Design, synthesis and evaluation of near-infrared fluorescent pH indicators in a physiologically relevant range

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Removal of a hydroxylsulfonylbutyl arm from indocyanine green dye produced a pH-sensitive near-infrared (NIR) fluorescent indicator that is useful at physiological range.

Biochemical processes frequently involve protonation and deprotonation of biomolecules with concomitant changes in the pH of the milieu. A common strategy to monitor the pH of biochemical events relies on color changes of pH indicators at visible wavelengths. However, in heterogeneous media where indicator concentration or visual observation is limited, highly sensitive pH indicators and methods to precisely measure their photoactivity are needed. This is especially true when monitoring tissue or intracellular pH using common pH indicators. To overcome these problems, a limited number of fluorescent pH probes have been developed to monitor diverse physiological and pathological processes. These include studies related to cancer,[1,2] cell proliferation,[3] endocytosis[4] and other physiological processes.[5–12]

Current fluorescent pH indicators for biomedical research have low sensitivity because their photoactivity is in the spectral window where endogenous chromophores either absorb light or autofluoresce.[13,14] Additionally, the synthesis of some fluorescent pH probes is difficult because of their complex chemical structure. These limitations attest to the need to develop new fluorescent pH indicators for biological applications. Near-infrared (NIR) fluorescent dyes are widely used in biomedical studies because tissue autofluorescence and light absorption by tissue between 700 and 900 nm is low, thereby increasing detection sensitivity and depth of light penetration in tissue. Therefore, development of NIR fluorescent pH indicators would enhance the imaging of molecular or physiological processes with high sensitivity.

Predicated on previous studies that norcarbocyanines are pH-sensitive[13,14] and carbocyanine dyes such as indocyanine green (ICG) and cypate are biocompatible,[15–17] we developed a simple method to prepare NIR fluorescent norcarbocyanines (H-ICG and H-cypate) based on ICG and cypate, respectively (Fig. 1). H-ICG was designed as a nonspecific pH indicator and H-cypate could be used as a target-specific pH probe by conjugating the free carboxyl group with biomolecules. The structural design involves removal of one arm of the N-substituents of either ICG or cypate to generate a protonable amino group within the chromophore core of the dye. Thus, the acidity of the medium would significantly alter the fluorescence emission of the probe.

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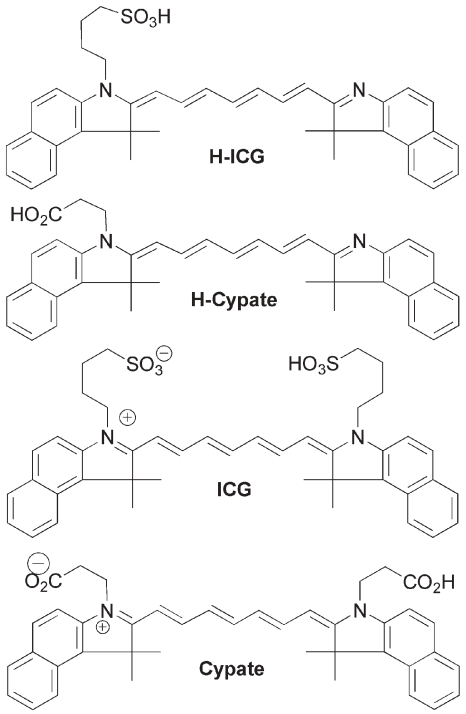
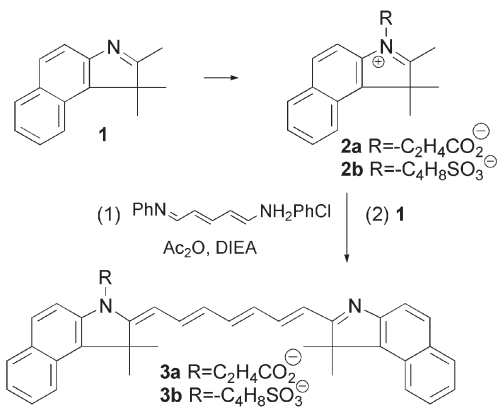


Fig. 1 Structure of H-ICG, H-cypate, ICG and cypate.

ICG was purchased from a commercial vendor and cypate was prepared by a literature method.[18] Compounds **2a** and **2b** (Scheme 1) were synthesized by reacting the benzoindole **1** with either bromopropionic acid or 1,4-butane sultone in 1,2-dichlorobenzene.[19] The resulting compounds were each added dropwise to



Scheme 1 Synthesis of H-ICG and H-cypate.

a mixture of glutaconic dialdehyde dianiline hydrochloride (benzenamine, *N,N′*-2-pentene-1,5-diylidenebis-, hydrochloride (9Cl)), Ac_2O and diisopropylethyl amine in dichloromethane under reflux. After 4 h, the reaction was quenched by adding water to the mixture and the crude product was precipitated in ether. Further purification by column chromatography afforded H-cypate (**3a**) or H-ICG (**3b**) in 10–50%. The variation in yield is due to differences in separation efficiency from one batch to another.

To measure their photoactivity, relatively concentrated ($\sim 5 \times 10^{-5}$ M) stock solutions of each of the four dyes in 20% aqueous DMSO were prepared. Aliquots of the stock solutions were added to phosphate pH buffer (5×10^{-3} M) to obtain dilute dye solutions (5×10^{-7} M) at different pH values. Buffer solutions at pH 4–10 were prepared from KH_2PO_4 and H_2PO_4. The solutions were sufficiently stirred before spectral measurements on a Beckman Coulter DU 640 UV-vis spectrophotometer (absorption) and a Jobin Yvon-Spex Fluorolog-3 spectrofluorometer (fluorescence).

The absorption maxima of ICG, cypate, H-ICG and H-cypate centered at 780 nm in 20% aqueous DMSO solution at pH < 4. A second peak at 560 nm began to emerge with concomitant decrease of the peak at 780 nm as the pH of the solution increased (Fig. 2, bottom). However, the intensity ratios of the 560 to the 780 nm peaks for ICG, cypate, and H-cypate were significantly small relative to that of H-ICG under similar conditions. This observation reflects the poor conversion of the compounds from one protic form to another and suggests that only H-ICG is highly sensitive to pH changes within the pH range studied. The molar absorptivities of ICG, cypate, H-ICG, and H-cypate at 780 nm and pH 2.5 are 3.8, 9.2, 5.8, and 5.1, respectively. These measurements were obtained in 20% aqueous DMSO in the presence of H_3PO_4 (5×10^{-3} M).

The fluorescence emission peaks of cypate and ICG (Fig. 3, bottom) showed slight but noticeable shifts at different pH values and significant intensity fluctuation at pH range close to the pK_a of the indole N atom. Expectedly, H-ICG has strong NIR absorption and fluorescence emission at pH < 4 (Fig. 2, top). Its response to changes in pH fits a sigmoid curve at a pH range of 4–10 (Fig. 3, top). A pK_a of 7.23 was calculated from Fig. 3 by using the equation $pH = pK_a + \log[A^-]/[HA]$.[20] In contrast, sigmoidal pH

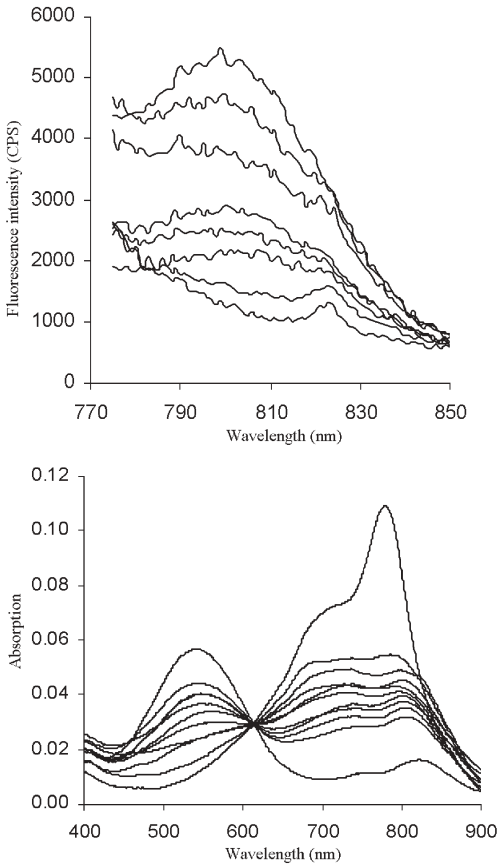


Fig. 2 Emission (top, excited at 760 nm) and absorption (bottom) of H-ICG at different pH buffers. Intensity of emission peak decreases as shown by the curves (from top to bottom) at pH = 4.87, 6.65, 7.40, 7.10, 7.62, 8.01, 9.50, 12.13. Absorption peak at 780 nm decreases as shown by the peaks (from top to bottom) at pH = 2.50, 4.87, 6.65, 7.62, 8.01, 8.14, 8.52, 9.50, 12.13. Dilute solutions ($\sim 5 \times 10^{-7}$ M) were used to minimize dye aggregation.

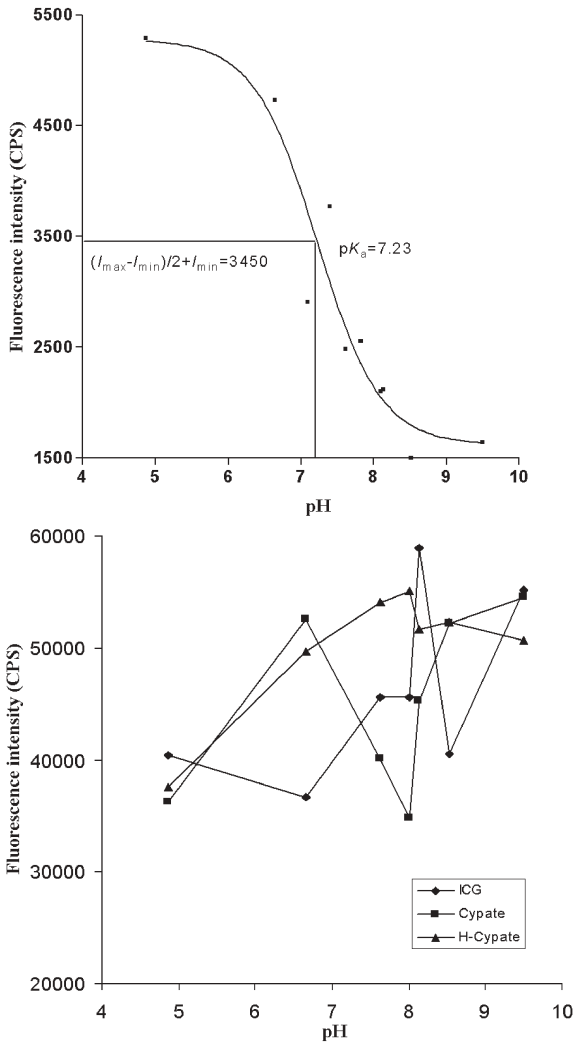


Fig. 3 Emission (800 nm, maximum ± 5 nm) of H-ICG (top) and ICG, cypate and H-cypate (bottom) at different phosphate pH buffers (excited at 760 nm, 20 nm lower than absorption maximum). Sample used is the same as in Fig. 2.

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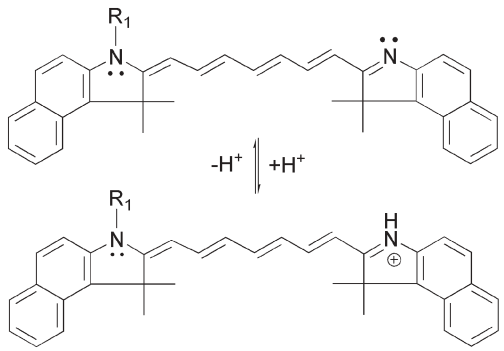

Scheme 2 pH responsive *via* protonation and deprotonation of H-ICG.

correlation with fluorescence intensity was not observed in the case of H-cypate at pH range between 4 and 10 (Fig. 3, bottom). Because the primary structural difference between H-ICG and H-cypate is the sulfonate and carboxylate groups, respectively, the results suggest that these functional groups affect the pH response of norcarbocyanines, although they are not directly attached to the fluorophore core of the molecules. Clearly, the ionization potentials of carboxylates and sulfonates are different and could affect the ease of protonating the amino group at different pH values. Apart from the pK_a differences between carboxylates and sulfonates, alternative explanations for the loss of fluorescence of H-cypate are conceivable. Synthesis of H-cypate derivatives with different carboxyl chain lengths are in progress. We anticipate that conversion of the carboxylate to an amide would produce a similar protonation pattern as H-ICG.

In conclusion, we have synthesized a new norcarbocyanine that is sensitive to pH changes at a physiologically relevant range. Its simple structural design, ease of synthesis, solubility in aqueous medium, and pK_a of 7.2 are the characteristic features of a good NIR pH indicator for biomedical research. Conjugation of biomolecules to H-cypate to explore its pH response within a physiologically relevant range is in progress.

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20 Curve was fitted using GraphPad Prism version 4.00 for Windows, GraphPad Softwarer, San Diego, California, USA.