorption of $Ru(bpy)_3^{2+}$ is required; and (c) high stability and the possibility of multiple measurements, because the solution containing the ECL label, $Ru(bpy)_3^{2+}$, can be used to make many ECL measurements without loss of signal.

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 ($AgBF_4$, 98%), and tri-*n*-propylamine (TPrA, 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 (EDAC, SigmaUltra), *N*-hydroxysuccinimide (NHS), fluorescein biotin (90%), 1-methylimidazole, and DNA hybridization buffer (PerfectHyb plus) from Sigma (St. Louis, MO); sodium hydroxide (GR), hydrochloric acid (GR), sodium chloride (GR), ethyl ether (anhydrous), acetonitrile (HPLC), tetrahydrofuran (THF, GR), and ethylenedinitrilotetraacetic acid (EDTA) from EM (Gibbstown, NJ); avidin (NeutrAvidin), *D*-biotin (ImmunoPure), and biotin-PEO-LC-amine from Pierce (Rockford, IL); and methanol (spectroanalyzed grade) from Fisher (Fairlawn, NJ) were used without further purification unless otherwise stated. Carboxylate polystyrene microspheres/beads (PSB, 10-μm diameter, 2.6% (w/w) aqueous suspension with $\sim 6.5 \times 10^4$ beads/μL) and streptavidin-coated superparamagnetic polystyrene beads (referred to as magnetic beads or MB of (a) 1.0-μm diameter, 10 mg/mL aqueous suspension with $\sim 9.5 \times 10^6$ beads/μL and (b) 2.8-μm diameter, 10 mg/mL aqueous suspension with $\sim 6.5 \times 10^5$ beads/μL) were purchased from PolySciences Inc. (Warington, PA) and Dynal Biotech Inc. (Lake Success, NY), respectively. Synthetic 23-mer single-stranded DNA (ssDNA) oligonucleotides derived from the *Bacillus anthacis* (Ba813)[18−20] were obtained from Qiagen Operon (Alameda, CA) and had the following sequences: (a) probe, 5′-[biotin-TEG]-AACGA TAGCT CCTAC ATTTG GAG-3′(p-ss-DNA, MW = 7617 g/mol); (b) target or complementary, 5′-[biotin-TEG]-CTCCA AATGT AGGAG CTATC GTT-3′ (t-ssDNA, MW = 7608 g/mol); (c) noncomplementary, 5′-[biotin-TEG]-TTAAC ACCTT AGCGA CGGCT AGT-3′ (nc-ssDNA, MW = 7593 g/mol); and (d) two base pair mismatched oligomer sequence, 5′-[biotin-TEG]-CTCCA AA***C***GT AGGAG ***T***TATC GTT-3′ (2-bp-m-ssDNA), in which the biotin-TEG contained a 16-atom mixed polarity spacer based on a triethylene glycol and was used to reduce the steric hindrance between the biotinylated DNA and surface-confined avidin/streptavidin interactions. 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. Tris(2,2′-bipyridyl)ruthenium(II) tetrakis(pentafluorophenyl)borate ($Ru(bpy)_3[B(C_6F_5)_4]_2$) was used as the ECL label in the present study, because, as shown in the next sections, this complex can be effectively loaded into polystyrene beads using a suitable organic solution and maintained entrapped within the beads during a series of modifications of the beads in aqueous solution. In other words, $Ru(bpy)_3[B(C_6F_5)_4]_2$ is soluble in organic solvents but completely insoluble in aqueous solutions. Another reason to choose $Ru(bpy)_3[B(C_6F_5)_4]_2$ as the ECL label was based on the fact that the $Ru(bpy)_3^{2+}$ moiety of the complex has a very high ECL efficiency. $Ru(bpy)_3[B(C_6F_5)_4]_2$ was prepared by a metathesis reaction between $Ru(bpy)_3Cl_2$ and $Li[B(C_6F_5)_4] \cdot nEt_2O$ ($n = 2-3$) in water. The precipitate was washed with water, recrystallized from an acetonitrile/water solution, and dried under vacuum.

Loading of the ECL Labels into Polystyrene Beads. Carboxylate polystyrene beads of 10-μm diameter were separated from an appropriate volume (0.10−1.0 mL) of a 2.6% (w/w) polystyrene bead suspension with an Eppendorf 5415D centrifuge

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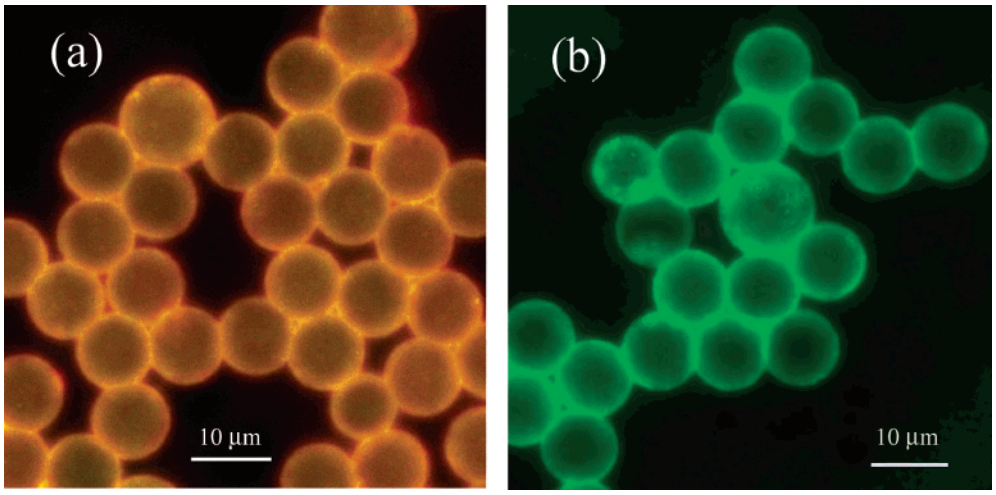

Figure 2. Fluorescent images of 10-μm diameter carboxylate polystyrene beads: (a) after entrapping of $Ru(bpy)_3[B(C_6F_5)_4]_2$ and (b) after covalent binding of avidin onto the surface of $Ru(bpy)_3[B(C_6F_5)_4]_2$-loaded beads. The exposure time was 30 s. The specimens were excited at λ_{ex} ~490 nm.

(Brinkmann Instruments, Inc., Westbury, NY) at ~10 000 rpm for 5 min, and then washed once with ~1 mL of water.

The beads were dried under vacuum at ~60 °C for 1 h, followed by addition of ~1.5 mL of the ECL label, a $Ru(bpy)_3[B(C_6F_5)_4]_2$-saturated (~0.7 mM) 5% THF−95% MeOH (v/v) solution into a 2-mL microcentrifuge tube containing PSB. The mixture was rotated with a Dynal sample mixer (Dynal Biotech Inc.) at ~20 rpm for 2 h, followed by centrifugation and washing with 50% MeOH−50% H_2O (v/v) twice. The resulting ECL label-containing yellowish polystyrene beads, designated as Ru(II)⊂PSB, were dried under vacuum at ~60 °C for 1 h. During the course of the above treatments, the polystyrene beads first swelled in the organic 5% THF−95% MeOH solution containing the ECL label, allowing the water insoluble ECL labels to diffuse into the polymer matrix, where they were entrapped when the organic solvents were removed from the beads by vacuum evaporation. The effective loading of the ECL labels into the polystyrene beads can be visually verified via their fluorescent images (Figure 2a) taken with a Nikon Eclipse TE 300 inverted microscope (Nikon Instruments Inc., Melville, NY) coupled with a Magnafire model S99806 Olympus America CCD camera (Olympus America, Melville, NY). A typical loading capacity of $\sim 7.5 \times 10^9$ $Ru(bpy)_3[B(C_6F_5)_4]_2$ molecules per bead was estimated on the basis of the ECL data obtained from Ru(II)⊂PSB dissolved in MeCN and a standard $Ru(bpy)_3[B(C_6F_5)_4]_2$ solution using TPrA as a coreactant.

Immobilization of Avidin on the Surface of Ru(II)⊂PSB. A layer of avidin was covalently attached to the surface of Ru(II)⊂PSB via the formation of amide bonds [i.e., Ru(II)⊂PSB-CO-NH-avidin] by immersing the beads in 1.5 mL of freshly prepared 25 μM avidin in a 0.10 M 1-methyl-imidazole buffer (pH 7) containing 0.10 M EDAC and 0.10 M NHS and rotating the mixture at ~40 rpm for 1 h. The newly formed avidin-coated Ru(II)⊂PSB, designated as Ru(II)⊂PSB/avidin, were centrifuged from the reaction solution at 5−10 000 rpm for 5 min and washed with 1 mL of 1× "B/W buffer" (1× binding/washing buffer, 5 mM Tris-HCl (pH 7.5) + 0.5 mM EDTA + 1.0 M NaCl) 3 times. The final Ru(II)⊂PSB/avidin product was resuspended in 1× B/W buffer solution that had the same volume as the starting PSB suspension (0.10−1.0 mL) and kept at ~4 °C until use. Approximately, 6.5×10^4 Ru(II)⊂PSB/avidin beads/μL can thus be estimated, with the assumption of no loss of the beads during the preparation of Ru(II)⊂PSB/avidin. Figure 2b shows the bright green fluorescent image of Ru(II)⊂PSB/avidin after the beads reacted with fluorescein biotin, suggesting that a uniform layer of avidin was formed on the surface of Ru(II)⊂PSB. In contrast, nonspecifically adsorbed fluorescein biotin on "bare" Ru(II)⊂PSB generated only a very weak fluorescent image (not shown). The binding capacity of Ru(II)⊂PSB/avidin for a biotinylated 23-mer ssDNA (p-ssDNA) was 0.565 nmoles (p-ssDNA)/mg PSB or 1.4×10^8 p-ssDNA molecules/bead, on the basis of fluorescein biotin titration experiments (see Figure S1a and Table S1 in the Supporting Information for details).

Attachment of ssDNA to the Surface of MB and Ru(II)⊂PSB/Avidin Beads and DNA Hybridization. (1) Probe DNA−MB Conjugates. Two different sizes of streptavidin-coated magnetic beads, namely, 1.0-μm and 2.8-μm diameter, were used as the probe DNA carrier. To form probe DNA−MB conjugates, 5.0 μL of 1.0-μm MB, or 10.0 μL of 2.8-μm MB, was first transferred into a 2-mL microcentrifuge tube, then separated from the original suspension with a magnet (Dynal MPC-S), followed by washing once with 200 μL of 2× B/W buffer and twice with 200 μL of 1× B/W buffer. The beads were then immersed in 100 μL of 2.5 μM biotinylated p-ssDNA and incubated for 30−60 min with gentle rotation at ~40 rpm. The probe DNA−MB conjugates formed were subsequently separated and washed with 200 μL of 1× B/W buffer 3 times, transferred to a new 2-mL microcentrifuge tube to avoid possible probe DNA adsorption on the wall of the previous tube, and resuspended in 20 μL of hybridization buffer. The conjugates produced in this way had a saturated probe DNA coverage of about 2.53 and 1.11 nmoles (p-ssDNA)/mg (beads), or 1.6×10^6 and 1.0×10^7 p-ssDNA molecules per bead, for 1.0- and 2.8-μm diameter MB, respectively (see parts b and c of Figure S1 and Table S1 in the Supporting Information for details).

(2) Target DNA−Ru(II)⊂PSB/Avidin Conjugates. An amount of 100 μL of an appropriate concentration of biotinylated t-ssDNA (1.0×10^{-8} to 1.0×10^{-15} M), or 1.0×10^{-9} M nc-ssDNA or 2-bp-m-ssDNA, was added to 25 μL of ~6.5×10^4 beads/μL

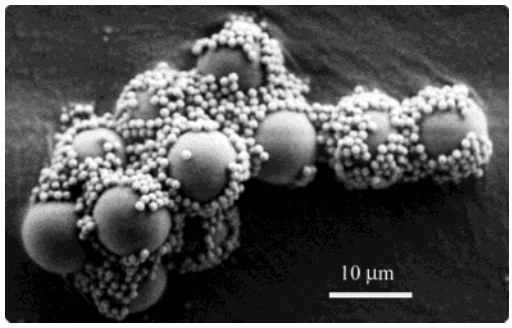

Figure 3. SEM image obtained after DNA hybridization between probe DNA−MB and complementary DNA−Ru(II)⊂PSB/avidin. The concentrations of both DNA were 5 μM, and the size of the PSB and the MB was 10 μm and 1.0 μm, respectively. The ratio of the MB to PSB was 29. Conductive carbon tape was used as the substrate, and the specimen was precoated with a 300-nm Cr thin film and imaged with a LEO 1530 scanning electron microscope at 4 kV.

Ru(II)⊂PSB/avidin in 1× B/W buffer and incubated for 1 h with gentle mixing at a rotation rate of ∼20 rpm, washed twice with 200 μL of 1× B/W buffer, centrifuged at 3000−5000−10 000 rpm for 5 min, and resuspended in 50 μL of the hybridization buffer.

(3) DNA Hybridization. The newly prepared target DNA−Ru(II)⊂PSB/avidin conjugates were transferred into 2-mL centrifuge tubes containing previously formed probe DNA−MB conjugates. An appropriate volume of the hybridization buffer was added into the tubes to make a total hybridization solution volume of 200 μL. After gentle mixing at ∼20 rpm for ∼1 h, the probe DNA−MB ↔ target DNA−Ru(II)⊂PSB/avidin aggregates were magnetically separated from the mixture containing free unbound Ru(II)⊂PSB/avidin beads, washed gently with 200 μL of 1× B/W buffer 3 times, and carefully transferred into a new centrifuge tube to minimize the possible adsorption of free Ru(II)⊂PSB/avidin beads on the wall of the tube. Such nonspecific adsorption can produce a significantly high background ECL level, since, along with the DNA hybridization aggregates, free Ru(II)⊂PSB/avidin beads on the wall will also dissolve in MeCN in the subsequent step. The aggregates were finally washed with 200 μL of water and 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. The formation of probe−DNA−MB ↔ target−DNA−Ru(II)⊂PSB/avidin aggregates after DNA hybridization can be clearly verified in the SEM image shown in Figure 3, in which both the probe DNA and the complementary DNA had a concentration of 5 μM, the initial ratio of MB/PSB = 29, and the size of the MB and the PSB was 1.0 μm and 10 μm, respectively.

Attachment of Biotin-PEO-LC-amine to Carboxylated Polystyrene Beads and the Poisson Distribution Test. Biotin-PEO-LC-amine (MW = 418.6 g/mol) is a water soluble cross-linker with a 22.9 Å poly(ethylene oxide) (PEO)-based spacer arm used to reduce the steric hindrance of biotin and avidin interactions.[21] Similar to the case described above for the immobilization of avidin onto Ru(II)⊂PSB, biotin-PEO-LC-amine molecules were covalently attached to the surface of carboxylated polystyrene beads via the formation of an amide bond between the primary amine group of the cross-linker and the carboxylate group of the beads in the presence of 0.10 M EDAC−0.10 M NHS in 0.10 M 1-methyl-imidazole buffer (pH 7). Typically, 1 mL of 5 mM biotin-PEO-LC-amine was used to react with the PSB separated from 250 μL of 2.6% PSB suspension for ∼2 h with a rotation rate of ∼40 rpm. The cross-linker-modified PSB, designated as PSB-CO-NH-LC-PEO-biotin, was subsequently separated and washed with 400 μL of 1× B/W buffer 3 times, resuspended in 250 μL of 1× B/W buffer, and then kept at ∼4 °C until use.

For the Poisson distribution measurements, in addition to 10-μm diameter PSB-CO-NH-LC-PEO-biotin beads, two different sizes of streptavidin/polystyrene-coated magnetic beads (1.0- and 2.8-μm diameter Dynal beads) were also used. When tens of thousands of PSB-CO-NH-LC-PEO-biotin beads are mixed with the same or a larger number of either 1.0- or 2.8-μm magnetic beads in solution, the probability that the PSB-CO-NH-LC-PEO-biotin beads will stick, via the irreversible reaction between the biotin of the PSB and the streptavidin of the MB, to one or more magnetic ones can be observed and compared to that predicted for a Poisson distribution. Experimentally, 50 μL of ∼6.5 × 10^4 beads/μL PSB-CO-NH-LC-PEO-biotin beads (∼3.3 × 10^6 PSB in total) were diluted with 175 μL of 1× B/W buffer and transferred to a 2.0-mL microcentrifuge tube containing a known number of 1.0-μm diameter streptavidin-coated magnetic beads that had been separated magnetically from the manufacturer's original suspension and washed once with ∼200 μL of 2× B/W buffer. The mixture was immediately shaken and rotated at ∼40 rpm for 1 h before the PSB−MB aggregates were magnetically separated from the mixture. The resulting supernatant contained unbound free PSB-CO-NH-LC-PEO-biotin beads, and their number was calculated on the basis of optical examination with an inverted microscope. Similar procedures were used for Poisson distribution studies of 2.8-μm MB. However, in this case, only half the amount of the PSB-CO-NH-LC-PEO-biotin beads, i.e., ∼1.6 × 10^6 beads, was used to react with a known amount of MB.

ECL and Electrochemical Measurements. A three-electrode cell was used, with a 2.2-mm diameter Pt disk, 2.0-mm diameter Au, or 3.0-mm in diameter glassy carbon (GC) 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 the working electrodes into a chromic acid solution (*CAUTION: Chromic acid solution is very corrosive and can react violently with organic materials and should be handled with extreme caution.*), polishing with a 0.05-μm alumina slurry (Buehler Ltd., Lake Bluff, IL), washing with copious amounts of water, rinsing with MeCN, and replacing the porous Vycor tip and the glass tube for the reference electrode. An ∼5-mL disposable glass vial served as the electrochemical cell. To exclude the possibility of the ECL signal being generated from a $Ru(bpy)_3^{2+}$-contaminated system, virgin glassware and electrodes were used whenever necessary. 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, Japan) installed under the

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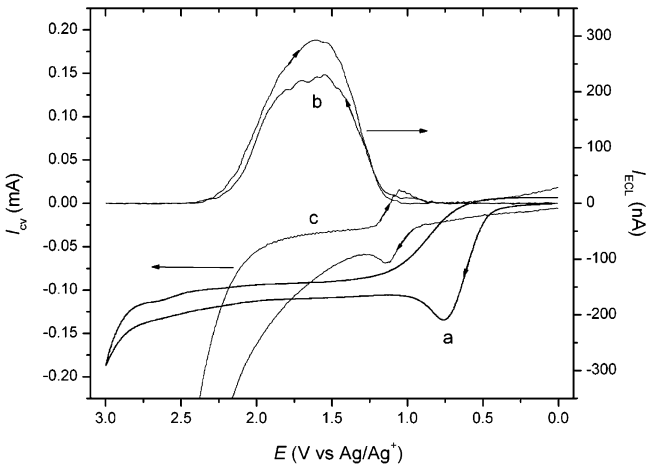

Figure 4. (a) Cyclic voltammetric response obtained from 0.10 μM $Ru(bpy)_3[B(C_6F_5)_4]_2$ in MeCN containing 0.10 M $(TBA)BF_4$ electrolyte−0.10 M TPrA coreactant at a 2.2-mm diameter Pt electrode with a scan rate of 50 mV/s. (b) ECL response during CV as in (a). For comparison, a CV of 1.0 mM $Ru(bpy)_3[B(C_6F_5)_4]_2$ in MeCN containing 0.10 M $(TBA)BF_4$ in the absence of TPrA is presented in (c), under the same experimental conditions as in (a) and (b), but the CV current shown was multiplied by 10.

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.

RESULTS AND DISCUSSION

Electrochemical and ECL Behavior of $Ru(bpy)_3^{2+}$ in MeCN Using TPrA as a Coreactant. The ECL of the $Ru(bpy)_3^{2+}$/TPrA system has been extensively studied in aqueous solution, and has been used in MeCN,[22,23] but to our knowledge, no detailed investigation has previously been reported in MeCN. The cyclic voltammetric and ECL responses of 0.10 μM $Ru(bpy)_3[B(C_6F_5)_4]_2$ in MeCN containing 0.10 M $(TBA)BF_4$ electrolyte−0.10 M TPrA coreactant at a Pt electrode at a scan rate of 50 mV/s are shown in Figure 4. TPrA starts to oxidize at potentials around 0.5 V versus Ag/Ag$^+$ and shows a maximum oxidation peak at about 0.75 V versus Ag/Ag$^+$ (Figure 4a). When the electrode is scanned to a potential more positive than ∼1.1 V versus Ag/Ag$^+$, where the $Ru(bpy)_3^{2+}$ is oxidized to $Ru(bpy)^{3+}$ (Figure 4c), ECL is produced (Figure 4b). The ECL intensity continuously increases with increasing potential, finally forming a broad peak with a half-width of about 700 mV and a peak potential at about 1.6 V versus Ag/Ag$^+$. On the reverse scan, a larger ECL intensity with a similar peak position is observed. Note that the oxidation potential of $Ru(bpy)_3^{2+}$ may be shifted slightly positive in the presence of TPrA, since the onset of ECL and the peak potentials are more positive than the potential for oxidation of $Ru(bpy)_3^{2+}$ in the absence of TPrA (parts b and c of Figure 4). As expected, a change in the counterions of the $Ru(bpy)_3^{2+}$ complexes, e.g., from $B(C_6F_5)_4^-$ to ClO_4^-, did not change the ECL behavior (not shown). In contrast to the case in aqueous solution, where a prewave ECL appeared

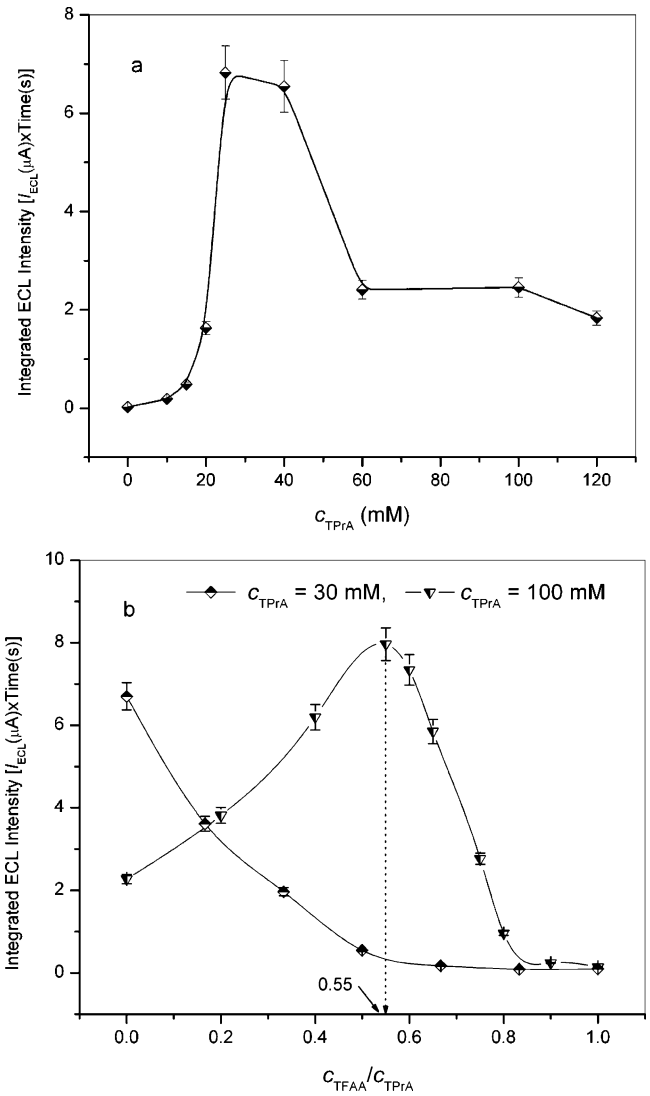

Figure 5. Effect of (a) TPrA and (b) TPrA-trifluoroacetic acid (TFAA) concentration on ECL intensity. All samples contained 0.10 μM $Ru(bpy)_3[B(C_6F_5)_4]_2$ and 0.10 M $(TBA)BF_4$ in MeCN. Working electrode: 2.2-mm diameter Pt. Scan rate: 50 mV/s.

in the potential region of TPrA oxidation due to the formation of excited state $Ru(bpy)_3^{2+*}$ on reaction of $TPrA^{\bullet+}$ with $Ru(bpy)_3^+$ (formed by reaction of $Ru(bpy)_3^{2+}$ with $TPrA^\bullet$) when a micromolar level of $Ru(bpy)_3^{2+}$ was used,[24] no noticeable corresponding ECL signal was found in MeCN. This suggests that under the present experimental conditions either the lifetime of $TPrA^{\bullet+}$ in MeCN is shorter than in neutral aqueous solutions or $TPrA^{\bullet+}$ is not energetically powerful enough to oxidize $Ru(bpy)_3^+$ to $Ru(bpy)_3^{2+*}$. However, in general, the ECL mechanism developed in aqueous solutions using TPrA as a coreactant[24] (where light is produced by reaction of $Ru(bpy)_3^{3+}$ with the reducing TPrA radical) is probably operative in MeCN.

The ECL intensity as a function of TPrA concentration is shown in Figure 5a, where the highest ECL intensity region corresponds to a TPrA concentration of ∼30 mM. As expected from the ECL−pH behavior in aqueous solution,[25] by adding trifluoroacetic acid

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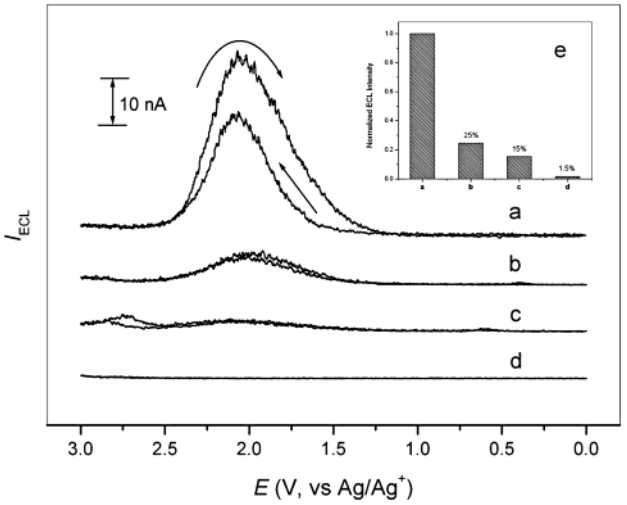

Figure 6. Elimination of ECL background for the TPrA−MeCN system: (a) 0.10 M TPrA−0.10 M (TBA)BF_4-MeCN; (b) (a) + 0.055 M TFAA; (c) same as (b) with the addition of 1.0% (v/v) H_2O; (d) 0.10 M (TBA)BF_4-MeCN in the absence of TPrA; (e) relative ECL intensities depicted in (a−d). Working electrode: 2.2-mm diameter Pt disk. Scan rate: 50 mV/s.

(TFAA) into a Ru(bpy)$_3^{2+}$/TPrA/MeCN solution, hence changing the acidity of the solution, the ECL intensity is changed (Figure 5b). A combination of 100 mM TPrA with 55 mM TFAA gave the largest ECL response.

The ECL intensity and stability were also affected by the electrode material used. Pt and Au electrodes showed similar responses; the ECL was stable over several potential cycles, with a slightly smaller photocurrent density found at Pt electrodes compared to Au. At a GC electrode, however, only the forward scan of the first potential cycle produced light. After polishing of the GC electrode, the light appeared again, suggesting the electrode surface had been blocked by a film of some kind that formed under the ECL reaction conditions. The relative ECL intensity, obtained from a MeCN solution containing 0.10 μM Ru(bpy)$_3$[B(C$_6$F$_5$)$_4$]$_2$−0.10 M TPrA−0.055 M TFAA−0.10 M (TBA)BF_4 during the first potential cycle between 0 and 3.0 V versus Ag/Ag$^+$ at a scan rate of 50 mV/s, at Au, Pt, and GC electrodes had a ratio of 100:93:61.

Interestingly, even in the absence of Ru(bpy)$_3^{2+}$, a TPrA in acetonitrile solution with 0.10 M (TBA)BF_4 shows an ECL signal (Figure 6a). Even with careful purification of the TPrA and MeCN by distillation, changing the electrolyte from $TBABF_4$ to $TBAClO_4$, using a newly opened electrochemical grade solvent, using virgin glassware and electrodes, and covering the Pt counter electrode with a glass tube coated with or without a layer of black plastic, an ECL signal was still observed. The ECL had a peak potential value of ~2.1 V versus Ag/Ag$^+$, which is a 500-mV shift compared to that obtained in the presence of 0.10 μM Ru(bpy)$_3^{2+}$ (Figure 4b). As shown previously in Figure 5a, in the absence of TPrA, a Ru(bpy)$_3^{2+}$ in MeCN solution with 0.10 M (TBA)BF_4 did not give an observable ECL response on the same intensity scale with a scan only to positive potentials. Thus, the ECL signals shown in Figure 6a must originate from TPrA and are probably due to the charge-transfer reaction inverse photoemission (CTRIP) associated with TPrA$^\bullet$ free radicals.[26−34] The integrated ECL intensity obtained from Figure 6a, which is about 12% of that in Figure 4b, is significant, since in the absence of TPrA, the residual photocurrent measured from a 0.10 M $TBABF_4$/MeCN solution is significantly less (Figure 6d). The background TPrA-related ECL signals can be suppressed dramatically by adding TFAA to the solution (Figure 6b), and a further elimination of the "unwanted" signals was achieved by an addition of 1% (v/v) H_2O into the 0.10 M TPrA−0.055 M TFAA−0.10 (TBA)BF_4 MeCN solution (Figure 6c). Details of the mechanism of this signal and the effect of TFAA and H_2O on the intensity are still unclear and bear further investigation. Figure 6e displays the relative ECL intensities obtained from Figure 6a−d.

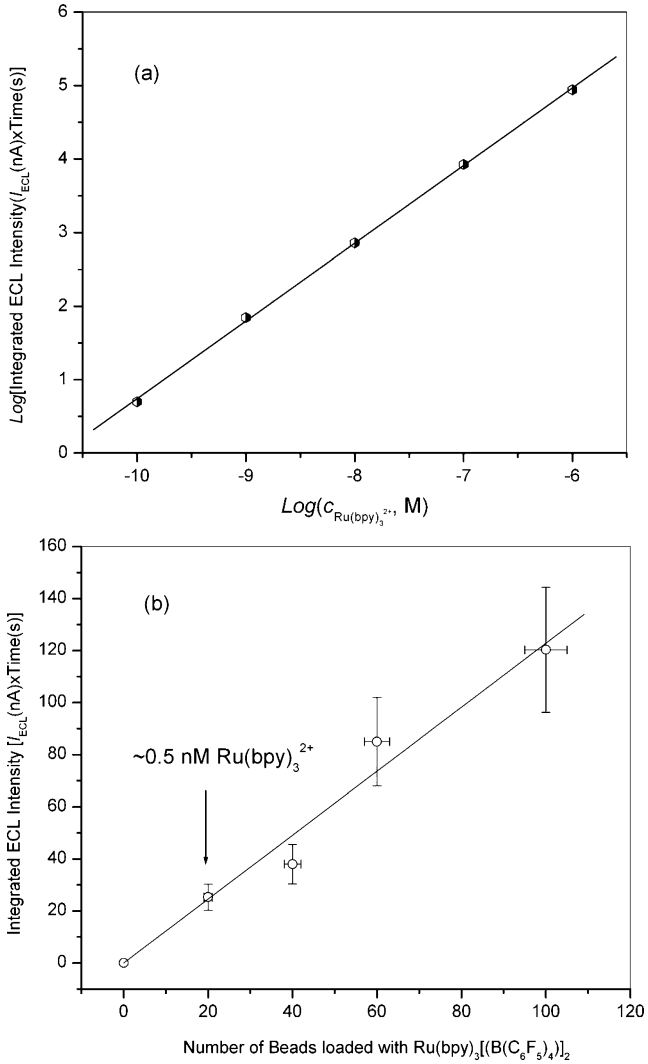

Figure 7. ECL intensity as a function of Ru(bpy)$_3$[B(C$_6$F$_5$)$_4$]$_2$ concentration (a) and the number of 10-μm diameter polystyrene beads loaded with Ru(bpy)$_3$[B(C$_6$F$_5$)$_4$]$_2$ (b). The experiments were carried out in 0.50 mL of 0.10 M TPrA−0.055 M TFAA−0.10 (TBA)BF_4 MeCN−1% H_2O at a 2.2-mm diameter Pt electrode by applying CV potential sweeps between 0 and 3.0 V vs Ag/Ag$^+$ at a scan rate of 50 mV/s. A background subtraction was applied to all data.

ECL Detection of DNA Hybridization. A linear relationship between the ECL intensity and the Ru(bpy)$_3$[B(C$_6$F$_5$)$_4$]$_2$ concentration in a range of 0.10 nM to 1.0 μM was found in 0.10 M TPrA−0.055 M TFAA−0.10 (TBA)BF_4 MeCN with the addition of 1% water (Figure 7a). Under the same experimental conditions, a good correlation between the ECL intensity and the number of 10-μm diameter polystyrene beads loaded with Ru(bpy)$_3$[B(C$_6$F$_5$)$_4$]$_2$

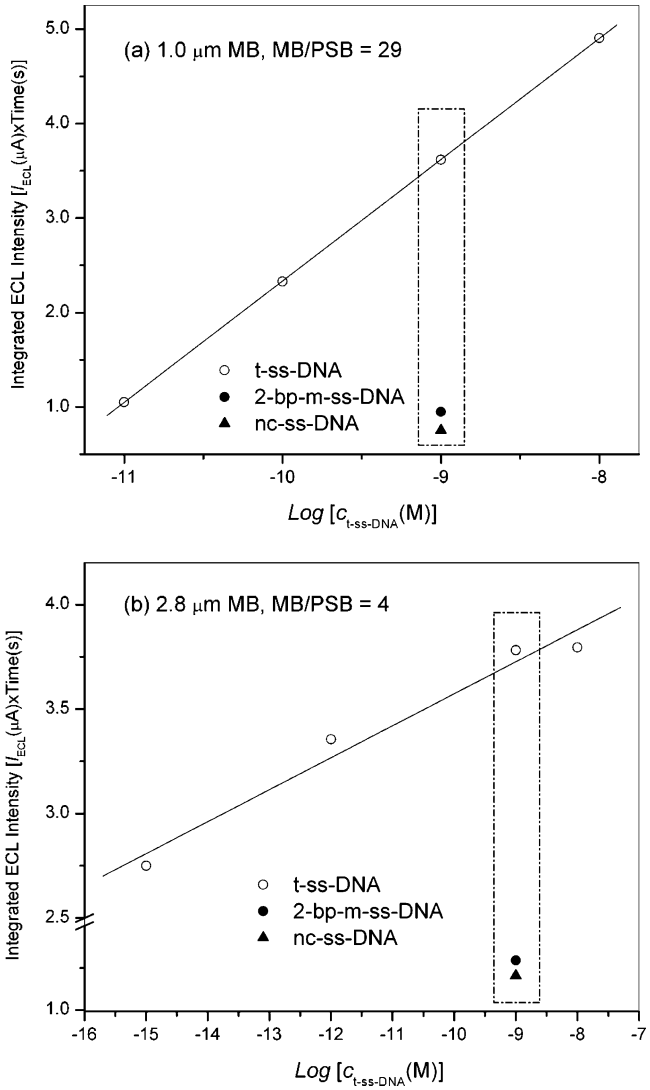

Figure 8. ECL detection of DNA hybridization. (a) The DNA hybridization occurred between probe DNA−MB (1.0 μm) and target DNA−Ru(II)⊂PSB/avidin (10 μm) at a ratio of MB/PSB = 29. (b) The DNA hybridization occurred between probe DNA−MB (2.8 μm) and target DNA−Ru(II)⊂PSB/avidin (10 μm) at a ratio of MB/PSB = 4. No background subtraction was made for the ECL intensity shown. ECL experiments were conducted in 0.50 mL of 0.10 M TPrA−0.055 M TFAA−0.10 M (TBA)BF_4 MeCN solutions between 0 and 3.0 V vs Ag/Ag^+ at a scan rate of 50 mV/s.

was also observed (Figure 7b). The beads were dissolved in 0.50 mL of electrolyte solution and showed that the polystyrene did not produce or affect the ECL signal. By comparing parts a and b of Figure 7, it is clear that the light intensity generated from 20 $Ru(bpy)_3^{2+}$-loaded beads is equivalent to that from 0.5 nM $Ru(bpy)_3^{2+}$, which leads to the loading capacity of the beads of ~7.5 × 10^9 $Ru(bpy)_3[B(C_6F_5)_4]_2$ molecules per bead. This result is consistent with the data obtained on the basis of the "bulk beads" ECL measurement.

Two sets of experiments were designed for the ECL detection of DNA hybridization. In the first, 1.0-μm diameter MB with a ratio of MB to PSB beads of 29 was used. As shown in Figure 8a, in this case, the ECL intensity was proportional to the target DNA concentration over a range of 10 pM to 10 nM, and the two base pair mismatched 2-bp-m-ssDNA and noncomplementary nc-ssDNA can be well distinguished from the complementary DNA hybridization. Note that unlike in the cases shown in Figure 7, no water was deliberately added to the electrolyte solution because the newly formed MB−PSB conjugates and the microcentrifuge tube already contained sufficient water. Note also that no background subtraction was made for the ECL intensity shown in Figure 8. By reducing the ratio of MB to PSB, and hence increasing the amplification factor of $Ru(bpy)_3^{2+}$ to t-ssDNA, DNA hybridizations at much lower concentrations of target DNA should be detectable. Moreover, we desire the maximum number of PSB to be magnetically isolated using a minimum number of MB from the hybridization media, as long as there are a sufficient number to carry out a magnetic separation. As shown in the next section, statistically, one 10-μm diameter PSB can be pulled out with one 2.8-μm diameter MB. As a result, ECL signals related to the DNA hybridization that use a very low target DNA concentration should be detectable when the combination of 2.8-μm MB and 10-μm PSB with a low ratio of MB to PSB is used. Figure 8b shows such an example, in which the ratio 2.8-μm MB/10-μm PSB = 4. The t-ssDNA can be detected at a concentration as low as 1.0 fM. Even with this condition, the obtained ECL intensity is still larger than that obtained from the DNA hybridization of 2-bp-m-ssDNA and nc-ssDNA when 1.0 nM of each species was used.

The Poisson Distribution Test. We thought it is of interest to study the statistical nature of the interaction of the MB and PSB. When a very large number of magnetic beads and polystyrene beads are mixed together, the probability $P(m, n)$ that these two kinds of beads collide and react irreversibly should follow a Poisson distribution that shows the relation between the following two parameters: (a) the initial ratio of magnetic beads to polystyrene beads m and (b) the number of magnetic beads bound to each polystyrene bead n. For example, if an equal number of MB and PS beads are mixed (m =1), the average number of MB bound to PS should be one. However, there will be a distribution, with 37% at $n = 0$ (no magnetic beads on a PSB), 37% at $n = 1$, 18% at $n = 2$, and 6% with $n = 3$ to lead to this average value. The relationship between $P(m, n)$, m, and n can be described by the Poisson distribution as follows:[35]

$$P(m,n) = e^{-m}[m^n/n!] \qquad n = 0, 1, 2, ...$$

Table S2 in the Supporting Information lists the $P(m, n)$ values for different values of m and n. With this table and the Poisson distribution test data (see the Experimental Section for details), one can estimate the minimum number of magnetic beads required to be added to the PSB to bind and pull out one single polystyrene bead from the reaction solution. For example, from

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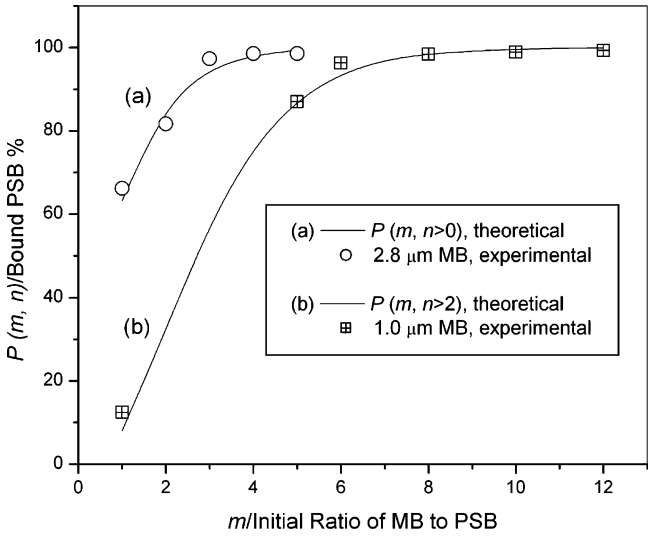

Figure 9. Poisson distribution test using (a) 2.8-μm and (b) 1.0-μm diameter streptavidin-coated MB reacting with 10-μm diameter biotinylated PSB. The "Bound PSB%" was calculated from the number of PSB found from the supernatant after the magnetic separation of MB−PSB conjugates from the reaction media.

the number of PSB collected from the supernatant, the total percentage of the PSB bound with one or more MB and pulled out magnetically from the reaction mixture can be calculated. The "bound PSB%" data with a number of known m values, e.g., m = 1, 2, 3, 4, 5, ..., can then be obtained experimentally. If the data fit the theoretical values of $P(m, n > j)$ (Table S2), where m is known from the experiments conducted, and j = 0, 1, 2, 3, ..., the minimum number of magnetic beads required to bind and pull out one single polystyrene bead must be $j + 1$. Figure 9 shows two examples of such a test. In the first example (Figure 9a), 2.8-μm MB and 10-μm PSB were used, and a minimum number of MB per PSB that can be separated, i.e., $n = 1$, can be deduced. That is, the results are consistent with all PSB that are bound by one or more MB being removed magnetically. In the second example (Figure 9b), instead of 2.8-μm MB, 1.0-μm MB were used to react with 10-μm PSB. In this case a higher minimum MB to PSB binding ratio of 3 for magnetic removal was obtained. These binding ratios can be utilized to optimize experimental conditions for the DNA hybridization between MB and PSB so that a minimum target DNA concentration could be detected.

Extensions. Although the studies described here were confined to DNA and protein−protein interactions, this same technique for ECL amplification can be used in many other formats, e.g., immunoassay in a sandwich format, or the binding of DNAs on the MB and PSB with a DNA strand in solution complementary to both. Similarly many other types of containers for the ECL tag, e.g., liposomes, can replace the PSB. Moreover, the nonaqueous format for the ECL analysis allows other tags, such as aromatic hydrocarbons, to be used as well as annihilation ECL approaches in addition to coreactant ones.

CONCLUSIONS

The ECL of $Ru(bpy)_3^{2+}$ species in MeCN using TPrA as a coreactant has been studied. The background ECL signals produced at a Pt electrode from TPrA alone in a MeCN solution (in the absence of $Ru(bpy)_3^{2+}$) was probably due to the inverse photoemission generated by $TPrA^{\bullet}$ free radical and can be significantly suppressed by adding TFAA and water to the electrolyte solution. ECL detection of DNA hybridization was carried out in 0.10 M TPrA−0.055 TFAA−0.10 M $(TBA)BF_4$ MeCN after the probe DNA−MB ↔ target DNA−Ru(II)⊂PSB/avidin aggregates were dissolved in the above solution. By adjusting the initial ratio of MB/PSB and using different sizes of MB, a very low concentration of t-ssDNA, e.g., 1.0 fM, could be detected and distinguished from 2-bp-mismatch-ssDNA and non-complementary-ssDNA. The interactions between streptavidin-coated MB and biotin-coated PSB beads were found to follow a Poisson distribution, and the minimum number of MB per polystyrene bead for magnetic separation from the reaction medium for two different magnetic bead diameters was also found.

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SUPPORTING INFORMATION AVAILABLE

Binding capacities of 10-μm PSB and 1.0- and 2.8-μm MB; Poisson distribution data obtained from $P(m, n) = e^{-m}[m^n/n!]$. This material is available free of charge via the Internet at http://pubs.acs.org.

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