Single- and multi-photon excited fluorescence from serotonin complexed with β-cyclodextrin

Roger H. Bisby[a]*, Stanley W. Botchway[b], Shakeela Dad[a] and Anthony W. Parker[b]

[a]Biosciences Research Institute, University of Salford, Salford M5 4WT, UK
[b]Lasers for Science Facility, Rutherford Appleton Laboratory, Chilton, OX11 0QX, UK

*Author for correspondence
Professor R H Bisby
Biosciences Research Institute
Peel Building
University of Salford
Salford M5 4WT, UK
Email:- r.h.bisby@salford.ac.uk Tel +(44)161 295 4912 Fax +(44)161 295 5015

Summary

The fluorescence of serotonin on binding with β-cyclodextrin has been studied using both steady-state and time-resolved methods. Steady state fluorescence intensity of serotonin at 340 nm showed ~ 30% increase in intensity on binding with K_a ~ 60 dm^3 mol^{-1} and the fluorescence lifetimes showed a corresponding increase. In contrast, the characteristic green fluorescence ('hyperluminescence') of serotonin observed upon multiphoton near-infrared excitation with sub-picosecond pulses was resolved into two lifetime components assigned to free and bound serotonin. The results are of interest in relation to selective imaging and detection of serotonin using the unusual hyperluminescence emission and in respect to recent determinations of serotonin by capillary electrophoresis in the presence of cyclodextrin. The results also suggest that hyperluminescence occurs from multiphoton excitation of a single isolated serotonin molecule.

Keywords:- serotonin, cyclodextrin, fluorescence lifetime, multiphoton, hyperluminescence

Abbreviations:- UV – ultraviolet, RBL – Rat basophilic leukaemia.

Introduction

Levels of the neurotransmitter serotonin have been linked to clinical depression and suicide.[1] This has consequently created considerable interest in the development of analytical methods for determining serotonin levels in biological samples such as cerebrospinal fluid.[2] A recent report describes the use of capillary electrophoresis in combination with hydroxypropyl-β-cyclodextrin for analyzing serotonin levels in brain microdialysis.[3] The importance of neurotransmitters has also stimulated research to develop methods to locate them within living cells[4] at physiological levels, in particular, and non-linear spectroscopic methods linked to microscopy have much potential in the field of neurobiology.[5] The peak UV absorption of the 5-hydroxyindole chromophore in both 5-hydroxytryptophan and serotonin occurs at a slightly longer wavelength than tryptophan. This has enabled the use of 5-hydroxytryptophan as a fluorescence probe to study conformation and mobility within proteins.[6] In RBL rat mast cells, confocal three-photon excitation using ultrafast near infrared laser pulses has enabled imaging of serotonin distribution and release by monitoring the UV fluorescence at 340 nm.[7,8] We and others are presently exploring the further possibility for selective intracellular imaging of serotonin by taking advantage of the specific green emission ('hyperluminescence') produced by multiphoton excitation of serotonin and 5-hydroxytryptophan.[9,10] However, the identity of the molecular photoproduct responsible for hyperluminescence remains elusive. The supra-linear dependence of hyperluminescence intensity on serotonin concentration in phosphate buffer[11] implies the possible involvement of a dimeric or higher aggregate photoproduct, although rapid capillary electrophoresis experiments indicate the photoproduct is of a similar size to serotonin itself.[12] The present experiments, in which a molecule of serotonin is isolated within a cyclodextrin cavity, seek to resolve some aspects of this fundamental issue.

Cyclodextrins, especially β-cyclodextrin (cycloheptaamylose), have found varied applications including drug solubilisation and delivery and in analytical chemistry procedures.[13] This wide range of applications comes from their ability to form inclusion complexes and act as a host for various guest molecular species. Cyclodextrins possess a relatively hydrophobic cavity with the more polar hydroxyl

groups arranged around the external rim. These features provide both isolation of the guest molecule from the surrounding environment as well as a means of improving aqueous solubility of hydrophobic compounds. Cyclodextrins are able to modify the behaviour of electronically excited states of organic molecules,[14] for example in permitting room temperature phosphorescence ,[15] enhancement of fluorescence quantum yields[16-19] and influencing the outcome of photochemical reactions.[20] This present work has sought to further the earlier work using capillary electrophoresis in combination with hydroxypropyl-β-cyclodextrin [3] and quantify the nature of the serotonin:β-cyclodextrin complex and characterise the photophysical properties of both UV fluorescent and multiphoton hyperluminescence from serotonin.

Experimental

All chemicals including serotonin and β-cyclodextrin were purchased from Sigma-Aldrich. Solutions were prepared in phosphate buffer (20 mmol dm^{-3}, pH 7.0) containing NaCl (0.15 mol dm^{-3}).

Fluorescence spectra were measured using a Spex Fluoromax spectrofluorimeter using the manufacturer's correction curve to obtain corrected spectra. Fluorescence lifetimes were measured using the time-correlated single photon counting technique using as the excitation source a Spectra-Physics Ti:Sapphire laser (120 fs pulses, 80 MHz) pumped by a 20 W argon ion laser (Spectra Physics Ltd). For one-photon excitation at 300 nm, the Ti:Sapphire laser output was shifted to 600 nm in a Mira OPO (Coherent) and doubled to 300 nm using a type 1 barium borate (BBO), delivering ~ 4mW to the sample. Fluorescence at 340 nm from a sample in a standard 1 cm quartz cuvette was selected by a monochromator (M500, IBH) and detected with a Becker & Hickl GmbH photon-counting photomultiplier (Model PMH-100) linked to a Becker and Hickel SPC700 multichannel analyser card. Fluorescence decays were deconvoluted with the instrument response function using Edinburgh Instruments software. Multiphoton excitation at 750 nm used the direct output of the Ti:Sapphire laser (~60 mW at the sample). A drop of the sample on a cover slip was placed on the stage of a fluorescence microscope (Nikon TE2000) and green emission selected with a 505 nm interference filter (500IU25, Comar, UK). Analysis of binding data by non-linear least squares analysis used the Grafit computer program.

Results and discussion

Ultraviolet fluorescence

Serotonin fluoresces in aqueous solution at pH 7 with a maximum at 334 nm on excitation at 300 nm. Addition of β-cyclodextrin to solutions of serotonin (5 μmol dm^{-3}) in solutions buffered with phosphate (20 mmol dm^{-3}, pH 7.0) and NaCl (0.15 mol dm^{-3}) resulted in a small enhancement of the fluorescence intensity (Figure 1). Neither the excitation nor emission spectra showed any significant changes in shape or peak wavelengths with the addition of β-cyclodextrin. This is consistent with the observation that the fluorescence properties of 5-hydroxyindole are relatively insensitive to solvent polarity.[21]

The binding parameters for the serotonin (S) - β-cyclodextrin (β-CD) complex were evaluated on the basis of formation of a 1:1 complex as has previously been established in similar cases:-[16,17]

$$S + \beta\text{-CD} \rightleftharpoons [S\text{-}\beta\text{-CD}] \quad \ldots\ldots. (1)$$

The association constant of the complex, K_A, was found by direct non-linear least squares fitting of plots of fluorescence intensity (F) versus β-cyclodextrin concentration according to the binding equation (2), where F_0 is the intensity in the absence of β-cyclodextrin and F_{max} is the maximal increase in intensity at infinite β-cyclodextrin concentration :-

$$F = F_0 + \frac{K_A F_{max} [\beta\text{-CD}]}{(1 + K_A [\beta\text{-CD}])} \quad \ldots\ldots.(2)$$

Intensities were obtained by integration of the fluorescence spectra on excitation at 300 nm and are shown plotted against β-cyclodextrin concentration in Figure 2. The best fit to equation (2) is shown by the line with $K_A = 53.2 \pm 17.3$ dm^3 mol^{-1} and the value of F_{max} corresponding to 33.8 ± 6.1% increase in fluorescence intensity of the fully complexed serotonin. (Note: The precision of the measurements of such a small K_A for serotonin: β-cyclodextrin binding is limited by the solubility of β-cyclodextrin and the comparatively small change in fluorescence intensity observed). It is

noteworthy that the value of K_A for binding of serotonin by β-cyclodextrin obtained here is less than those for tryptamine[17] and tryptophan[16] (160 ± 30 and 184 ± 18 dm^3 mol^{-1} respectively) but the overall values for increases in fluorescence intensity on binding are similar.

Time-resolved fluorescence decays of serotonin, excited at 300 nm and detected at 340 nm, were measured using the time-correlated single photon counting technique. In the absence of cyclodextrin, the fluorescence decay was satisfactorily deconvoluted ($\chi^2 < 1.2$) as a single exponential with a lifetime of 3.65 ns, in good agreement with a previous determination of 3.80 ns at pH 7.[22] Addition of β-cyclodextrin resulted in an increase in fluorescence lifetime (Figure 4), reaching a value of 4.24 ns in the presence of 20 mmol dm^{-3} β-cyclodextrin. The increase in fluorescence lifetime (16%) closely matches the rise of steady state fluorescence intensity (18%) at 20 mmol dm^{-3} β-cyclodextrin recorded above.

Time-resolved hyperluminescence

Multiphoton excitation of serotonin using an ultrafast near-infrared laser is known to produce the green fluorescence at 505 nm ('hyperluminescence'). This corresponds to a 6-photon process, involving the initial formation of photochemical intermediate in a four-photon step, followed by excitation of the intermediate by a further two photons.[9] For this work we used a focussed laser beam (750 nm, 130 fs pulses, 80 MHz, 56 mW at the sample) produced by a Ti:sapphire laser. The decay of hyperluminescence from solutions of serotonin (1 mmol dm^{-3}, pH 7.0) was biexponential. Due to the inherently weaker emission in the hyperluminescence experiments, a higher serotonin concentration (1 mmol dm^{-3}) was used. However over most of the range of cyclodextrin concentrations used (2.5 to 20 mmol dm^{-3}), the cyclodextrin is present in modest excess (≥ 5:1 molar ratio) and analysis of data using equation (2) is justified. Representative time-resolved decays are shown in Figure 3 and analysis shows they contain a short lifetime (τ_1) of 710 ± 17 ps that is found to be independent of cyclodextrin concentration. However, the lifetime of a second component (τ_2) increased from 1.65 ns in the absence of β-cyclodextrin to a limiting value of 3.4 ± 0.3 ns above β-cyclodextrin concentrations of 10 mmol dm^{-3} (Figure 4).

More complex analysis of the data was not attempted and the increase in the long lifetime component to this limiting value is taken to indicate the eventual predominance of the longer lifetime component due to serotonin to β-cyclodextrin. The fractional intensity of the longer lifetime component (f_2), defined by equation 3 where α_1 and α_2 are the pre-exponential factors from the fitting analysis,

$$f_2 = \frac{\alpha_2 \tau_2}{\alpha_2 \tau_2 + \alpha_1 \tau_1} \qquad \text{..... (3)}$$

increased with increasing β-cyclodextrin concentration (Figure 5). The variations of the fractional intensities of the two lifetime components versus β-cyclodextrin concentration are shown in Figure 5 with the solid lines representing best fits to the equivalent of equation (2) with K_A = 59.4 ± 11.2 and 53.8 ± 10.6 $dm^3\ mol^{-1}$ for the short and long component intensities respectively. These values are consistent with the binding constant (53.2 ± 17.3 $dm^3\ mol^{-1}$) obtained from the steady state fluorescence experiments described above. Extrapolation of the data shows that at saturating β-cyclodextrin concentrations only emission from bound serotonin is observed with the percentage intensity of the lifetime for the bound form of 120 ± 11% and that for the free form of 15 ± 10%.

As similarly reported in a study of fluorescence lifetimes measured on the binding 2-amino-5,6-dimethylbenzimidazole to β-cyclodextrin,[19] the hyperluminescence decays from serotonin clearly contain exponential components attributable to bound and free species. For the hyperluminescence the principal lifetimes of bound and free serotonin are 3.4 ns and 0.71 ns respectively. It might have been anticipated that differentiation of bound and free serotonin would be observed in the single photon-excited ultraviolet (340 nm) fluorescence decays from serotonin. However since both intensity and lifetimes show equivalent small increases of only 16-18% over the available β-cyclodextrin concentration range, the two lifetimes may not have been identified within the deconvolution process. Hyperluminescence from 5-hydroxytryptophan has been shown to have a similarly short lifetime in aqueous solution of 0.91 ns.[10] Unlike the fluorescence lifetime of the normal ultraviolet (340 nm) fluorescence of 5-hydroxytryptophan,[21] hyperluminescence lifetimes were found

to be very responsive to solvent,[10] increasing up to 3.1 ns in 90% ethanol in water, a value rather similar to the lifetime identified here with the hyperluminescent species bound within the β-cyclodextrin cavity.

Hyperluminescence of molecules such as serotonin and 5-hydroxytryptophan offers new opportunities in the detection[11] and imaging[23] of these compounds. The transient species responsible for hyperluminescence remains unidentified, although new routes to multiphoton excitation of serotonin have been explored recently.[24] Capillary electrophoresis experiments have been used to eliminate dimer or higher multimers of serotonin as being responsible for the intermediate that produces the hyperluminescence.[12] In biological systems there is the possibility that serotonin may be bound to proteins such as albumin, and that binding may affect attempts to quantify or image serotonin using hyperluminescence. The results show that whilst native ultraviolet fluorescence may be relatively unaffected by binding, the hyperluminescence lifetime is more sensitive and might be used as an indicator of such interactions.

In the present experiments there are two possibilities leading to hyperluminescence from serotonin with the lifetime of 3.4 ns. This value is characteristic of the fluorophore being located within an environment with a polarity lower than water. Thus the first possibility is the photochemical conversion of serotonin to the hyperluminescent intermediate arises from the 4-photon excitation (at 750 – 830 nm) of a serotonin molecule already complexed within the β-cyclodextrin cavity. This intermediate whilst still complexed may then be further excited by a further two photons to generate the hyperluminescence. In this case the hyperluminescence comes from an isolated single serotonin molecule. An alternative mechanism for the hyperluminescence with 3.4 ns lifetime is that the initial intermediate is formed by the four-photon process in solution. This may then associate with cyclodextrin during its lifetime (>100 microseconds)[9] before being excited to give hyperluminescence from within the cyclodextrin cavity. In the latter case the similarity of the association constants in the static fluorescence observed at 340 nm generated by UV excitation and the time-resolved hyperluminescence experiments indicate similar sizes and polarities of the serotonin molecule and the intermediate species. These arguments

support the hypothesis that hyperluminescence occurs from a monomer species derived from serotonin, and that it should be possible to observe hyperluminescence from a single residue of 5-hydroxytryptophan within a protein.

Acknowledgements

The authors thank the Biological and Biotechnological Research Council (BBRSC) for a grant in support of this work, CCLRC for access to the Central Laser Facility and Mathew Dillingham for technical assistance.

References

1. A.S.Elhwuegi, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2004, **28**, 435-451.

2. M.Asberg, Ann. *N.Y. Acad. Sci.*, 1997, **836**, 158-181.

3. N.Beturquia, F.Couderc, V.Sauvinet, C.Ordset, S.Parrot, C.Bayle, B.Renaus and L.Denoroy, *Electrophoresis*, 2005, **26**, 1071-1079.

4. S.Okumoto, L.L.Looger, K.D.Michever, R.J.Reimer, S.J.,Smith and W.B.Frommer, *Proc.Natl.Acad.Sci.U.S.A.*, 2005, **102**, 8740-8745.

5. J.Merz, *Curr.Opin.Neurobiol.*, 2004, **14**, 610-616

6. J.B.A.Ross, A.G.Szabo and C.W.V.Hogue, *Methods Enzymol*., 1997, **278**, 151-190.

7. S.Maiti, J.B.Shear, R.M.Williams, W.R.Zipfel and W.W.Webb, Science, 1997, **275**, 530-532.

8. R.M.Williams, J.B.Shear, W.R.Zipfel, S.Maiti and W.W.Webb, *Biophys.J*., 1999, **76**, 1835-1846.

9. J.B.Shear, C.Xu and W.W.Webb, *Photochem.Photobiol*., 1997, **65**, 931-936.

10. R.H.Bisby, M.Arvanitidis, S.W.Botchway, I.P.Clark, A.W.Parker and D.Tobin, *Photochem.Photobiol.Sci*., 2003, **2**, 157-162.

11. M.L.Gostkowski, J.Wei and J.B.Shear, *Anal.Biochem.*, 1998, **260**, 244-250.

12. M.J.Gordon, E.Okerberg, M.L.Gostkowski and J.B.Shear, *J.Am.Chem.Soc.*, 2001, **123**, 10780-10781.

13. J.Szejtli, *Chem.Rev*., 1998, **98**, 1743-1753.

14. P.Bortolus and S.Monti, *Adv.Photochem*., 1996, **21**, 1-119.

15. S.Scypinski, and L.J.C.Love, *Anal. Chem.*,1984, 56, 331-336.

16. A.Orstan and J.B.A.Ross, *J.Phys.Chem.*, 1987, **91**, 2739-2745.

17. R.E.Galian, A.V.Veglia and R.H. de Rossi, *Analyst*, 1998, **123**, 1587-1591.

18. C.Donze, E.Rizzarelli and G.Vecchio, *Supramol.Chem.*, 1998, **10**, 33-42.

19. M.A.El-Kemary and I.M.El-Mehasseb, *Talanta*, 2004, **62**, 317-322.

20. K.S.S.P.Rao, S.M.Hubig, J.N.Moorthy and J.K.Kochi, *J.Org.Chem.*, 1999, **64**, 8098-8104.

21. K.Lotte, R.Plessow and A.Brockhinke, *Photochem.Photobiol.Sci.*, 2004, **3**, 348-359.

22. A.Chattopadhyay, R.Rukmini and S.Mukerjee, *Biophys.J.*, 1996, **71**, 1952-1960.

23. S.B.Botchway, K.Brindle and A.W.Parker, *Biochem. J.*, 2005 in press (doi:10.1042/BJ20050648).

24. M.L.Gostkowski, R.Allen, M.L.Plenert, E.Okerberg, M.J.Gordon,and J.B.Shear, *Biophys.J.*, 2004, **86**, 3223-3229.

Figure legends

FIGURE 1 Fluorescence spectra from serotonin (5 μmol dm^{-3}) excited at 300 nm in solutions at pH 7 containing:- 0 (curve 1); 5 (curve 2); 10 (curve 3) and 20 (curve 4) mmol dm^{-3} β-cyclodextrin.

FIGURE 2 Increase in fluorescence intensity from solutions of serotonin (5 μmol dm^{-3}) at pH 7 on addition of β-cyclodextrin. The solid line indicates the fit with Ka= 53.2 dm^3 mol^{-1}, as described in the text.

FIGURE 3 Nanosecond time-resolved fluorescence decays at 505 nm following multiphoton excitation at 750 nm of serotonin (1 mmol dm^{-3}) in solutions (pH 7) containing β-cyclodextrin:- none (trace 1); 5 mmol dm^{-3} (trace 2); 10 mmol dm^{-3} (trace 3) and 20 mmol dm^{-3} (trace 4).

FIGURE 4 Fluorescence (hyperluminescence) lifetimes of the biexponential decay from serotonin (1 mmol dm^{-3}) on multiphoton excitation at 750 nm and pH 7 with increasing β-cyclodextrin concentration. Emission was observed at 505 nm. Increasing the β-cyclodextrin concentration had no effect on the short lifetime component (■), whilst the lifetime of longer component increased (□). Also shown are the single exponential lifetimes of the UV fluorescence at 340 nm, excited at 300 nm (▲).

FIGURE 5 Intensity contributions in the biexponential decay of serotonin fluorescence (hyperluminescence) at 505 nm after multiphoton excitation at 750 nm with increasing β-cyclodextrin concentration. Relative intensity (percentage) contributions are shown for the short lifetime component (□) and the long lifetime component (■).

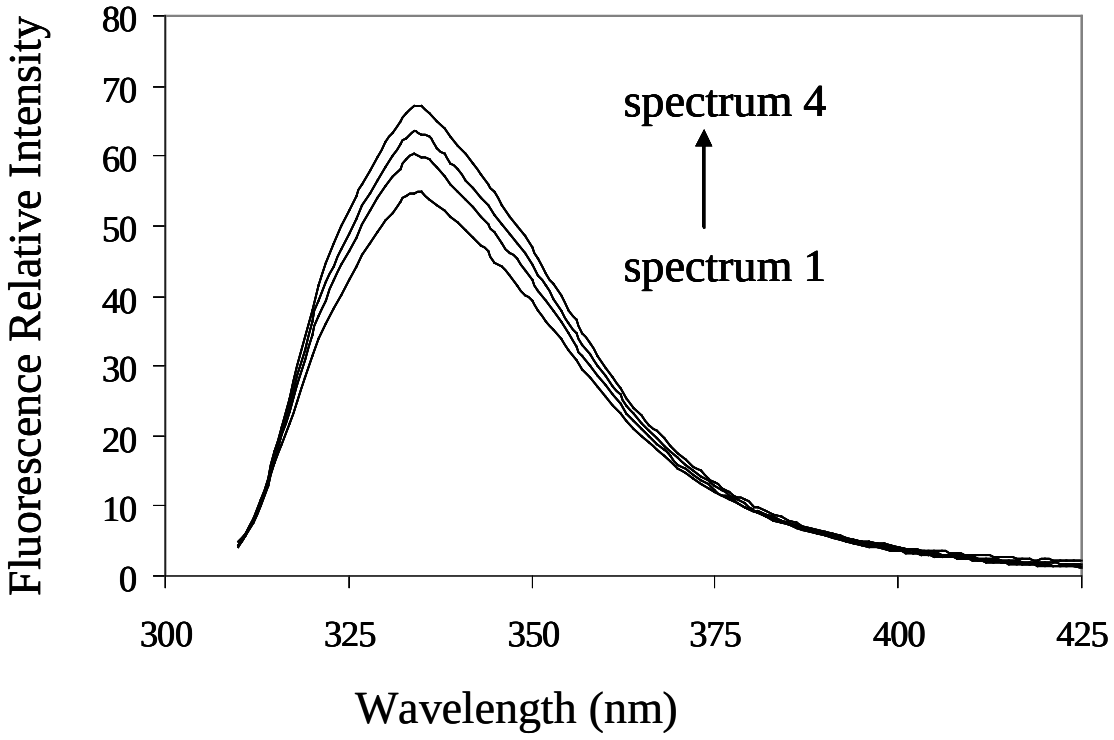

FIGURE 1

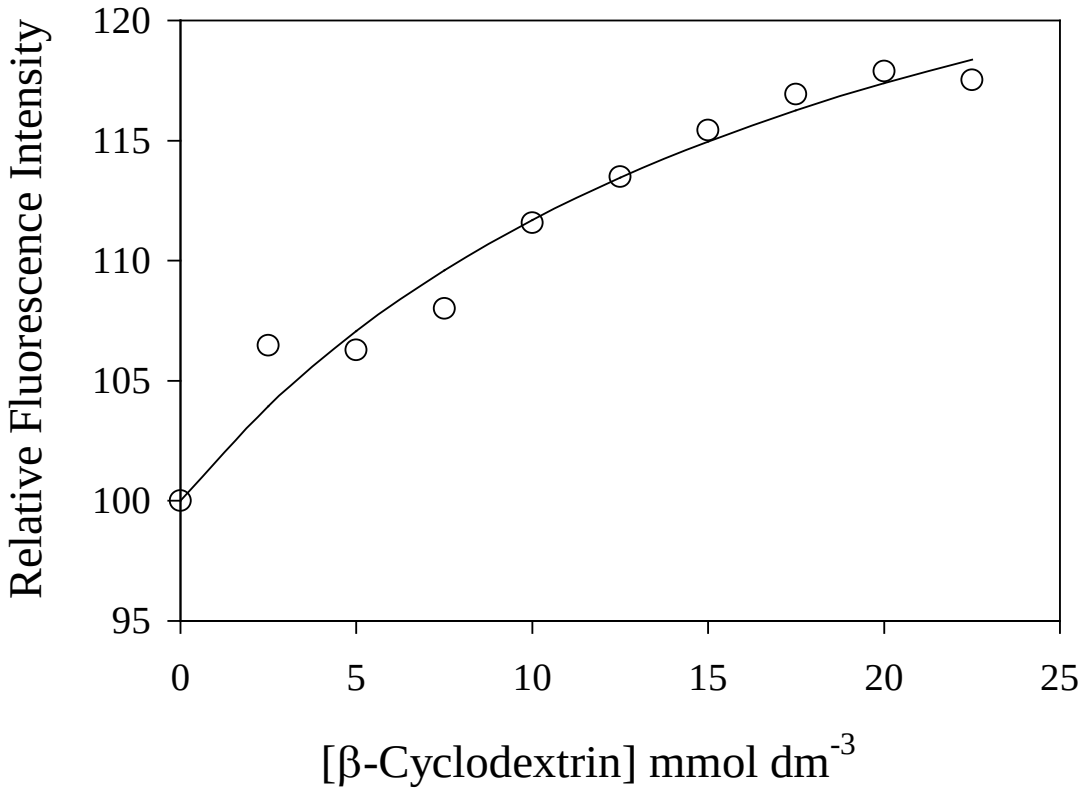

FIGURE 2

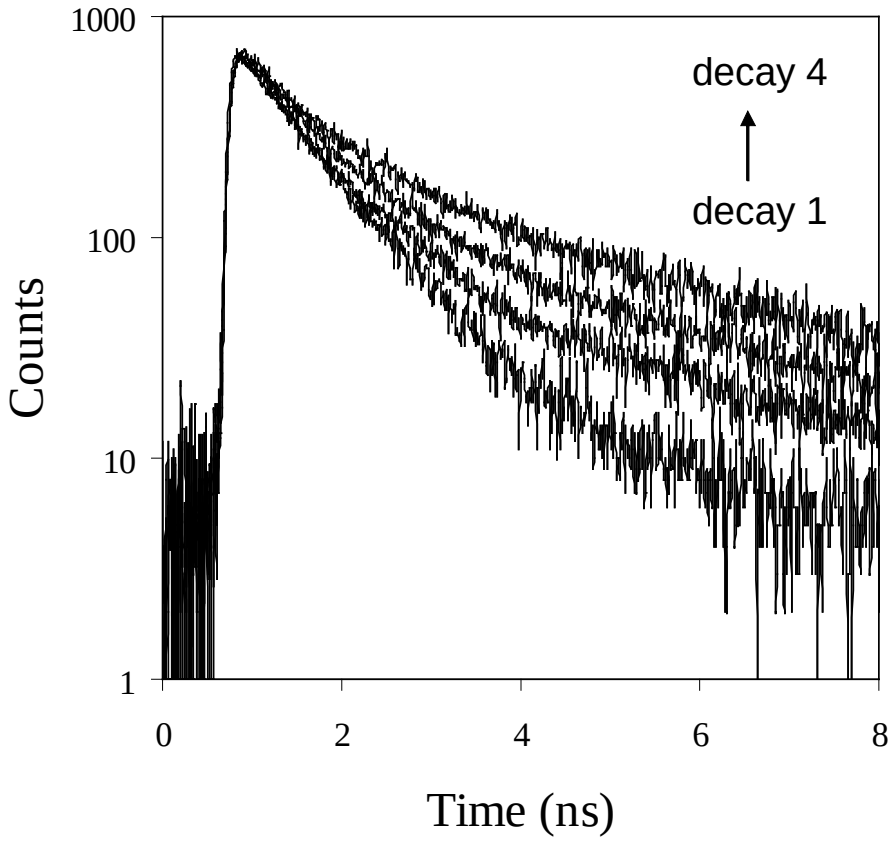

FIGURE 3

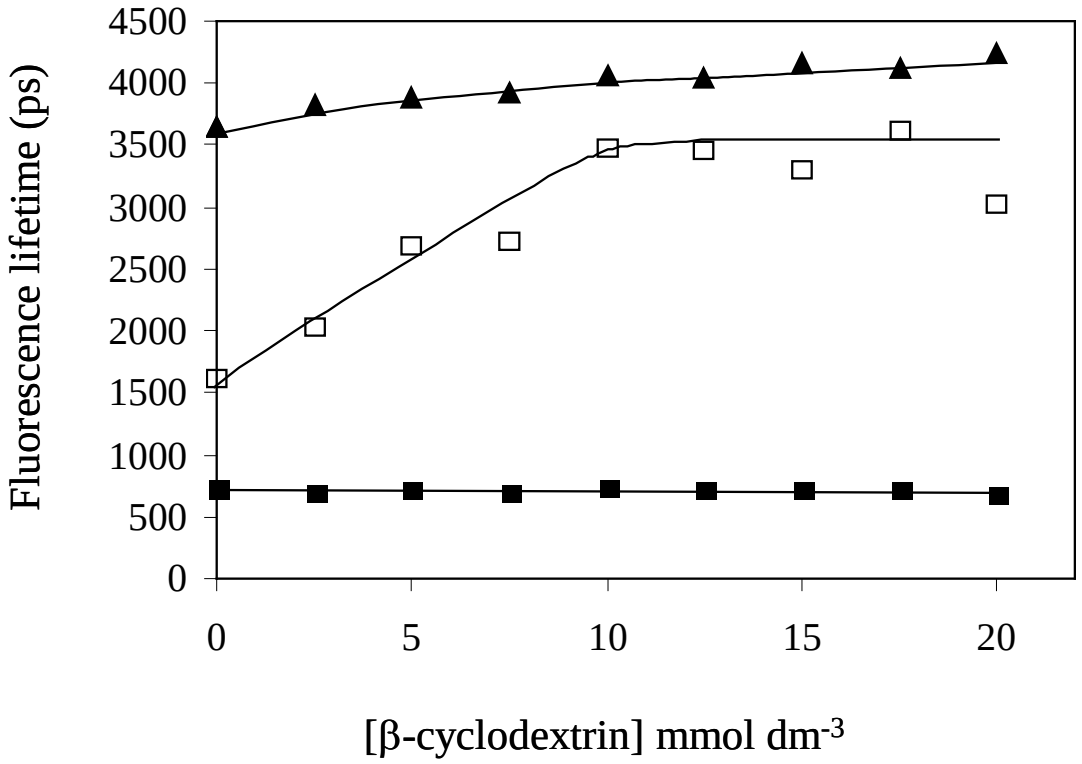

FIGURE 4

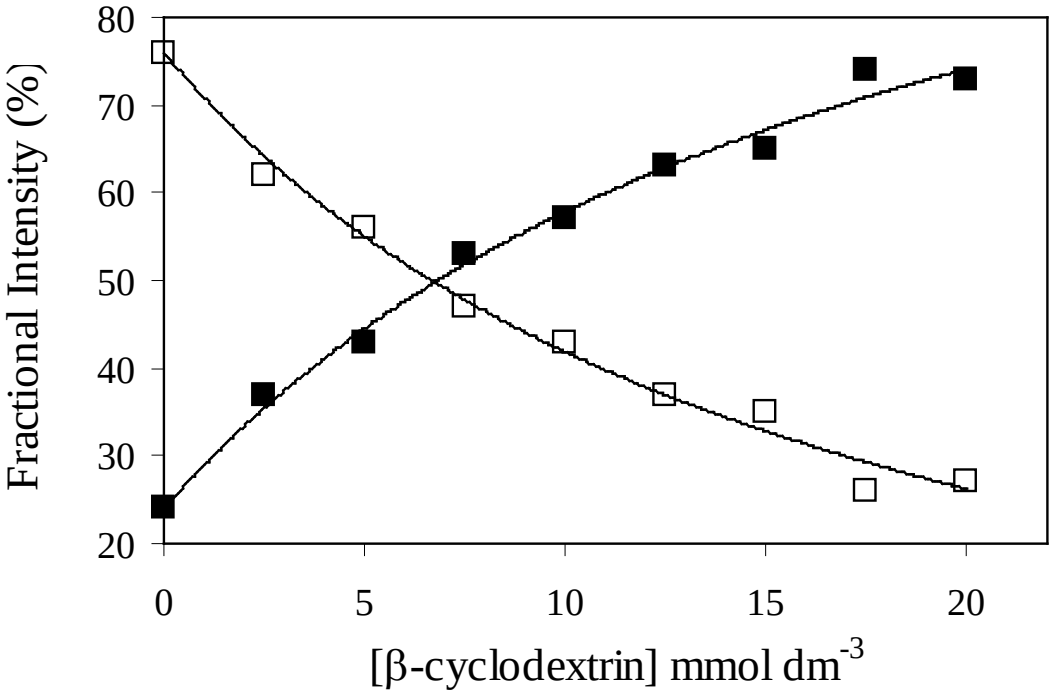
Fractional Intensity (%)
80
70
60
50
40
30
20
0
5
10
15
20
[β-cyclodextrin] mmol dm-3

FIGURE 5