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Electrogenerated Chemiluminescence Detection in Reversed-Phase Liquid Chromatography

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Electrogenerated chemiluminescence (ECL) has been developed as a detection method for liquid chromatography. The radical cation of tri-*p*-tolylamine (TPTA) is used as a common electron acceptor for the electrogenerated radical anions of a variety of organic analytes. ECL is accomplished with a high-frequency potential pulse program applied to a microelectrode immersed in the column eluent. ECL detection is demonstrated with reversed-phase liquid chromatography. Selectivity at the ECL detector is shown to be tunable based on differing electrochemical conditions and excited-state energetics. Low minimum detection limits in ECL are attributed to the dependence on the photon detector shot noise, allowing a limit of detection of 0.14 nM for perylene in the presence of 0.1 mM TPTA. A derivatization agent useful for ECL detection is demonstrated by the use of naphthalene-2,3-dicarboxaldehyde. This reagent, which does not itself result in ECL, forms ECL candidates following reaction with primary amines.

Chemiluminescence (CL) detection is a highly sensitive method for use following liquid chromatographic separations.[1−3] The sensitivity of CL detection arises because the emissive species is excited through a chemical reaction rather than through laser excitation.[1,4,5] Thus, noise in typical luminescent measurements associated with the illumination source (Raman and Raleigh scattering, source fluctuations) is avoided, and detection is limited only by chemical noise and the electronic noise of the detector.[1,4,5] In addition to its high sensitivity, CL detection requires inexpensive and simple equipment and offers a wide linear dynamic range.[1,4,6]

Electrogenerated chemiluminescence (ECL), where emissive excited states are produced by reactions between electrogenerated intermediates, offers several further advantages over CL detection.[6,7] Since the reactive intermediates that yield the emissive excited state are generated at the electrode surface, emission is localized in a well-defined area. This facilitates placement of the photon detector. In many ECL schemes, starting materials are regenerated and can be recycled to produce multiple photons per analyte molecule. Careful control of the applied potential also introduces a degree of selectivity over the CL experiment. In addition, the reagents for ECL are not restricted to stable species as in CL.

ECL from polyaromatic hydrocarbons (PAHs) can arise from the following sequence of reactions.

$$A + e^- \rightarrow A^{\bullet -} \quad (1)$$

$$D - e^- \rightarrow D^{\bullet +} \quad (2)$$

$$A^{\bullet -} + D^{\bullet +} \rightarrow {}^1A^* + D \quad (3)$$

$${}^1A^* \rightarrow A + h\nu \quad (4)$$

where the electron acceptors (A) and electron donors (D), which can be the same species, are alternately reduced and oxidized at the electrode surface to form radical ions (reactions 1 and 2). When the radical ions diffuse together, electron transfer can produce an excited state (reaction 3). Formation of two ground-state molecules is kinetically slow, lying in the Marcus inverted region. Formation of excited-state triplets may occur if the energetics are insufficient to form the singlet excited state.[8] In the case of triplet formation, singlets can arise from annihilation of two excited-state triplets (reaction 5):

$${}^3A^* + {}^3A^* \rightarrow {}^1A^* + A \quad (5)$$

The excited-state singlet, whether formed directly in the singlet route mechanism (reaction 3) or through triplet−triplet annihilation (reaction 5), relaxes giving photon emission (reaction 4).

ECL has been used for detection of PAHs following chromatographic separation.[9−11] However, macroelectrodes were used with extreme overpotentials and dc or low-frequency potential steps. Bubble formation was a significant problem, and light production occurred predominately near the anode, presumably due to the instability of the radical anions being formed. Microelectrodes, with fast potential steps, can dramatically improve the apparent ECL quantum efficiency since the short reaction time can

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discriminate against competitive side reactions.[12−15] Microelectrodes also simplify the experimental setup since a three-electrode potentiostat is unnecessary. This paper describes ECL utilizing microelectrodes and rapid potential steps and its use as a detector for liquid chromatography.

EXPERIMENTAL SECTION

Electrodes. Electrode fabrication has been reported previously.[12,16] Briefly, microelectrodes were constructed by attaching leads to fine platinum or gold wires and to silver foil (0.28 mm thick) and then sealing them in an insulating epoxy (Epon 828 with 14% *m*-phenylenediamine, Miller Stephenson, Danbury, CT). Sanding with successively finer grit sandpaper, and finally polishing with diamond paste, revealed a flat disk working electrode (WE) and silver band quasi-reference electrode (QRE).

ECL Flow Cell. The ECL flow cell for this work was modified from similar cells reported previously for flow injection analysis.[12,16] A thin polyethylene spacer (0.002 in., U-PZBT-2/3, Small Parts Inc., Miami Lakes, FL) placed against the epoxy-sealed electrode defined the flow cell dimensions and yielded a cell volume of ∼4 μL. Two narrow-bore (0.02 in.) channels were used to flow solution through the flow cell. An optical window (glass) was placed opposite the electrode to allow light collection. The flow cell was placed inside an aluminum light-tight box and wire mesh Faraday cage.

Methods. Cyclic voltammetry studies employed a Ag/AgCl reference electrode (leakless, EE009, Cypress Systems, Lawrence, KS, or RE-5B, BAS, West Lafayette, IN, in a sidearm electrochemical cell). Steady-state cyclic voltammetry was acquired with a commercial potentiostat (CV-37, BAS). Fast-scan cyclic voltammetry was acquired using two electrodes, and the current was measured with a fast current-to-voltage converter built in-house and a digital oscilloscope (TDS 3052, Tektronix, Wilsonville, OR). In fast-scan cyclic voltammetry or ECL generation, the applied potential was controlled with an arbitrary waveform generator (1050, HP, Englewood, CO). For ECL generation, a continuous square wave was applied to the cell and the potential was controlled to alternately generate radical cations or anions with an overpotential of 200−500 mV based on prior cyclic voltammograms.

ECL photons were detected with a detector placed near the optical window of the ECL flow cell. Either a fiber-optic spectrometer (S2000, Ocean Optics Inc., Dunedin, FL) or photon-counting (R4632 or R2295, Hamamatsu, Bridgewater, NJ) photomultiplier tube (PMT) was used. Pulses from the PMT were evaluated with a multichannel scaler (Turbo-MCS, EG&G Ortec, Oak Ridge, TN). Unless otherwise noted, photons were binned at a sampling rate of 10 Hz, and excess noise was removed with a digital low-pass filter (Componentworks, National Instruments, Austin, TX) with a 0.2-Hz cutoff.

In flow injection mode, a stainless steel syringe pump (SFC-500, Isco, Lincoln, NE) directed mobile phase through a loop injector (7010, Rheodyne, Rohnert Park, CA) to the ECL cell. For chromatography, an ODS column (00F-4114-E0, Phenomenex, Torrance, CA) was inserted between the injector and detection cell. Postcolumn addition of reagents (10:1) was accomplished with a second syringe pump (μ-LC500, Isco) and a mixing T immediately following the column. In some cases, an absorbance detector (33120A, HP) operated at 358 nm was used. The absorbance was digitized (PCI 1200, National Instruments) under software control (Visual Basic, Microsoft). Solutions used in chromatography were filtered (0.2-μm PTFE, Nalge Nunc International, Rochester, NY). All solutions were thoroughly deoxygenated prior to electrochemical experiments by purging with nitrogen.

Derivatization. Naphthalene-2,3-dicarboxaldehyde (NDA) was used to derivatize butylamine as reported previously.[17] Briefly, NDA was reacted with *n*-butylamine and sodium cyanide in methanol to produce the cyanobenzisoindole derivative (CBI_{buN}). The reaction mixture was then purified on a silica gel column with methylene chloride as the eluent. Further recrystallization from methylene chloride−hexane yielded the pure product. Melting point analysis, NMR, UV−visible absorbance, liquid chromatography, and fluorescence experiments established the authenticity of the derivative (data not shown).[17,18] CBI_{buN} was stored at −15 °C in the dark.

Reagents. Diphenylanthracene (DPA, Fluka Chemika, Milwaukee, WI) and 9-phenylanthracene (9-PA, Aldrich, Milwaukee, MI) were recrystallized twice from hot ethanol. Tetrabutylammonium hexafluorophosphate ($TBAPF_6$, Fluka) was dried in a vacuum oven at 60 °C for 4 h. Tri-*p*-tolylamine (TPTA, Aldrich), perylene (Aldrich), naphthylene-2,3-dicarboxaldehyde (NDA, Molecular Probes, Eugene, OR), and butylamine (Fluka Chemika), which was stored under nitrogen, were used as received. All compounds were stored in a dry, dark environment prior to use. Acetonitrile (MeCN, UV grade, Burdick and Jackson, Muskegon, MI), methanol (Lab Grade, Mallinckrodt, Paris, KY), dichloromethane (Analytical Reagent, Mallinckrodt), and hexane (Spectrophotometric Grade, Aldrich) were used as received. Sodium cyanide (ACS Reagent, Aldrich) is highly toxic and should only be used in a chemical fume hood with chemical-resistant gloves.

RESULTS AND DISCUSSION

Rapid Potential Step ECL for PAHs. For ECL generation, the potential of a microelectrode was rapidly stepped negative and positive to produce the radical anions and cations in sequential steps. The ECL flux, which is governed by the generation of these radical ion precursors, is largest immediately following each potential step and then decays as shown in Figure 1 (inset) for 9-PA. Photons are produced by the following reaction:

$$9\text{-PA}^{\bullet -} + 9\text{-PA}^{\bullet +} \rightarrow {}^{1}9\text{-PA}^{*} + 9\text{-PA} \qquad (6)$$

which has sufficient energy (3.2 eV) to produce the excited state directly and light of equal amplitude is seen on each step. This behavior matches simulated behavior reported previously.[8,19] Emission from several steps can be binned to give a dc signal.

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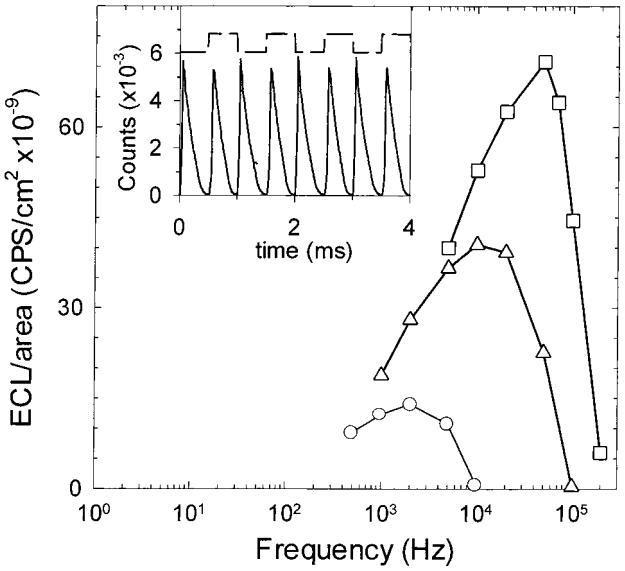

Figure 1. Light flux in rapid potential step ECL experiments. The transient flux (inset) shows pulses of light (solid) concurrent with each step of the applied square wave (dashed) for a 38-μm-radius disk electrode in a 1 mM 9-PA solution. Light was collected in 10-μs bins, and 100 traces were summed. The time-averaged ECL signal (main panel, normalized by electrode area) is shown for 38- (circle), 13- (triangle), and 5-μm (square)-radius disk electrodes in a 0.5 mM 9-PA solution where light was collected for 1 s to generate each point. In both cases, ECL was generated by stepping the electrode potential ~500 mV more positive or negative than radical cation or anion formation, as judged by cyclic voltammetry immediately prior to the ECL experiment.

The intensity of the dc level of the ECL depends on the frequency of the potential step program as well as the electrode size as shown in Figure 1 (main panel). In this figure, the ECL signal is normalized by the electrode area. In each case, the ECL signal increases with frequency as expected for a diffusion-controlled process. However, due to the delay caused by double-layer charging, the electrode potential does not reach the potentials necessary for radical ion production within the potential step at very high frequencies. Under these conditions, the ECL intensity decreases.[12] Thus, there is an optimum frequency for each electrode size, with smaller electrodes being "tuned" for higher frequencies because of their smaller cell time constant. Although higher frequencies improve the ECL efficiency by discriminating against non-light-producing reactions, larger electrodes give a brighter ECL response because of their larger area. For the ECL detection presented here, a 38-μm-radius electrode was chosen and was operated at a frequency of 1 kHz.

The mode of ECL used to generate light in Figure 1 (radical cation and anion generation from the same species) can be used to detect many PAHs (e.g., DPA, 9-PA, perylene).[20] However, since the rate of excited-state formation depends on the concentration of both the radical anion and radical cation of the same species, the dependence of the ECL intensity on the neutral analyte concentration is expected to be complex. Indeed, a logarithmic plot of the ECL intensity versus [9-PA] gave a slope of 1.65 (data not shown).

Selective Detection of PAHs. An alternate approach is to use a separate reaction partner, either the donor or acceptor, in excess. This simplifies the dependence on the analyte concentration, giving a first-order response in the case of a singlet mechanism or a second-order response for triplet−triplet annihilation.[8] The use of a reaction partner at very high concentrations also improves the ECL sensitivity by ensuring that every analyte radical ion formed will encounter a reaction partner, increasing the yield of emissive excited states (reaction 3). For this work, the radical cation of TPTA was used as a common electron acceptor to partner with multiple analytes. TPTA was chosen because it does not emit during ECL, is commercially available, reversibly forms a radical cation, and is reduced at a potential much more negative than any of the analytes studied here. The reaction yielding excited states is

$$PAH^{\bullet -} + TPTA^{\bullet +} \rightarrow PAH^* + TPTA \qquad (7)$$

In this case, the excited state is either a triplet or an excited singlet of the PAH.

ECL detection was coupled to reversed-phase chromatography by directing the column eluent through the ECL flow cell. TPTA was present in both the mobile phase and injected sample, so TPTA radical cations were constantly generated throughout the experiment. However, ECL was only seen as PAHs (i.e., 9-PA, DPA, and perylene) passed through the flow cell as shown in the solid trace of Figure 2B. ECL emission from systems similar to TPTA/DPA and TPTA/9-PA has been shown to arise from triplet−triplet annihilation (eq 5) because the reaction energy of eq 7 is insufficient to form excited singlets directly.[8] In the case of TPTA/perylene, however, the ECL response was found to be first order with respect to perylene concentration, indicating direct population of perylene excited singlets (2.83 eV[21]). The higher ECL sensitivity to perylene as compared to 9-PA and DPA is attributed to the energy-sufficient mechanism. Increased sensitivity and a first-order ECL response seem to indicate that ECL detection holds more promise when emission occurs via the singlet route.

Because electrochemical reduction is necessary for ECL detection, the selectivity can be adjusted by varying the applied potential. The dashed trace in Figure 2B shows ECL detection for an injection of the same PAHs where the electrode potential was insufficient to generate the radical anions of 9-PA or DPA, although perylene was reduced. Under these conditions, 9-PA and DPA are undetected. The slight decrease in the perylene response is consistent with the decrease in reduction overpotential.

Derivatization for ECL Detection. ECL detection is only useful for a limited number of compounds. Most substrates lack either the appropriate electrochemistry or accessible, emissive excited states. To circumvent this restriction, derivatization was explored as a method to allow ECL detection of primary amines. ECL of derivatives has been demonstrated previously by using a derivatizing agent that was itself detectable by ECL.[22] However, analysis was complicated by multiple chromatographic peaks attributed to excess derivatizing reagents. Here an alternative derivatization scheme for ECL is proposed using NDA. NDA reacts rapidly with primary amines in the presence of CN^- to form a cyanobenzisoindole (CBI) derivative, and the derivative of butylamine (CBI_{buN}) was synthesized for these experiments as a representative primary amine.[17] The derivative was purified in this

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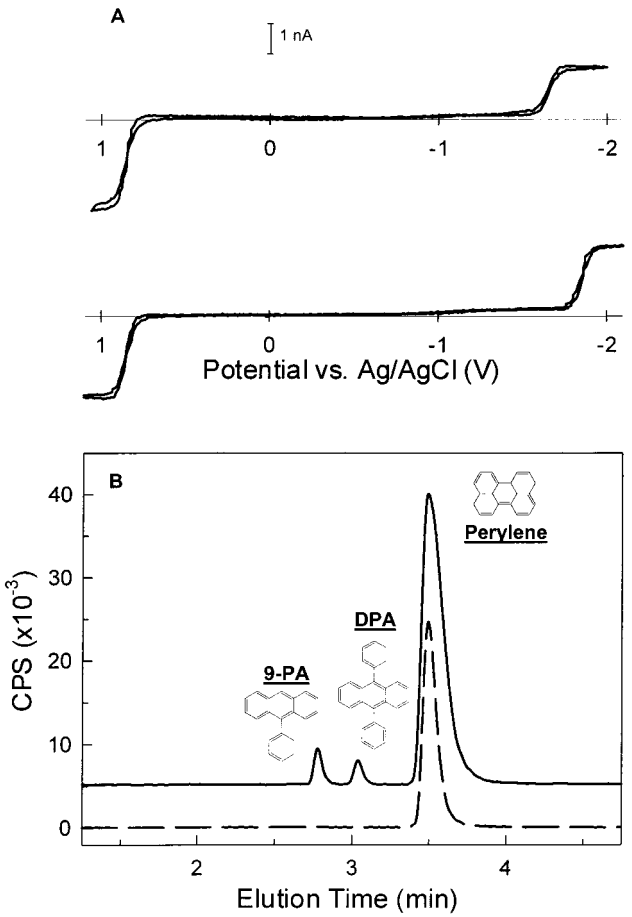

Figure 2. Cyclic voltammetry and ECL detection of PAHs with TPTA radical cation as a common electron acceptor. Cyclic voltammetry of 1 mM TPTA and 0.46 mM perylene (top) or 0.77 mM DPA (bottom) is attained at a 5-μm-radius gold disk electrode at $\nu = 0.1$ V/s. (9-PA electrochemistry was identical to that of DPA and is not shown here.) Reversed-phase LC separation is shown with ECL detection for an injection of a mixture containing 1 nmol of 9-PA, DPA, and perylene. TPTA (0.1 mM) and $TBAPF_6$ (0.1 M) were in both the mobile phase (0.6 mL/min) and sample. The ECL response was obtained at a 38-μm-radius platinum disk electrode stepped between 1.4 and −2.3 V (solid, offset by 5 kcounts/s) or −2.0 V (dashed) at 1 kHz.

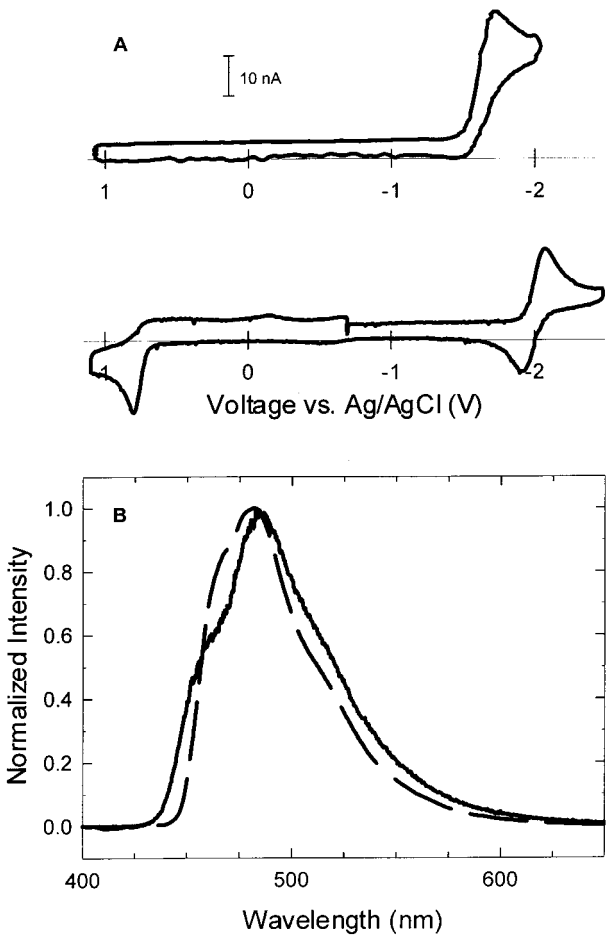

Figure 3. Electrochemistry and spectra for CBI_{buN}. Cyclic voltammetry for 3 mM NDA (top) and 2 mM CBI_{buN} (bottom) is attained at a 5-μm-radius gold disk electrode ($\nu = 50$ V/s). Fluorescence spectra for 0.5 mM CBI_{buN} (dashed) closely matches the spectra for ECL from 0.25 mM CBI_{buN} and 2.5 mM TPTA (solid). ECL was generated by stepping a 38-μm-radius electrode at 1 kHz between −2.3 and 1.3 V.

work to facilitate electrochemical characterization, though it would not be necessary in routine chromatographic analyses. The electrochemistry of NDA and CBI_{buN} is shown in Figure 3A. The irreversible oxidation of the derivative has been used previously for oxidative amperometric detection.[23] However, the reversible reduction of CBI derivatives ($E_{pc} = -2.0$ V for CBI_{buN}) has not been previously reported. Although ECL generation using CBI_{buN} as both an electron donor and acceptor gave a stable luminescent response (data not shown), the signal was improved by coupling the more stable CBI_{buN} reduction product with TPTA radical cations. Comparison of the potential separation of CBI_{buN} reduction and TPTA oxidation (2.79 eV) to the excited-state singlet energy for CBI_{buN} (2.75 eV estimated from absorbance and fluorescence spectra in ref 18) predicted that ECL would proceed via the singlet mechanism:

$$CBI_{buN}^{\bullet -} + TPTA^{\bullet +} \rightarrow CBII_{buN}^{*} + TPTA \qquad (8)$$

This was confirmed experimentally by a logarithmic plot of the ECL emission intensity versus CBI_{buN} concentration which gave a first-order dependence (data not shown). A spectrum of the ECL emission following TPTA oxidation and CBI_{buN} reduction compared favorably with a spectrum of CBI_{buN} fluorescence, as shown in Figure 3B, confirming that CBI_{buN}* was the emissive species in both cases. The detection limit for CBI_{buN} was determined to be 1 nM in the presence of 0.25 mM TPTA. No ECL response was detected for similar analysis of any of the derivatization reagents (NDA, butylamine, sodium cyanide).

ECL detection of derivatized butylamine following reversed-phase liquid chromatography is demonstrated in Figure 4. Absorbance detection of NDA and CBI_{buN} (Figure 4a,b) indicated similar retention times. When both species were injected, the response to CBI_{buN} was indistinguishable from the much larger NDA absorbance. However, since NDA lacks an accessible excited state,[24] it gives no ECL emission even though it exhibits reductive electrochemistry (Figure 4c). Since CBI_{buN} does give an ECL response (Figure 4d), detection of the derivative was facilitated. When both species were simultaneously injected, the CBI_{buN} emission was easily detected; however, the presence of excess

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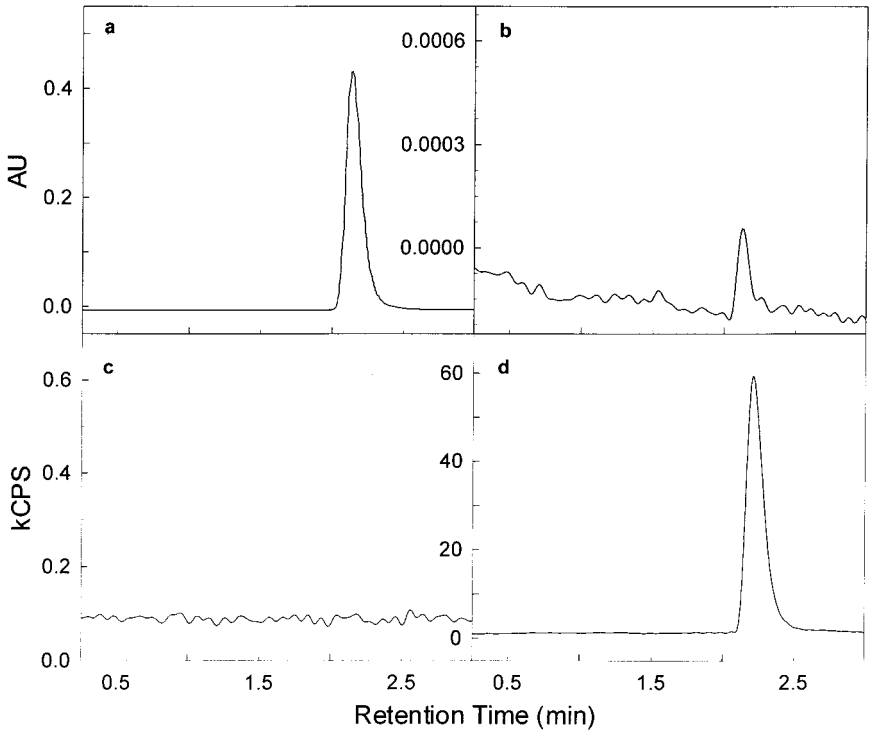

Figure 4. Absorbance (a, b) and ECL (c, d) detection of CBI_{buN} and NDA eluting from a reversed-phase LC column. Injections contained 20 nmol of NDA (a, c) or 580 pmol of CBI_{buN} (b, d). ECL detection employed a 38-μm-radius platinum electrode stepped between −2.4 and 1.4 V at 1 kHz. The mobile phase was MeCN with a flow rate of 0.6 mL/min; for ECL detection, postcolumn addition give final concentrations of 0.1 M $TBAPF_6$ and 0.25 mM TPTA.

NDA resulted in an attenuation (∼50%) of the CBI_{buN} ECL response. This is attributed to homogeneous electron transfer between the reduced CBI_{buN} and neutral NDA in bulk solution as might be expected from the cyclic voltammetric data (Figure 3A).

ECL Sensitivity. Since there is no source of luminescence in ECL apart from the electrogenerated reaction, ECL detection can be very sensitive. Detection of individual molecular reactions has been accomplished previously via ECL.[25] The predominant source of interfering noise in the fast potential step ECL experiment arises from the shot noise of the PMT. In the experiments shown, the background was ∼100 counts/s, as seen in Figure 4c. In this experiment, the PMT and ECL cell were separated by 60 mm. With a head-on PMT (R2295) placed ∼3 mm from the ECL source, the sensitivity was greater by nearly 3 orders of magnitude, giving a limit of detection by flow injection analysis of 140 pM for perylene in the presence of 0.1 mM TPTA. This represents much lower detection limits than reported in previous ECL detection schemes.[10]

The presence of an electrolyte at high concentrations (∼0.1 M) in the chromatographic mobile phase can limit separation efficiencies and shorten column life. To avoid this condition, postcolumn addition of TPTA and TBAH was explored. A 1:10 dilution allowed ECL detection with minimal (12%) loss of sensitivity. Ongoing work is directed at the development of smaller ECL flow cells compatible with capillary columns.

CONCLUSIONS

The ability of high-frequency potential pulses at microelectrodes to provide efficient ECL signals without the traditional requirements for exceedingly pure solvents and chemicals allows ECL to be used as a detection method for chromatographic analyses. The sensitivity of ECL, which is attributed to its dependence on the shot noise of the photon detector, can be improved by addition of a reaction partner (e.g., TPTA) in excess concentrations. This has the added benefit of giving a well-defined dependence of the ECL response on the analyte concentration. Selectivity in ECL detection arises from the double requirement that analytes must possess both appropriate electrochemistry and accessible emissive excited states. Chromatographic separation, coupled with ECL detection of PAHs that exhibit a native ECL signal, is demonstrated with TPTA as a common electron donor for multiple analytes. Replicate injections with different applied potentials at the microelectrode demonstrates the ability to tune ECL detection to discriminate between analytes based on their electrochemistry. ECL detection of butylamine, which exhibits an ECL response only when derivatized, shows that whole new classes of analytes can be investigated. Detection of CBI_{buN} in the presence of excess NDA demonstrates ECL selectivity based on the accessibility of emissive excited states.

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