AppliedPhotophysics

Ultrasensitive Spectroscopy for the Life Sciences

Abstract: Applied Photophysics Ltd is offering a range of light emitting diode (LED) light sources for use in stopped-flow spectroscopy in the UV-Vis range. These light sources provide an alternative to the xenon arc-lamp and monochromator of the SX20, offering high light intensity, high stability, long lifetime and robustness, and at a fraction of the cost. This application note underlines the suitability of these LED light sources for stopped-flow applications.

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SX SERIES APPLICATION NOTE

LED Light Sources for Stopped-Flow Spectroscopy

INTRODUCTION

LED technology has progressed dramatically in the last few years, to the point that there are a wide variety of high brightness monochromatic LEDs at wavelengths from the UV (~260nm) to the NIR. These new light sources have a number of advantages over traditional monochromatic light sources like the xenon arc-lamp and monochromator of the standard SX20 stopped-flow spectrometer. These advantages are: a low cost, high light intensity, high light stability, long lifetime, robustness and small footprint.

For laboratories conducting stopped-flow at specific wavelengths that do not require the flexibility of the xenon arc-lamp and monochromator, Applied Photophysics Ltd is offering a range of single wavelength LED light sources in the UV-Vis spectral range. These light sources enable the measurement of single wavelength fluorescence and absorbance data with data quality equivalent to or better than the xenon arc-lamp and monochromator of the SX20.

In this application note, we demonstrate the capability of the LED light source option to successfully carry out standard fluorescence and absorbance stopped-flow applications.

KEYWORDS

▶ Light Emitting Diodes, LED	▶ Stopped-flow spectroscopy
▶ Fluorescence	▶ High intensity
▶ Absorbance	▶ Low cost
▶ Kinetics	▶ SX20 stopped-flow spectrometer

A very common application of stopped-flow fluorescence is the monitoring of intrinsic protein fluorescence. This is most often observed from fluorescence of tryptophan amino acids with excitation peak at 280nm. So an LED with a peak emission intensity of 280nm will handle the majority of applications using intrinsic fluorescence.

A good example of this type of application is the analysis of protein unfolding and refolding using chemical denaturants. The trace in Figure 1 shows the refolding of lysozyme using a 280nm LED. Unfolded lysozyme (2.0mg/mL) in 6M guanidinium hydrochloride was rapidly mixed with phosphate buffer, pH 7.2, in a 1:10 volume ratio using an SX20 stopped flow spectrometer with a 280nm LED used for excitation. A 320nm cut-off filter was used to block excitation light and fluorescence was measured with a photomultiplier tube.
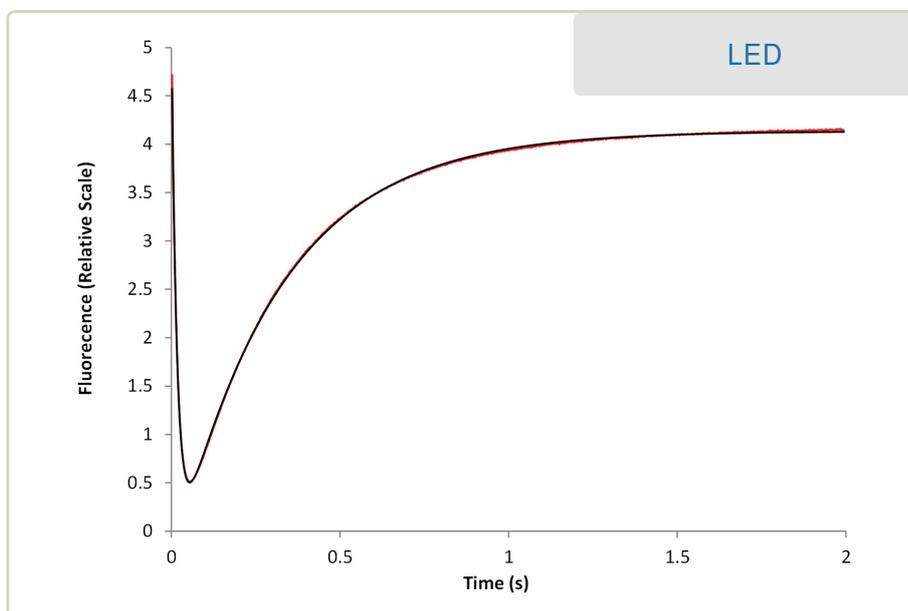

Figure 1. Lysozyme refolding kinetics with a 280nm LED as the excitation source. The red line shows the raw data and the black line shows the fit.

The trace can be fitted to a double exponential equation which yielded rate constants of 62 and 3.2 s^{-1} for the fast and slow phases respectively. For comparison, an equivalent trace recorded with the traditional xenon arc-lamp and monochromator is shown in Figure 2. This fit produced very similar data, with similar rate constants of 56 and 3.5 s^{-1}.

The refolding of lysozyme is known to involve a number of intermediates[1], and the above multiphasic reaction kinetics is consistent with this. In a complete study the final denaturant concentration would be varied, from which we could derive a chevron plot. By extrapolation back to 0 denaturation concentration rate constants for folding and unfolding of the protein in buffer can be derived.
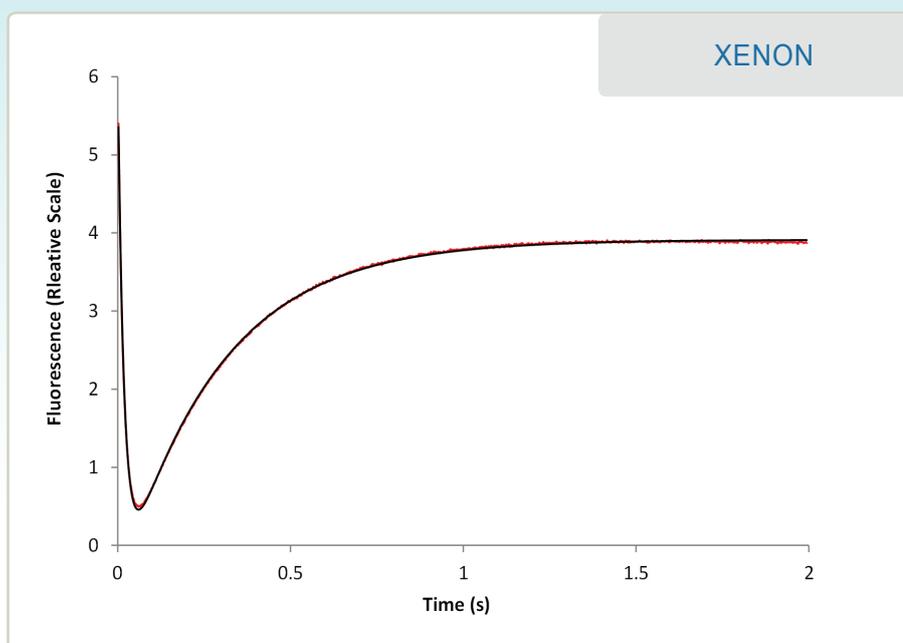
Figure 2. Lysozyme refolding kinetics recorded with a xenon arc-lamp light source and a monochromator set to 280nm. The red line shows the raw data and the black line shows the fit.

Although this simple experiment is a small snap shot of what would be a much larger study, it demonstrates that LED light sources can be used for stopped-flow studies probing protein intrinsic fluorescence, with wide applications in protein folding and refolding[1] [2], protein ligand binding [3], and protein-protein interactions [4].

Application 2: Ligand Binding by Fluorescence

Ligand binding kinetics and protein-protein interactions is a core application area for stopped-flow spectroscopic studies. It allows the calculation of on and off rates, and also it can identify multistep binding mechanisms.

Here we show a simple ligand-protein stopped-flow binding experiment, with the fluorophore 8-Anilino-1-naphthalene-sulphonic acid (1µM) (ANS) binding to the protein bovine serum albumin (10µM). On binding of the ANS into a hydrophobic binding pocket, the quenching of fluorescence is relieved and the fluorescence signal of ANS goes up significantly.

ANS has an excitation spectrum with a peak ~360nm, with an emission peak >395nm. A very sensitive ANS stopped-flow fluorescence detection system was setup using an excitation LED with a peak wavelength of 360nm, and a 395nm cut-off filter was placed in front of the detector to attenuate scattered excitation light. The result of a single binding reaction of ANS to BSA is shown in Figure 3.
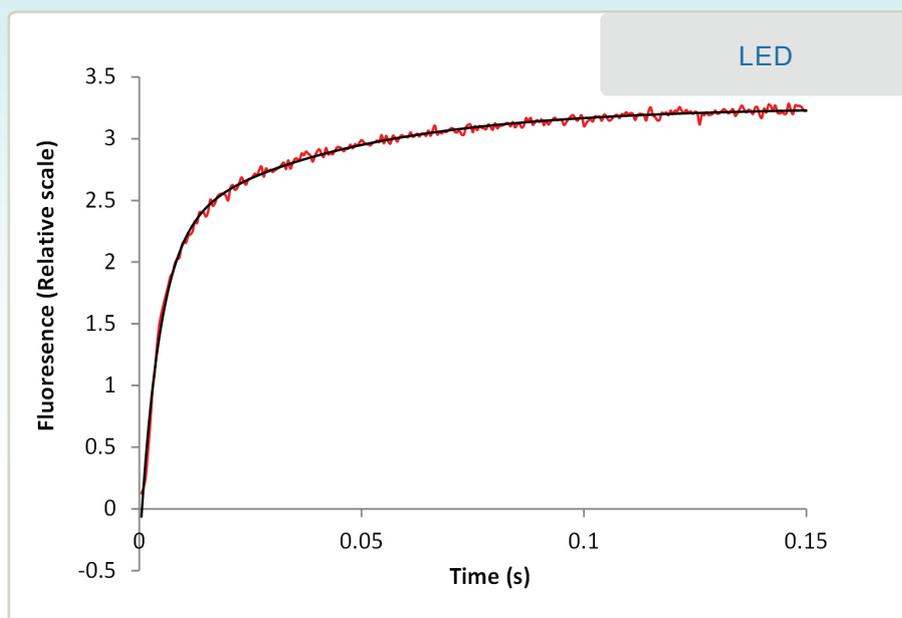
Figure 3. 8-Anilino-1-naphthalene-sulphonic acid binding to bovine serum albumin. 360nm light source used for excitation. The red line shows the raw data and the black line shows the fit.

ANS binds to multiple binding sites on BSA, which exhibits multiphasic binding kinetics. The above kinetic trace has to be fitted with 2 exponentials, with rate constants of 227 and 25.0 s^{-1} respectively. The complex kinetics is due to the multiple binding sites of ANS to BSA, resulting in multiple parallel binding rates.[5]

An LED is suitable for analysis of simple and complex ligand binding reactions, using both fluorescence and absorbance optical probes. The quality of the data compares very favourably with that obtained using the xenon arc-lamp and monochromator as a light source as is shown in Figure 4.
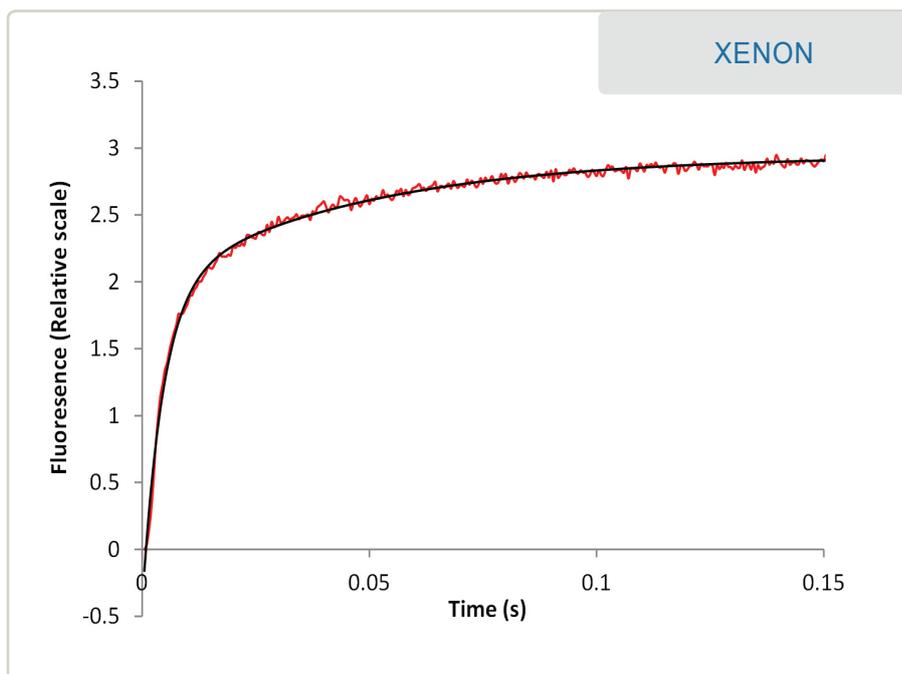

Figure 4. 8 -Anilino-1-naphthalene-sulphonic acid binding to bovine serum albumin recorded with a xenon arc-lamp light source and a monochromator set to 360nm. The red line shows the raw data and the black line shows the fit.

These simple examples show the utility of the LED light-source for following changes in fluorescence. More sophisticated experiments such as Fluorescence resonance energy transfer (FRET) measurements are also possible with an LED light source.[6]

A key advantage at a number of wavelengths is the combination of very high light intensity and stability, which work together to dramatically reduce signal to noise of the experiment.

ABSORBANCE SPECTROSCOPY APPLICATIONS

Application 3: Enzymatic hydrolysis of p-nitrophenyl acetate by α-chymotrypsin

Stopped-flow spectroscopy is used widely to understand enzymatic reaction kinetics, probing changes in substrate or cofactors during a reaction with absorbance and fluorescence measurements.

Here is an example of the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin probed by absorbance changes at 400nm (Figure 3). This experiment rapidly mixed 30µM α-chymotrypsin (Phosphate buffer, pH7.2, 20% Isopropyl alcohol) with 3mM p-nitrophenyl acetate (Phosphate buffer, pH7.2, 20% isopropyl alcohol). An LED with a peak intensity of 400nm was used as the light source. The hydrolysis is observed by measuring the increased absorbance seen by the released p-nitrophenolate.
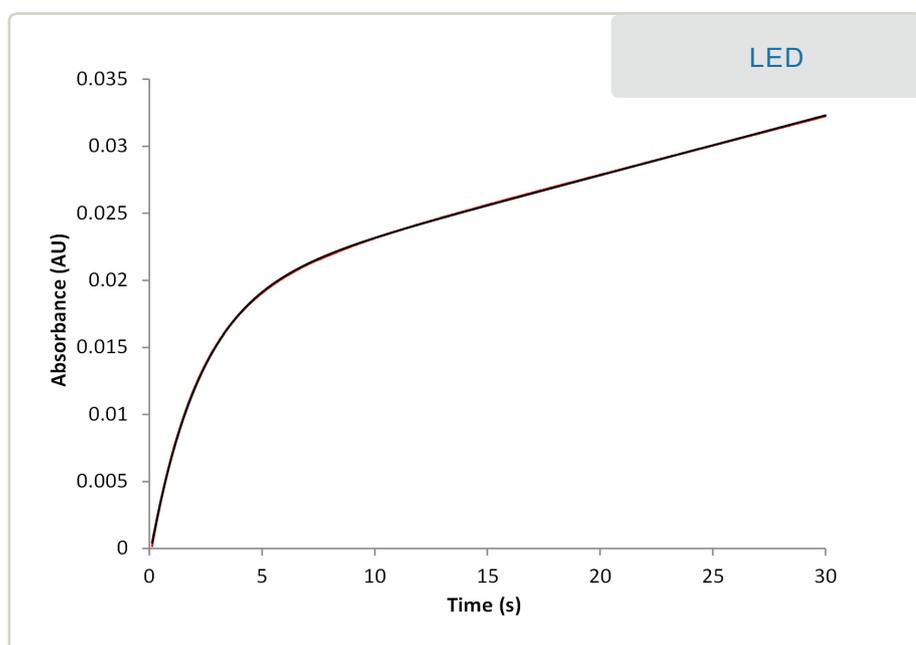

Figure 5. Hydrolysis of p-nitro-phenyl acetate catalysed by α-chymotrypsin. 400nm light source used. The red line shows the raw data and the black line shows the fit.

The trace in Figure 5 is typical for mechanisms of a serine proteases, which have been studied extensively[7]. Serine proteases are ubiquitous throughout life where they carry out their essential function in peptide bond hydrolysis. All serine proteases execute their catalytic role at an active site consisting of a catalytic triad.

The triad invariably contains a histidine, serine, and an aspartic acid residue. The serine residue acts as a nucleophile on the target carbonyl group whereas the histidine and aspartic acid residues involve themselves in hydrogen transfer and hydrogen bonding. The steps in the catalysed hydrolysis of p-nitrophenyl acetate by α-chymotrypsin and trypsin are shown in Figure 2.
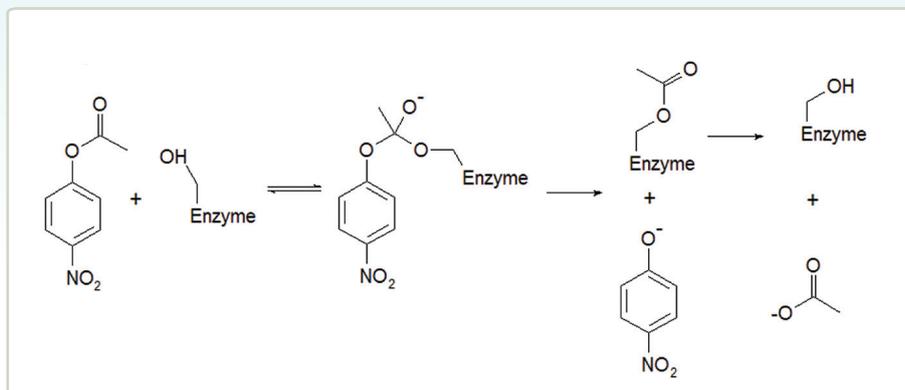

Figure 6. Reaction scheme for the enzyme catalysed hydrolysis of p-nitrophenyl acetate.

The initial binding step between the enzyme and substrate is a pre-equilibrium that occurs much faster than the subsequent two steps. The formation of acylated enzyme intermediate is accompanied by the loss of the first product p-nitrophenolate. It is this step that can be observed in the stopped flow experiment due to the intense absorption of light at 400 nm by p-nitrophenolate.

The shape of the curve in Figure 5 can be explained if the observable step occurs rapidly compared to the final deacylation step. The initial observed rate occurs when the enzyme-substrate concentration is at its highest. At this point, the rate of formation of p-nitrophenolate depends only on the first two steps of the mechanism. This gives rise to the initial observable 'burst phase' of the reaction. As the reaction proceeds, the acylated enzyme reaches an equilibrium concentration so that the rate becomes dependent only on the final deacylation step, giving rise to the linear part of the trace. (Please refer to application note 4140Q010 for a more complete description of how kinetic and thermodynamic parameters can be obtained from such experiments.)

The trace can be described by a single exponential plus a linear slope. The fast phase of the reaction, which includes the first 2 reaction steps, was calculated to be $0.38s^{-1}$ and the slow steady state linear phase was determined to be $0.02s^{-1}$. For comparison, the experiment was repeated with a xenon arc-lamp and monochromator set to 400nm (Figure 7).
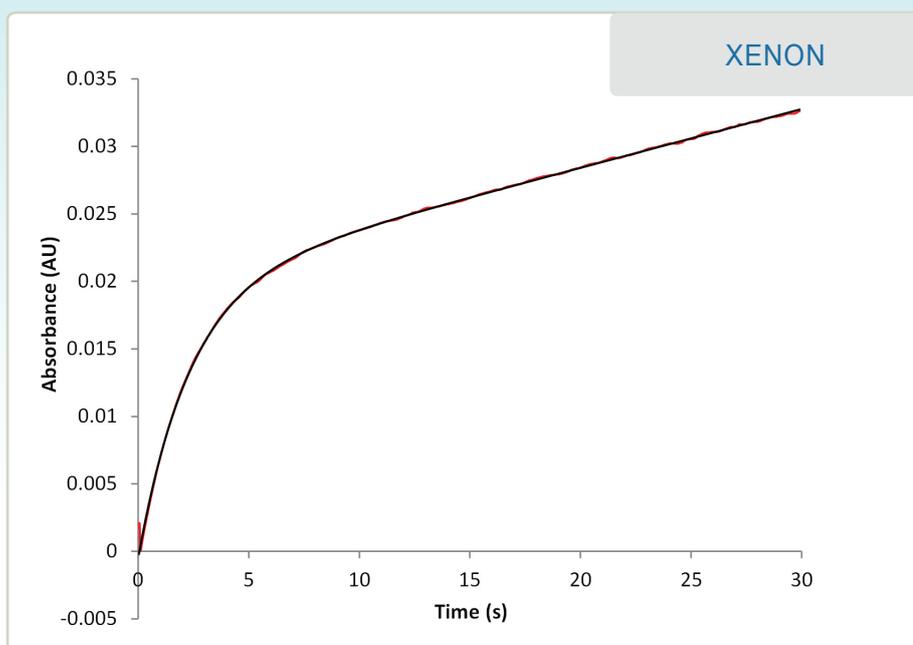
Figure 7. Hydrolysis of p-nitro-phenyl acetate catalysed by α-chymotrypsin recorded with a xenon arc-lamp light source and a monochromator set to 400nm. The red line shows the raw data and the black line shows the fit.

SUMMARY

The LED light source is able to be used in the majority of standard single wavelength fluorescence and absorbance stopped-flow applications, with a number of advantages:

- ▶ Simple robust design
- ▶ Low cost
- ▶ Long light-source life
- ▶ High light intensity
- ▶ High light stability
- ▶ Compact instrument footprint

The much reduced cost is also a considerable bonus and the LED light source is likely to be of interest in teaching labs, start-up labs and established kinetics labs wishing to extend their resources.

NOTE: It should be kept in mind that, in the absence of a monochromator, the bandwith is effectively set by the LED light source that is used. This is typically in the range 10-30 nm. Naturally, this is a consideration when assessing the most appropriate filter in a fluorescence experiment.

ORDERING INFORMATION
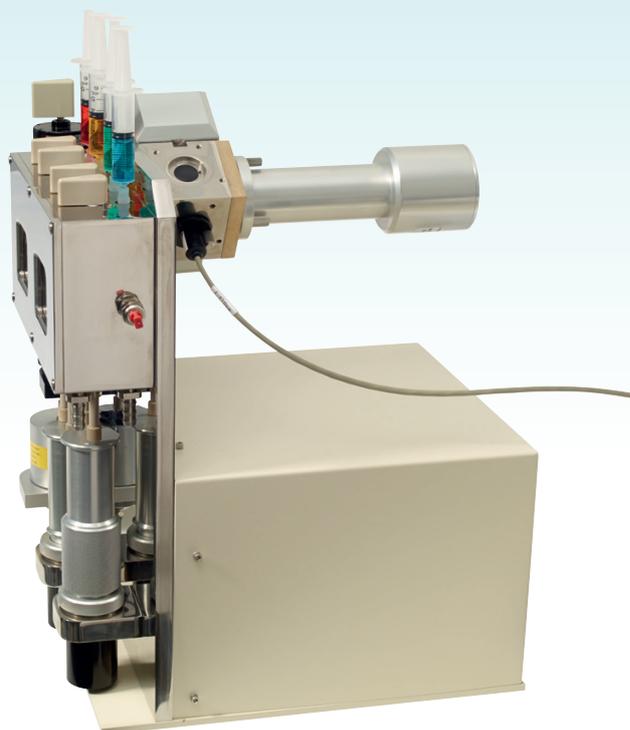
The SX20 stopped-flow sample handling unit (SHU) equipped with an LED light source.

These high intensity, high stability light sources are available in a number of specific wavelength outputs and may be used to complement or replace the original xenon arc-lamp.

The LEDs are especially suitable for fluorescence experiments and may also be used in absorbance measurements. The wavelength range currently offered is listed in Table 1, alternative wavelengths may be available on request.

Peak Wavelength	Bandwidth, FWHM (Approx.)
280	11
320	10
340	10
360	21
400	16
435	19
450	20
470	20
490	28
505	28
535	34
572	14
590	13
610	15
650	20
660	19
680	20

Table 1. Peak emission wavelengths and bandwidths of LED light sources.

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