APPLICATION NOTE

Red-Edge Excitation Spectroscopy of Proteins with the FS5 Spectrofluorometer

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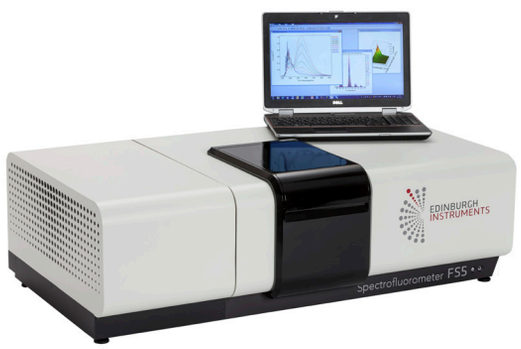
Introduction

Red-edge fluorescence spectroscopy of polar fluorescence probes is a powerful method to study the structure and dynamics of phospholipid membranes, micelles and proteins.[1-4] This method takes advantage of the possibility to selectively excite at the red edge of the absorption band, a small fraction of fluorescence probes with red-shifted emission spectrum. Position of the emission maximum of these red-shifted fluorescence probes yields information about the polarity and dynamics of a protein's microenvironment.



FS5 Spectrofluorometer

Experiments and Results

The method requires a rather modest spectral resolution of ~5 nm. However, it needs to effectively discriminate Rayleigh scattering of the excitation light and requires high sensitivity. Double-monochromator spectrometers have traditionally been used to provide efficient discrimination of the excitation light. Sensitivity of a double-monochromator spectrometer is substantially better than that of a single-monochromator spectrometer.

The novel optical design of the FS5 Spectrofluorometer developed by Edinburgh Instruments, harmonises these two contradictory requirements. The optical system of the FS5 employs only one monochromator in both the excitation and emission channels. The FS5 provides virtually the same discrimination of excitation light as the best double-monochromator spectrometers on the market but still possesses remarkably high sensitivity. In addition, the correction of intensity fluctuations of the excitation light source (both slow drift and high frequency) that is available, enables accurate subtraction of Rayleigh and Raman spectral components as well as background emission from raw data (Fig. 1).

Figure 1 shows the raw fluorescence spectrum of a 50 µM porcine eye lens protein sample in 7 M urea (blue) measured at red-edge excitation (315 nm), where tryptophan extinction drops by about three orders of magnitude compared with that at 280 nm. The protein emission is caused mostly by fluorescence of the tryptophan, however, the water Raman and fluorescence background significantly distort the spectral shape. The background spectral curve (green) can be subtracted from the raw data, when measured at the same experimental conditions from a blank sample. This restores the true shape of tryptophan/protein emission spectrum (inset).

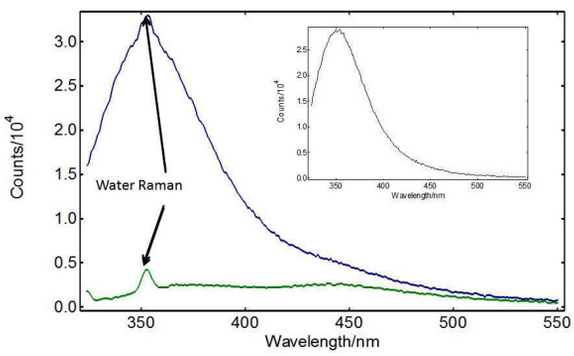
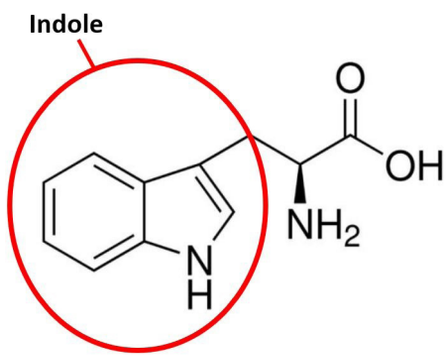


Figure 1: Subtraction of emission background and Raman scattering from raw fluorescence data. Spectrally corrected emission spectrum of porcine eye lens proteins in 7 M area (50um) (blue) and background (green) at 315 nm excitation wavelength. Protein emission spectrum after background subtraction (inset).

Tryptophan fluorescence has found a broad scope of applications in life sciences. Tryptophan (amino acid) is a derivative of indole, which is a polar dye molecule with a large permanent dipole moment in the ground (2.2 D) and excited (5.7 D) states.[2] This makes tryptophan fluorescence sensitive to polarity and viscosity. In proteins, each tryptophan site chain is situated in a cavity (pocket) with a unique polarity and molecular dynamics. The tryptophan fluorescence pattern of a protein, containing only one tryptophan residue, shows similar properties as a viscous polar dye solution. This can be rationalised by a dynamic model of inhomogeneous broadening of electronic spectra,[5] which accounts for a spectral relaxation of the solvated molecule in the excited state.



Tryptophan

In multi-tryptophan proteins, fluorescence pattern has features of both a dynamic and static model accounting for spectral relaxation in each tryptophan pocket and its unique micropolarity.[2] The maximum of the total steady-state fluorescence spectrum is given by a superposition of fluorescence spectra of all individual tryptophan residues and usually falls into the 320 nm - 340 nm spectral range depending on the proportion of hydrophobic and hydrophilic tryptophan pockets in the

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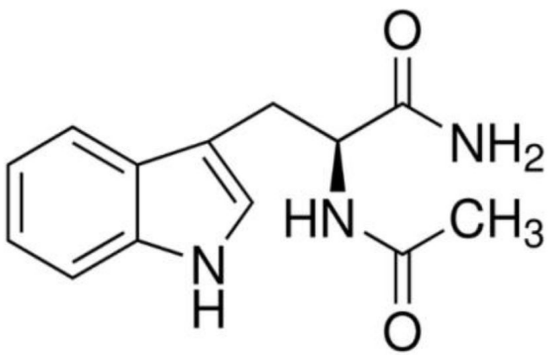
protein. Tryptophan emission of denatured proteins or peptides has maximum at 350 nm.



N-Acetyl-L-tryptophanamide

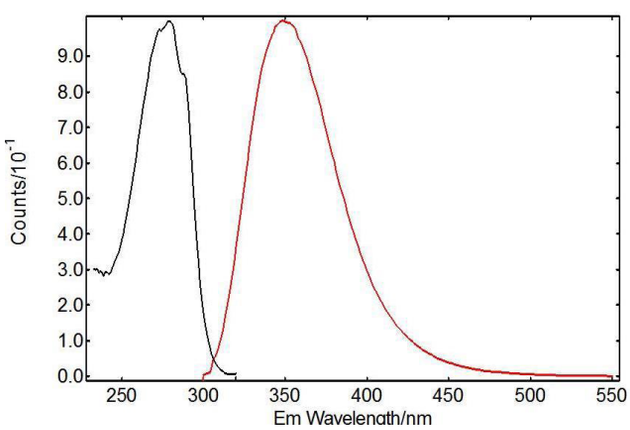


Figure 2: Corrected emission (red) and excitation (black) spectra of NATA.

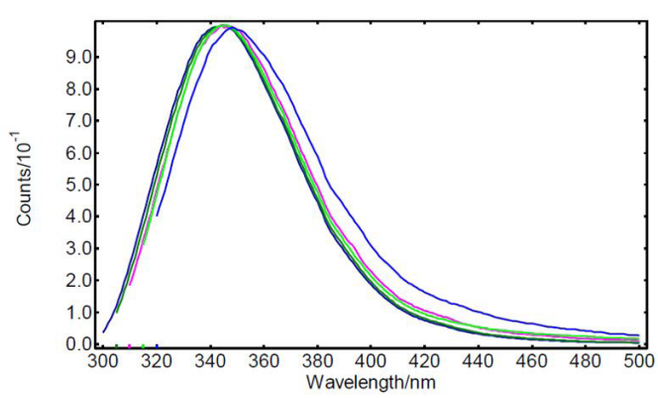


Figure 3: Emission spectra of NATA in glycerol at -10°C. Excitation wavelength 295 nm, 300 nm, 305 nm, 310 nm and 315 nm.

A bathochromic shift of emission spectrum is observed in a NATA/glycerol solution at -10°C (Fig. 3). This shift is caused by a relatively high viscosity of glycerol, which makes the rate of the S_1 state comparable with the rate of deactivation of the excited state by photon emission ($S_1 \rightarrow S_0$). As a result NATA molecules emit from a partially relaxed S_1 level.[2]

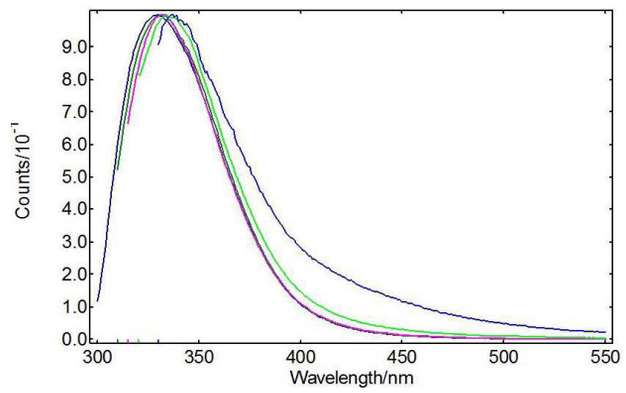


Figure 4: Emission spectra of a pig's eye lens protein sample in PBS. Excitation wavelengths are 295 nm, 300 nm, 305 nm, 310 nm, 315 nm.

A similar bathochromic shift is also observed in a sample of eye lens protein dissolved in PBS (Fig. 4). (A distortion of the red slope of the emission spectrum at 315 nm excitation (blue line) is apparently caused by the presence of non-tryptophan emitting specious in the protein).

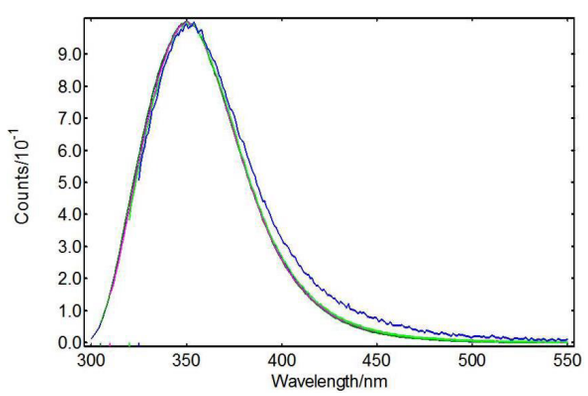


Figure 5: Emission spectra of a pig's eye lens protein sample in 7 M urea.

The bathochromic shift completely disappeared when this protein was denatured in 7M urea (Fig. 5). This makes all tryptophan side-chains exposed to an aqueous environment with a similar viscosity as seen in PBS. As a result, tryptophan fluorescence of the denatured protein becomes similar to the emission of NATA in PBS.

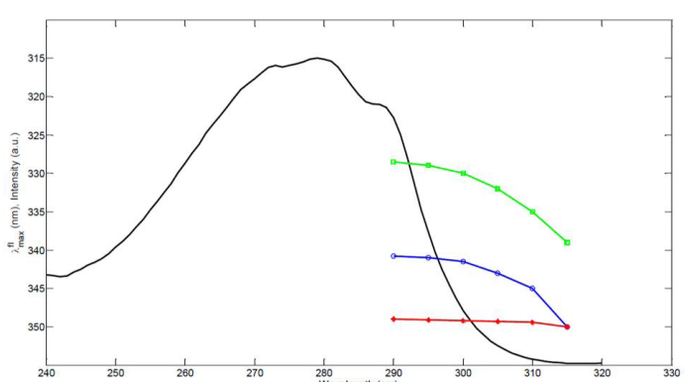


Figure 6: Fluorescence emission spectral shift functions of NATA/glycerol solution at -10°C blue), crystalline /PBS sample (green) and crystalline proteins/7 M urea (red). Excitation spectrum of NATA (black).

Measuring Charge Carrier Lifetime in Halide Perovskite Using Time-Resolved Photoluminescence Spectroscopy

The positions of the fluorescence maxima of the above experiments as a function of excitation wavelength are shown in Fig. 6.

Conclusion

Red-edge fluorescence spectroscopy of proteins is a powerful tool to study protein structure and dynamics. It is a steady-state method and does not require expensive instrumentation. With the FS5 Spectrofluorometer it is possible to work with micro-cuvettes and still maintain high sensitivity of fluorescence detection. This was a necessity with this method as it requires very small amounts of protein. The new low cost FS5 is a powerful instrument and is ideally suited for this type of application

References

[1] Demchenko, AP. The red-edge effects: 30 years of exploration. *Luminescence* **17**, 19–42 (2002).
[2] Demchenko, AP. Ultraviolet spectroscopy of proteins. (Springer, 2008).
[3] Gakamsky, DM et al. Selective steady-state and time-resolved fluorescence spectroscopy of an HLA-A2-peptide complex. *Immunol. Lett.* **44,** 195–201 (1995)
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[5] Nemkovich, NA et al. *In:* Topics in Fluor. Spec. v. 2. (Ed. Lakowicz JR) 367–432 (1992).

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