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Optimization of native fluorescence detection of proteins using a pulsed nano laser excitation source

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Abstract

We present a mathematical description of the S/N ratio in a fluorescence-based protein detector for capillary electrophoresis that uses a pulsed UV laser at 266 nm as an excitation source. The model accounts for photobleaching, detector volume, laser repetition rate, and analyte flow rate. We have experimentally characterized such a system, and present a comparison of the experimental data with the predictions of the model. Using the model, the system was optimized for test analytes tryptophan, tyrosine, BSA, and conalbumin, producing detection limits (3σ) of 0.67 nM, 5.7 nM, 0.9 nM, and 1.5 nM, respectively. Based on the photobleaching data, a photobleaching cross section of $1.4 \times 10^{-18} \text{ cm}^2$ at 266 nm was calculated for tryptophan.

Keywords

Protein Fluorescence; Pulsed Laser Excitation; Photobleaching; Capillary Electrophoresis

Introduction

Proteomics is a large developing field that studies and analyzes proteins and their activity. The analysis includes the separation and detection of proteins. Laser induced fluorescence (LIF) is a sensitive and well understood method for detecting fluorescently labeled proteins. In the case where labeling is undesirable, native fluorescence of the aromatic amino acids, tryptophan (trp), tyrosine (tyr) and phenylalanine (phe), is an appealing alternative. Extensive research on the use of laser induced native fluorescence (LINF) for protein detection has been conducted and documented.¹⁻¹⁵ In many cases, LINF is coupled to capillary electrophoresis (CE).^{6-13, 16} LINF has also been used more recently with microcapillary electrophoresis (μ -CE) devices.¹³⁻¹⁵ Using laser induced native fluorescence, researchers have achieved sensitivities adequate to detect low-abundance proteins.¹⁴

Methods for improving detection based on LIF in microanalytical systems are discussed in an excellent review by Johnson et al.¹⁷ Among the works cited is a study done by Mathies et al in which optimization of fluorescence signal is obtained by adjusting the transit time of the molecules through the detector and the intensity of the laser.¹⁸ The transit time through the detector is determined by the linear velocity of the molecule and the distance of the probe volume through which the analyte must flow. In CE, the linear velocity of the molecule depends on the conditions of the separation, and the length of the probe volume is determined by the illumination and collection optics. Typically separation quality is paramount, and an operator is not likely to compromise separation parameters to improve detector sensitivity. At the low linear velocities used in capillary CE, the signal magnitude is affected by photobleaching. Adjusting the laser power is a more practical way to optimize S/

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N. At low laser powers the signal and background both are proportional to laser power. At higher powers the signal typically exhibits some form of saturation while the background and its associated noise continue to increase. Johnson *et al* suggest that a maximum S/N can be obtained by tuning the laser power to just before the roll off in signal.¹⁷ In short, the optimum S/N depends on the photophysical properties of the molecule.

Of the studies mentioned by Johnson *et al*, none pays particular attention to the differences in transit time and laser power (peak power) that are inherent in a pulsed laser system. Paquette *et al.* showed that an increase in pulse rate, in proportion to average excitation power, results in a lower LOD for trp.⁸ Schulze *et al.* also dealt with a pulsed laser and compared its performance to a CW laser with the same average power.¹⁵ They demonstrated superior S/N with the CW system. Among these and other studies, none, to the best of our knowledge, has included a discussion on optimization of S/N using a pulsed laser. In a review on LIF detection of peptides and proteins in CE, the authors summarize many works in CE-LIF and the lasers used in these studies.¹³ Particular mention is made of diode-pumped solid state lasers being used with greater frequency, as they are compact, relatively inexpensive and provide sufficient power for LIF detection. Chan *et al* used a relatively inexpensive and compact pulsed solid state UV laser to achieve LOD (3σ) of 3.2 nM for tryptophan, 12 nM for BSA, and 8.8 nM for conalbumin.⁹ A similar laser was used in our work.

In this paper we provide a detailed analysis of S/N optimization for LINF of proteins using a pulsed microchip laser operating at 266 nm as an excitation source. We consider the effects of laser power, flow rate, and signal averaging on signal and background magnitudes. The analysis gives particular emphasis to the effect of photobleaching with 266 nm excitation on detector performance.

Theory

Tryptophan photophysics

The photophysical properties of trp with UV excitation have been extensively studied and documented.^{1, 19, 20} This paper is not an attempt to add to the fundamental understanding of tryptophan photophysics. It is important, however, to note some photophysical characteristics of the molecule that are relevant to its use as a fluorophore for analytical determinations. The quantum yields for fluorescence and photoionization both exhibit strong dependencies on pH and temperature. The fluorescence quantum yield is at a maximum at pH 11. At pH 11, it decreases monotonically over the temperature range from 0 to 80 °C. There is a corresponding rise in the photoionization quantum efficiency over the same temperature range.¹⁹ With 266 nm excitation, photoionization, which is the dominant photobleaching mechanism, proceeds by a combination of one- and two-photon processes.¹ Following photoionization, there is some recombination of the solvated electrons with the trp radical cation, so not all photoionization leads to permanent bleaching of the molecule. In the following discussion, we will not concern ourselves with the photobleaching mechanism. Rather, we will simply attribute any decrease in the fluorescence magnitude from one laser shot to the next to photobleaching.

Model development

At low flow rates through a capillary, a plug of molecules flowing through the detector volume will be probed multiple times by the laser. Each time the population is probed, a fraction of the molecules is photobleached, or a fraction remains. This is schematically shown in Fig. 1. The number of laser shots a given molecule will see is lR/v , where l is the length of the detector volume, v is the linear flow velocity and R is the laser repetition rate.

The signal produced by any laser shot is proportional to the total number of unbleached molecules in the detector volume. That number is given by the number density of molecules times the following sum,

$$V_{eff} = \frac{\nu}{R} A_{cap} \left[\sum_{k=0}^{n_{int}-1} f_{nb}^k + n_{frac} f_{nb}^{n_{int}} \right] \quad (1)$$

where A_{cap} is the cross sectional area of the capillary, n_{int} is the integer part of the ratio lR/ν , n_{frac} is the fractional part of the same ratio, and f_{nb} is the fraction of the molecules not bleached by a single laser pulse. This formula holds until the velocity is high enough that the entire volume of the detector is cleared between pulses. Then the effective volume is just the volume of the detector. The sum can be approximated by the integral

$$A_{cap} \int_0^1 f_{nb}^{(n)} dx \quad (2)$$

where n is xR/ν . The solution of this integral is

$$V_{eff} = \frac{\nu}{R} A_{cap} \frac{1}{\ln(f_{nb})} \left(f_{nb}^{\frac{Rl}{\nu}} - 1 \right) \quad (3)$$

Equations 1 and 3 both approach a maxima as the velocity reaches the value where the detector volume is replaced on each laser shot. A representative plot of Eq. 3 is shown in Fig 2.

The picture becomes more complicated when signal averaging is considered. The number of shots that can be averaged without degrading the resolution of a separation is approximately equal to the number of shots hitting a molecule as it passes through the detector. The number is inversely proportional to the flow velocity, i.e. lR/ν .

Assuming that the noise is limited by counting statistics, S/N is proportional to

$$\left(\frac{l\nu}{R} \right)^{\frac{1}{2}} A_{cap} \frac{1}{\ln(f_{nb})} \left(f_{nb}^{\frac{Rl}{\nu}} - 1 \right) \quad (4)$$

A representative curve is shown in Fig 3. As the curve shows, maximum S/N is obtained at relatively low linear velocities, close to those used in CE.

Experimental

Sample/Solution Prep

A 50 mM stock borate buffer, pH 8.9, was made by dissolving 7.29 g of sodium tetraborate 10 H₂O and 3.09 g of boric acid in 1 L Millipore water (18 MΩ). A 10 mM sodium phosphate buffer, pH 11, was made by dissolving 1.7991 g of dibasic, sodium phosphate, 12-hydrate in about 150 ml of Millipore water (18 MΩ), then adding 0.1 N NaOH until pH reached 11. The buffer was then diluted to 500 ml. All buffer solutions were photobleached for more than 4 hrs before use in solution preparation or before use as a running buffer.

LIF Setup

Excitation radiation was produced by a passively Q-switched diode pumped solid state Nd:YAG (NG-10320-100, JDS Uniphase, USA) which was frequency doubled to 532 nm inside the commercial package. The 532 nm light was frequency doubled to 266 nm by a beta-barium borate doubling crystal (BBO, Caston Inc., FuzhouFujian, China) mounted external to the laser package. The 532 nm and 266 nm light were separated by a prism, and the 532 nm light was used to trigger detection electronics. The 266 nm light was focused by a microscope objective (LMU-10X-266, OFR, Newton, NJ) onto a fused silica capillary, 50 μm i.d., from which the polyimide coating had been removed. Fluorescence was collected at 90° by a reflective objective (50102-01, Newport Corporation, Irvine, CA), NA of 0.52, and focused back 160 mm through a Semrock bandpass filter (FF01, Semrock, Rochester, NY) and 2.5 mm diameter iris to a PMT (H6780-03, Hamamatsu, Hamamatsu, Japan). The pulsed fluorescence signal was integrated with a gated boxcar integrator (SR250, Stanford Research Systems, Sunnyvale, CA). The gate and signal were monitored on a LeCroy Waverunner digital oscilloscope (LT372, LeCroy, Chestnut Ridge, NY). The 25-nsec width of the gate was set to include the entire fluorescence decay of each laser pulse. The output of the gated integrator was recorded using a National instruments multifunction card (PCI-6034E, National Instruments, Austin, TX) and LabVIEW software.

Continuous Flow Measurements

The solutions used to validate the model, and those used to obtain a saturation curve were introduced by a Harvard Apparatus syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA), using a 250 μl syringe (Gas Tight #1725, Hamilton Company, Reno, NV). The syringe was connected to the capillary using a 28 gauge PTFE needle (Hamilton Company, Reno, NV). The LabVIEW software used for these measurements was programmed to sample at the frequency of the laser pulse.

Capillary Electrophoresis

Separations were done using a Groton Biosystems CE (GPA100, Groton Biosystems, Boxborough, MA) at 30 kV using a 50 $\mu\text{m} \times 75$ cm (55 cm to detector) fused silica capillary. Samples were injected by 100 mbar pressure for 7.2 sec. The capillary was rinsed for 2 min after every run with running buffer (10 mM borate buffer, pH 9).

Experimental data used to test the model were obtained using trp fluorescence. The syringe pump introduced a 377 nM trp solution into the capillary at flow rates ranging from 0.01 to 30 $\mu\text{l}/\text{min}$ (0.085-25.5 cm/s). The molecules flowing through the probe volume received anywhere from 0 to more than 2800 shots from the laser. The number of shots received was determined by the probe volume length of 50 μm , the flow rate, and the repetition rate of the laser. The repetition rate of the free running laser fluctuated, depending on warm up time, between and 4.8 kHz and 5.6 kHz. The pulse rate was continuously monitored by the oscilloscope. Because the data were pulse rate dependent, it was important that the fluorescence signal be monitored at the repetition rate of the laser. In order to do this, a LabVIEW software interface was employed that used an external clock derived from the laser pulse. Essentially, an integrated fluorescence signal was recorded for every laser pulse. Fluorescence signal data were recorded for 30,000 shots (about 6 sec) at each flow rate. Background signal was also taken in the same fashion, at varying flow rates, for 30,000 shots. The experiment was repeated four times.

Photobleaching decays

Photobleaching decays for trp were measured by irradiating a static solution of trp in a 50 μm capillary with the laser and recording the fluorescence intensity for each laser shot.

Curves were recorded for a range of laser powers, controlled with neutral density filters and measured with a power meter (3A-SH, Ophir Optonics, Jerusalem, Israel). Solutions were introduced by syringe pump and then stopped. The inlet end of the capillary was completely closed to the atmosphere by the syringe pump, preventing any gravity-induced flow through the capillary during the experiment. The laser was defocused to a diameter of about 100 μm so that solution in a length of capillary twice that visible to the detection optics was bleached. This measure minimized the effects of diffusion on the decay curves.

Results and Discussion

Model Validation

The experimental data for the flow rate dependent, background subtracted signal are shown in Fig. 4. The curve shown in the figure is based on Eq. 3. The equation was scaled to fit the data, with f_{nb} as the only adjustable parameter. The fit suggests a f_{nb} value of 0.956. In other words, only 4.4% of the molecules in the probe volume are bleached each laser shot.

The small differences between the calculated and experimental data are not surprising. The calculated curve is based on the assumption that the detector volume is an ideal cylinder illuminated by a rectangular laser with a perfectly homogeneous beam. The calculations also ignore diffusion effects. In practice the detection volume does not have flat ends, it is not uniformly illuminated, and diffusion certainly affects the results on the time scale and dimensions of the experiment. Given the limiting assumptions, the agreement between model and experimental data is quite good.

It is instructive to compare the value of f_{nb} obtained by the fit in Fig. 4 with that obtained more directly from photobleaching curves. Figure 5 shows a plot of the fraction bleached per laser shot as a function of laser fluence. Extrapolation of the linear data to the fluences provided by the tightly-focused beam (the open square in fig. 5) yields a fraction bleached of 0.029, or f_{nb} of 0.971. It is not surprising that the fraction bleached is lower than the value of 0.044 derived from the fit in Fig. 4. With increasing fluence, the contribution from two-photon photoionization becomes increasingly important, and the linear extrapolation in Fig. 5 leads to an underestimate of the correct value. The data in Fig. 5 also yield a photobleaching cross section at low fluence (σ_{pb}) of $1.4 \times 10^{-18} \text{ cm}^2$ for trp at 266 nm, determined from the slope of the curve. In comparison the absorption cross section (σ_a) for trp at 266 nm is $1.85 \times 10^{-17} \text{ cm}^2$.²¹ The two cross sections combined yield a photobleaching quantum efficiency of 0.076, which is within the range that would be expected based on the photoionization quantum efficiencies published by Stevenson, *et al.*¹

Optimizing S/N

The S/N data shown in Fig. 6 were produced by appropriate averaging of the background as discussed in the model. The number of data points that is averaged together is equivalent to the number of shots that a molecule would receive from the laser (from 1 to more than 2800). This type of averaging causes no loss in detector resolution for a separation. The experimental data correspond well with the model, producing a maximum S/N at lower flow rates. The maximum S/N for our laser repetition rate is obtained when linear velocities are around 0.42 cm/s. Another way of demonstrating this improvement in S/N is by obtaining a LOD for trp at two different flow rates. The calibration curves in Fig. 7 are obtained for trp running at linear velocities of 0.42 cm/s and 25.5 cm/s. The standard deviations used for the LOD calculations are a result of the appropriate averaging allowed by the model. The LODs obtained for trp under these conditions are 1.2 nM for trp flowing at 0.42 cm/s, and 3.1 nM for trp flowing at 25.5 cm/s. Here we see that despite the increase in sensitivity due to

minimized effects of photobleaching, appropriate averaging allows for the decreased LOD at lower linear velocities.

The model also suggests a correlation between linear velocity at which the maximum S/N can be found and the laser pulse rate. According to the model, the maximum S/N shifts to higher linear velocities for higher pulse rates, and to lower linear velocities with lower pulse rate. This behavior can be used to explain the results obtained by Paquette et al, who saw a decrease in LOD for trp with increased laser repetition rate.⁸ Substituting the parameters from their work into the model, we see that as they increased the repetition rate of the laser, the maximum S/N shifted to higher linear velocities, getting closer to their operating linear velocity of about 0.4 cm/s for trp. This could be the explanation for the decrease in LOD for trp that they saw with increasing laser repetition rates. Other results that can be explained by the model are those obtained by Schulze et al.¹⁵ Their experimental conditions suggest a large theoretical shift of maximum S/N to higher linear velocities, far from their experimental linear velocity of about 0.1 cm/s that we estimate from their data. A decrease in pulse rate in this situation might produce an improvement in the S/N for the measurement. This ability to best match the pulse rate to the ideal linear velocity is limited to those who have the ability to select the repetition rate of their laser.

Although the model does not appear to take laser intensity into account, it is clear that the fraction of molecules that are not bleached by the laser pulse, represented by f_{nb} , depends on laser fluence. It was important, therefore to determine the laser pulse energy at which maximum S/N was obtained. To do this, a solution of trp was run through the capillary at 30 μ l/min. Faster flow rates were needed in this experiment to minimize the effects of photobleaching. A double filter wheel and a combination of neutral density filters were used to attenuate the laser. The pulse energy was monitored by a laser energy meter. The results in Fig. 8 show compact solid state nanolasers have the power to produce a roll off in signal. This is likely due to the high peak power. Figure 8 also shows that a slight increase in S/N is achieved by attenuation of the laser from 440 nJ/pulse to 90 nJ/pulse. Attenuation minimized the photobleaching and gave us the best S/N for this laser.

CE Separations

The model is based on the assumption that resolution in the separation is to be limited by the detector volume, and that signals are to be averaged only to the extent that the averaging does not increase the effective detector volume. For example, an analyte traveling at 0.5 cm/s through a probe volume 50 microns long, being probed at 5 kHz, would receive 50 shots while in the probe volume, allowing for only 50 samples to be averaged together. This type of resolution is often not required in separations having well separated peaks, or where factors other than detector volume limit resolution. In cases where proteins are being separated in CE, peaks can be more than 10 seconds wide. With a pulse rate of about 5 kHz, there are more than 50,000 points across the peak. Desiring at least ten time constants across a peak, we can safely average 5,000 samples together without degrading the resolution of the separation or the signal. This is two orders of magnitude more data points to be averaged than suggested by the model. The width of the peaks for amino acids, like trp and tyr, can be a second or less, but are still significantly wider than the ~0.1 second that it takes a molecule to flow through the detector. One second allows for around 500 samples to be averaged together. In the separations that we performed, the amino acids trp and tyr were separated in one run and the proteins BSA and conalbumin were separated in another run. These analytes were used mainly to demonstrate the utility of a detector based on LINF. For the proteins, BSA and conalbumin, 3000 samples were averaged. For the amino acids, 300 samples were averaged. The proteins were nearly baseline separated and the amino acids were very well separated as seen in Figs. 9 and 10.

LOD's for BSA, conalbumin, and trp were 0.9, 1.5, and .67 nM respectively (3σ). This is an improvement over other LOD obtained using similar lasers by over and order of magnitude for BSA, more than a factor of 5 for conalbumin, and almost a factor of 5 for trp.⁹ The LOD for tyr was 5.7 nM.

Conclusions

A mathematical description of the S/N ratio in a LINF-CE system that uses a pulsed laser has been presented. The model was validated using a pulsed, diode pumped, solid-state nanolaser at 266 nm, and trp fluorescence. The optimal settings for our current setup were determined by the model and the LINF-CE was used to detect sub-nanomolar concentrations of trp. The experimental data suggest that only a small fraction of trp is bleached each pulse (4.4%). The model suggests that the maximum S/N for our laser repetition rate can be obtained when analyte linear velocities are about 0.42 cm/s. This value is close to those linear velocities seen in capillary and microcapillary electrophoresis. In such micro analytical systems where pulsed lasers are applied, this model can be useful in determining the optimal settings for analyte linear velocity, laser repetition rate and attenuation.

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Figure 1. Schematic representation of a photobleaching gradient as analyte flows through the probe volume

Figure 2.

Calculated plot of effective volume vs linear flow velocity. For this plot a capillary internal diameter of 50 μm , a detector length of 50 μm , a laser repetition rate of 5 kHz, and a fraction not bleached of 0.956 were used.

Figure 3. Calculated plot of S/N vs linear flow velocity. Conditions were the same as used for Fig. 2. The scaling of the vertical axis is arbitrary.

Figure 4. Experimental flow rate dependent trp fluorescence data (symbols) and a calculated fit, suggesting a f_{nb} of .956.

Figure 5. Photobleaching data obtained from fits to trp photobleaching decay curves (◆), and a value extrapolated to the laser fluence used in fig. 4, representing a f_{nb} value of 0.971 (◇).

Figure 6.
Experimental S/N as a function of linear velocity

Figure 7. Comparative calibration curves for trp at linear velocities of 0.42 cm sec^{-1} (◆) and 25.5 cm sec^{-1} (◇).

Figure 8.

a) Signal (◇) and background (◆) vs laser pulse energy. b) S/N vs laser pulse energy.

Figure 9.
Separation of 40.4 nM trp and 316 nM tyr.

Figure 10.
Separation of 202 nM conalbumin and 129 nM BSA