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# pH-Sensitive Cyanine Dyes for Biological Applications

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A series of functionalized, water-soluble, pH-sensitive pentamethine cyanine (Cy™5) dyes has been designed and synthesized. These probes are fluorescent in acidic media but are non-fluorescent in an alkaline environment. Subtle changes to the structure of these probes can lead to pronounced changes in the pKa of these probes. These probes have been utilized in a cellular environment to detect localized changes in pH using the IN Cell Analyzer, a confocal imager formatted for imaging of cell-based assays.

**KEY WORDS:** Fluorescent; cyanine; pH sensitive; confocal microscopy, IN Cell Analyzer; CypHer™5.

## INTRODUCTION

The use of fluorescent labeling reagents for biological applications has expanded rapidly in recent years [1]. In particular the use of fluorescent cyanine dyes [2–7] has increased since the introduction of the sulfoindocyanine succinimidyl esters. The excitation and emission spectra of these probes span the visible and NIR spectrum from beyond 450 nm, where autofluorescence from biomolecules is greatly reduced, up to 800 nm, beyond which Rayleigh scattering is greatly increased and fluorescence emission detectors are more specialized. These fluorescent probes have wide-ranging applications [8,9], including DNA sequencing, flow cytometry, and high throughput screening. The existing sulphonated cyanine dyes have quantum yields greater than 0.1, good aqueous solubility, and high extinction coefficients (e.g.; ε for Cy™5 > 250,000). We recently reported the synthesis of a pH-sensitive pentamethine sulfoindocyanine dye [10]. Further progress has now resulted in the development of a series of pH-sensitive sulfoindocyanine dyes for biological applications.

The concentration of protons within a cell can greatly affect the biological pathways and the kinetics of many intracellular processes. pH plays an important role in many intracellular biological processes, including cell growth, differentiation, and proliferation [11]. The proton concentration within mammalian cells can vary from 10 n*M* to 1000 n*M* [12], and many biological interactions and processes can be monitored by measuring local intracellular fluctuations in pH. There are a number of techniques available to measure proton flux within cells including classical suspension techniques and colorimetry [13]. In particular, fluorescence microscopy [14,15] is a widely employed technique that provides information regarding local proton concentrations and the biochemical pathways involved. Furthermore, such assays may be performed in a relatively non-destructive manner and with minimal disturbance and perturbation of the local environment [16–19].

There has been an increase in the use of pH-sensitive fluorescent probes for intracellular studies [20–22] in recent years. These probes include fluorescent [23] and benzo[c]xanthene [24] based probes such as the seminaphthorhodafluors (SNARFs) and seminaphthofluoresceins (SNAFLs). For the most part these probes provide a signal decrease in response to increasing the acidity of the local environment. Other examples include *bis*(morpholi-

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nylmethyl)-anthracene probes such as Lysotracker-blue™ [20]. These are often employed to monitor receptor mediated endocytosis and provide an increase in fluorescence emission upon increased proton concentration. In general, most commercially available pH-sensitive fluorescent probe's emit between 350 and 550 nm and do not contain any functionality for biolabeling.

A more practical approach for some applications would utilize a pH-sensitive probe that is fluorescent between 600 and 800 nm. Few endogenous substances absorb photons at these longer wavelengths, and autofluorescence will be reduced in cell samples leading to improved resolving power and a greater signal to noise ratio. Furthermore, at longer-excitation wavelengths there is deeper penetration into cellular tissue and the generation of radicals is reduced, leading to lower phototoxicity and less damage to tissue. To this end, a range of pH-sensitive pentamethine cyanine dyes has been developed. It is known that addition of mineral acid to alcoholic solutions of certain anhydronium bases (Fig. 1) yields structures with absorption properties indistinct from cyanine dyes [25]. A range of functionalized, water-soluble pentamethine cyanine dyes has been designed and synthesized. These probes have comparable properties to previously described [2] cyanine dyes in acidic media, but are non-fluorescent in an alkaline environment. One of the key advantages to these probes is that they contain a reactive functional group, allowing bioconjugation. The reactive group employed is an *N*-hydroxy-succinimidyl ester, allowing the pH probe to be covalently linked to a range of aliphatic amino groups ubiquitous to proteins, such as antibodies or amino groups present on the outer cell membrane. This offers a series of fluorescent dye–labeled biomolecules that are responsive to areas of localized proton concentration change. Furthermore, the probe labeled adduct can also be tracked within a cellular environment. Unlike many other pH-sensitive probes, these dyes may also be specifically designed to give an increase in fluorescent emission in response to increasing proton concentration (low pH) and are essentially non-fluorescent in environments of high pH.

## EXPERIMENTAL

Dye purification was performed on a Gilson 805 HPLC system equipped with either a Vydac analytical reverse phase $C_{18}$ column (4.6 × 250 mm, inner diameter) or a semi-preparative reverse phase $C_{18}$ Dynamax −60 Å column (21.2 × 250 mm, inner diameter). All separations were performed using acetonitrile-water mixtures containing 0.1% acetic acid. Proton NMR analysis was carried out using a Varian 300 MHz FT-NMR spectrometer using $d_6$-DMSO or $CDCl_3$ (Aldrich, UK). NMR signals are described by use of *s* for singlet, *d* for doublet, *t* for triplet, *q* for quartet, and *m* for multiplet and are expressed in δ with TMS as the internal standard and J coupling values are expressed in Hertz. Data were collected on a Sun UNIX System and processed using VNMR software, Fluorescence measurements were performed using a Perkin-Elmer LS50B in fluorescence mode using 2.5-nm excitation and emission slit widths. All measurements were performed in a 2-mL quartz cuvette of 1-cm pathlength. The cuvettes were acid-washed with 1-M HCl, rinsed with distilled deionized water, and dried between each measurement. All spectra were collected using a Gateway 2000 PS-120 PC and analyzed using Perkin-Elmer Winlab software. UV and visible absorption measurements were performed upon a Hewlett Packard 8453 UV/Vis spectrophotometer with a diode array detector. Data were collected using an HP Vectra XA PC and analyzed using HP 845× UV/Vis software. Mass spectroscopy was performed using a Kratos analytical Kompact probe MALDI-TOF mass spectrometer using α-cyano-4-hydroxy cinnamic acid (10 mg/mL water/acetonitrile 1:1) as the matrix. Data were collected on a Compaq Deskpro EP series PC and analyzed using Kompact V1.2.2 analytical processing software employing insulin as the low molecular weight calibration marker. Negative infusion electrospray accurate mass spectroscopy was performed on a Q-Tof-2 (Micromass) using an isoleucine enkephalin marker of known molecular weight. Labeled antibodies were purified using Econo-Pac® 10DG columns (Biorad) buffered with phosphate buffered saline (pH 7.4, 100 m*M*) (Sigma).

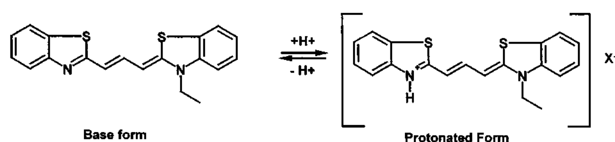



**Fig. 1.** pH sensitivity of cyanine dye and complementary anhydronium base.

### Synthesis

The synthesis of CypHer 5, the preferred pH-sensitive fluorescence probe is outlined below and exemplifies the synthetic methods employed.

#### CypHer 5: 1-(5-Carboxypentyl)-2-[1*E*,3*E*)-5-(3,3-Dimethyl-5-Sulfo-1,3-Dihydro-2*H*-Indol-2-Vlidene)-1,3-Pentadienyl]-3,3-Dimethyl- -3*H*-Indolium-5-Sulfonate (V)

1-(5-Carboxy-pentyl-1,3,3-trimethyl indolium-5-sulfonate (100 mg, 0.28 mmol), 5-sulfo-2,3,3-trimethylindolenine (73 mg, 0.28 mmol), malonaldehyde *bis*-

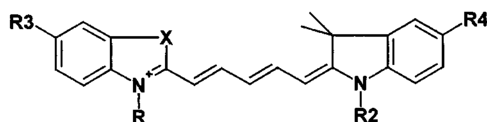
**Table I.** Spectral Properties of pH Sensitive Cy5 Probes

| Dye | Abs (max) pH 4.67 | Abs (max) pH 9 | Em (ex = 630 nm) | pKa |
|---|---|---|---|---|
| I | 645 nm | 484 nm | 665 nm | 7.5 |
| II | 650 nm | 515 nm | 665 nm | 6.4 |
| III | 640 nm | 531 nm | 670 nm | 6.85 |
| IV | 653 nm | 501 nm | 660 nm | 7.5 |
| V (CypHer 5) | 655 nm | 500 nm | 665 nm | 6.1 |

(phenylimine) (79 mg, 0.31 mmol), benzoic acid (68 mg, 56 mmol) and benzoic anhydride (127 mg, 0.56 mmol) were dissolved in acetonitrile (5 mL), and the solution was stirred at 80°C for 2 hr. The dark blue solution was cooled and the solvent removed *in vacuo*. The resulting solid was dissolved in DMSO and the solution purified by reverse phase HPLC using a Rainin Dynamax 60Å C18 column at 10 mL/min with a solvent gradient of 15% B for 5 min ramping from 15% to 50% B over 75 min. Where A = $H_2O$ (0.1% acetic acid) and B = acetonitrile (0.1% acetic acid). The retention time of VII was 41 min (UV/Vis detection at 650 nm). Yield 36 mg, 21%. $^1$H-NMR ($d_6$-DMSO). δ 7.9 (1H, m), δ 7.76 (d, 1H), δ 7.49 (s, 1H), δ 7.43 (d, 1H), δ 7.42 (d, 1H), δ 7.13 (d, 1H), δ 6.5 (m, 1H), δ 6.3 (m, 1H), δ 6.15 (m, 2H), δ 4.06 (m, 2H, $CH_2$), δ 3.21 (m,2H, $CH_2$), δ 2.19 (m,2H, $CH_2$), δ 1.65 (s, 6H, $(CH_3)_2$), δ 1.57 (s, 6H, $(CH_3)_2$), δ 1.394 (m,2H, $CH_2$), δ 1.27 (m, 2H, $CH_2$) MS (electrospray, negative mode) m/z (%) = 627.1835 (M, 100). UV?Vis ($H_2O/H^+$) $\lambda_{max}$ = 653 nm, ($H_2O/OH^-$) $\lambda_{max}$ = 501 nm.

### General Synthesis of *N*-Hydroxy Succinimidyl Esters

A typical conversion to the *N*-hydroxy succinimidyl ester involved the reaction of the dye (in this case V) (5 mg) in DMSO (500 μL) with PyBOP (1 eq), *N*-hydroxy-succinimide (1 eq) and diisopropylethylamine (1 eq). The solution was stirred for 1 hr to give quantitative conversion. TLC analysis (10% methanol/DCM [0.1% acetic acid] on $SiO_2$) observed a fast-running blue spot. For example, for probe V, $R_f$ = 0.60. $R_f$ of the free acid starting material = 0.41. Hydrolysis of this material with sodium carbonate buffer (pH = 9.22) gave the free acid by TLC. NHS ester product MS (MALDI-TOF) m/z (%) = 576 (M, 100).




I: R = H, R2 = $(CH_2)_4SO_3H$, R3 = $SO_3H$, R4 = $CH_2CO_2H$, X = $C(CH_3)_2$
II: R = H, R2 = $CH_2CH_3$, R3 = $SO_3H$, R4 = $SO_3H$, X = $C(CH_3)_2$
III: R = $(CH_2)_5CO_2H$, R2 = H, R3 = H, R4 = $SO_3H$, X = S
IV: R = H, R2 = $(CH_2)_5CO_2H$, R3 = $SO_3H$, R4 = $SO_3H$, X = $C(CH_3)_2$
V: R = H, R2 = H, R3 = $CO_2H$, R3 = $SO_3H$, X = $C(CH_3)_2$

**Fig. 2.** Range of Cy5 pH-sensitive fluorescent probes.

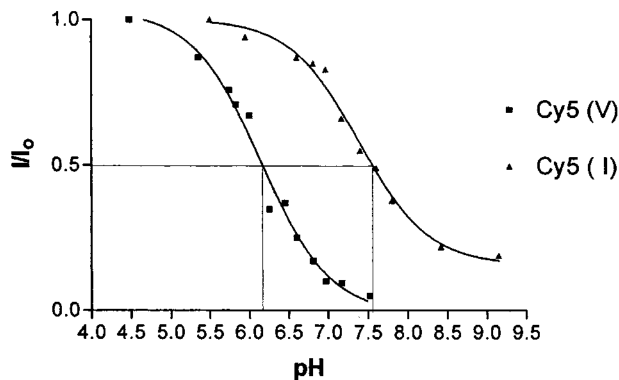



**Fig. 3.** Comparison of the pKa for Probes I and V (CypHer5).

## RESULTS

Table I details the spectroscopic properties of these probes. Table I illustrates how subtle changes in dye structure can lead to marked changes in pKa. pKa may be defined as the pH, whereby 50% of the dye population in solution is protonated. This is more apparent when comparing the spectral data of the dyes as a function of pH. Figure 3 compares the pKa of probes I and V where:

$$I/I_0 = \frac{\text{Emission of Probe at pH n}}{\text{Emission where Probe is 100\% protonated}}$$

Where $n$ = integer between 4.5 and 9.5.

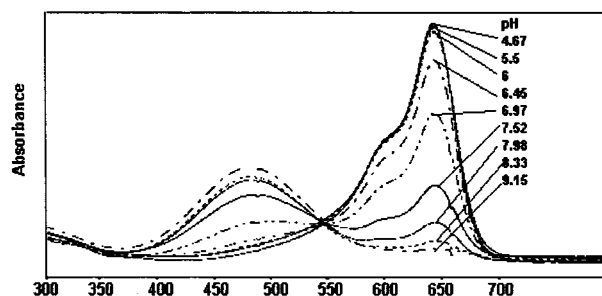



**Fig. 4.** Absorption characteristics of Probe I in a range of phosphate buffers.

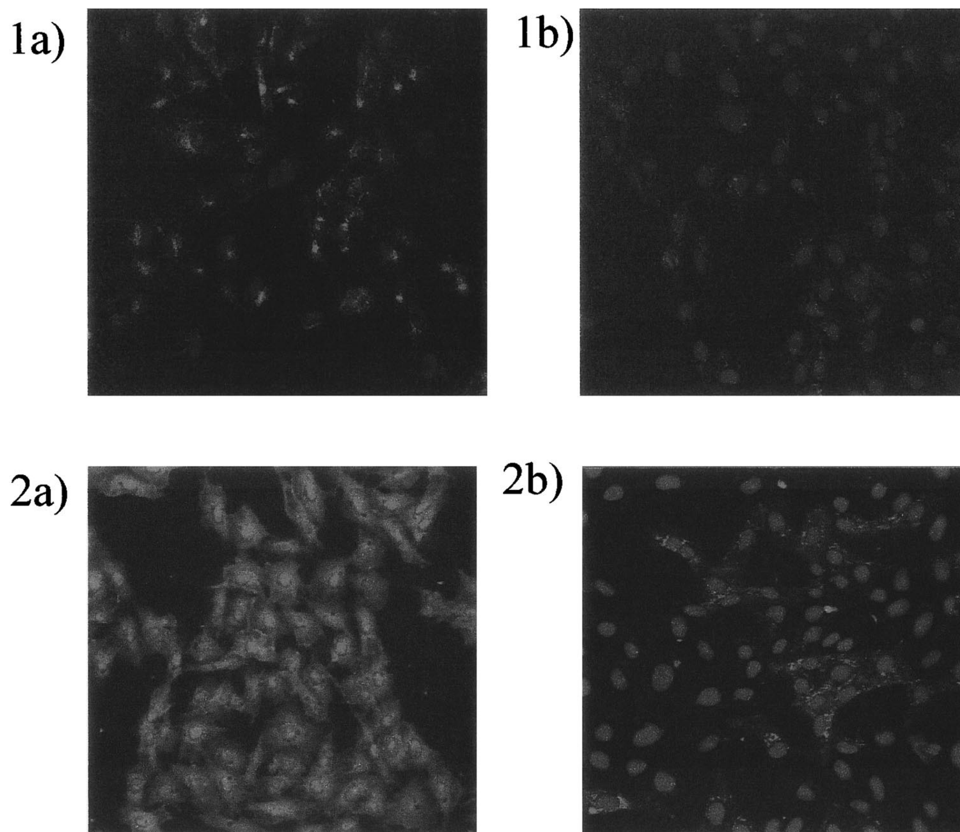



**Fig. 5.** c-*myc*-δ-opioid receptor expressing CHO cells treated with (a) 100 nM DADLE (D-ala, D-leu enkephalin), (b) no DADLE for 20 min. 2. VSV-TRHR expressing CHO cells treated with (a) 10 μM TRH, (b) no TRH for 30 min.

pKa can be calculated whereby $I/I_0 = 0.5$ for a range of phosphate buffers [26] of equal ionic strength. With reference to Table I, it can be observed how the different functional groups directly linked to the chromophore can affect the basicity of the indolenine moiety and hence the pKa of fluorescent probe. Furthermore the disappearance of the absorption band at ~650 nm characteristic of pentamethine cyanine dyes is coupled with the appearance of a new absorption band at 450–520 nm characteristic of the base form of these dyes. The typical UV/Vis absorption profile is illustrated for probe I in Fig. 3. Of note is the isobestic point at 545 nm. This single crossover point of all spectra is indicative of each buffer solution being of equal ionic strength [27] and therefore minimizes the effect that others ions may have on the spectral properties of the probe in relation to protons.

## THE APPLICATION

This range of pH-sensitive fluorescent pentamethine cyanine dyes has been synthesized for use on the IN Cell Analyzer (Amersham Biosciences). This is a multichannel, line scan confocal imager formatted for imaging of cell-based assays. Figure 3 compares the pKa of probes I and V, illustrating how structural differences can affect pKa. Probe V (CypHer™ 5) has the most desirable pKa for monitoring GPCR-ligand interactions via constitutive endocytosis.

The pKa profile, as illustrated in Fig. 3 observes that at a pH > 7 CypHer 5 is >95% deprotonated with subsequent change in the absorption spectrum such that it cannot be excited with a 633-nm laser. CypHer 5 is therefore suitable for GPCR-ligand monitoring. The *N*-succinimidyl ester moiety facilitates the fluorescent labeling of the outer membrane of a cell or a cell surface receptor, for example, a G-protein coupled receptor (GPCR). Agonist activation of this class of receptor almost invariably results in the internalization of the receptor from the plasma membrane (pH 7.4) to the endosomal pathway (pH 5–6) within the cell. Thus an increase in fluorescence will be observed on internalization as the probe becomes protonated and the fluorescence switches on.

By N-terminally tagging a GPCR with an epitope, and labeling an antibody to that epitope with a pH-sensitive dye, the internalization of a GPCR can be monitored within the cell [28]. In this case two cell lines were used—CHO (Chinese hamster ovary) cells expressing the c-*myc*-δ-opioid receptor and CHO cells expressing the thyrotropin-releasing hormone receptor (VSV-TRHR). We have preincubated these cells with 0.01 mg/mL CypHer 5-labeled anti c-*myc* antibody (clone 9E10) and anti-VSV glycoprotein antibody (clone P5D4), respectively. Agonist was then added to each cell line and the fluorescence, corresponding to receptor internalization, measured confocally after the appropriate time interval using IN Cell Analyzer. Figure 5 observes agonist activation of both cell lines resulting in a clear signal increase when compared with cells that had not been treated with agonist.

## CONCLUSIONS

A range of novel water-soluble pH-sensitive fluorescent probes has been designed and synthesized for biological applications. These probes also contain an *N*-succinimidyl ester group facilitating facile bioconjugation. Furthermore, unlike the majority of protonophores, these probes furnish an increase in fluorescent emission within an environment of higher proton concentration and therefore provide excellent reagents for signal increase assays for localized acidity within a turbid environment, for example, mammalian cells. Another great advantage of these probes is that they provide a fluorescent signal at a wavelength where little else within a biological environment emits. This greatly enhances the signal to background ratio, permitting high resolution and good sensitivity. Furthermore, by adjusting the auxochromic groups present upon the fluor, the pKa can be tuned for a particular application providing a pH-sensitive switch that can be adapted to monitor the physiological pH range desired.

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