ChemComm

A pH sensitive fluorescent cyanine dye for biological applications

Mark S. Briggs, D. Dougal Burns, Michael E. Cooper* and Susan J. Gregory

Drug Discovery, Amersham Pharmacia Biotech, Forest Farm, Whitchurch, Cardiff, UK CF14 7YT. E-mail: michael.cooper@eu.apbiotech.com

Received (in Cambridge, UK) 1st September 2000, Accepted 10th October 2000
First published as an Advance Article on the web 9th November 2000

A pentamethine cyanine dye has been synthesised that is fluorescent when protonated, becoming non-fluorescent upon proton abstraction. The probe has a pK_a of 7.5, with observable changes in the fluorescent emission properties across a pH range of 6.0–8.0, therefore providing a useful probe for studying pH in biological media.

Cyanine dye[1] fluorescent probes are useful markers for flow cytometry,[2] sequencing assays[3] and high throughput screening.[4] These probes have high extinction coefficients (> 200000 l mol^{-1} cm^{-1}), can be designed with long excitation and maximum fluorescent emission wavelengths (> 600 nm) and with lower molecular weights than the majority of commercially available natural fluorophores.[5] These fluorescent cyanine dyes also have the added value of containing reactive functional groups, such as an *N*-hydroxysuccinimidyl ester or an isothiocyanate facilitating a covalent link to a biomolecule. More recently, attention has also focussed on the synthesis of more specialised probes,[6] or "functional dyes", that can act as molecular and optical switches.[7] Indeed, many of these dyes are designed to be environmentally sensitive, acting as ionophores for protons, Ca^{2+}, Na^+ or K^+ or as small molecule sensors for cyclic adenosine monophosphate[8] or inositol phosphate.[9]

One subject of ongoing interest is acidity regulation in mammalian cells.[10] Monitoring small changes in proton concentration is important for the study of numerous signal transduction pathways and ligand interactions with G-protein coupled receptors. The majority of commercially available pH sensitive fluorescent dyes employed in intracellular studies[5] provide a reduced fluorescent signal in acidic media or alternatively the pK_a of the dye is outside the critical intracellular pH window of between 5–8 pH units.

We now report an example of a fluorescent pentamethine cyanine dye (Cy5™) that is sensitive to proton concentration over a pH range of 6–9 (Fig. 1). The intensity of the fluorescent emission (665 nm) for this probe increases with increasing proton concentration. This probe is analogous to a number of previously reported benzothiazolium[11] trimethine cyanine dyes with pH sensitive UV/visible absorption properties, although in addition, the design of our fluorescent probe includes several other important elements. The presence of a sulfonic acid functional group aids solubility in aqueous media and the position of such functional groups is also known to reduce probe aggregation in solution.[12] There is also a pendant carboxylic acid group that can be easily converted to the reactive *N*-hydroxysuccinimidyl ester for biolabelling. Furthermore, unlike previously reported cyanine dyes,[1] the pH sensitivity is introduced *via* a non-*N*-alkylated indolium moiety whereupon protonation and deprotonation occurs depending upon the localised hydrogen ion concentration.

The probe exists in two forms, as either the fluorescent cyanine dye or the complementary non-fluorescent base (Fig. 1). The intense cyanine dye absorption and emission properties are largely due to a resonance effect between the two nitrogen atoms of the two indole rings *via* the conjugated pentamethine bridge. Abstraction of a proton from this system destroys this resonance, and subsequently leads to the non-fluorescent base form. Moreover, this property provides excellent resolution at long wavelengths (> 600 nm) where little else in a biological sample is likely to absorb.

The probe was synthesised by standard cyanine dye methods.[1] 1-Ethyl-2,3,3-trimethyl-3*H*-indol-1-ium-5-sulfonate[1] and 5-carboxymethyl-2,3,3-trimethyl-2,3-dihydroindole[13] were condensed with malonaldehyde bis(phenylimine) in the presence of acetic acid, acetic anhydride and pyridine at 70 °C . The product was purified by reverse phase HPLC[14] and conversion to the NHS (*N*-hydroxysuccinimidyl) ester is facile by standard methods. A typical conversion to the NHS ester involves reacting the dye in DMSO with 1 eq. of PyBOP (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate, *N*-hydroxysuccinimide and 1.1 eq. of diisopropylethylamine. Stirring for 1 h gives quantitative conversion. TLC analysis (10% methanol–DCM on SiO_2) observes a fast running blue spot R_f = 0.60. R_f of the free acid = 0.41. Hydrolysis of this material gives the free acid by TLC.

The fluorescent properties of this dye can be seen in Fig. 2. Equimolar solutions of the dye were made up in phosphate buffers[15] over a pH range of between 4.5–9. It can be observed that the fluorescent characteristics of the probe are greatly reduced as the buffers become less acidic in nature. This is due to increased deprotonation of the cyanine dye and leads to a larger population of the non-fluorescent base species. Similar experiments with a commercial bis(*N*-alkylated) sample of

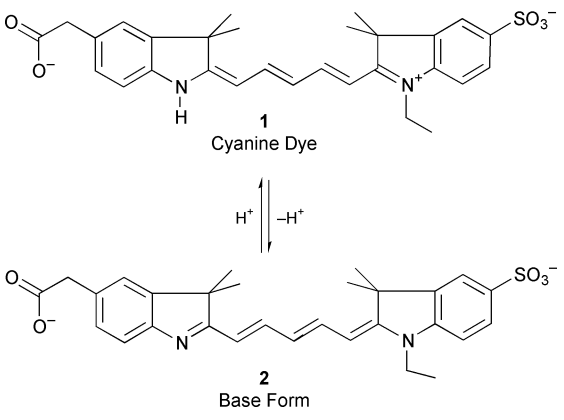

Fig. 1 The cyanine dye-base form of the pH sensitive fluorescent probe.

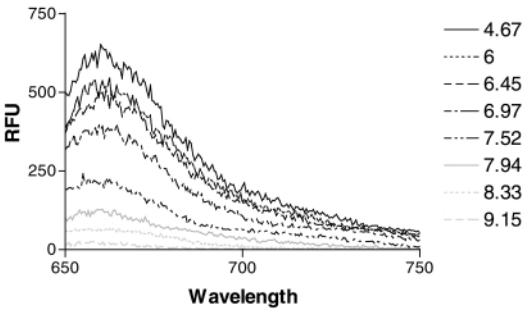

Fig. 2 Fluorescence characteristics of the pH sensitive probe as a function of pH (~ 0.1 μmol of complex, λ_{ex} = 630 nm, 295 K. Readings were made using a Perkin-Elmer LS50B fluorimeter in fluorescence mode, with excitation and emission slits set at 2.5 nm and with 3 ml quartz cuvettes).

Cy5™ showed no change in the emission properties of the probe over the same pH range. Furthermore, the photostability of the fluorescent form of this probe was comparable to the non-pH sensitive cyanine pentamethine dyes (Cy5™) routinely used in biological studies.

Further proof of this acid–base equilibrium can be achieved by observation of the UV/visible spectra of **1** in phosphate buffers of different pH (Fig. 3). It can be seen that as the buffer solutions become less acidic, the characteristic absorption maximum for the pentamethine cyanine dye at 645 nm is greatly reduced as a new peak evolves at 480 nm. This absorption peak is due to the increased presence of the base form (**2**) of the probe by proton abstraction from the pentamethine cyanine dye. A further observation is the presence of an isosbestic point at 545 nm. This gives a good indication that the solutions are all of similar ionic strength.[16] It is therefore likely that the proton concentration alone is responsible for the different observed spectra, and that this phenomenon cannot be attributed to the differing concentrations of any other ions present in the buffer solution.

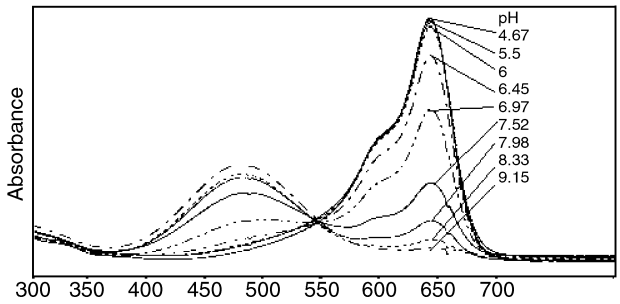

Fig. 3 UV/visible absorption spectra of **1** (and **2**) as a function of pH (from a Hewlett Packard 8453 UV/vislble spectrophotometer with diode array detector, 1 cm pathlength quartz cuvettes).

Fig. 4 illustrates the pH response of probe **1** as a function of I/I_{max} *vs.* pH, where I is the measured fluorescent emission at that pH, and I_{max} is the maximum output of the probe. It can be seen that a regular sigmoidal response is observed for this probe in response to pH. The pK_a of **1** can be estimated where I/I_{max} is 0.5. This provides a pK_a value of 7.5 (296 K) for this probe.

A current limitation in the application of many current pH sensitive fluorescent probes is due to lack of any specific labelling functionality present on the dye such as an NHS ester. The presence of these groups allows specific biomolecules or particular functional groups, *e.g*. primary amines to be targeted, and a subsequent range of bioassays to be performed using the dye-labelled biomolecule. The presence of a pendant carboxylic acid allows rapid conversion to the *N*-hydroxysuccinimidyl ester and therefore facilitates the labelling of biological molecules such as proteins or antibodies.[17] The reactive form of this probe has been employed to label aliphatic amine groups present on the outer membrane of a CHO cell. Upon constitutive endocytosis the probe becomes internalised into acid vesicles with a subsequent increase in fluorescent emission.[18] Furthermore, the pK_a of the probe when bioconjugated did not change implying that the carboxylic acid present in the free form of the probe does not affect the pH sensitive fluorescent properties. Details of these cellular studies will be reported elsewhere.

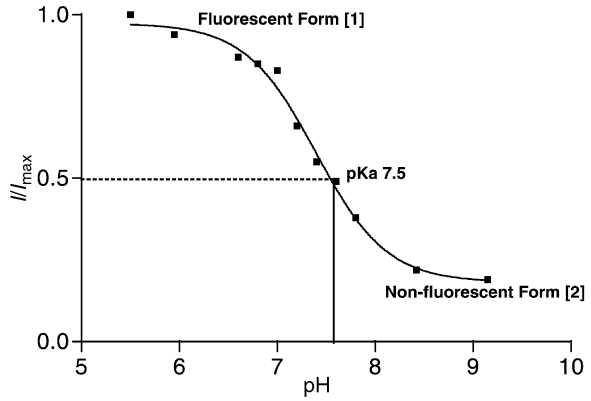

Fig. 4 pH Dependence of **1** (I/I_{max}) at 295 K in phosphate buffers of differing pH (λ_{exc} = 630 nm λ_{em} = 665 nm. Readings were made using a Perkin-Elmer LS50B fluorimeter in fluorescence mode, with excitation and emission slits set at 2.5 nm and with 3 ml quartz cuvettes. Each point is the average of 10 measurements).

Furthermore many other probes offer a reduced fluorescent emission in acidic conditions and the spectral data acquired often requires deconvolution due to both protic and non-protic forms being fluorescent and thus, these probes are of interest for ratiometric pH studies. In contrast, this pH sensitive cyanine probe has only one fluorescent form and is therefore useful for high throughput studies whereby a change in fluorescent emission can be easily and rapidly observed. The long emission wavelength of this pentamethine pH sensitive cyanine dye is also of value for biological studies, due to the increased light penetration into cell tissue of the excitation lasers. A range of homologous pH sensitive fluorescent pentamethine cyanine dyes is now being synthesised and evaluated and will be reported in subsequent communications.

MEC thanks Adam Colebrook and Tony Rees for the accurate mass spectroscopic analysis and J. Tony Smith and Martyn Birch for stimulating discussion.

Notes and references

1 R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis and A. S. Waggoner, *Bioconjugate Chem.*, 1993, Vol. 4, No. 2., 105–111.

2 L. A. Ernst, R. K. Gupta, R. B. Mujumdar and A. S. Waggoner, *Cytometry*, 1989, **10**, 11.

3 Z. Zhu, J. Chao, H. Yu and A. S. Waggoner, *Nucleic Acids Res.*, 1994, **22**, 3418.

4 M. E. Cooper, *Fluorescence Microscopy and Fluorescent Probes 3*, ed. A. Kotyk, Espero Publishing, Prague, 1999, 37–57.

5 R. P. Haugland, *Handbook of Fluorescent Probes Seventh Edn.*, Molecular Probes Inc., Oregon, USA, 1999.

6 W. T. Mason, *Fluorescent and Luminescent Probes for Biological Activity, 2nd edn.*, ed. W. T. Mason, Academic Press, 1999.

7 (*a*) M. P. Lowe and D. P. Parker, *Chem. Commun.*, 2000, 707; (*b*) L. Fabbrizzi, M. Licchelli, L. Parodi, A. Poggi and A. Taglietti, *Eur. J. Inorg. Chem.*, 1999, 35; (*c*) I. García-Ochoa, M.-Á. Díez López, M. H. Viñas, L. Santos, E. Martínez Atáz, F. Amat-Guerri and A. Douhal, *Chem. Eur. J.*, 1999, 5, 3, 897.

8 P. Turkewitsch, B. Wandelt, G. D. Darling and W. S. Powell, *Anal. Chem.*, 1998, **70**, (13), 2771.

9 K. Niikura, A. Metzger and E. V. Anslyn, *J. Am. Chem. Soc.*, 1998, **120** (33), 8533.

10 T. J. Rink, R. Y. Tsien and T. Pozzan, *J. Cell. Biol.*, 1982, **95**, 189.

11 L. G. S. Brooker, R. H. Sprague, C. P. Smyth and G. L. Lewis, *J. Chem. Soc.*, 1940, **62**, 1116.

12 S. R. Mujumdar, R. B. Mujumda, C. M. Grant and A. S. Waggoner, *Bioconjugate Chem.*, 1996, **7**, 356.

13 P. L. Southwick, J. G. Cairns, L. A. Ernst and A. S. Waggoner, *Org. Prep. Proced. Int.*, 1988, **20** (3), 279.

14 NMR data: 200 MHz ^{1}H-NMR, (d_6-DMSO), δ 8.3–8.1 (m, 2H, β,β′-protons in bridge), 7.76 (d, 1H, *J* = 4H-Ar), 7.62 (d, 1H, 7H-Ar, *J* = 8.3), 7.45 (s, 1H, 4H-Ar′), 7.27 (d, 1H, 7H-Ar, J = 7.8) 7.19 (d 6H-Ar, *J* = 8.3) 7.14 (d, 1H, Ar-6H, *J* = 7.8), 6.49 (t, 1H, γ′-proton in bridge), 6.27 (d, 1H, α-proton in bridge) 6.16 (d, 1H, α′-proton in bridge) 4.09 (q, 2H, α-CH_2, 1.63 (s, 6H, (-CH_3)$_2$, 1.56 (s, 6H, −CH_3)$_2$), 1.25 (t, 3H, β-CH_3). MS (electrospray, negative mode) *m*/*z* (%) = 521.2110 (100%).

15 *U.S. Pharmacopeia. The National Formulary*, Mach Printing Co., Easton, PA, USA, 1990, pp. 1784–1785.

16 V. Good, *pH Measurements, Their Theory and Practice*, Methuen Publ., London, 1956.

17 M. Aslam and A. Dent, *Bioconjugation. Protein Coupling Techniques for the Biomedical Sciences*, Macmillan Reference Ltd Publ., London, 1998.

18 E. Adie, *et al.*, *Measurement of Receptor Translocation using pH Sensitive Cyanine Dyes*. Presented at *Society for Biomolecular Screening 6th Annual Conference*, Vancouver, 2000.