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Multilabeling Biomolecules at a Single Site. 1. Synthesis and Characterization of a Dendritic Label for Electrochemiluminescence Assays

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Ultrasensitive bioanalytical assays are of great value for early detection of human diseases and pathogens. The sensitivities of immunoassays and DNA probing can be enhanced by multilabeling the biorecognition partner used for affinity-based assays. However, the bioreactivity of biomolecules is affected by a high degree of multilabeling at multiple functional sites. It is proposed that dendritic scaffoldings be used to link multiple signal-generating units to a single site with potentially minimum impact on the bioaffinity. A prototype label, a zeroth-generation dendron, bearing three $[\text{Ru}(\text{bpy})_3]^{2+}$ units for electrochemiluminescence (ECL) assays was synthesized and characterized preliminarily by spectroscopic, electrochemical, and ECL methods. No evidence of interaction between the neighboring $[\text{Ru}(\text{bpy})_3]^{2+}$ units in the label molecule was found from these characterizations. Both the photoluminescence and ECL of the prototype label have features very similar to those of mononuclear $[\text{Ru}(\text{bpy})_3]^{2+}$ compounds. Labeling a model protein with a triad of $[\text{Ru}(\text{bpy})_3]^{2+}$ at one NH_2 position was demonstrated. The results reported here provide support to applying the proposed multilabeling strategy to affinity-based bioanalytical assays.

Affinity-based bioanalytical assays, such as immunoassay and DNA probing, rely largely on the labeling technique, by which signal-generating units are linked to some functional groups of biomolecules that selectively bind to the analytes. For a high signal level in immunoassay, multilabeling at multiple accessible sites (e.g., NH_2) of a protein molecule is normally practiced. However, a high degree of multilabeling may result in the loss of biological activity of the biomolecules,¹ thus high nonspecific binding of the antianalytes and eventually the low signal-to-noise ratio. For some monoclonal antibodies, multilabeling may even lead to the precipitation of proteins.¹ One approach to introduce a large number of label molecules at as few sites as possible is to use carrier proteins.^{2,3} However, this approach involves complicated

Chart 1. Multilabeling a Biomolecule at a Single Site with a Dendritic Label Bearing Multiple Signal-Generating Units

biochemical processes and the carriers themselves are big in size and mass. Here we show, through the synthesis of a prototype label, a concept of multilabeling biomolecules at a single site (Chart 1) using a dendritic platform bearing a plurality of signal-generating units. It is expected that the single-site multilabeling strategy enhances signal intensity with potentially minimum perturbation of the biobinding affinity of the labeled biomolecules and thus helps reduce nonspecific binding in affinity-based bioanalytical assays.

Use of dendrimer⁴ technology for various signal amplification processes in genomic research and clinical diagnostics has been demonstrated by several authors^{5–9} and was reviewed recently.¹⁰ In our proof-of-concept research, a zeroth-generation dendron, succinimidyl-functionalized trinuclear ruthenium(II) tris(bipyri-

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dine) complex (**4** in Scheme 1), was synthesized. The reason for choosing $[\text{Ru}(\text{bpy})_3]^{2+}$ as a signal-generating unit of the prototype multilabeling reagent is 3-fold. First, $[\text{Ru}(\text{bpy})_3]^{2+}$ derivatives are used not only as valuable probes^{11–14} in biological systems such as nucleic acids, peptides, and proteins but also as labels for

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probing.^{16,17} In particular, [Ru(bpy)₃]²⁺-based ECL has been developed into a mature, highly sensitive bioanalytical technology widely employed in basic research laboratories, pharmaceutical industry, clinical settings, and homeland security.^{16–20} For an ECL immunoassay, typically, [4-(*N*-succinimidylcarbonylpropyl)-4'-methyl-2,2'-bipyridine] bis-(2,2'-bipyridine)ruthenium (II) dihexafluorophosphate^{16a} serves as a protein labeling reagent, which, upon electrochemical oxidation and follow-up chemical reduction by the deprotonated tripropylamine radical, generates luminescence through its metal-to-ligand charge-transfer (MLCT) excited state.^{21–24} The trinuclear prototype label with similar [Ru(bpy)₃]²⁺ units and a succinimidyl linking group provides a direct access to the well-formulated assays developed for the commercially available instruments. Second, [Ru(bpy)₃]²⁺, its derivatives, and its other N–N chelating analogues have played a central role in areas of photochemistry, photophysics, photoelectrochemistry, and photoinduced electron and energy transfer for the last two decades.^{25,26} Their multiple, reversible redox states and long-lived photoemission are very useful for us to characterize the prototype assembly and compare it with the available database of the [Ru(bpy)₃]²⁺ family. Third, in addition to ECL, the electrochemical and photoluminescent signals can be utilized as supplementary data to evaluate the multilabeling effect in our research even though these signals are not currently exploited for practical assays.

Although previous studies have demonstrated that, unlike some di- and polynuclear systems with electronically delocalized bridging ligands,^{26,27} the appended [Ru(bpy)₃]²⁺ units at the peripheries of star scaffoldings are noninteracting and retain

their redox and photophysical properties,^{28,29} a concern about the feasibility of the prototype label is still associated with the possible interaction among the very closely packed [Ru(bpy)₃]²⁺ units. In this preliminary account, we report the synthesis, characterization, and ECL of the prototype label and demonstrate multilabeling a model protein with the prototype label at a single NH₂ position.

EXPERIMENTAL SECTION

Chemicals. For synthesis, reagent grade solvents and reactants were used as received unless otherwise specified. For characterization, Ru(bpy)₃Cl₂·6H₂O (Aldrich), tetrabutylammonium hexafluorophosphate (TBAPF₆, Fluka, electrochemical grade), tri-*n*-propylamine (TPA, 99+% Aldrich), bovine serum albumin (BSA, lyophilized powder, Sigma), anhydrous acetonitrile (Aldrich), phosphate buffered saline (PBS, in the form of tablet for preparing solution of pH 7.4, Sigma), and deionized water (18 MΩ) were used as received.

Synthesis. Synthesis of 4-Chloro-2,2'-bipyridine and *cis*-Ru(bpy)₂Cl₂·2H₂O. The procedures were described previously.²⁹

Synthesis of 1. A 7.5-g (5.51 × 10^{−2} mol) aliquot of pentaerythritol and 3 g of KOH were stirred in 15 mL of DMSO for 15 min. A 1.5-g (5.66 × 10^{−3} mol) aliquot of 11-bromoundecanoic acid was dissolved in 5 mL of DMSO and added in eight portions to the pentaerythritol/KOH mixture in a period of 8 h (1 portion/h). The reaction mixture was kept stirred under argon at room temperature for 14 h (total 22 h). The oil-like liquid was poured into 150 mL of water, and the solution was acidified with 1 N HCl to pH 1–2. The precipitate was filtered, washed, and dried to afford 1.38 g of white powder (yield 76%): ¹H NMR (400 MHz, acetone-*d*₆) δ 10.4 (b, 1 H), 3.62 (s, 6 H, 3 CH₂O), 3.46 (s, 2 H, CH₂O), 3.40 (t, 2 H, OCH₂), 2.28 (t, 2 H, CH₂), 1.59 (q, 2 H, CH₂), 1.54 (q, 2 H, CH₂), 1.32 (b, 12 H, 6 CH₂).

Synthesis of 2. A 0.303-g (9.45 × 10^{−4} mol) aliquot of **1** and 2 g of KOH were stirred in 10 mL of DMSO for 10 min. A 0.645-g (3.38 × 10^{−3} mol) aliquot of 4-chloro-2,2'-bipyridine was added. The reaction mixture was kept stirred under argon at 50 °C for 22 h. After reaction, the mixture was poured into 30 mL of water. Extraction with 100 mL of CH₂Cl₂ was tried when the solution was highly alkaline, but it was found difficult to separate the two phases. After evaporation of CH₂Cl₂, the oil was purified by chromatography (silica gel treated with 20% triethylamine in hexane, elution 5–10% methanol in CH₂Cl₂, and pure methanol) and vacuum-dried to afford a sticky transparent product. This was dissolved in methanol and precipitated in acidified water to afford 52 mg of white powder. The remaining water phase was adjusted to pH 8 with NH₃·H₂O. The solution was further extracted with CH₂Cl₂ until no more bipyridine derivatives could be detected by TLC. After evaporation of CH₂Cl₂, the oil was purified by chromatography (silica gel treated with 20% triethylamine in hexane, elution 5–10% methanol in CH₂Cl₂, and pure methanol), vacuum-dried, and precipitated in acidified water to afford 223 mg of product.

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The yield for the combined product is 37%. ^1H NMR (400 MHz, CDCl_3) δ 8.63 (d, 3 H), 8.45 (d, 3 H), 8.32 (d, 3 H), 7.4–8.2 (b, 4 H, NH_4), 7.97 (d, 3 H), 7.76 (t, 3 H), 7.26 (t, 3 H), 6.84 (dd, 3 H), 4.39 (s, 6 H, 3 CH_2O), 3.72 (s, 2 H, CH_2O), 3.38 (t, 2 H, OCH_2), 2.20 (t, 2 H, CH_2), 1.53 (q, 2 H, CH_2), 1.45 (q, 2 H, CH_2), 1.0–1.2 (b, 12 H, 6 CH_2).

Synthesis of 3. 2 (0.102 g, 1.275×10^{-4} mol) and *cis*-Ru(*bpy*) $_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (0.252 g, 4.843×10^{-4} mol) were mixed with 10 mL of methanol and 3 mL of water and refluxed under nitrogen for 24 h. After being cooled to room temperature, the solution was rotoevaporated. The remaining solid was dissolved in 10 mL of water and filtered to remove unreacted *cis*-Ru(*bpy*) $_2\text{Cl}_2$. The filtrate was rotoevaporated and redissolved in 20 mL of water. Three drops of concentrated HCl were added, and the solution was left overnight. The water was rotoevaporated, and the acidification process was repeated with three drops of concentrated HCl in 5 mL of water. The solution was again filtered, rotoevaporated, and dried to afford 0.262 g of dark brown solid **3-Cl** (yield 92%).

The remaining small amount of unreacted *cis*-Ru(*bpy*) $_2\text{Cl}_2$ was further washed out by CH_2Cl_2 . **3-PF₆** was prepared by adding a large excess of saturated NH_4PF_6 /water solution to **3-Cl** water solution. The orange precipitate was filtered, washed with water, and dried. The dried solid was redissolved in acetonitrile and treated with 60% HPF_6 aqueous solution (**Caution**. HPF_6 is corrosive! Incompatible with glass! Avoid breathing fumes!) and then precipitated in dry diethyl ether. After centrifugal separation and vacuum-drying, very pure **3-PF₆** was obtained: ^1H NMR (400 MHz, acetonitrile- d_3) δ 8.59 (d, 3 H), 8.49 (d, 12 H), 8.16 (s, 3 H), 8.04 (m, 15 H), 7.77 (d, 3 H), 7.72 (m, 12 H), 7.46 (d, 3 H), 7.38 (m, 15 H), 6.98 (d, 3 H), 4.46 (s, 6 H, 3 CH_2O), 3.71 (s, 2 H, CH_2O), 3.35 (t, 2 H, OCH_2), 2.13 (t, 2 H, CH_2), 1.35 (m, 4 H, 2 CH_2), 0.95–1.15 (b m, 12 H, 6 CH_2).

Pure **3-Cl** was prepared by replacing PF_6^- with Cl^- . The preparation was carried out by adding an excess of tetrabutylammonium chloride saturated in acetone to the acetone solution of **3-PF₆**, followed by acidification with hydrochloric acid, filtration, and vacuum-drying: ^1H NMR (400 MHz, acetonitrile- d_3) δ 9.19 (d, 3 H), 8.80 (m, 3 H), 8.62 (d m, 12 H), 8.05 (m, 15 H), 7.78 (m, 3 H), 7.70 (m, 12 H), 7.45 (d, 3 H), 7.38 (m 15 H), 7.05 (d, 3 H), 4.62 (s, 6 H, 3 CH_2O), 3.71 (s, 2 H, CH_2O), 3.40 (t, 2 H, OCH_2), 2.19 (t, 2 H, CH_2), 1.34 (m, 2 H, CH_2), 1.28 (m, 2 H, CH_2), 0.90–1.10 (b m, 12 H, 6 CH_2); ^1H NMR (400 MHz, D_2O) δ 8.52 (m, 15 H), 8.25 (m, 3 H), 7.98 (m, 15 H), 7.53–7.80 (m, 18 H), 7.15–7.40 (m, 15 H), 7.06 (m, 3 H), 4.50 (m, 6 H, 3 CH_2O), 3.70 (m, 2 H, CH_2O), 3.39 (t, 2 H, OCH_2), 1.90 (t, 2 H, CH_2), 1.29 (b, 2 H, CH_2), 0.82 (b, 4 H, 2 CH_2), 0.71 (b, 2 H, CH_2), 0.52 (b, 4 H, 2 CH_2), 0.38 (b, 2 H, CH_2), 0.23 (b, 2 H, CH_2).

Synthesis of 4-PF₆. *N,N*-Dicyclohexylcarbodiimide (DCC, 2.31 mg, 1.10×10^{-5} mol) and *N*-hydroxysuccinimide (NHS, 1.36 mg, 1.15×10^{-5} mol) were mixed with **3-PF₆** (16.1 mg, 5.56×10^{-6} mol) in 0.4 mL of acetonitrile and stirred overnight at room temperature. The reaction mixture was injected into 10 mL of dry diethyl ether through a 0.2- μm syringe filter. The orange precipitate was collected by centrifuging and vacuum-dried to afford 11.2 mg of product (yield 67%): ^1H NMR (400 MHz, acetonitrile- d_3) δ 8.68 (d, 3 H), 8.48 (d, 12 H), 8.25 (s, 3 H), 8.04 (m, 15 H), 7.77 (d, 3 H), 7.72 (m, 12 H), 7.45 (d, 3 H), 7.37 (m, 15 H), 6.96 (d, 3 H),

4.47 (s, 6 H, 3 CH_2O), 3.70 (s, 2 H, CH_2O), 3.35 (t, 2 H, OCH_2), 2.76 (s, 4 H), 2.48 (t, 2 H, CH_2), 1.46 (q, 2 H, CH_2), 1.35 (q, 2 H, CH_2), 0.9–1.2 (b m, 12 H, 6 CH_2).

Synthesis of 5. Pentaerythritol (0.07 g, 5.2×10^{-4} mol) and 1 g of KOH were stirred in 5 mL of freshly dried DMSO. The mixture was evacuated for 10 min and saturated with argon. 4-Chloro-2,2'-bipyridine (0.49 g, 2.57×10^{-3} mol, 1.24 equiv/ OH) was added, and the heterogeneous mixture was stirred at 40 °C for 60 h. The resulting suspension was poured into 50 mL of water and neutralized with 1 N HCl. Filtering, washing with pentane, and vacuum-drying afford 0.33 g of white powder **5** (84% yield): ^1H NMR (400 MHz, CDCl_3) δ 8.66 (d, 4 H), 8.50 (d, 4 H), 8.46 (d, 4 H), 8.07 (d, 4 H), 7.83 (t, 4 H), 7.33 (m, 4 H), 6.92 (m, 4 H), 4.61 (s, 8 H, CH_2O).

Synthesis of 6. 5 (0.11 g, 1.46×10^{-4} mol) and *cis*-Ru(*bpy*) $_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (0.413 g, 7.94×10^{-4} mol) were refluxed in 20 mL of absolute ethanol for 44 h. After being cooled to room temperature the solution was filtered. The filtrate was evaporated and redissolved in a small amount of ethanol. The ethanol solution was dropped into dry ether. Centrifuging and drying at 58 °C for 6 h afforded 0.377 g of product **6-Cl** (95% yield): ^1H NMR (400 MHz, methanol- d_4) δ 8.86 (d, 4 H), 8.70 (m, 16 H), 8.51 (d, 4 H), 8.11 (m, 20 H), 7.88 (d, 4 H), 7.79 (m, 16 H), 7.54 (d, 4 H), 7.47 (m, 20 H), 7.24 (dd, 4H), 4.77 (s, 8 H, CH_2O).

6-PF₆ was prepared by adding a large excess of saturated NH_4PF_6 -water solution to **3-Cl** water solution. The orange precipitate was filtered, washed with water and vacuum-dried at 58 °C overnight. ^1H NMR (400 MHz, acetone- d_6) δ 8.78 (m, 16 H), 8.68 (d, 4 H), 8.32 (d, 4 H), 8.18 (m, 16 H), 7.96–8.1 (m, 24 H), 7.71 (d, 4 H), 7.54 (m, 20 H), 7.13 (dd, 4 H), 4.80 (s, 8 H, CH_2O).

Labeling of Protein. **4-PF₆** was dissolved in anhydrous DMF (**Caution**. DMF is toxic and flammable!) and then mixed with BSA in a PBS with a pH of 7.4 at room temperature for 4 h under stirring. The resulting mixture was then transferred to a dialysis tube with cutoff molecular weight of 10 000 for dialysis against PBS solution. After 3 days of dialysis at 277 K, the attachment of the ruthenium assembly to BSA, via a stable amide linkage, was readily visualized by the remaining color in the dialysis tube and further evidenced by the strong orange fluorescence of BSA solution under UV. A comparison dialysis experiment with only **4-PF₆** in PBS showed that there were essentially no ruthenium compounds left in the dialysis tube after 3 days of dialysis against PBS.

Characterization. ^1H NMR spectra were obtained on a Varian Inova (400 MHz) spectrometer. UV–visible spectra were recorded on a Hewlett-Packard 8453 spectrophotometer. Emission spectra were obtained with a Fluorolog spectrofluorometer. Both absorption and emission measurements were carried out in argon-saturated acetonitrile or PBS solutions in a quartz cell with a 1-cm optical path at room temperature. Cyclic voltammetry was performed in a three-electrode configuration in a specially designed electrochemical cell.³⁰ A Pt disk (diameter 1 mm, area 0.785 mm²) sealed in a soft glass rod was employed as the working electrode. It was polished with diamond polishing paste (0.25 μm), rinsed thoroughly with acetone and acetonitrile, and dried by a warm air flow. Pt and Ag wires were used as counter and quasi-reference

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electrodes, respectively. Potentials versus the Ag quasi-reference electrode were then calibrated with the ferrocene/ferrocenium (Fc/Fc^+) redox couple by taking $E^\circ_{\text{Fc}/\text{Fc}^+} = 0.35$ V versus Ag/AgCl (4 M KCl saturated with AgCl). A Parstat 2263-2 Advanced Electrochemical System with PowerSUITE software was used for electrochemical measurement and control. For ECL measurement, a pair of parallel platinum foils (6×24 mm) and Ag/AgCl were employed as working, counter, and reference electrodes in a quartz cell with the working electrode facing the photomultiplier tube of the Fluorolog spectrofluorometer. The TPA-saturated PBS solution for ECL measurement was prepared by adding TPA (nominal concentration 0.2 M) to PBS (pH 7.4) followed by vigorous shaking. The resulting solution has a pH value of ~ 9.0 . Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were recorded on a Perseptive Biochemistry Voyager-DE STR MALDI spectrometer operated in linear mode using sinapinic acid as the matrix.

RESULTS AND DISCUSSION

Synthesis. In the past, we attempted to introduce a free arm in a carbosilane dendrimer system in order to build a bioconjugatable assembly with multiple $[\text{Ru}(\text{bpy})_3]^{2+}$ at the periphery of a second-generation dendritic scaffolding.²⁹ This was unsuccessful due to multiple protective steps required by the synthesis procedures. The present simpler supramolecular assembly with three $[\text{Ru}(\text{bpy})_3]^{2+}$ units (**4** in Scheme 1) is based on a pentaerythritol platform.³¹ First, a symmetric, nonbioconjugatable tetranuclear $[\text{Ru}(\text{bpy})_3]^{2+}$ assembly **6** was synthesized following the procedure described by Astruc et al.^{28b,c,29} The succinimidyl group introduced in the trinuclear assembly **4** is capable of linking the three luminophores to NH_2 sites of proteins through peptide formation under mild conditions. The long C_{11} spacer arm is designed for alleviating steric hindrance between the bulky molecule and protein molecules.

1 was prepared by the reaction of 11-bromoundecanoic acid with a large excess of pentaerythritol in DMSO in the presence of KOH, followed by precipitation in water under acidic conditions. The structure of **1** was confirmed by ^1H NMR, which shows the characteristic features of the two singlets of pentaerythritol-derived methylene groups in a ratio of 3:1 at δ 3.62 and 3.46 ppm in CDCl_3 . These structural features remain in compounds **2–4** and are detected in their NMR spectra. The ligand **2** and trinuclear assembly **3** were synthesized by the same approaches as for **5** and **6**, respectively. However, due to the amphipathy of **2**, which possesses both basic and acidic groups, the separation of **2** from the reaction mixture was difficult. By adjusting pH, we were able to extract and chromatographically purify **2** with a yield of 37% (compared to 84% yield of **5**). Since the cationic assembly of **3** was synthesized in a methanol/water mixture, care had to be taken to ensure that the carboxylic acid group of **3** remains after the formation of metal complex. Hydrochloric acid was added to the aqueous solution of the chloride form (**3-Cl**). For the same reason, acidification of **3-PF₆**, with hexafluorophosphoric acid (**Caution.** HPF_6 is corrosive! Incompatible with glass! Avoid breathing fumes!) was carried out after **3-PF₆** was precipitated by ammonium hexafluorophosphate from the aqueous solution of **3-Cl**.

^1H NMR of **3-PF₆** in deuterated acetonitrile is displayed in Figure 1A. The integration and position of aromatic and aliphatic peaks match the structure perfectly. The assignment of the individual aromatic peaks is difficult due to the existence of diastereoisomers as a result of the three chiral octahedral ruthenium centers.³²

The quantitative high-yield conversion of the carboxylic acid group into an NHS ester is usually performed in DMF in the presence of DCC at low temperatures. We have found that this condition—in DMF, at 0 °C for more than 20 h—resulted in yields lower than 75% according to the NMR spectrum analysis. Because of the difficulty of separating **4** from **3**, a higher conversion rate is required so that further characterization and application can be unambiguously carried out.

The incomplete conversion of the carboxylic acid group into the NHS ester is due probably to the bulky $[\text{Ru}(\text{bpy})_3]^{2+}$ triad, which limits the mobility of the reactive carboxyl group at a lower temperature. To gain a better understanding of the reaction, we performed a kinetic study of the esterification at room temperature in acetonitrile- d_3 by monitoring NMR spectral changes in situ. As can be seen in the NMR spectra provided in the Supporting Information, shifts of two peaks are significant during the reaction, i.e., the singlet peak of CH_2CH_2 in the succinimidyl group shifts from 2.53 (NHS) to 2.76 ppm (ester) and the triplet peak of CH_2 adjacent to the carboxyl group shifts from 2.13 (acid) to 2.48 ppm (ester). The NMR kinetic study demonstrated that the reaction started immediately after mixing **3-PF₆** with NHS and DCC (both in excess). In ~ 95 min, half of **3-PF₆** was converted into **4-PF₆**, and in 5 h, $\sim 80\%$ of **3-PF₆** was converted. After 20 h, complete conversion was evidenced by the disappearance of the triplet at 2.13 ppm and by the equal split of the multiplet at 1.35 ppm (see Figure 1B for the NMR of the isolated **4-PF₆**). To isolate product **4-PF₆**, we dropped the reaction mixture through a $0.2\text{-}\mu\text{m}$ syringe filter into anhydrous diethyl ether. The urea byproduct was filtered out and the unreacted DCC and NHS dissolved in ether, while **4-PF₆** precipitated in ether and was separated by centrifuging. Although some small peaks of impurities can still be found in the NMR spectrum of the isolated **4-PF₆** shown in Figure 1B, these impurity peaks are easily traceable, as they can be found also in the NMR spectra of the kinetic study. As a result of the precipitation in diethyl ether, a small amount of ether, as indicated in Figure 1B, remains in the precipitated solid even after 2 days of vacuum-drying at room temperature.²⁹

Absorption and Emission. The absorption spectra of **4-PF₆** and $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$ in acetonitrile are compared in Figure 2. Like the nonbioconjugatable dendrimer analogues, such as hexa- and octanuclear $[\text{Ru}(\text{bpy})_3]^{2+}$ assemblies with spherical architecture,^{28c,29} a strong MLCT absorption band was found. The absorption maximum of **4-PF₆** is slightly red-shifted, with respect to $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$, from 451 to 456 nm and the extinction coefficient based on Ru unit concentration ($\epsilon_{456} = 13\,900\text{ M}^{-1}\text{ cm}^{-1}$) is slightly lower than that of $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$ ($\epsilon_{451} = 14\,720\text{ M}^{-1}\text{ cm}^{-1}$) but the same as that of the octanuclear $[\text{Ru}(\text{bpy})_3]^{2+}$ dendritic supramolecule.²⁹ As confirmed previously in the octanuclear $[\text{Ru}(\text{bpy})_3]^{2+}$ dendritic systems, the shifts of spectra are due to the tuning effect of the substitution of the bipyridine ligands at the 4-position. The emission spectrum, with a maximum also slightly red-shifted to

(31) Constable, E. C.; Housecroft, C. E.; Cattalini, M.; Phillips, D. *New J. Chem.* **1998**, 193–200.

(32) Keene, F. R. *Chem. Soc. Rev.* **1998**, 27, 185–193.

Figure 1. ^1H NMR in acetonitrile- d_3 of (A) **3**-PF $_6$ and (B) **4**-PF $_6$. The calculated number of aromatic protons is 69.

Figure 2. UV–visible absorption and emission spectra (455 nm excitation) of **4**-PF $_6$ and $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$ recorded in acetonitrile at 293 K. The Ru unit concentration is 40 μM for both compounds.

$\lambda_{\text{max}} = 613 \text{ nm}$ from 605 nm for $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$, is also shown in Figure 2. The intensity at 613 nm is $\sim 75\%$ of the peak intensity of $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$. This value is the same as those obtained from the octanuclear $[\text{Ru}(\text{bpy})_3]^{2+}$ assembly and a model monomer with a similar 4-position substitution reported previously.²⁹ Based on the extinction and relative emission intensity, the emission efficiency of the Ru unit in **4**-PF $_6$ is therefore close to 90% of that of $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$.³³ The good wavelength separation between the MLCT adsorption and emission of $[\text{Ru}(\text{bpy})_3]^{2+}$ prevents largely the occurrence of fluorescence quenching, which is often the case for fluorescent dyes with a small Stokes' shift. The 613-nm emission lifetime is 870 ns, comparable with the published data,

which are in the range from 850 to 1100 ns^{26a} for $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$ under various experimental conditions. As a reference compound closely related to the prototype label molecule, **6**-PF $_6$ has absorption and emission spectra completely identical to those of **4**-PF $_6$ at the same concentration of Ru unit.

Electrochemistry. The cyclic voltammogram (Figure 3) of **4**-PF $_6$ recorded in acetonitrile exhibits a fully reversible metal-centered oxidation wave of process $[\text{Ru}(\text{bpy})_3]^{2+} \rightarrow [\text{Ru}(\text{bpy})_3]^{3+}$ with $E_{1/2}$ at 1.185 V ($\Delta E_p = 61 \text{ mV}$) and three successive reduction waves with E_{pc} at -1.450 , -1.610 , and -1.890 V (vs Ag/AgCl) corresponding to the process of $[\text{Ru}(\text{bpy})_3]^{2+} \rightarrow [\text{Ru}(\text{bpy})_3]^+$, $[\text{Ru}(\text{bpy})_3]^+ \rightarrow [\text{Ru}(\text{bpy})_3]^0$, and $[\text{Ru}(\text{bpy})_3]^0 \rightarrow [\text{Ru}(\text{bpy})_3]^-$, respectively. As previously observed in the octanuclear dendritic system, the reduction process of $[\text{Ru}(\text{bpy})_3]^0$ to $[\text{Ru}(\text{bpy})_3]^-$ shows a very sharp peak as a result of the accumulation³⁴ of the neutral product on the electrode. After the Ru units undergo the third reduction, the $[\text{Ru}(\text{bpy})_3]^-$ units in the compound are, upon potential scan

(33) For practical ECL-based immunoassay and DNA probing, a derivative of $[\text{Ru}(\text{bpy})_3]^{2+}$, such as $[4(N\text{-succinimidylloxycarbonylpropyl})\text{-}4'\text{-methyl-}2,2'\text{-bipyridine}]\text{bis}-(2,2'\text{-bipyridine})\text{ruthenium(II) dihexafluorophosphate}$, is used to label a biomolecule that recognizes the analyte. Since no comparison between $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$ and the commercial labels is readily available, the comparative data related to $\text{Ru}(\text{bpy})_3(\text{PF}_6)_2$ in this paper are meant to provide information only and not intended to give the impression that the prototype label will produce 2.7-fold stronger luminescence in any case. Due to the effect of the substitution in one of the bipyridine rings, meaningful intensity comparison for the absorption and emission can only be made between the $[\text{Ru}(\text{bpy})_3]^{2+}$ triad and a mononuclear $[\text{Ru}(\text{bpy})_3]^{2+}$ with an OR group at the 4-position of a bipyridine ring. This has been discussed and done in ref 29.

(34) Flanagan, J. B.; Margel, S.; Bard, A. J.; Anson, F. C. *J. Am. Chem. Soc.* **1978**, *100*, 4248–4253.

Figure 3. Cyclic voltammogram of **4**-PF₆ (0.40 mM) 1.2 mM Ru unit) in comparison with Ru(bpy)₃(PF₆)₂ (1.0 mM, with added ferrocene as a reference) in acetonitrile at 293 K. Supporting electrolyte, 0.1 M TBAPF₆. Scan rate, 100 mV s⁻¹.

Figure 4. Cyclic voltammograms of **6**-Cl (0.2 mM or 0.8 mM Ru unit) in acetonitrile at 293 K. Supporting electrolyte, 0.1 M TBAPF₆. Scan rate, 50 mV s⁻¹.

reversal, reoxidized in an overall three-electron process to [Ru(bpy)₃]²⁺ showing a wave with the peak potential at -1.420 V. However, if the potential scan is reversed immediately after the first reduction wave, no electroprecipitation but a normal reoxidation peak is observed. If the potential is reversed immediately after the second reduction, a combined two-electron oxidation process leading to [Ru(bpy)₃]²⁺ is observed also at the peak potential of -1.420 V.

The tetranuclear [Ru(bpy)₃]²⁺ assembly **6** displays very similar voltammetrical behavior with a small difference when the potential is reversed after the second reduction wave leading to the accumulation of the redox product bearing four neutral [Ru(bpy)₃]⁰ units. Instead of a combined two-electron wave found in the case of **4**-PF₆, a sharp peak ([Ru(bpy)₃]⁰ → [Ru(bpy)₃]⁺) and a separated normal wave ([Ru(bpy)₃]⁺ → [Ru(bpy)₃]²⁺) can be seen. As an example, the voltammograms of **6**-Cl are shown in Figure 4, in which the redox wave next to the [Ru(bpy)₃]^{2+/3+} wave is associated with Cl^{-/0},³⁵ which does not exist in the compounds with PF₆⁻ as a counterion.

The redox potentials available are compared in Table 1. These values and the discussed features are very much the same, within experimental error, as those reported for the octanuclear dendritic system.²⁹

(35) (a) Tokel, N. E.; Bard, A. J. *J. Am. Chem. Soc.* **1972**, *94*, 2862–2863. (b) In our work, the chloride ion wave was further confirmed by the voltammogram of tetrabutylammonium chloride in acetonitrile.

Table 1. Redox Potentials vs Ag/AgCl in Acetonitrile at 293 K^a

compound	$E_{1/2,ox}$	$E_{pc,rc}$		
		first	second	third
4 -PF ₆	1.185 (0.061)	-1.450	-1.610	-1.890
6 -Cl	1.185 (0.051)	-1.395	-1.545	-1.915
Ru(bpy) ₃ (PF ₆) ₂	1.248 (0.082)	-1.380 (0.058)	-1.570 (0.058)	-1.815 (0.066)

^a The numbers in parentheses are the separation of the peak potentials (ΔE_p) for the completely reversible waves. All the potential values for Ru(bpy)₃(PF₆)₂ are taken as $E_{1/2}$.

The spectroscopic and electrochemical studies of **4**-PF₆ reported here demonstrate that the independence of [Ru(bpy)₃]²⁺ units, as investigated before with several symmetrical systems,^{28,29} holds also for the asymmetric assembly with three closely packed [Ru(bpy)₃]²⁺ units. This noninteracting nature is the foundation for the single-site multilabeling strategy introduced in the beginning of the paper.

Electrochemiluminescence. The ECL of [Ru(bpy)₃]²⁺ and its analogues can be generated in both organic^{27d,29} and aqueous^{16–24} electrolyte solutions in the presence of TPA following the mechanisms proposed for TPA/[Ru(bpy)₃]²⁺ coreactant systems.^{21–24} Among polynuclear metal complexes, dinuclear [Ru(bpy)₃]²⁺ systems with electronically delocalized bridging ligands^{27d} and an octanuclear dendritic system²⁹ with appended [Ru(bpy)₃]²⁺ units have been reported to be able to generate ECL in TPA/ acetonitrile electrolyte solution. For the sake of utilization in the practical bioanalytical settings, the ECL of the trinuclear assembly **3**-Cl³⁶ and tetranuclear assembly **6**-Cl was first examined by using buffer solution (ProCell, pH 6.8) specially designed for commercial ECL immunoassay systems. The ECL spectra of both **3**-Cl and **6**-Cl were found to be the same as their photoluminescence spectra. However, due to the presence of some undisclosed additives in the commercial ECL assay solutions, for the purpose of primary characterization, we prefer to report here the similar ECL measurement results in TPA saturated PBS solutions.³⁷

The potential step method was employed to generate the ECL. The typical waveform is set from initial potential -0.05 V for 5 s to a series of predeminted potentials for 7 s and then to -0.3 V for 5 s. It was found that applying the negative potentials after and before the potential step at the predeminted potentials regenerates the electrode surface state and keeps it constant from experiment to experiment. The values of -0.05 and -0.3 V were determined from the voltammogram of the solution prepared for ECL (see Supporting Information). Figure 5A demonstrates the ECL intensities as a function of time when different potential steps are applied to the working electrode. In

(36) In consideration of both the solubility and the hydrolysis stability of NHS ester, **3**-Cl, instead of **4**-Cl and **4**-PF₆, was used for the measurement in this work.

(37) (a) As one reviewer of this paper pointed out, the generation of the ECL from this solutions with pH ~9.0 may also follow a mechanism, in addition to those discussed in ref 23, involving the reduction of [Ru(bpy)₃]³⁺ to [Ru(bpy)₃]^{2+*} by hydroxyl ion. See ref 38b, in which [Ru(bpy)₃]³⁺ in 9 N sulfuric acid was allowed to react with 9 N NaOH and chemiluminescence was observed. (b) Hercules, D. M.; Lytle, F. E. *J. Am. Chem. Soc.* **1966**, *88*, 4745–4746.

Figure 5. ECL generated by potential step method. (A) Intensity of ECL emission maximum as a function of time and applied potential and (B) chronoamperometric response of **3-Cl**. Solutions: **3-Cl** (0.275 mM or 0.825 mM Ru unit) and Ru(bpy)₃Cl₂ (0.865 mM) in TPA saturated PBS (pH ~9), respectively; reference electrode, Ag/AgCl; background photon counting, <1000.

most cases ($E > 1.05$ V in Figure 5A), the ECL reaches the highest level once the potentials are stepped. It decays with time, due possibly to electrode oxidation³⁸ and the depletion of reactants.³⁹ The current responses recorded for the potential step experiments are given in Figure 5B. By comparing Figure 5A and Figure 5B, one can easily see that although both ECL intensities and currents decay with time, the decay patterns are not the same for all the potential steps and an optimal potential (~ 1.15 V in the experiment of Figure 5A) for a maximum ECL emission exists. At the lowest applied potential (0.95 V in Figure 5A), a slightly higher than background photon counting remains constant over the measurement period. A similar nondecaying behavior was also found for the measurement at 1.05 V. Since the complication of many potential-dependent ECL reaction pathways^{23,37} and Pt electrode surface change³⁸ in aqueous solutions, the time transient profile and the nonmonotonic potential dependence reported here

cannot be thoroughly explained without more comprehensive investigation. It is very likely that different ECL mechanisms^{23,37} exist at different potentials. However, discussion on these issues is beyond the scope of this paper. The results reported here just confirm the ECL activity of the triad.

Before performing the ECL experiments, it was expected that Ru(bpy)₃Cl₂ might have a higher level of ECL emission than that of **3-Cl**—without considering any other factor—simply because of the presumed higher diffusion coefficient of Ru(bpy)₃Cl₂ due to the size difference. However, after repetitive experiments, it was reproducibly confirmed that there is no significant difference in ECL intensities of **3-Cl** and Ru(bpy)₃Cl₂ at the same concentration of Ru unit, no matter whether the ECL experiments were performed in the TPA-saturated PBS or in the commercial ECL assay buffers with unknown ingredients. As comparison, the ECL of Ru(bpy)₃Cl₂ at a potential of 1.15 V is given in Figure 5A. Note that the concentration of Ru(bpy)₃Cl₂ in Figure 5A is slightly higher than the Ru unit concentration of **3-Cl**, and so is the emission intensity. We have to recognize again that the course of generating ECL with TPA involvement is a fairly complicated one and there are many factors governing the cascade of electrochemical/chemical reactions and photophysical process,^{21–24,37}

(38) (a) Anson, F. C.; Lingane, J. J. *J Am. Chem. Soc.* **1957**, *79*, 4901–4904. (b) Angerstein-Kozłowska, H.; Conway, B. E.; Sharp, W. B. A. *J Electroanal. Chem.* **1973**, *43*, 9–36.

(39) Although [Ru(bpy)₃]²⁺ undergoes a circulation, [Ru(bpy)₃]²⁺ → [Ru(bpy)₃]³⁺ → [Ru(bpy)₃]^{2+*} → [Ru(bpy)₃]²⁺, the coreactant TPA is consumed in a series of irreversible electrochemical and chemical reactions. For the details of the mechanism, see refs 21–24 and references therein.

Figure 6. ECL spectra of **3-Cl** (0.275 mM or 0.825 mM Ru unit) and $\text{Ru}(\text{bpy})_3\text{Cl}_2$ (0.865 mM) in TPA-saturated PBS (pH ~ 9). Recorded after the emission became steady at the potential of 1.15 V vs Ag/AgCl.

which lead finally to the luminescence. The surfactant-like structure of **3-Cl** may also have some favorable effects⁴⁰ on its ECL.

A 5-s scan was required to collect ECL emission spectra from 500 to 800 nm in our experimental setting. Therefore, the ECL spectra were recorded when the ECL intensities became relatively steady, normally 4 s after the potentials were stepped to the predetermined levels (see Figure 5A). As expected, within experimental error, the ECL spectra obtained for **3-Cl** at different electrode potentials have the same features as the photoluminescence spectra of **4-PF₆** in acetonitrile (Figure 2), **3-Cl**, and Ru-labeled BSA in the same PBS solutions (Supporting Information), because the emission is the result of the decay of the same MLCT excited state $[\text{Ru}(\text{bpy})_3]^{2+*}$, generated by either photo- or electrochemical excitation. In addition, these spectra are also the same as that of **6** with the same four $[\text{Ru}(\text{bpy})_3]^{2+}$ units. Figure 6 shows the ECL spectrum of **3-Cl** compared with that of $\text{Ru}(\text{bpy})_3\text{Cl}_2$ generated at the potential of 1.15 V.

Labeling of Protein. Protein labeling experiments were carried out by using BSA as a model protein, which is commonly employed as a protein standard in bioanalytical assays and as a molecular weight standard (66 431⁹) for gel permeation chromatography. BSA contains 59 lysines, and 30–35 of these are primary amines capable of reacting with the succinimidyl conjugation group.⁴¹ It should be noted that the chlorides of **3**, **4**, and **6** are very soluble in water. However, due to the generally possible slow hydrolysis of NHS ester in aqueous solutions, we used **4-PF₆**, instead of the water-soluble **4-Cl**, to prepare stock solution for the labeling experiment. Like other hexafluorophosphate salts, **4-PF₆** is very soluble in polar organic solvents such as acetone, acetonitrile, methanol, DMF, and DMSO, but insoluble in water.

The UV–visible absorption of the labeled BSA in PBS solution has the ligand-centered transition absorption at 286 nm and the MLCT absorption at 458 nm, which is slightly red-shifted with respect to its MLCT absorption band in acetonitrile. The average number of $[\text{Ru}(\text{bpy})_3]^{2+}$ units attached to a BSA molecule was determined by the absorbance peaks at 286 and 458 nm, assuming the extinction coefficients for the free and BSA-bound trinuclear

assemblies are the same. **3-Cl** (extinction coefficients in PBS based on Ru unit are $\epsilon_{456} = 11\,500\text{ M}^{-1}\text{ cm}^{-1}$ and $\epsilon_{286} = 57\,400\text{ M}^{-1}\text{ cm}^{-1}$) was used as a reference in PBS. In one labeling experiment with the initial molar ratio of **4-PF₆** to BSA set as 5.1:1, we found that on average four triads, i.e., 12 $[\text{Ru}(\text{bpy})_3]^{2+}$ units were bound to a BSA molecule.

The binding of the prototype label to the BSA and the number of bound $[\text{Ru}(\text{bpy})_3]^{2+}$ units were further confirmed by MALDI-TOF mass spectrum. The mass spectra in Figure 7 demonstrates the BSA triply labeled with $[\text{Ru}(\text{bpy})_3]^{2+}$ at a single site. Compared to the measured BSA mass of 66 503 Da, the peak with m/z at 68 481 Da indicates the labeled BSA has a mass increase of ~ 1978 Da, which—assuming that all six PF_6^- moieties were lost during the ionization process—is in excellent agreement with the calculated value of 2005.25 Da within the general mass error of 0.5% for protein MALDI-TOF mass spectra. For the purpose of internal reference in Figure 7, the BSA used for labeling was in excess (the molar ratio of BSA to **4-PF₆** was 1.2:1). However, a shoulder at $\sim 70\,551$ Da (4048 Da shift from 66 503 Da) is apparent, indicative of a small amount of BSA labeled with two $[\text{Ru}(\text{bpy})_3]^{2+}$ triads, i.e., six $[\text{Ru}(\text{bpy})_3]^{2+}$ units (calculated mass increase 4010.50 Da, assuming 12 PF_6^- moieties lost). The mass spectrum of the pristine BSA is also exhibited in Figure 7, showing a single peak at 66 503 Da and ruling out any concern about the existence of impurities in the displayed mass scale. The MALDI-TOF mass spectra in Figure 7 represent direct and clear evidence for the successful multilabeling with $[\text{Ru}(\text{bpy})_3]^{2+}$ triad at a single site of a protein molecule.

ECL was observed also from the Ru-labeled BSA in a TPA/PBS solution, indicating that the $[\text{Ru}(\text{bpy})_3]^{2+}$ attached to protein remain ECL active. Although we have not conducted a comparative ECL immunoassay with a multireporter label and a similar monoreporter label (see ref 33 for the concern of a meaningful comparison), which will need much more effort, including establishing well-defined labeling procedures for controlled labeling levels and careful characterization of the labeled antianalytes, we hope that using a dendritic platform bearing multiple signal-generating units for multilabeling biomolecules will enhance signal intensity by increased number of reporters or reduce nonspecific binding by using fewer functional groups of biomolecules while maintaining the same labeling level.

CONCLUSIONS

For the purpose of multilabeling biomolecules at a single site in bioanalytical science, a dendritic prototype label with three $[\text{Ru}(\text{bpy})_3]^{2+}$ linked to a succinimidyl group was synthesized and characterized by structural, photophysical, and electrochemical methods. The confirmed independence of each $[\text{Ru}(\text{bpy})_3]^{2+}$ unit, the covalent attachment of the trinuclear $[\text{Ru}(\text{bpy})_3]^{2+}$ assembly to BSA in PBS, and the generation of ECL in tripropylamine-containing aqueous buffer solution substantiate the applicability of the novel multilabeling strategy to the established ECL assays. Although the prototype label is demonstrated only for ECL-based assays in this preliminary work, it is likely applicable to other analytical techniques based on $[\text{Ru}(\text{bpy})_3]^{2+}$ labeling such as fluorescence polarization immunoassay and fluorescence lifetime imaging. Moreover, the concept of single-site multilabeling of biologically active substances for enhanced signal with minimum impact on bioreactivities could be generally utilized for other

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(41) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, 1996; p 423.

Figure 7. MALDI-TOF mass spectra of Ru-labeled BSA mixed with excess BSA (black) and pristine BSA (red). The labeled BSA mixture used for mass spectrum analysis was further desalted after dialysis against PBS. Sinapinic acid was used as matrix.

labeling-dependent technologies, which use radioactive isotopes, fluorescent dyes, or other redox-active or luminescent metal complexes as signal-generating units. The increasing interests in dendritic and supramolecular systems have resulted in many synthetic strategies for assembling more signal-generating units into suitable molecular platforms. Based on the availability of the triad label and the preliminary characterization, further research work is currently under way in our laboratories.

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SUPPORTING INFORMATION AVAILABLE

NMR kinetic data, absorption and emission spectra of other related compounds, and cyclic voltammogram of ECL solution. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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