Selective excitation of tryptophan fluorescence decay in proteins using a subnanosecond 295 nm light-emitting diode and time-correlated single-photon counting

Colin D. McGuinness
Photophysics Research Group, Department of Physics, University of Strathclyde, 107 Rottenrow, Glasgow G4 0NG, United Kingdom

Kulwinder Sagoo and David McLoskey
Horiba Jobin Yvon IBH Limited, Skypark 5, 45 Finnieston Street, Glasgow G3 8JU, United Kingdom

David J. S. Birch[a)]
Photophysics Research Group, Department of Physics, University of Strathclyde, 107 Rottenrow, Glasgow G4 ONG, United Kingdom

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We demonstrate an AlGaN light-emitting diode (LED) giving pulses of ~600 ps full width half maximum, 0.35 μW average power, 0.6 mW peak power, and ~12 nm bandwidth at 295 nm. This source is ideal for protein intrinsic tryptophan fluorescence decay research without the unwanted excitation of tyrosine and paves the way to lab-on-a-chip protein assays using fluorescence decay times. Fluorescence decay and anisotropy decay measurements of human serum albumin are reported and the usefulness of the 295 nm LED demonstrated in comparisons with a nanosecond flashlamp and LEDs with nominal wavelength emission of 280 nm. © *2005 American Institute of Physics*. [DOI: 10.1063/1.1984088]

Recently, there has been much focus on the area of deep ultraviolet (UV) nitride light-emitting diodes (LEDs). AlGaN LEDs have been fabricated with wavelength emission at 280 nm (Ref. 1) and 237 nm.[2] In previous work, we reported a subnanosecond pulsed LED at 280 nm and its application to protein research, which is key to understanding much of molecular biology.[3] Others have reported the application of a pulsed UV LED operating in the 340 nm wavelength region, and its application to time-resolved fluorescence spectroscopy of nicotinamide adenine dinucleotide (NADH),[4] an important indicator of cell metabolism. These UV LED sources are of particular interest as they excite such important biomolecules conveniently, reliably, and inexpensively. Previously, the study of time-resolved fluorescence of amino acids and NADH, required the use of optical sources, such as synchrotrons,[5] mode-locked lasers,[6] and flashlamps,[7] all totally incompatible with miniaturized technologies. UV nitride semiconductor devices have the potential to replace such high cost or high maintenance sources in biomolecular fluorescence research and have been the missing element in the search to achieve lab-on-a-chip assays based on fluorescence lifetimes. Semiconductor devices for single-photon counting and timing have been available for some time in the form of avalanche photodiodes.[8] When combined with proteins entrapped in a biocompatible porous matrix, such as a silica sol gel,[9] to exclude high molecular weight interferents and transmit low molecular weight metabolites, the way is clear for the fabrication of lab-on-a-chip fluorescence decay time-based biosensors. Fluorescence decay time assays are well known to have advantages over conventional fluorescence assays with respect to independence of fluorophore concentration, ease of calibration, discrimination against artifacts and photon counting sensitivity. Typical of the range of fluorescence lifetime assays, which might now be incorporated on a chip include glucose sensing[10] for diabetes management using the glucose transport protein hexokinase and iron monitoring using transferrin.[11] Here, we report the characteristics and application to fluorescence decay studies of a subnanosecond pulsed LED operating at 295 nm, again based on AlGaN fabrication technology.[12] Our recently reported 280 nm LED is ideal for exciting tyrosine, however, it is well known that the kinetics are simplified by selectively exciting tryptophan, the most widely used amino acid probe, using an excitation wavelength of 295 nm.[5,13,14] We believe this source will be of interest to researchers wishing to investigate intrinsic protein tryptophan fluorescence decay in

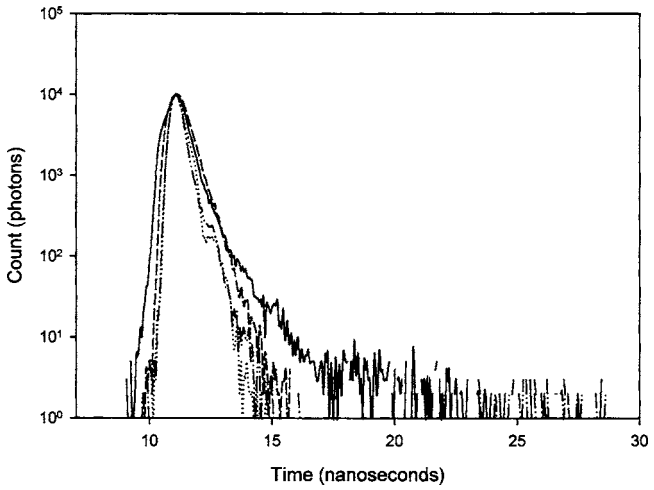

FIG. 1. 295 nm LED, 5000 F flashlamp, latest 280 nm LED and prototype 280 nm[3] LED pulse widths shown on a semilogarithmic scale. Legend is as follows:——5000 F Flashlamp, ·····—295 nm LED, – – – – ——latest 280 nm LED, and –·–·–·—prototype 280 nm LED.

[a)]Also at: Horiba Jobin Yvon IBH Limited, Skypark 5, 45 Finnieston St., Glasgow G3 8JU, UK; electronic mail: djs.birch@strath.ac.uk

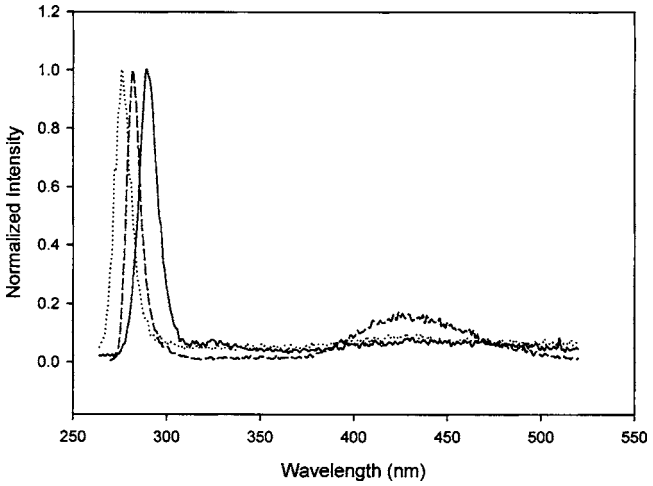

FIG. 2. Full spectral profiles of 295 nm LED, latest 280 nm LED, and prototype 280 nm LED[3]. Legend is as follows: — 295 nm LED (actual peak 296 nm), ····· latest 280 nm LED (actual peak 279 nm), and – – – prototype 280 nm LED (actual peak 282 nm).

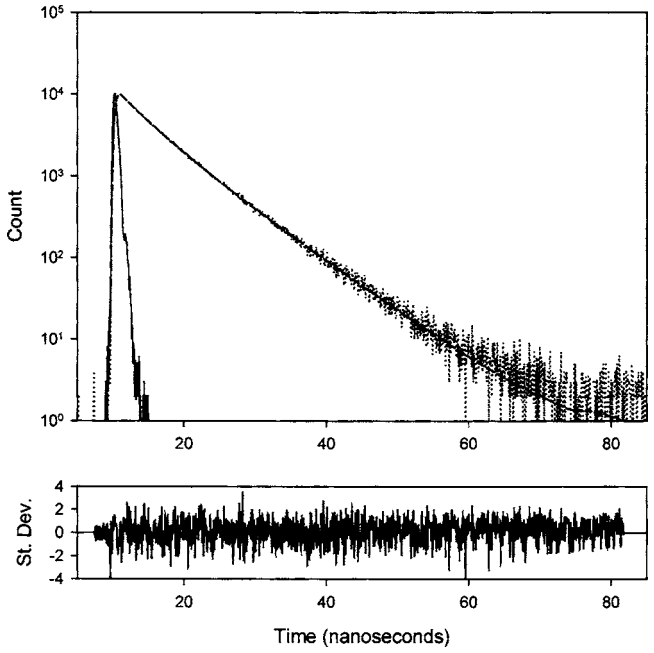

FIG. 3. HSA fluorescence decay including fitted function and residuals.

spectroscopy, sensing, microscopy and imaging.

The fluorescence and anisotropy decay parameters of human serum albumin (HSA) in phosphate buffered saline (PBS) (pH 7.4) at a concentration of 2.5 mg/ml were measured in order to demonstrate the source. HSA contains a single tryptophan residue that simplifies the kinetics, but at 280 nm, as well as direct tryptophan excitation, energy transfer from tyrosine to tryptophan also takes place, complicating the kinetics and leading to fluorescence depolarization other than by fluorophore mobility. To measure protein fluorescence decays, we have used the time-correlated single-photon counting (TCSPC) technique[15] to record, and IBH reconvolution software to analyze, the fluorescence decays. Here, the 295 nm LED has been installed and configured in the IBH NanoLED drive circuitry operated at 1 MHz to produce LED pulsing and TCSPC synchronization. The data accumulation rate in TCSPC is proportional to the source repetition rate up to ~2% if pile-up effects are to be avoided.[15]

Figure 1 shows the pulse duration of the 295 nm LED, compared with a coaxial nanosecond hydrogen flashlamp,[7] prototype 280 nm LED (Ref. 3) and the latest production version of the pulsed 280 nm LED technology, recorded using the IBH TBX-04 detector under TCSPC conditions. The instrumental full width half maximum (FWHM) of the 295 nm LED, including the detector response, is ~640 ps. Given the impulse response of the detector is ~200 ps, this leads to an estimate of the LED optical pulse duration, using addition in quadrature, to be ~600 ps. This compares favorably with the flashlamp (~900 ps) and is comparable to the 280 nm LED.[3] The LED's faster pulse is a great advantage over flashlamps, as it allows shorter lifetimes to be resolved. The log scale clearly shows the sharp LED pulses free from afterglow, before or after pulses.

Figure 2 shows the full spectral response of the 295 nm LED recorded using a SPEX FluoroMax2 at 2 nm spectral bandwidth. The spectral FWHM is ~12 nm and the power output of the 295 nm LED is typically ~0.6 mW peak power and 0.35 μW average power when driven from the IBH NanoLED drive circuitry (1 MHz repetition rate) with the output optics removed and the power meter (Hamamatsu Model No. S1277-1010BQ) placed close to the LED. The spectrum shows the response to be free from long wavelength emission, which was previously observed with the prototype 280 nm devices.[3]

Fluorescence decay measurements were carried out using the IBH 5000U fluorescence lifetime system incorporating a TBX-04 picosecond photon detection module, and ex-

TABLE I. Source power and the HSA decay parameters obtained. Errors are quoted to three standard deviations.

Source	τ_1 (ns)	Relative intensity	τ_2 (ns)	Relative intensity	τ_3 (ns)	Relative intensity	χ^2	Flourescence count rate	Average power	Peak power
5000 F[a]	0.92±0.41	3%	4.27±0.31	44%	7.45±0.11	53%	1.07	700	0.45 μW[b]	12 mW[b]
295 nm LED[a]	0.45±0.26	2%	3.95±0.27	38%	7.25±0.08	60%	1.08	13 000[d]	0.35 μW	0.6 mW
Latest 280 nm LED[a]	0.87±0.14	5%	4.12±0.47	42%	7.22±0.11	53%	1.04	62 000[d]	0.70 μW	1.2 mW
Prototype 280 nm LED[c]	0.63±0.08	6%	3.82±0.42	41%	7.16±0.10	53%	1.13	900	0.42 μW	0.7 mW

[a]Fluorescence count rates obtained with excitation and emission monochromators configured to 12 nm and 16 nm bandwidth, respectively.
[b]The flashlamp powers are integrated from 200 nm to 1000 nm.
[c]Fluorescence count rate obtained with both excitation and emission monochromator bandwidths at 32 nm. The lower count rate, obtained from the prototype 280 nm diode compared with the 295 nm diode, despite its higher-power output and greater bandwidth, is due to less efficient collection optics on the diode.
[d]The difference between the fluorescence rates as compared with the powers for the 295 nm LED and latest 280 nm LED is caused by the preferential excitation of the larger number of tyrosines with the 280 nm LED.

TABLE II. Comparison of rotational decay parameters measured using the 295 nm and latest 280 nm LED. Errors are quoted to three standard deviations.

Source	τ_{r1} (ns)	Relative intensity	τ_{r2} (ns)	Relative intensity	Initial anisotropy	χ^2
295 nm LED	1.04±0.68	0.4%	32.28±1.96	99.6%	0.198	0.98
Latest 280 nm LED	0.72±0.32	1.3%	20.56±1.28	98.7%	0.136	0.95

citation and emission $f/3$ monochromators incorporating a holographic grating in a Seya Namioka geometry. Table I compares the HSA decay parameters recorded with the 295 nm LED and the IBH 5000F coaxial hydrogen flashlamp. The excitation monochromator was tuned to 295 nm, and the emission monochromator tuned to 335 nm to select fluorescence. A factor of $\sim 20\times$ higher protein fluorescence count can be obtained from the 295 nm LED when compared to the flashlamp. This equates to a collection time of <2 min for the LED compared with ~ 30 min for the flashlamp, counting over 2048 channels with 0.057 ns per multichannel analyzer channel and collecting to 10 000 counts in the peak channel. The data are fitted to a three exponential model with a χ^2 goodness of fit criterion. These decay parameters can be attributed to the widely accepted tryptophan conformer model.[16] The data obtained from the LED are in good agreement with the data obtained from the flashlamp within the error of three standard deviations. Also included in Table I are the decay parameters obtained from the latest, and lower power prototype[3] 280 nm LED, again fitted to a three-exponential model. The decay parameters obtained with all four devices are in good agreement. Figure 3 shows the fluorescence decay, including fitted function and residuals, of HSA in PBS buffer, recorded with the 295 nm LED and is shown to be free from systematic errors such as radio-frequency interferences, scattered excitation light, temporal instabilities, etc.

Fluorescence anisotropy measurements were also carried out using the IBH 5000U fluorescence lifetime system, collecting until a difference of 10 000 counts is obtained between horizontal and vertical emission polarizer orientations. Table II compares the rotational decay parameters recorded by fitting to the fluorescence anisotropy decay with the 295 nm and latest 280 nm LED. In both cases, the Brownian rotation of the whole protein is the dominant parameter (longest decay component). As expected, the 280 nm LED yields shorter rotational times, and lower initial anisotropy, as energy transfer from the 18 tyrosines to the single tryptophan takes place, thus contributing to the more rapid fluorescence depolarization in addition to that caused by tryptophan local motion. Fluorescence anisotropy measurements on proteins using a flashlamp are well known to be quite time consuming and close to the limit of what is comfortably measurable. However, using pulsed UV LED technology, fluorescence anisotropy measurements can be obtained with much less effort, higher stability, and in a fraction of the time (c.f. Table I).

In measurements on HSA, we have demonstrated selective excitation of tryptophan fluorescence decay in proteins with a 295 nm LED for the first time. While our previously reported excitation of protein fluorescence using a 280 nm LED was a significant step, the use of a 295 nm LED for exciting tryptophan fluorescence means the ideal source for protein lab-on-a-chip fluorescence assays is now available.

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