Hyperluminescence from multiphoton excitation of serotonin complexed with β-cyclodextrin and imaged within mammalian cells

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Introduction

Cyclodextrins, especially β-cyclodextrin (β-CD, cyclohepta-amylose) form complexes with many aromatic molecules with hydrophobic character [1]. The guest molecule bound within the cyclodextrin cavity occupies a relatively isolated and hydrophobic environment. There have been many photochemical studies of cyclodextrin complexes that indicate significant effects of complex formation on the outcome of photochemical reactions, excited state lifetimes and quantum yields [2].

Previous studies [3] at the LSF have reported on the characteristic green emission ('hyperluminescence'), together with the normal ultraviolet fluorescence, that results from multiphoton excitation of 5-hydroxyindoles (the amino acid 5-hydroxytryptophan and the neurotransmitter serotonin) using ultrafast near-infrared laser pulses. Hyperluminescence was originally reported in single colour experiments by Shear *et al.*[4] to involve the formation of a photochemical intermediate by 4 photon absorption at 830 nm, and excitation of the intermediate by a further 2 photons. We have subsequently investigated effects of solvent on intensity and lifetime [3] and found that polarization of hyperluminescence is a useful probe of local viscosity [5]. The attraction of using hyperluminescence for imaging of serotonin is that near-infrared illumination may be more selective than ultraviolet excitation, resulting in lower background and higher sensitivity. Furthermore, the green emission is removed at least partially from the intrinsic blue emission observed from tissues and cells with UV excitation. However sensitivity using 830 nm in a six photon process is limited by the low cross-section. We have found that use of shorter wavelengths may bring improvements by needing fewer photons – for example at 600-630 nm excitation is a 4 photon process and only 2 photons are required at 315 nm [5].

The nature of the green-emitting intermediate remains uncertain, although it has been found to behave similarly to the parent hydroxyindole in capillary electrophoresis experiments[6]. In contrast, the supralinear effect of concentration on emission intensity in phosphate buffers[7] suggests possible dimer formation. Formation of 1:1 inclusion complexes of serotonin with a cyclodextrin suggests that the photochemistry of a relatively isolated molecule may be studied.

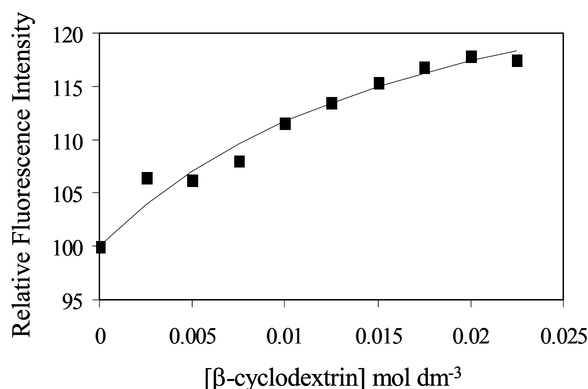

Figure 1. Effect of β-cyclodextrin concentration on fluorescence intensity of serotonin (5 μmol dm^{-3} in phosphate buffer, 20 mmol dm^{-3}, pH 7.0), excitation at 300 nm.

Interaction between serotonin and β-cyclodextrin

The addition of β-cyclodextrin to solutions of serotonin in phosphate buffer, pH 7.0, results in a small increase in steady-state ultraviolet (334 nm) fluorescence intensity from serotonin indicating formation of a complex, although both emission and excitation spectra appear essentially unchanged. Figure 1 shows a plot of fluorescence intensity (F) versus β-cyclodextrin concentration ([β-CD])with the curve indicating the best fit of the data to equation (1), with an association constant (K_A) of 53.2 ± 17.3 dm^3 mol^{-1}, and the intensity at saturation (F_{max}) equivalent to a 33.8 ± 6.1% increase in fluorescence intensity of the fully complexed serotonin. The fluorescence lifetime (τ) of serotonin in the absence of β-cyclodextrin was mono-

$$F = F_0 + \frac{K_A F_{\max} [\beta\text{-CD}]}{(1 + K_A[\beta\text{-CD}])} \qquad (1)$$

exponential with τ 3.65 ns (literature value 3.8 ns [8]) increasing to 4.24 ns when bound to β-cyclodextrin (Figure 2), therefore showing a similar increase as the intensity.

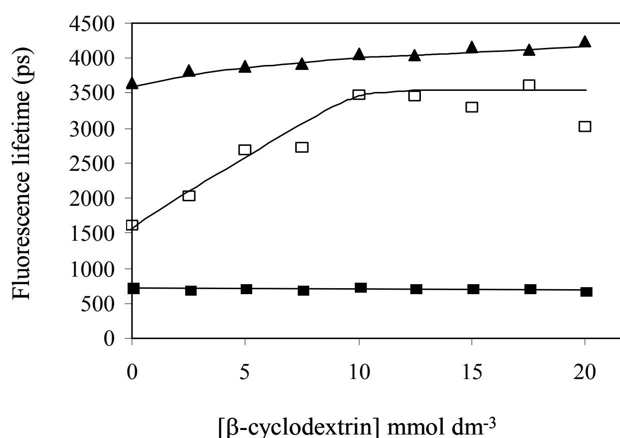

Figure 2. Effect of β-cyclodextrin concentration on liftimes of UV (340 nm) fluorescence excited at 300 nm (▲), and the short (■) and long (□) fluorescence lifetime components of the green (505 nm) hyperluminescence from multiphoton excitation at 750 nm.

The fluorescence lifetime of the green emission (505 nm) from serotonin on multiphoton excitation at 750 nm was found to analyze as a double exponential. In the absence of β-cyclodextrin the component of the decay with greater fractional intensity had τ 0.71 ns, similar for that from 5-hydroxytryptophan (0.91 ns [3]). This lifetime remained constant with increasing β-cyclodextrin concentration, whereas the longer lifetime component increased from 1.6 ns to 3.5 ns (Figures 2 and 3). The fractional intensities of the two lifetime components of the hyperluminescence are shown in Figure 4. Analysis of this data by the equivalent of equation (1) gave K_A

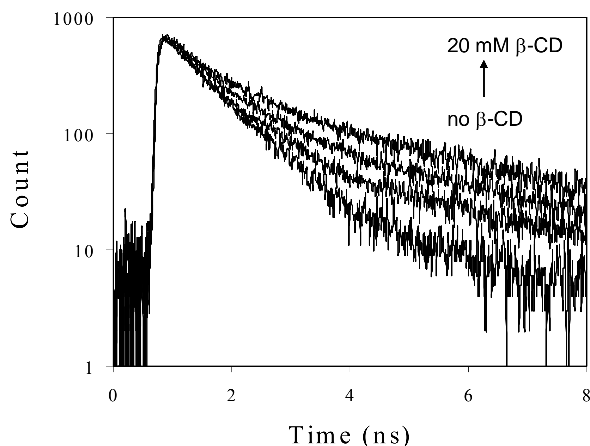

Figure 3. Fluorescence decays at 505 nm after multiphoton excitation of serotonin at 750 nm in solutions of serotonin alone (bottom), 5, 10 and 20 mmol dm^{-3} (top) β-cyclodextrin.

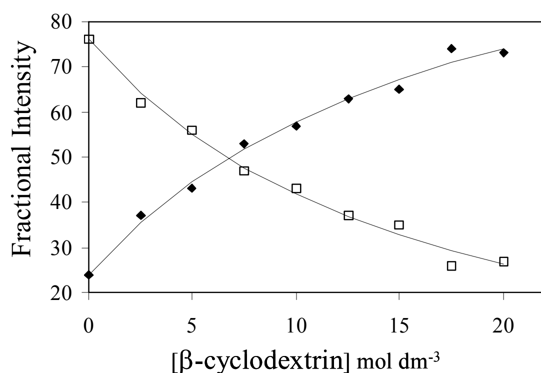

Figure 4. Fractional intensities of the long (♦) and short (□) lifetime components in the decay of 505 nm fluorescence from multiphoton excitation (750 nm) of serotonin with increasing β-cyclodextrin concentration.

values of 59.4 ± 11.2 and 53.8 ± 10.6 dm^3 mol^{-1} for the short and long component intensities respectively.

The hyperluminescence (505 nm) decays clearly identify the free (short lifetime) and bound (long lifetime) states of serotonin in solutions containing β-cyclodextrin. The increase in lifetime in the bound state corresponds with previous data in which the lifetime of hyperluminescence increased with decreasing solvent polarity, reaching 3.1 ns in ethanol:water (90:10 v/v) [3)]. The bound emission might arise either from the intermediate fluorescing state formed in solution or already in the complexed form, since it has a lifetime of >100 μs [4)]. If the former, then the data in Figure 4 shows that the intermediate associates similarly to serotonin itself, and therefore much have similar size and polarity. If the latter, then the results demonstrate hyperluminescence from a single bound molecule in the cyclodextrin cavity. In either case the results suggest that a single residue of 5-hydroxytryptophan in a protein should be capable of exhibiting hyperluminescence.

Imaging of serotonin in cells

Mast cells sequester serotonin within intracellular granules. Serotonin release from such granules may be triggered by stimulation by antigen and imaging of serotonin uptake and release has previously been achieved by the Webb group[9-11)] using three photon excitation (750-850 nm) of the ultraviolet fluorescence of serotonin. Use of these wavelengths is also capable of exciting tryptophan, thereby potentially increasing the background signal and decreasing contrast in serotonin imaging. There appears to be no report of imaging of hyperluminescence. Using the Ti:sapphire laser and OPO available in the Confocal Microscopy Lab, serotonin granules in rat basophilic leukemia cells (RBL-2H3) were imaged. In the first experiments, 2-photon excitation of UV emission (340 nm interference filter) was observed. Images with 630 nm excitation provided better contrast than when using 600 nm, consistent with the more specific excitation of the 5-hydroxyindole chromophore at this slightly longer wavelength. Images were also obtained using 750 nm excitation and 500 nm emission. Figure 5 shows images of control cells (right) and of cells previously loaded by incubation with serotonin (left).

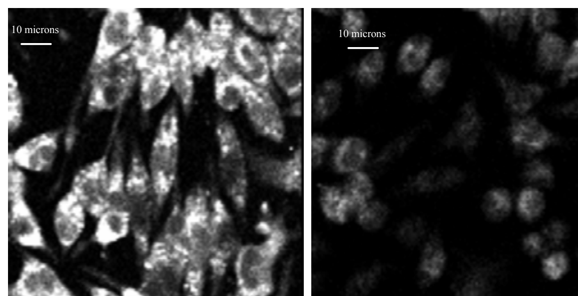

Figure 5. Images of rat RBL-2H3 mast cells obtained with 750 nm excitation and emission observed through a 500 nm interference filter. Laser power ca. 50 mW. Left - after overnight incubation with serotonin (250 μmol dm^{-3}); right – control cells.

The serotonin-containing granules are clearly seen with the loaded cells, and imaging of the green hyperluminescence in this way provides at least as good contrast as with the 340 nm/630 nm combination. These results indicate the potential for higher resolution confocal 3-D imaging of serotonin granules in these cells.

Acknowledgements

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