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# Understanding dichromic fluorescence manifested in certain indocyanine green (ICG) analogs*

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*Abstract*: Fluorescence has advanced our understanding in various aspects of biological processes. Fluorescence in the near infrared (NIR) region avoids background autofluorescence from biological samples, leading to improved image quality. In searching for indocyanine green (ICG) analogs that can be attached to biomolecules, we observed that dichromic fluorescence manifested in some mono reactive-group-functionalized ICG analogs. The two emission bands are distinctively separate from each other, making it a unique feature of fluorescent probes found in biological studies. We further demonstrated that the dichromism comes from the structure and is transferable from dye to its bioconjugates. In this paper, we used resonance theory and molecular orbital theory to explain the fluorophore photochemistry in an effort to understand the general fluorescence feature of ICG analogs and provide understanding of the secondary emission band.

*Keywords*: biooptics; dichromism; fluorescence; near infrared; resonance.

The merit of near infrared (NIR) over shorter-wavelength fluorescent biooptic probes is that NIR probes emit signal away from that of endogenous fluorophores [1,2]. Indocyanine green (ICG) derivative cypate [3] has been an important NIR dye in that it retains the optical feature of its structural analog ICG while making it possible to attach biomolecules such as short peptides at designated positions to give tumor avid optical bioprobes [1,4–6]. The symmetrical difunctionality of cypate could produce unwanted by-products in the bioconjugation of peptide molecules. The presence of another reactive carboxyl group in their mono-bioconjugates could also potentially be a source of instability on storage. In addition, cypate is significantly less hydrophilic than ICG, which is unfavorable in aqueous formulations. In our search for novel hydrophilic dyes that principally provide only one reactive center, we synthesized a mono-amino-functionalized ICG analog, $NH_2$-ICG (Fig. 1).

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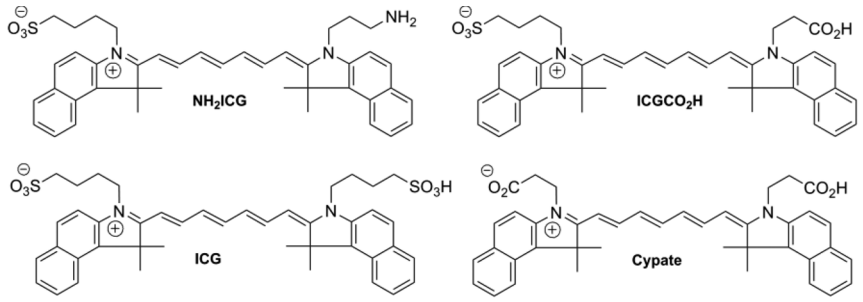


**Fig. 1** Chemical structures of $NH_2$-ICG and structural comparison with its analogs ICG-$CO_2H$, ICG, and cypate.

Interestingly, the dye not only emits fluorescence at the expected wavelength, it also gives a secondary fluorescence band of about equal intensity at a shorter wavelength after excitation (Fig. 2). The two emission bands are distinctively separated from each other, making it a unique feature of these indocyanine dyes. We initially thought that the shorter wavelength emission originated from impurities, solvent effect or decomposed fragments present in the sample. Repeated studies with purified samples led us to believe that the dual fluorescence originated from the distinct structural features of the molecules. To our knowledge, no NIR dichromic fluorescent indocyanine dyes have been reported prior to our work [7]. A closely related dichromic molecular construct involved chemical unification of two dyes [8] and the resultant dichromism established FRET (fluorescence resonance energy transfer) technology when the excitation and emission profiles of the two dyes are matched [9]. We believe the dichromism presented in the single-dye $NH_2$-ICG could have important applications not only in the area of biooptical imaging but also in other areas of optics.

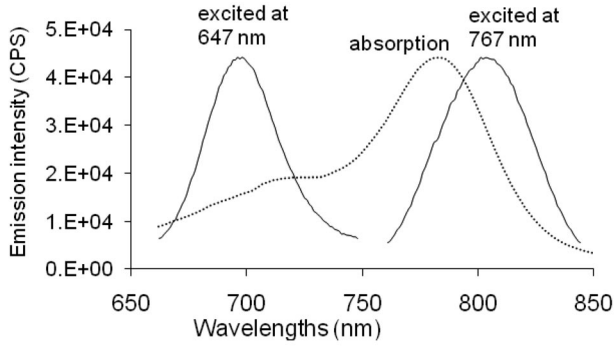


**Fig. 2** Absorption and emission spectra of $NH_2$-ICG in 20 % aq. dimethyl sulfoxide (DMSO). Absorption and shorter wavelength emission curves are normalized to the same intensity of the principal emission band. Emission taken at absorbance of $A = 0.10$ at $\lambda_{max}^{abs}$. The actual shorter wavelength emission intensity is comparable to the principal emission at the optimal excitation wavelength.

The nature of the observed dichromism is not clearly understood at this time. However, structure analysis clearly established that the mono bioreactive functionalization altered the structural symmetry of the original dyes. In the case of ICG and cypate, the molecules are structurally symmetric. The positive charge is delocalized in the entire fluorophore. The structure of $NH_2$-ICG, on the other hand, raises a possibility that the positive charge could be preferentially localized on one of the indole N atoms in the ground state. The charge localization could further be stabilized by the sulfate anion nearby. This is readily understandable from resonance structures shown in Fig. 3. The nonsymmetrical character of

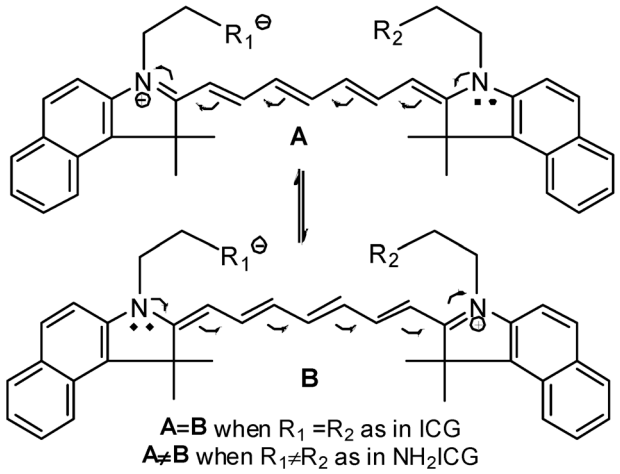


**Fig. 3** Differentiation of functional groups causes non-equivalency in terms of charge location in resonance theory.

$NH_2$-ICG is also supported by NMR studies. The two gem-dimethyl groups in cypate showed a singlet at upfield [10] while they split into two singlets in $NH_2$-ICG proton spectrum. Furthermore, the corresponding 1st, 3rd, 5th, and 7th vinyl protons of the heptamethine bridge showed a doublet and a triplet in cypate, while it appeared to have four distinguishable doublets and triplets in $NH_2$-ICG. The presence of seven methylene groups, $-(CH_2)_3-$ and $-(CH_2)_4-$ from the two indole N-substituents and the seven methine protons at the vinyl bridge of $NH_2$-ICG clearly showed up in NMR studies. Their correlations are confirmed by 2D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser enhancement spectroscopy (NOESY) spectra.

The understanding that dichromism could have resulted from nonsymmetrical charge distribution led us to re-investigate the spectral features of ICG and cypate. Both ICG [11] and cypate showed the same absorption and principal emission bands as $NH_2$-ICG in 20 % aq. DMSO. As expected, we did not observe any significant secondary emission in the region where $NH_2$-ICG shows up secondary emission. This suggests that the positive charge on the fluorophore is fully and symmetrically delocalized in ICG and cypate. It is this electronic configuration that is responsible for the absorption and the principal emission band associated with ICG, cypate, and $NH_2$-ICG. It is interesting to point out that the two dyes indeed showed little emission in the region, which could have been interpreted as originating from impurities. The secondary emission of ICG is relatively more intense than that of cypate. Even in the ICG case, the intensity of the secondary emission, however, is much smaller (<10 %) than that of $NH_2$-ICG, excited at optimal wavelengths (Fig. 4). The insignificant secondary emission observed in ICG and cypate cases is thus from the situation where a fraction of the molecules exists with localized positive charge.

For a fluorescent dye, the ability to emit fluorescence arises from the presence of an excited electronic singlet state ($S_1$) between excited state and ground state. Excited molecules first release partial energy as heat from the excited state to $S_1$. The remaining energy is subsequently released as photons as the molecule returns to the ground state ($S_0$). The stability of $S_1$ and the relative energy difference between $S_1$ and $S_0$ define the quality of a fluorescent dye [12]. According to Jablonski theory [13], fluorescence wavelength remains unchanged while the intensity of the emission changes depending on the excitation wavelength. The maximum emission intensity is attained when the molecules are excited at maximum excitation wavelength. This has been well supported from our spectral studies of ICG, cypate, and the principal emission of $NH_2$-ICG. Interestingly, the secondary emission band of $NH_2$-ICG also showed similar dependence. No wavelength shifts were observed when excited at different wavelengths.

 

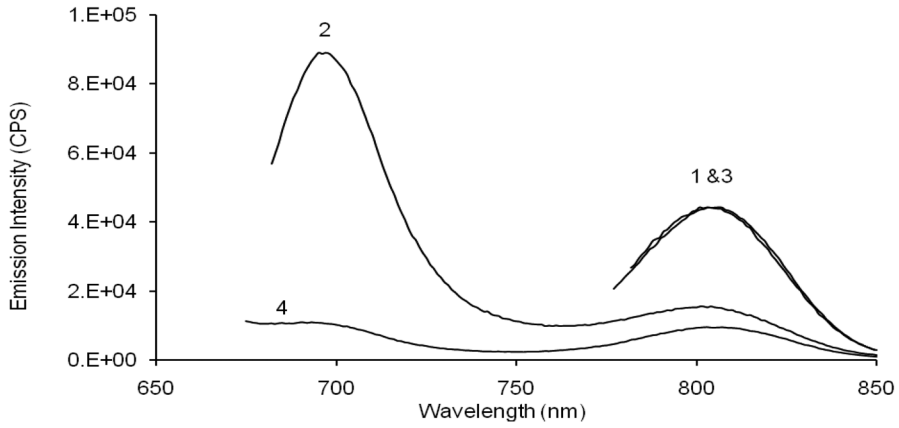


**Fig. 4** Comparative emission spectra of $NH_2$-ICG and ICG excited 20 nm and 120 nm below $\lambda_{max}^{abs}$. Emission of ICG at $\lambda_{max}^{abs}$-20 nm excitation (curve 3) is normalized to the same intensity of emission of $NH_2$-ICG on $\lambda_{max}^{abs}$-20 nm excitation (curve 1). For comparative purpose, emission of ICG at $\lambda_{max}^{abs}$-120 nm excitation (curve 4) is normalized by the same factor as in the normalization of the emission at $\lambda_{max}^{abs}$-20 nm excitation. Curve 2 is $NH_2$-ICG emission at $\lambda_{max}^{abs}$-120 nm excitation.

In addition to the symmetry collapse upon functional group differentiation and the potential localization of the positive charge, it is important to observe that the three dyes are chemically intramolecular salts similar to classic zwitterions. The position of the attached anion thus directly influences the distribution of positive charge on the fluorophore. Symmetrical positioning, as in ICG and cypate, exerts no influence on the positive charge delocalization, while nonsymmetrical positioning, as in $NH_2$-ICG, enhances charge localization. A more significant influence could have occurred when the anion is positioned close to the fluorophore N atom where positive charge is localized. It is thus clear that the molecules could have two excited states from two sets of molecular orbitals. The only difference between the two structures is full or partial delocalization of the positive charge. Interestingly, $NH_2$-ICG did not show strong absorption around the secondary emission band. A possibility is that passive absorption is not strong enough to excite $NH_2$-ICG from ground state to the excited state in charge localized case.

To further confirm our finding, we synthesized ICG-$CO_2$H (Fig. 1) where the aminopropanyl group in $NH_2$-ICG was replaced with a hydrocarbonylethyl group. ICG-$CO_2$H shares an identical fluorophore as $NH_2$-ICG, ICG, and cypate. ICG-$CO_2$H is a nonsymmetrical molecule similar to $NH_2$-ICG. ICG-$CO_2$H showed strong secondary emission band at our expected position under the same condition used in the study of $NH_2$-ICG, ICG, and cypate. The relative intensity of the emission band is comparable to that of $NH_2$-ICG. Again, as we expected, the absorption and principal emission of ICG-$CO_2$H are not different from that of $NH_2$-ICG, ICG, and cypate. It is interesting to point out that the fluorescent dichromism is transferable from parent dye to its peptidyl bioconjugates. We have attached ICG-$CO_2$H to a variety of peptides targeting specific receptors manifested on cancer cells. Studies on ICG-$CO_2$H-based NIR optical bioprobes will be reported elsewhere.

In conclusion, indocyanine NIR dichromic fluorescent emitters were developed. The dyes have only one bioreactive functional group that makes them selectively attachable to biomolecules for biological studies. Their unique dichromic feature could also be used in optical applications other than the life sciences.

 

## ACKNOWLEDGMENTS

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