Excitation of fluorescence decay using a 265 nm pulsed light-emitting diode: Evidence for aqueous phenylalanine rotamers

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The authors describe the characteristics and application of a 265 nm AlGaN light-emitting diode (LED) operated at 1 MHz repetition rate, 1.2 ns pulse duration, 1.32 μW average power, 2.3 mW peak power, and ~12 nm bandwidth. The LED enables the fluorescence decay of weakly emitting phenylalanine to be measured routinely, even in dilute solution. For *p*H of 6–9.2, the authors find evidence for a biexponential rather than monoexponential decay, providing direct evidence for the presence of phenylalanine rotamers with a photophysics closer to the other two fluorescent amino acids tryrosine and tryptophan than has previously been reported. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2245441]

Progress in the fabrication of deep ultraviolet (UV) AlGaN light-emitting diodes (LED) has been considerable in recent years.[1] In biomolecular research this technology is providing researchers with cheaper, more reliable and simpler means to achieve excitation of important fluorophores such as amino acids, protein, and nicotinamide adenine dinucleotide (NADH). Previously we demonstrated the excitation of protein fluorescence decay using pulsed 280 nm (Ref. 2) and 295 nm (Ref. 3) LEDs with time-correlated single-photon counting (TCSPC). Peng *et al.* have shown the excitation of NADH using a 340 nm pulsed LED and TCSPC,[4] while Barbieri *et al.* have demonstrated the excitation of protein fluorescence using 280 and 300 nm LEDs and phase-modulation fluorometry.[5] Here we report fluorescence decay studies of phenylalanine excited with a nanosecond pulsed LED with peak emission at 265 nm, possibly the shortest LED wavelength yet demonstrated for this type of application, our previously reported devices being ideal for exciting the amino acids tyrosine and tryptophan. Hitherto the excitation of phenylalanine fluorescence decay required the use of bulky, expensive or high maintenance pulsed sources such as synchrotrons,[6] mode-locked lasers,[7] or flashlamps[8] and/or working at potentially problematical high concentration ($>10^{-4}$ mol l^{-1}) where self-absorption or excimers can occur. Phenylalanine is one of three fluorescent amino acids in proteins; however, its low fluorescence quantum yield[9,10] (~0.02) and short wavelength of excitation have to date limited its use in protein structural studies. Nonetheless, a 265 nm LED is shown here to excite measurable fluorescence decays of this weakly fluorescent amino acid even in dilute solution. The fluorescence decay of aerated phenylalanine (Fluka >99% purity) in distilled water at *p*H of 6.0, phosphate buffered saline (PBS) at *p*H of 7.4, and borate buffer (*p*H of 9.2) at a concentration of 3×10^{-6} mol l^{-1} (0.5 mg/ml) was recorded using TCSPC (Ref. 11) and analyzed using reconvolution on an IBH Model 5000U fluorometer. The 265 nm LED (Ref. 12) (Sensor Electronic Technology Inc.) was integrated into the standard IBH NanoLED drive circuitry operating at a repetition rate of 1 MHz.

Figure 1 shows the pulse of the 265 nm LED and that of a typical hydrogen nanosecond flashlamp[8] to be free from pre- or after pulses. The instrumental full width of half maximum (FWHM) of the 265 nm LED, including the detector response, is ~1.2 ns. This is a little broader but comparable to the flashlamp (~0.9 ns) and other UV LEDs.[2,3]

Figure 2 illustrates the spectral width of the 265 nm LED recorded using a SPEX FluoroMax 2 with 2 nm spectral bandwidth. The spectral FWHM observed is ~12 nm, once again consistent with other LEDs.[2,3] The spectrum also

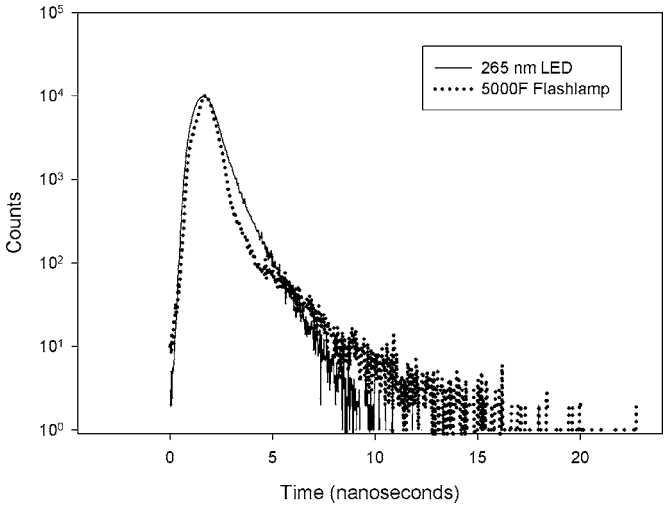

FIG. 1. Pulse profiles of the 265 nm LED and IBH 5000F nanosecond flashlamp at their respective 1 MHz and 40 kHz repetition rates under which they were operated.

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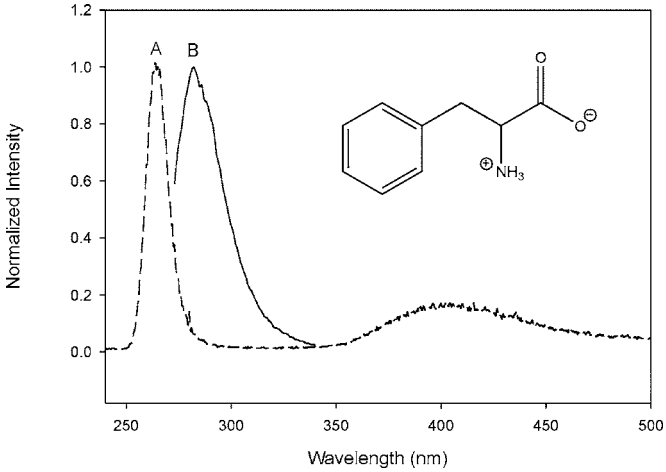

FIG. 2. (A) Emission spectral profile of 265 nm LED with 2 nm bandwidth. The FWHM of the main peak is ~12 nm. (B) Fluorescence spectrum of 3×10^{-6} mol l^{-1} phenylalanine at *p*H of 7.4 excited using the 265 nm LED. The structure of phenylalanine is also shown.

shows a weaker broadband emission at ~400 nm, a feature reminiscent of our first report with a 280 nm LED.[2] For this reason the source output needs to be prefiltered to ensure that this longer wavelength is not detected along with the Stokes-shifted fluorescence. This long wavelength emission cannot be used for exciting other fluorophores due to its long decay time.[2] The LED operated stably with an output power of typically ~1.3 μW average and ~2.3 mW peak at 1 MHz repetition rate (Hamamatsu power meter type no. S1227-1010 BQ placed as close as possible to the LED). The output of the 265 nm LED compares favorably with the previously reported nanosecond 280 nm LED,[2] 295 nm LED,[3] and hydrogen flashlamp[8] (Table I).

Fluorescence decay curves were accumulated to 10 000 counts in the peak channel of 4096 channels at 29 ps channel width. Data rate in TCSPC is proportional to the source repetition rate up to ~2% if photon pileup effects are to be avoided.[11] The LED gave a fluorescence count rate of 6.4 kHz at *p*H of 6 and 8 nm monochromator bandpass, reducing the measurement time to just a few minutes and minimizing the susceptibility to systematic errors such as temporal drift. In comparison, the flashlamp gave 30 Hz and required correction for the detection of scattered excitation.

Figure 2 also shows the steady-state fluorescence spectrum of phenylalanine at 3×10^{-6} mol l^{-1} concentration (*p*H of 7.4) recorded using the 265 nm LED under TCSPC conditions. Phenylalanine's well-known fluorescence spectrum is replicated without distortion or artifacts.[9,10] Initially we fitted the fluorescence decay of 3×10^{-6} mol l^{-1} (0.5 mg/ml) phenylalanine in PBS (*p*H of 7.4) to a monoexponential function as used by others at higher (~10^{-3} mol l^{-1}) concentration.[9,10] Using reconvolution with a χ^2 goodness of fit criterion yields a decay time of 7.47±0.01 ns consistent with previous results.[6,9] Although at first glance the low χ^2 (1.10) of our monoexponential analysis suggested little possibility of a second excited species, a second less intense decay component, becoming more evident at higher *p*H, is also evident in both the flashlamp and LED data (Table II). Fluorescence from both the water and buffer was negligible.

TABLE I. Source average and peak output powers. Note that the flashlamp power is integrated between 200 and 900 nm.

Source	Average power (μm)	Peak power (mW)
IBH 5000F flashlamp	0.45	12
265 nm light-emitting diode	1.32	2.3
280 nm light-emitting diode	0.70	1.2
295 nm light-emitting diode	0.35	0.6

TABLE II. Fluorescence decay times of 3×10^{-6} mol l^{-1} phenylalanine in water at *p*H of 6 and buffered at *p*H of 7.4 and 9.2 fitted to monoexponential (1E) and biexponential (2E) models using LED and flashlamp excitations at 265 nm.

*p*H	τ_1 (ns)	α_1 (%)	τ_2 (ns)	α_2	χ^2
LED excitation					
6.0 (1E)	6.95±0.02	100	···	···	1.21
6.0 (2E)	7.12±0.04	95.19	3.61±0.10	4.81	1.08
7.4 (1E)	7.47±0.01	100	···	···	1.10
7.4 (2E)	7.53±0.03	98.25	3.86±0.13	1.25	1.08
9.2 (1E)	7.29±0.02	100	···	···	2.34
9.2 (2E)	7.31±0.05	68.76	4.43±0.24	31.24	1.04
Lamp excitation					
6.0 (1E)	7.33±0.02	100			1.24[a]
6.0 (2E)	7.47±0.04	94.85	4.01±0.10	5.15	1.09[a]
7.4 (1E)	7.43±0.02	100	···	···	1.29[a]
7.4 (2E)	7.60±0.03	96.10	2.89±0.54	3.90	1.02[a]
9.2 (1E)	6.00±0.02	100	···	···	2.48[a]
9.2 (2E)	6.72±0.05	73.70	3.76±0.23	26.30	1.05[a]

[a]Corrected for excitation light detected using reconvolution.

The fluorescence decay of phenylalanine at 265 nm excitation and 280 nm emission (6 nm bandpass throughout) in *p*H of 9.2 buffer and fitted functions for one and two exponential components are illustrated in Fig. 3. It is clear from the χ^2 value that one decay component is insufficient to describe the kinetics and a two-component model is required.

Unlike the other two fluorescent amino acids tyrosine and tryptophan, previous studies on aqueous phenylalanine over a range of *p*H (including *p*H of 6 reported here) and in other homogeneous solvents,[6,9,10] seem only to have reported evidence for a monoexponential fluorescence decay. On reflection this is in some ways surprising given that the identical side-chain containing carboxyl and amino groups (Fig. 2) occur in all three fluorescent amino acids. Moreover, the complex multiexponential fluorescence decay kinetics of tyrosine and tryptophan have been interpreted in terms of rotamers of this side chain, which can perturb according to *p*H the phenyl π electrons responsible for the fluorescence.[13,14] The rotamer model for tyrosine and tryptophan photophysics is now widely accepted, but the existence of rotamers in phenylalanine has hitherto lacked supporting evidence because of its apparent monoexponential decay in solution.[6,9,10] Our findings differ from these previous results and confirm that there is much more commonality between the photophysics of all three fluorescent aromatic amino acids than has hitherto been recognized. Our data suggest that there are at least two ground state rotamers of phenylalanine, which coexist on a time scale longer than their fluorescence decay times. In this context it should be noted that a triexponential fluorescence decay of phenylalanine embedded in a peptide sequence has been reported previously.[6]

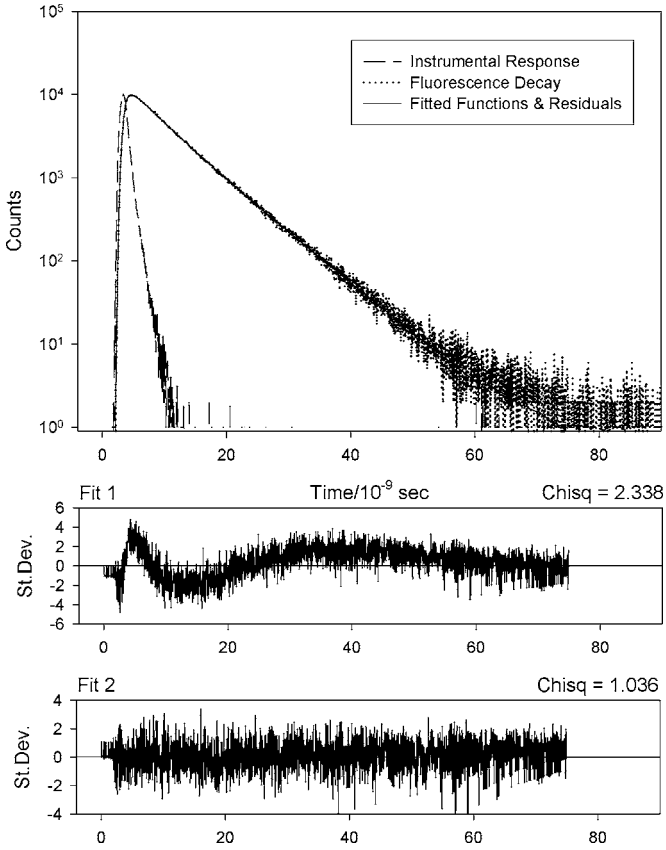

FIG. 3. LED instrumental pulse, fluorescence decay of 3×10^{-6} mol l^{-1} phenylalanine (*p*H of 9.2), fitted function, and weighted residuals for a biexponential (fit 2) model. The weighted residuals for a monoexponential fitted function (fit 1) and chi-squared values are shown for comparison.

Although phenylalanine fluorescence is difficult to detect in most proteins, due to its low quantum yield and resonance energy transfer from phenylalanine to tyrosine and tryptophan, the convenience of the 265 nm LED may well take protein photophysics in new directions, for example, by making use of this resonance energy transfer or by observing phenylalanine fluorescence directly in specific proteins where resonance energy transfer is inefficient. While the 265 nm LED completes the semiconductor wavelengths available for exciting all three fluorescent amino acids, it should be noted that other fluorescent systems, for example, organic polymers such as polystyrene, skin chromophores such as urocanic acid, and other small aromatic molecules such as toluene, can also be efficiently excited at 265 nm. Finally, the 265 nm LED further increases the opportunities for miniaturized sources in terms of the already demonstrated hand held remote sensing capability,[15] imaging, and the future scope for laboratory-on-a-chip assays.

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