Subattomole Fluorescence Determination of Catecholamines in Capillary Electrophoresis Effluent Streams

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Capillary electrophoresis (CE) is a valuable tool for characterizing multicomponent femtoliter to nanoliter samples, including those encountered in quantitative assays of individual cells.[1] Chemical characterization of individual neurotransmitter secretory vesicles—a goal critical to understanding communication between neurons—is particularly challenging because each structure is likely to contain only 10^3 to 10^6 molecules. Of the various high-sensitivity measurement approaches that have been coupled to electrophoretic separations, amperometry and laser-induced fluorescence (LIF) have been the most successful for measuring extremely small quantities of neurotransmitters.

Although numerous neurotransmitter species can be analyzed in circumstances that permit electrogenic or fluorogenic derivatization, only the monoamines have been measured at low levels in their native chemical states. End-column electrochemical oxidation of both the *catecholamines* (tyrosine derivatives) and *indoleamines* (tryptophan derivatives) after fractionation with CE provides detection limits at the attomole level.[2] Ultraviolet (UV) excited fluorescence detection with CE also has approached attomole detection limits for the indoleamine serotonin,[3] but is orders of magnitude less sensitive for measuring catecholamines because of the poor photophysical properties of these species (small excitation cross sections for $S_0 \rightarrow S_1$ transitions, small Stokes shifts, and short emission wavelengths).

Here, we describe an alternate strategy for measuring catecholamine fluorescence after CE fractionation that relies on two-photon excitation (2PE) of relatively intense $S_0 \rightarrow S_2$ electronic transitions[4] ($\Delta E \approx 6$ eV) while suppressing photoionization reactions that accompany S_2 excitation. Mass detection limits achieved with this approach, <1 amol for epinephrine and 1.7 amol for dopamine, are more than 100-fold lower than previously attained for spectroscopic measurements of catecholamines.[5] These large improvements arise from several advantages of two-photon absorption for excitation of "ultraviolet" chromophores. First, the excitation source in these studies operates in the blue region of the spectrum instead of the far UV, making it feasible to use high numerical aperture (NA) microscope objectives to focus the excitation light to sub-μm dimensions (thereby minimizing scatter and fluorescence background from solution).[6] Importantly, the same objective can be used for high NA light *collection* when incorporated in an epi-illumination geometry. In addition, the quadratic dependence of the excitation rate on laser intensity (in the limit of negligible bleaching and saturation) limits 6 eV excitation events to spatial coordinates in proximity to the focal point (i.e., in the sample solution), preventing autofluorescence from ancillary structures such as the separation capillary, lenses, and filters. Moreover, the long wavelength of the excitation light makes it possible to virtually eliminate specular scatter from reaching the detector using glass filters with extremely high UV transmission.

In these studies, catecholamines (and the indoleamine serotonin) are fractionated using a field of ~770 V/cm in 2.1-μm i.d. fused silica capillaries, a channel size well-matched for future sampling of secretion from single cells. At the capillary outlet, neurotransmitter molecules intersect a tightly focused beam produced by second-harmonic generation (SHG; $\lambda_{SHG} \approx 414$ nm) from a mode-locked titanium:sapphire (Ti:S) laser. Ultraviolet fluorescence is collected through the same high NA objective used to focus the excitation light, and the counterpropagating emission ($\lambda_{max} \approx 305$ nm for catecholamines) is separated from the optical path using a UV dichroic mirror that reflects wavelengths in the approximate range, 275−375 nm. Residual laser scatter is filtered from the collected fluorescence using UV-transmissive glass, and the signal is measured with a photomultiplier tube connected to a photon counter.[7] Figure 1A demonstrates the capabilities of CE-2PE fluorescence for analyzing extremely small quantities of catecholamine and indoleamine mixtures. Approximately 4 pL from a standard mixture of epinephrine (60 μM) and serotonin (10 μM) is electrophoretically separated and detected. On the basis of the concentrations needed to produce a signal three times larger than the RMS noise level, limits of detection (LODs) are 1.7 amol (440 nM) for epinephrine and 380 zmol (90 nM) for serotonin. Although dopamine comigrates with serotonin in the citrate separation buffer, the detection limit for dopamine in a separate electrophoretic run is 2.3 amol (Figure 1B). The buffer pH (~5) used in these studies was selected on the basis of observations that 2PE fluorescence intensities of the monoamines are somewhat higher in moderately acidic solutions, decreasing to a small extent in neutral to slightly basic solutions (this trend is qualitatively similar to, but less dramatic than, that found by others[5] using a 275-nm laser to excite $S_0 \rightarrow S_1$ transitions through single-photon absorption). It is important to recognize that the primary advantage of 2PE fluorescence resides in its ability to detect extremely small *quantities* of neurotransmitters in small volumes; UV (single photon) excitation may be more appropriate to detect trace *concentrations* of these species because of its capacity to probe larger focal volumes.

Previously, a number of groups have noted a decrease in the fluorescence quantum yield at (single photon) excitation wavelengths shorter than ~240 nm for the chromophoric building blocks of the monoamine transmitters, phenol and indole (and for the biological precursors of the monoamines, tyrosine and tryptophan).[9] This decrease is generally attributed to the formation

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(6) Nie, S.; Chiu, D. T.; Zare, R. N. *Anal. Chem.* **1995**, *67*, 2849−2857. Given a fixed number of molecules, fluorescence can be measured against a smaller background when less solvent is present in the probe volume, i.e., in *smaller* probe volumes.

(7) With the exception of buffers containing 2-propanol, solutions are filtered with 0.2-μm cellulose acetate filters to remove particulates. SHG is produced from a Coherent Mira 900B Ti:S oscillator using an LBO crystal. The coupling of capillary electrophoresis and multiphoton excitation is achieved using an end-column geometry similar to that described previously.[8] A Zeiss Ultrafluar 100× (1.2 NA) objective ($\lambda_{trans} > 250$ nm) is used to focus excitation light and collect fluorescence, and three Schott Glass UG-11 filters (each 2-mm thick) are used to discriminate fluorescence from background. The linear dynamic range in these studies—more than 2 orders of magnitude—is defined by the limit of quantitation and the level that produces photon-counting saturation.

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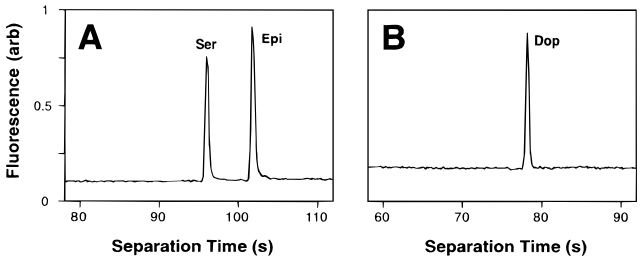

Figure 1. (A) Analysis of epinephrine and serotonin in 10 mM citrate buffer (pH 5.1). Electrokinetic injection volumes, calculated on the basis of analyte mobilities, are 3.9 and 4.2 pL for epinephrine and serotonin, respectively. The separation is performed in a 26-cm long capillary using a field of ~770 V/cm, and fluorescence is excited using ~35 mW of 414-nm light focused on the capillary effluent stream. Peak height reproducibility is estimated to be ~10−12% RSD (N = 10). (B) Electropherogram of dopamine in citrate buffer. Electropheresis is performed in a 23-cm long capillary using a field of ~850 V/cm. The sample volume is ~4.1 pL, and fluorescence is excited using ~40 mW.

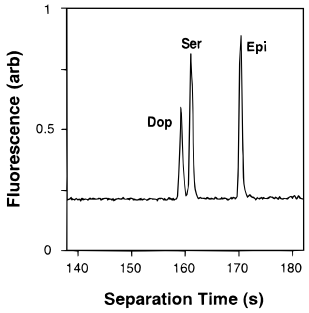

Figure 2. Separation of epinephrine, dopamine, and serotonin in 10 mM citrate buffer (pH 5.1) with 10% 2-propanol. Injection volumes are calculated to be 2.3 pL for epinephrine and 2.5 pL for dopamine and serotonin. Laser power is ~25 mW. Peak height RSD for 5 separations is ~10%.

of charge-transfer states from S_2 that lead to ionization; the use of solvent mixtures with low dielectric constants (e.g., ethanol, methanol, and 2-propanol) was found to be effective at suppressing this deactivation pathway. In preliminary experiments, we have found that incorporation of 2-propanol in aqueous separation buffers improves the fluorescence signal for both the catecholamines and serotonin. Figure 2 shows the fractionation of dopamine (30 μM), epinephrine (30 μM), and serotonin (5 μM) using a citrate electrophoresis buffer prepared in 10% (by volume) 2-propanol. The injection volumes for these species are ~2.5 pL, corresponding to LODs of 0.8 amol (360 nM) for epinephrine, 1.7 amol (670 nM) for dopamine, and 180 zmol (70 nM) for serotonin. These results demonstrate that a relatively low 2-propanol content can decrease mass LODs 2-fold for epinephrine and serotonin, and by ~30% for dopamine. Although we have not yet performed extensive studies, other low dielectric constant solvents (ethanol and methanol) also appear to be effective at reducing photoionization rates following 2PE of the monoamine transmitters.

It is important to note that improved detection limits are achieved despite an increased background for measurements in 2-propanol solutions. Although excitation powers are ~30% lower to maintain relatively low background, the measured baseline levels for separations in 10% 2-propanol are almost 2-fold higher than for separations using only H_2O as the solvent. The cause of the additional background has not been established, but two characteristics are apparent. First, the initial baseline level can be stable for several hours and then increase rapidly to prohibitively large count rates; the duration of the usable period appears to be dependent on the intensity of the excitation light at the capillary outlet. Second, fluctuations in the measured baseline level can appear non-Poissonian, with periods of high-frequency spikes, especially following the onset of rapid increases in the baseline. Because the 2-propanol−H_2O citrate buffers have not been filtered using sub-μm pore size membranes, it is likely that these solutions contain greater particulate contamination than buffers comprised of H_2O and citrate only. Filtration (or other purification) of various low dielectric constant solvents may elucidate the role played by contaminants in increased background levels, allowing higher mole fractions of nonaqueous solvents to be examined in 2PE fluorescence measurements with CE. Preliminary experiments indicate that higher fluorescence signals can be achieved with higher 2-propanol levels. However, these gains are accompanied by a decreased stability in the measured background level (i.e., a prohibitively high noise level) and short capillary lifetimes. The use of 10% 2-propanol solutions in these studies, therefore, represents a compromise that currently yields the best *signal-to-noise* ratios.

The use of sub-μm electrophoresis channels that better match the small dimensions of the two-photon focal spot would facilitate more efficient detection of fluorophores in the effluent stream. In separate studies,[10] we have performed reproducible measurements of various fluorophores at the outlet of 0.6-μm channels, using the Ti:S fundamental, and currently are investigating optical aberrations that have made it difficult to align focused SHG with sub-μm channels. The current results demonstrate that fluorescence-based assays for catecholamines can provide mass detection limits comparable to those previously achieved by CE amperometry. Improvements in both measurement approaches are possible, making it uncertain which strategy ultimately will provide the best detectability. Regardless, there are applications in which fluorescence could offer advantages to electrochemistry, such as detection for parallel separations,[11] analysis using buffers/solvents incompatible with high-sensitivity amperometry, and detection of UV fluorophores (e.g., neuropeptides with aromatic amino acids) that can be challenging to measure using electrochemistry. Although some improvements still may be necessary to use CE-2PE fluorescence to measure the contents of *single* neuronal vesicles, work currently is underway to characterize catecholamine secretion at individual release sites on cultured cells.

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