Electrophoretic Characterization of Dynamic Biochemical Microenvironments

Mary Jane Gordon and Jason B. Shear*

Department of Chemistry & Biochemistry and The Institute for Cellular & Molecular Biology The University of Texas, Austin, Texas 78712

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Spectroscopic measurements can provide valuable information on the state of a dynamic chemical system, but may be insufficient when information is sought regarding *multiple* components in a mixture or important participants in reactions do not have characteristic spectroscopic signatures. In such cases, molecular speciation can be achieved via fractionation, but separations traditionally have not been useful for tracking rapid chemical dynamics because of relatively long analysis times. Jorgenson et al. described a high-speed capillary electrophoresis (CE) technique that relies on optical sample gating to continually profile fluorescent components in a dynamic environment.[1] Analytes are drawn into a short microbore channel under the influence of a large electric field, and are photobleached near the inlet using a focused laser beam. By transiently blocking this beam at a fixed frequency, a train of fluorescent sample plugs is allowed to pass the gating site. Rapid fractionation of these discrete samples yields an electrophoretic "movie" that reveals sub-second chemical changes. However, because this approach requires analytes to be *pre-labeled* with a brightly fluorescent dye, it is useful mainly in specialized applications. Kennedy et al. have shown that fast CE can be extended to a more general set of amine-bearing analytes by using on-column fluorogenic tagging, but at a cost of reduced temporal resolution.[2]

Here, we report the use of multiphoton-excited (MPE) phototransformation to periodically *create* packets of fluorescent derivatives from biological analytes as a means for optically gating high-speed capillary separations. The components in these packets—representative of the biological species from which they were formed—fractionate through an extremely short capillary in tens-to-hundreds of milliseconds, thereby providing sequential snapshots of the chemical state of a biological sample solution without the need for fluorogenic tagging. We previously have described the use of multiphoton excitation to transform biological molecules (monoamine neurotransmitters, proteins) within microseconds into photoproducts that emit in the visible spectral region, and detailed studies were performed on 5-hydroxyindoles.[3] By tightly focusing a titanium/sapphire (Ti:S) laser beam at the outlet of a CE channel, fractionated bands of hydroxyindoles were phototransformed and assayed with low zeptomole detection limits. The identities of photoproducts are unknown.

In the current system (Figure 1), a large electric field causes analytes to continuously enter and migrate through a several-millimeter-long capillary. A Ti:S gating beam is focused periodically at the capillary inlet to generate fluorescent sample packets. Packet components fractionate in the capillary, and are detected individually at the outlet using two-photon-excited fluorescence. Because multiphoton excitation is confined to within micrometers of a tightly focused beam waist,[4] photochemistry is localized to the precise region where gating is desired: directly on the channel inlet.

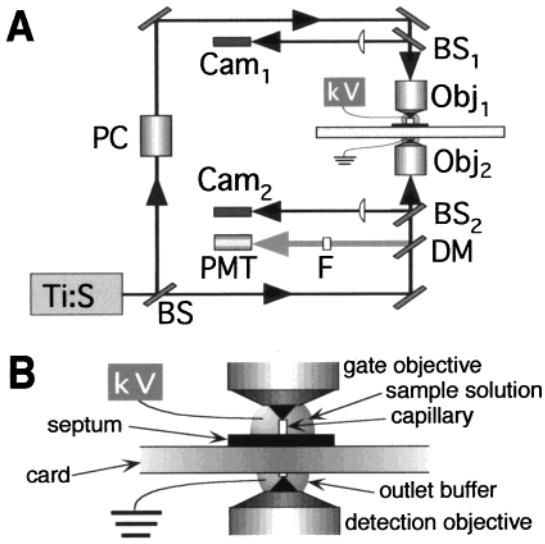

Figure 1. Fast capillary electrophoresis instrument. (A) Overview of the system. A titanium/sapphire (Ti:S) laser beam is split at BS. One portion of the beam is directed through a Pockel's cell (PC) that rejects the beam except during optical gating. This beam is focused at the capillary inlet using a water-immersion microscope objective (Obj_1; Zeiss 63×, NA = 0.9). The second portion of the Ti:S beam is focused at the capillary outlet using water-immersion Obj_2 (Olympus 60×, NA = 0.9). Epi-fluorescence is split from the beam path using a long-pass dichroic mirror (DM), and is transmitted through absorption filters (F) to a photon counter. Alignment of the gating and detection focal spots is guided by imaging the capillary inlet and outlet onto video cameras (Cam_1, Cam_2). (B) Closeup of the separation system. Capillaries several millimeters long are mounted in a ∼375-μm diameter hole through a 1.6-mm-thick plexiglass card. A rubber septum electrically isolates the two sides of the card, and the immersion media (∼50−100 μL) comprise the sample/electrophoresis solutions. To avoid significant changes in these low-volume solutions (e.g., caused by evaporation and electrochemistry), individual experiments are typically restricted to a several-minute period.

Figure 2A demonstrates the ability of this system to rapidly analyze spectroscopically similar MPE photochemical products of the serotonin metabolite, 5-hydroxyindole acetic acid (5HIAA; Figure 2B, left structure). After a 25-ms optical gate, electrophoretic analysis of the 5HIAA photoreaction packet is achieved in ∼0.5 s using an ∼8 kV cm^{-1} field (capillary length ≈ 4.9 mm). The effective sample volume for this separation is ∼25 pL and the detection limit falls in the low femtomole range. Substantial improvements in sensitivity are anticipated by independently optimizing the photogating and fluorescence-excitation wavelengths and by using smaller channels that better match the focal spot sizes.

In Figure 2A, CE-MPE photochemical analysis of 5HIAA reveals two fluorescent products that are thermally stable for a sub-second fractionation period. Analysis of 5HIAA photoreaction packets reproducibly generates *at least* these two photoproduct peaks; in some cases, additional species can be observed. Although changes in capillary wall surface conditions can modify the resolving capabilities of CE, it also is possible that actual differences exist in the number of components arriving at the capillary outlet. The relative production efficiency of different species, for example, may depend sensitively on the photogating laser intensity. Moreover, because thermally labile photoproducts

(1) Monnig, C. A.; Jorgenson, J. W. *Anal. Chem.* **1991**, *63*, 802−807. Moore, A. W.; Jorgenson, J. W. *Anal. Chem.* **1993**, *65*, 3550−3560.

(2) Tao, L.; Thompson, J. E.; Kennedy, R. T. *Anal. Chem.* **1998**, *70*, 4015−4022. Thompson, J. E.; Vickroy, T. W.; Kennedy, R. T. *Anal. Chem.* **1999**, *71*, 2379−2384.

(3) Shear, J. B.; Xu, C.; Webb, W. W. *Photochem. Photobiol.* **1997**, *65*, 931−936. Gostkowski, M. L.; Wei, J.; Shear, J. B. *Anal. Biochem.* **1998**, *260*, 244−250. Gostkowski, M. L.; Curey, T. E.; Okerberg, E.; Kang, T. J.; Vanden Bout, D. A.; Shear, J. B. *Anal. Chem.* **2000**, *72*, 3821−3825. Wei, J.; Okerberg, E.; Dunlap, J.; Ly, C.; Shear, J. B. *Anal. Chem.* **2000**, *72*, 1360−1363.

(4) Shear, J. B. *Anal. Chem.* **1999**, *71*, 598A−605A.

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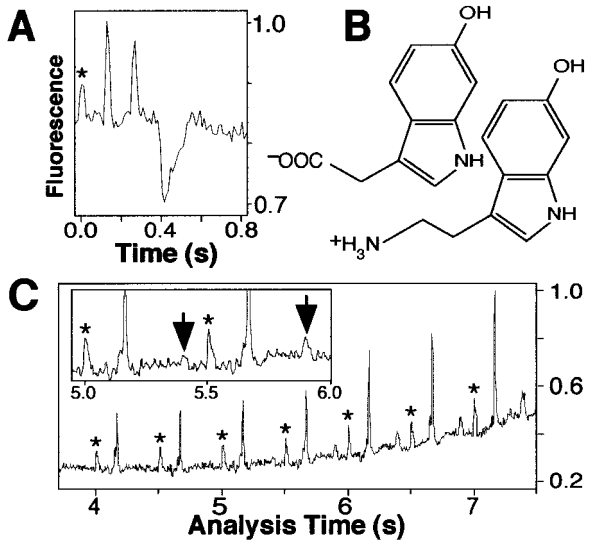

Figure 2. (A) Electrophoretic analysis of 5HIAA photochemistry. The first peak is a spike from the optical gate ($t = 0$; see asterisk), which is followed by a positively charged photoproduct ($t \approx 130$ ms) and a nearly neutral photoproduct ($t \approx 270$ ms). Analysis conditions: capillary i.d. = 5 μm; laser wavelength = 780 nm; gating power ≈ 320 mW; detection power ≈ 50 mW; data bin width ≈ 10.5 ms; [5HIAA] = 500 μM; buffer = 5 mM Hepes, pH 7. (B) Structures of 5HIAA (left) and 5HT (right). (C) Analysis of a dynamic solution. Continual (2 Hz) fractionations of a solution initially containing only 500 μM 5HT show two peaks every 0.5 s: a spike from the gating beam (e.g., at $t = 4.0$ s, 4.5 s, etc.; see asterisks) and a 5HT photoproduct peak ~170 ms after each gating spike. Several seconds into the analysis, ~10 μL of 4 mM 5HIAA solution is added to the sample using a Hamilton syringe, and the late-migrating 5HIAA photoproduct peak grows into the data plot ~400 ms after each gate spike (see downward arrows in the inset). All analysis conditions are similar to those described in panel A, except that data are collected in ~2.6-ms bins and are subjected to a three-point running average. Differences in 5HIAA photoproduct migration times between panels A and C likely reflect electroosmotic changes caused by modifications to the capillary surface.

degrade at distinct rates, the number of species detected could depend on how rapidly analytes are transported through the capillary. We are exploring these possibilities, and the possible use of fast CE for probing the generation and longevity of MPE photoproducts.

Because the detection power is great enough to cause some photoderivatization of native hydroxyindoles at the outlet, peaks are detected in the presence of fluorescence background. This effect reduces detectability, but provides opportunities for gauging the migration velocity of parent compounds—valuable reference points for interpreting photoproduct mobilities. The baseline dip at ~0.4 s (Figure 2A) appears to represent a 5HIAA concentration "hole" (created by photobleaching during sample gating)—a feature that would be expected to have the same velocity as 5HIAA. On the basis of the mobility of this concentration hole and those produced by neutral and positive hydroxyindoles (data not shown), the early peak in Figure 2A can be attributed to a positively charged product and the later peak to a neutral (or slightly positive) species. While it is not immediately clear what reaction pathways would yield fluorescent products that maintain either positive or neutral charges, we expect this electrophoretic result will complement other photochemical data to elucidate MPE photochemical reaction mechanisms.

Even faster CE separations can be performed using this strategy by reducing the capillary length and increasing the separation field. For example, electrophoretic transport of a serotonin (5HT; Figure 2B, right structure) photoproduct can be accomplished in ~50 ms when using an 11 kV cm^{-1} field and a 2.8-mm separation capillary (data not shown). Our design for mounting capillary segments across a plexiglass card should be amenable to the use of much shorter (perhaps < 0.5 mm) capillaries. Moreover, the small inner diameters of the capillaries used in these studies (~5 μm) should accommodate fields several times greater than those examined here.[5] As a result, prospects are promising for photoproduct analyses on much faster time scales.

The ability of this electrophoretic strategy to track dynamic biochemical environments is shown in Figure 2C. A low-volume (50 μL) 5HT solution is continually analyzed using a 2-Hz optical gate and a separation field of ~8 kV cm^{-1}. Several seconds into the analysis, 5HIAA solution is added to the sample using a syringe positioned several millimeters from the capillary entrance. The slow-migrating 5HIAA photoproduct peak is initially detected in the separation that begins at $t = 5$ s, and continues to grow as 5HIAA equilibrates throughout the sample solution (the fast-migrating 5HIAA photoproduct comigrates with the 5HT photoproduct, causing this initial peak to increase as the analysis proceeds). In addition, because the total hydroxyindole concentration entering the capillary increases as 5HIAA equilibrates, the fluorescence background grows with time.

In preliminary studies, we have examined other biological indoles (tryptophan, 5-hydroxytryptophan, 4-hydroxyindole, melatonin, N-acetylserotonin) using this new analysis approach. We also have determined that some phenolic compounds form visible-emitting products via an MPE photoderivatization process,[3] suggesting that this technique could be useful for optically gating broad classes of aromatic molecules. Because electrophoretic data can be summed from many separations (data not shown), it should be feasible to characterize the electrophoretic properties of photoproducts that degrade on millisecond or faster time scales without limitations imposed by poor photoconversion or fluorescence properties. Moreover, an ability to track dynamic biochemical microenvironments on a millisecond-to-second time scale will prove valuable in various applications, including kinetic studies of enzyme-driven reactions and analysis of temporal variations in the secretory output of excitable cells and tissues. The approach for fast CE described in this work will enable one to *translate* the sampling capillary within an environment under microscopic control—a capability that should offer new opportunities for characterizing temporally and *spatially* heterogeneous samples.

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(5) Jacobson, S. C.; Culbertson, C. T.; Daler, J. E.; Ramsey, J. M. *Anal. Chem.* **1998**, *70*, 3476−3480.