Near-Infrared Probes: Design and Applications

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ABSTRACT

Near-Infrared (NIR) absorbing chromophores have been valuable in analytical and bioanalytical chemistry. NIR probes and labels have been used for several applications, including solvent polarity, hydrophobicity, DNA sequencing, immunoassays, CE separations, etc. The NIR region (700-1100 nm) is more advantageous for the bioanalytical chemist due to the inherently lower background interference and the high molar absorptivities. NIR dyes can be used as simple probes to investigate biomolecule properties or just simply to detect the presence of biomolecules. Another typical application is the use of NIR fluorophores as labels. In these applications covalent labeling is the preferred method but it requires NIR dyes with appropriate reactive moieties. Due to the hydrophobic nature of NIR chromophores non-covalent labeling may be a viable alternative. For this purpose novel bis(carbocynines) have been developed in our laboratories. These dyes form intramolecular H-aggregates in polar solvents, even at very low concentrations. Spectral properties of this intramolecular dimer greatly depend on the properties of heterocyclic moieties and the length, the location and/or flexibility of the connecting chain. This form of the dye can be described as a clamshell complex with two interacting hydrophobic carbocyanine moieties. This intramolecular H-aggregate has a low extinction coefficient and fluorescence quantum yield. Upon opening the clamshell that can be facilitated by changing microhydrophobicity (i.e., binding to biomolecules) the H-and D- bands are decreased and the monomeric band is increased, with concomitant increase in fluorescence intensity. The main analytical utility of these bis(carbocyanines) is that the free dye (i.e., not complexed to an analyte) has negligible fluorescence in a typical aqueous buffer environment. Examples of different applications of these bis(carbocyanines) are given including forensic applications.

Keywords: Near-Infrared, probes, microhydrophobicity, bis(carbocyanines)

1. INTRODUCTION

Advances in chemistry and laser diode technology now allow for the development of new approaches to near-infrared (NIR) spectroscopy in analytical chemistry. NIR spectroscopy affords low detection limits and opens up new analytical possibilities. By moving detection from a visible region to the longer wavelengths, e.g., 750-1100 nm, the background interference from the complex matrix is greatly lowered, thereby reducing scatter and shifting the Raman line even further from the spectral region of interest. Very low detection limits can be achieved using NIR fluorescence. In addition to the extremely low background interference, the low detection limits are also due to high molar absorbtivity and high quantum yield of NIR dyes as well as to high efficiency of semiconductor laser and detection systems. Since the NIR region is inherently a region of low interference, it is well suited for analytical techniques using high complexity samples, without any need for preseparation. Unfortunately, most of the NIR applications require the use of fluorophores that are of limited commercial availability or are not commercially available at all. Accordingly, this paper pertains to the design and synthesis of the desired NIR fluorophores. These dyes exhibit absorption and fluorescence wavelengths of up to 1100 nm. Following the design, synthesis and spectral characterization of the dyes, their fundamental analytical and bioanalytical applications are discussed.

Our research groups have been active in NIR dye applications for over a decade, and during this time a large number of dyes have been synthesized by using classical methodologies and/or novel chemistry developed by us.[1,2] Our initial efforts focused on the synthesis of monomeric cyanine dyes substituted with a chemically reactive functionality for covalent binding with macromolecules, such as proteins, or substituted with groups that enhance non-covalent binding with the biomolecules. Many reporter dyes of these two types have been developed. Additional studies involved NIR dyes having a moiety that recognizes a particular analyte molecule of interest with a concomitant change in the dye spectra that can be detected. More recently, for the first time we have introduced bis-cyanines as novel NIR indicators for analytical and

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bioanalytical applications. Depending on their microenvironment, the bis-cyanines can exist as an intramolecular dimer with the two cyanines in a stacked form or in a linear conformation in which the two subunits do not interact with each other. The two conformations of the bis-cyanines show dramatically different spectral properties.

2. SYNTHESIS OF NIR DYES

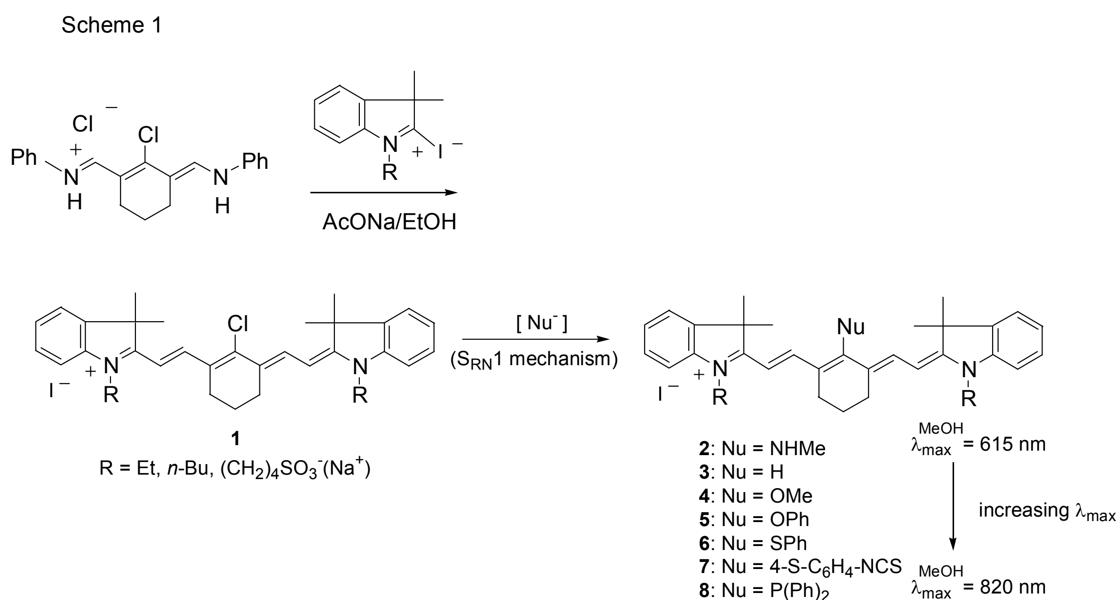
2.1 Monomeric cyanines

There are many classical approaches to the synthesis of cyanine dyes, and the preparation of an important heptamethine cyanine **1** is illustrated in Scheme 1. The indolium derivative **1** shows absorption around 780 nm, and the absorption can be extended up to 1100 nm by choosing appropriate end-heterocyclic subunits and substitutents at the chromophore.

One of the convenient ways to introduce reactive or recognition moieties is a chemical modification of the NIR dye molecule. For the recognition moiety, an additional requirement is that the binding event should change the electronic energy levels for easy detection. This feature can be achieved by introducing a moiety that is conjugated in the polymethine chain of the dye as illustrated by structures **2-8** in Scheme 1. Specifically, we have shown for the first time that the central chloro substituent in indolium heptamethine cyanines **1** and their benzindolium analogs is easily replaced via an $S_{NR}1$ pathway by a number of nucleophiles (Nu) that are good electron donors. The reaction proceeds only in solvents that support a single-electron-transfer (SET) process, such as *N,N*-dimethylformamide or dimethyl sulfoxide. The resultant Nu-substituted derivatives, such as **2-8**, can also undergo a similar replacement reaction in these solvents but are stable in aqueous solution because water strongly inhibits the SET process. Accordingly, the modified dyes are stable under aqueous conditions of bioanalytical experiments. A number of NIR dyes prepared by us using this approach and published in the open literature are currently being offered commercially. This pathway can be used for synthesizing both covalent labels and probe molecules.

Another methodology for functionalization of cyanine dyes, introduced by us for the first time, is illustrated in Scheme 2 by the synthesis of methoxycarbonyl substituted cyanines **11** and **12**. The key element to this preparation is the synthesis of a novel Vilsmeier-Haack reagent **10** starting with readily available keto ester **9**. As a result, the dyes can be synthetically modified not only at the central position of the chromophore but also at the trimethylene bridge. This is illustrated in Scheme 2 by the preparation of the NIR reagent **14** for labeling of biomolecules at an amino group.

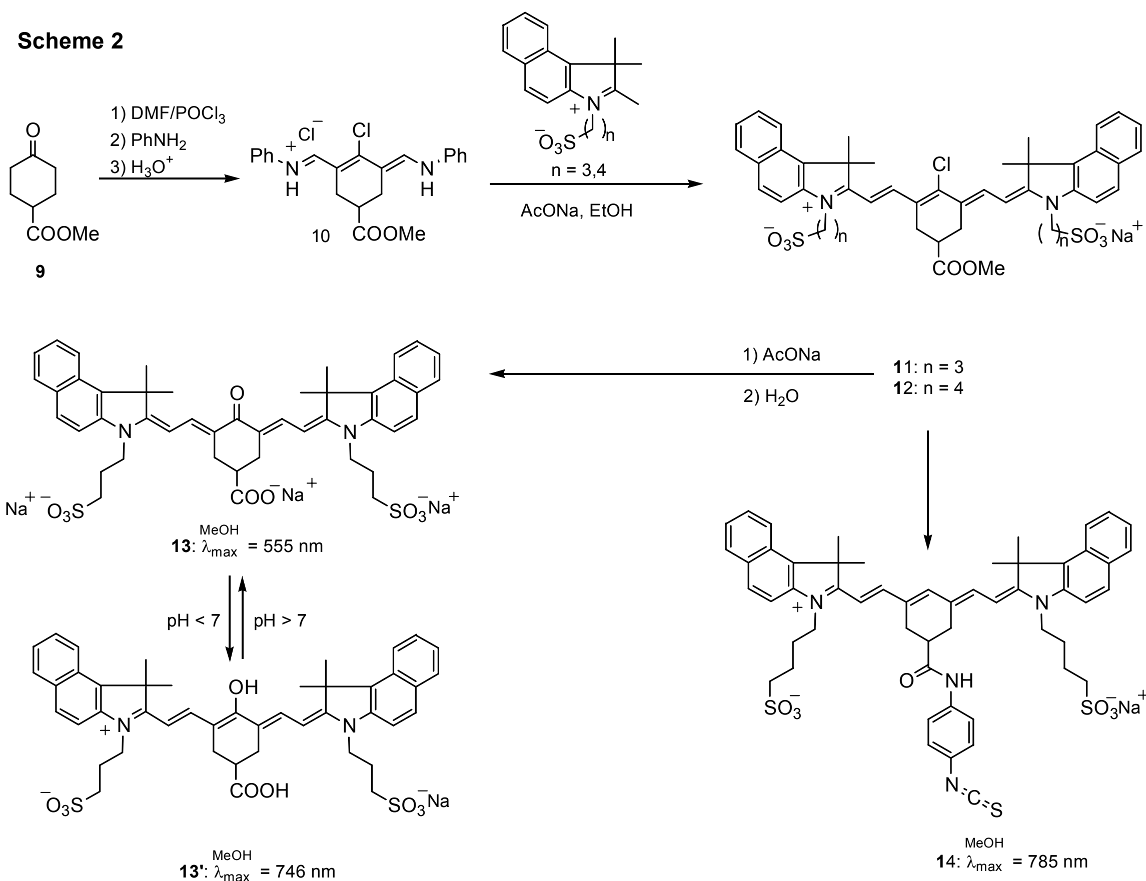
Scheme 1



In addition, stable bis(aminodien)ones were synthesized, such as **13** (Scheme 2 and **15** (eq. 1), the visible absorption of which undergoes a shift to an NIR region upon protonation. The transition ketone ↔ cyanine is fully reversible and depends solely on pH conditions. Several independent approaches to the synthesis of the ketone/cyanine systems were developed in our laboratories.[3] These dyes are excellent pH probes both in vitro and in vivo applications. It should be noted that the transition range is suitable for distinguishing normal cells from cancerous ones.

Several metal-ion complexing NIR dyes were synthesized by placing appropriate functionalities at the indolinium moieties or the phenoxy group at the center of the chromophore. The *ortho*-hydroxy-carboxy substituted dyes are selective for aluminum and show substantial changes in fluorescence upon complexation. Crown-ether substituted dyes are suitable for other biomedically important metal ion detection. Of these a calcium sensitive dye needs to be mentioned.

Although significant portion of our research efforts was focused on tailoring the absorption of NIR dyes to the

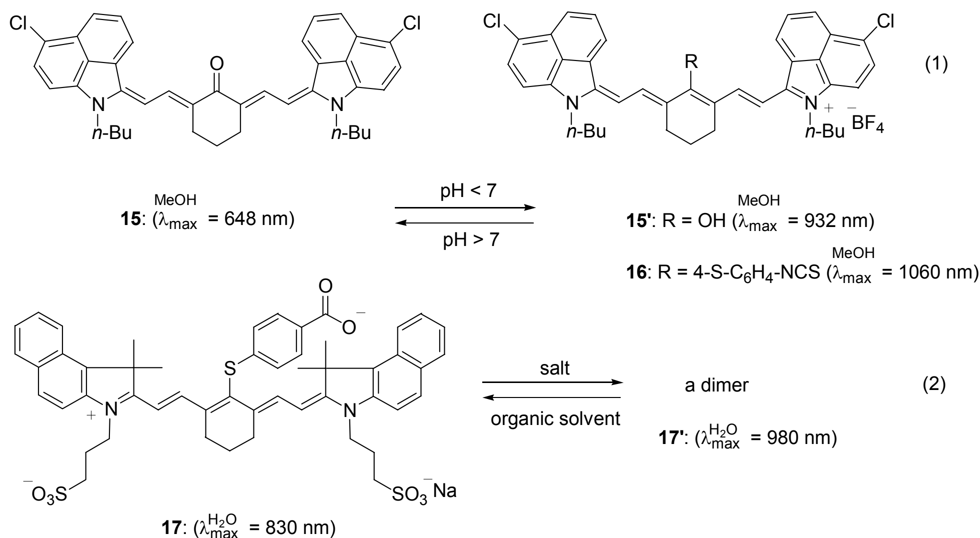


wavelength of 785 nm that is the range of the most prevalently available and least expensive members of the commercially available GaAlAs laser diodes, recently our work is being extended to the design and synthesis of stable NIR dyes that absorb and fluoresce in the region beyond 900 nm. In addition to the pH-sensitive dye system **15/15'** that absorbs at 932 nm under acidic conditions, a new stable dye **16** (λ_{max} = 1060 nm) was synthesized (structure in eq. 1). A large number of appropriately substituted heptamethine cyanines are extremely stable, and the desired absorption beyond 900 nm can be attained by structural substitution and/or modification of the chromophore, as already discussed.

We also synthesized a dye **17** (eq. 2) the monomeric form of which exhibits λ_{max} = 830 nm in aqueous solution. The dye **17** undergoes dimerization (stacking) upon addition of a salt (K_2HPO_4, KBr or CH_3COONa), which is accompanied by disappearance of the absorption at 830 nm and appearance of an even stronger, narrow band centered at 980 nm. The dimer is stable up to the tested pH of 12 but undergoes dissociation upon addition of an organic solvent or acidification

of the aqueous solution. The latter result demonstrates that the carbocyclic acid group of **17** is ionized in the dimer **17'**. In full agreement with this conclusion the analog of **17** devoid of the COOH group does not exhibit the behavior discussed above.

A large number of additional NIR dyes have been synthesized by our research groups. These dyes have been made to fully characterize how different substituents influence dye spectral properties as well as stability. All these studies laid the foundation for novel analytical applications.

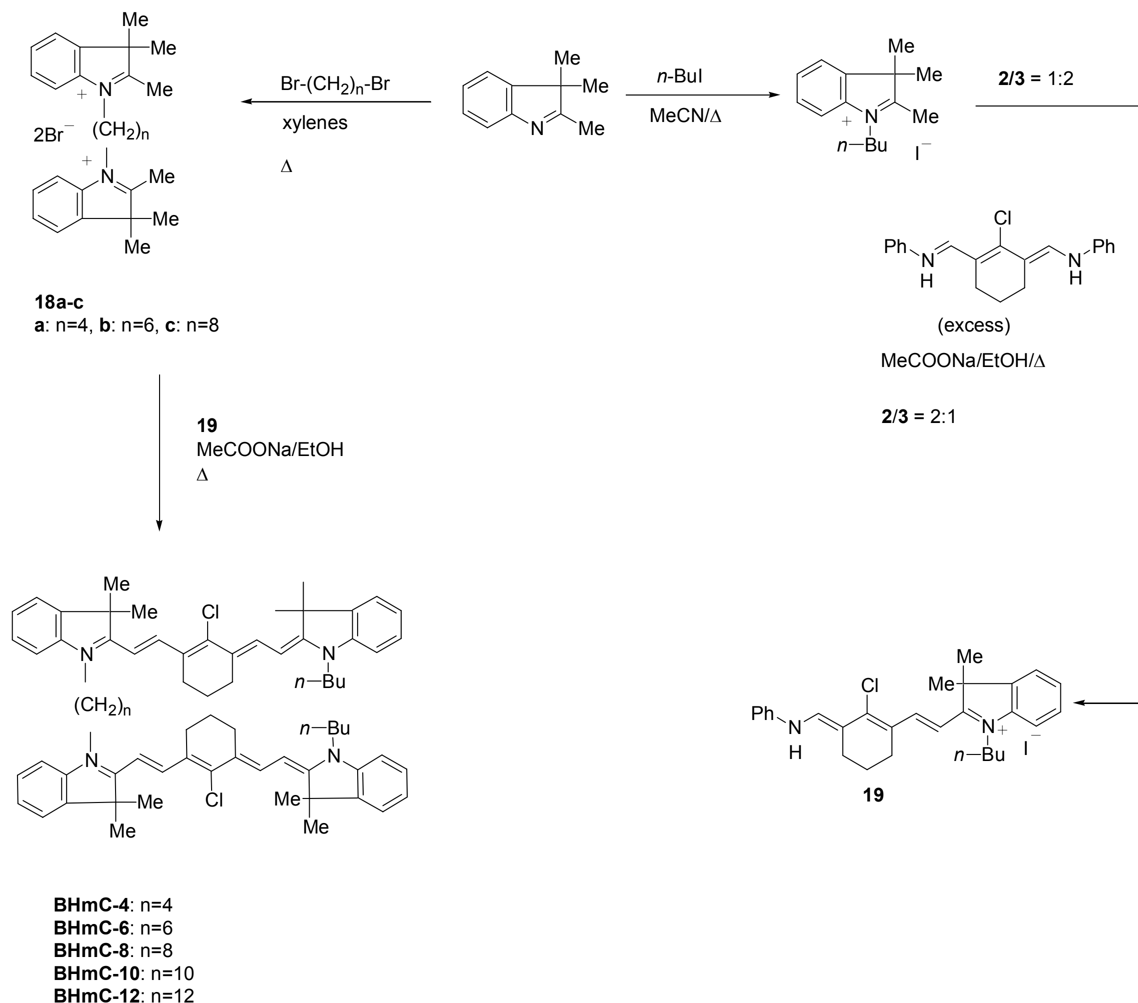


2.2 Stability

Some NIR dyes show limited stability in the presence of molecular oxygen and visible light that can limit biomedical applications. An enormous increase in the photooxidative stability is observed for NIR dyes substituted with a long group (at least four atoms in the chain) at the indolium nitrogen atom in comparison to that of *N*-ethyl- and *N*-methylindolium derivatives. For example, dyes **11-17** (Scheme 2) do not show any spectral changes for solutions (organic or aqueous) exposed to air and sunlight for at least a week. It is known that photodecomposition of cyanine dyes in the presence of oxygen involves the terminal methine groups that are directly connected to the heterocyclic subunits of the dye molecule. The long, flexible substituents, such as in **13-16**, may effectively shield the dye chromophore from contact with molecular oxygen. Since photodecomposition is especially effective for aggregated dyes, the long substituents may also hinder the aggregation, thereby increasing the stability of dyes. In a full agreement with this suggestion the introduction of several anionic groups (in general, sulfonate groups) into the molecule greatly decreases the aggregation of the resultant negatively charged dye and, at the same time, greatly increases stability of the dye.

2.3 Dimeric cyanines

This novel class of dyes has been introduced by us very recently.[4-6] As shown in Scheme 3, the molecule consists of two cyanines linked by a flexible polymethylene chain that can vary in length. The abbreviation of a particular dimer is **BHmC** [**B**is(**H**epta**m**ethine **C**yanine)] followed by a number of methylene units in the linker. Their synthesis involves three distinct steps. First, an indolium substrate is quaternized by the reaction with an α,ω-dibromoalkane to give a bis-indolium salt **18**. Second, a mono-indolium salt is allowed to react with an excess of the Vilsmeier-Haack reagent to furnish a half-dye **19**. Dye **BHmC** is produced by condensation of **18** and **19** under standard conditions. Although this procedure appears to be straightforward, the purification of the intermediate compounds and the product is tedious and the yield of the final analytically pure product is low. Currently we are working on improving efficiency of this synthesis.



3. ANALYTICAL STUDIES

3.1 Monomeric cyanines

Analytical applications of these dyes may be divided into two separate approaches. The first study is comprised of research related to the application of NIR fluorophores as probes; the second study deals with the application of NIR fluorophores as reporting labels. Related work resulted in a paper reporting on the determination of solvent hydrophobicity using carbocyanine dyes.[7] These studies used the aggregation properties of NIR dyes to estimate solvent hydrophobicity. Another interesting application of NIR probes is the use of hydrophobicity probes for the determination of surface and/or binding site hydrophobicity on mammalian serum albumins.[8] This new approach for determining binding site hydrophobicity on mammalian serum albumins and globulins is superior to the previously used methods that frequently perturb the system under study. Additional utility of NIR hydrophobicity probes was demonstrated by determining CMC of surfactant utilizing the low background interference of the NIR spectral region. To fully utilize the NIR technology in bioanalytical applications, we studied covalent and non-covalent interactions between NIR dyes and biomolecules.[8] The analytical use of NIR fiber optic probes and NIR fluorophores was evaluated in a comprehensive work.[9] NIR immunochemistry was utilized for environmental applications and bioanalytical applications by several research groups. Additional utility of NIR fluorophores was presented by using them to characterize polymers by determining functional groups in polymer matrices. Calcium sensitive NIR dyes containing crown ether moieties expanded the applications into cell biology and inorganic

analytical chemistry.[10] The 0.8 amol detection limits were achieved in CE applications using NIR laser induced fluorescence detection. Non-covalent labeling applications of NIR dyes have been discussed in some recent publications from several laboratories. Recently, these dyes have been found useful in forensic analytical chemistry. The very latest of our research efforts focus on the analytical applications of novel bis-heptamethine cyanines.[4-6] Promising results are reported for near-IR dyes and near-IR fluorescent detectors for capillary electrophoresis with laser induced fluorescence detection (CE-LIF) using silicon photodetectors.[11-13] It has been reported that the use of monocarbocyanines has the disadvantage of a relatively high background signal when the dye is not bound to the biomolecule.[14-20] The use of bis-carbocyanines can significantly reduce the background noise.

3.2 Dimeric cyanines

Up to now, visible bis-type dyes have been used for the DNA analysis by intercalating bis-dyes to DNA templates. Our novel class of NIR dimeric dyes can be used in much broader applications, e.g., DNA, protein, and micromolecule analysis. Several researchers reported in the past non-covalent interactions of cyanine dyes to biomolecules using carbocyanines. Carbocyanines exhibit fluorescence enhancement and some spectral shift upon binding to biomolecules. Based on this phenomenon NIR dyes can be used as probe molecules mostly reporting on the binding site properties of the biomolecule or as a non-covalent label to the biomolecule. Non-covalent labeling can replace the labor-intensive and time-consuming conventional covalent labeling methods that require proper reactive functional moieties on the biomolecules. Significant improvement can be achieved by using bis(carbocyanines) that strongly form H-type intramolecular dimers that exhibit negligible fluorescence in the absence of biomolecules. In addition to non-covalent labeling applications, bis(carbocyanines are advantageous as probe molecules. These type of probe molecules utilize the enormous spectral changes that observed when carbocyanines form different aggregates. Of these aggregates, H-aggregates have the most useful utility as probe molecules.

H-aggregates exhibit hypsochromic shift relative to monomer band in the absorbance spectra with negligible fluorescence. In contrast to H-aggregates, J-aggregates exhibit bathochromic shift having sharp absorption spectra features and strong fluorescent enhancement. The geometries of these aggregates are usually defined as head-to-head and head-to-tail formation for H- and J-aggregates, respectively. In previously published reports, we proposed that there could be strong intra and intermolecular stacking between two dye subsystems linked by various alkyl chains. These newly synthesized bis(carbocyanine) dyes provide convenient and effective bioanalytical applications in aqueous environment. Earlier publications discussed several of these dyes and their bioanalytical and biomedical utilities. Of these dyes in Scheme 3, BHmC-12 has not been discussed yet.

In aqueous solutions, BHmC-12 has characteristic absorption H-bands at 720 nm (Fig. 1), and Fig. 2a shows the advantage of bis-carbocyanines for biomolcule detection without generating background fluorescence caused by the monomer counterpart of BHmC-12. Fig. 2b shows its response on biomolecule with various concentrations. Its flexible alkyl linker and intrinsically hydrophobic moieties make it possible to form intra- or/and inter-molecular aggregates immediately after sampling it in aqueous solution, and those stacked forms are deaggregated by hydrophobic or electrostatic interaction with the biomolecule. The sensitivity of this kind of bis-dye on biomoclues is dependent on the flexibility and length of its linker arm. It should be noted that its conformational changes in different environments is a function of hydrophobicity.

Recently BHmC-12 has been used in capillary electrophoresis with near-infrared laser induced fluorescence detection (CE-NIR-LIF) for the complex biomolecule separations and also as a forensic tool for latent fingerprint detection. Fig. 3 shows the separation of a biomolecule complex under different environmental conditions. In methanol, BHmC-12 indicates the presence of possible H-type intramolecular formation that exhibits negligible or low fluorescence in the electrophoresis running buffer. The low peak intensity indicates that in aqueous buffer the dye in a closed clam-shell form that opens up in methanol with concomitant increase in its fluorescence. In phosphate buffer, fluorescence is completely quenched which makes its use for protein detection advantageous due to the reduced background. Hence only the biomolecule-dye complex is detected as BHmC-12 binds to HSA.

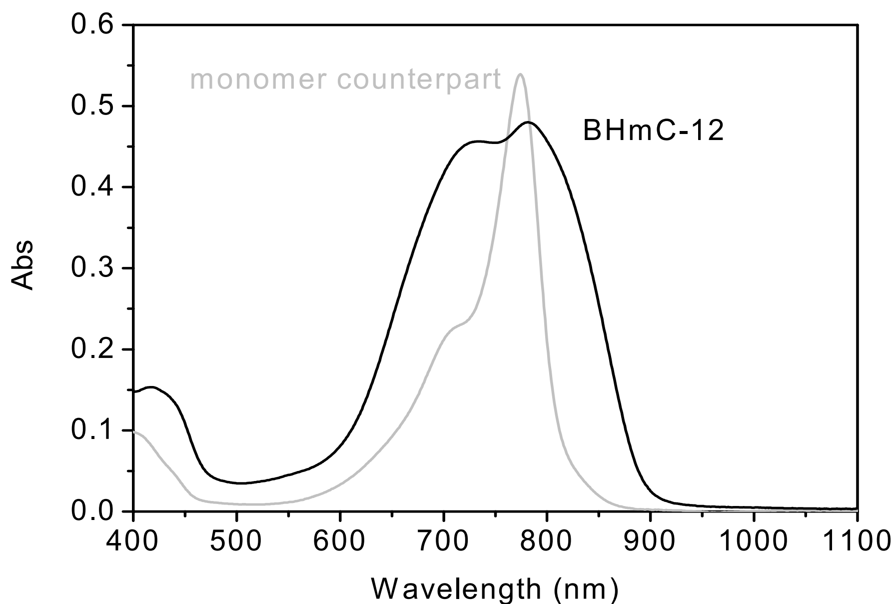


Figure 1. Absorbtion spectra of BHmC-12 and its monomeric counterpart at dye concentrations of 10 uM in 20 mM phosphate at pH 7.2.

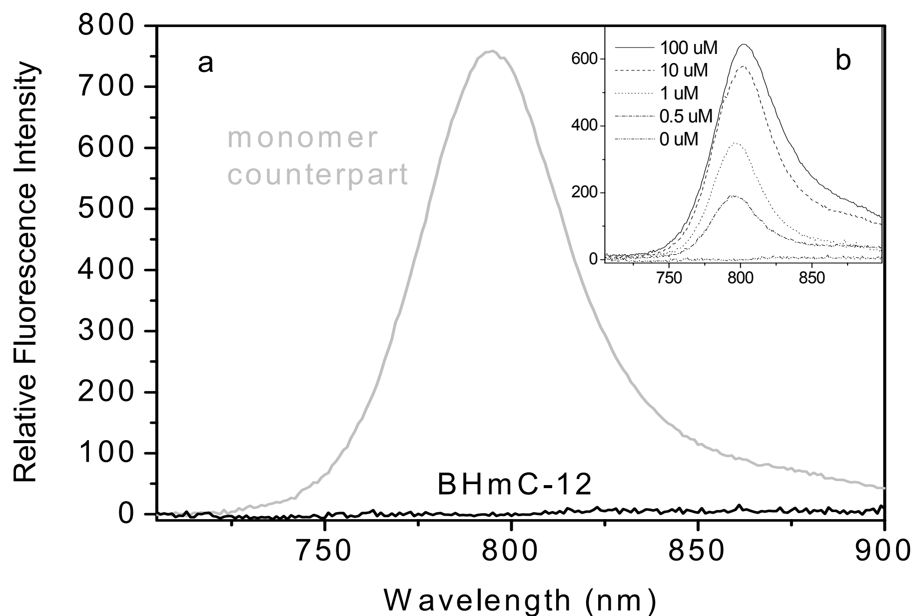


Figure 2. a; Fluorescence spectra of BHmC-12 and its monomeric counterpart at dye concentrations of 10 uM in 20 mM phosphate at pH 7.2. b; BHmC-12 in absence and presence of HSA. Dye and HSA are prepared in 20 mM phosphate buffer at pH 7.2, and various HSA concentrations are added to dye having constant concentration at 10 uM.

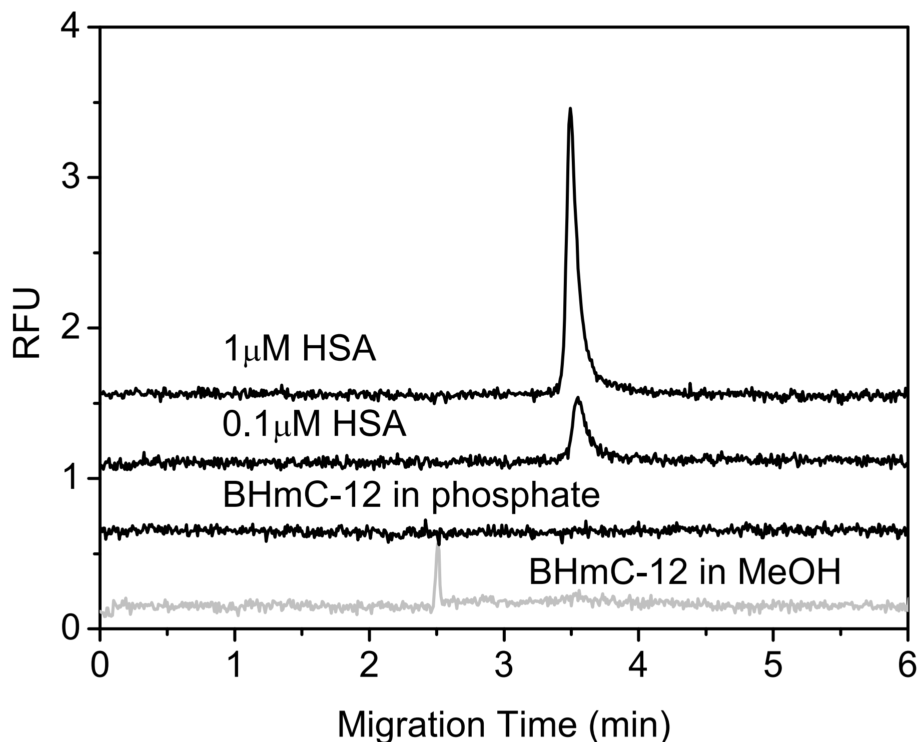
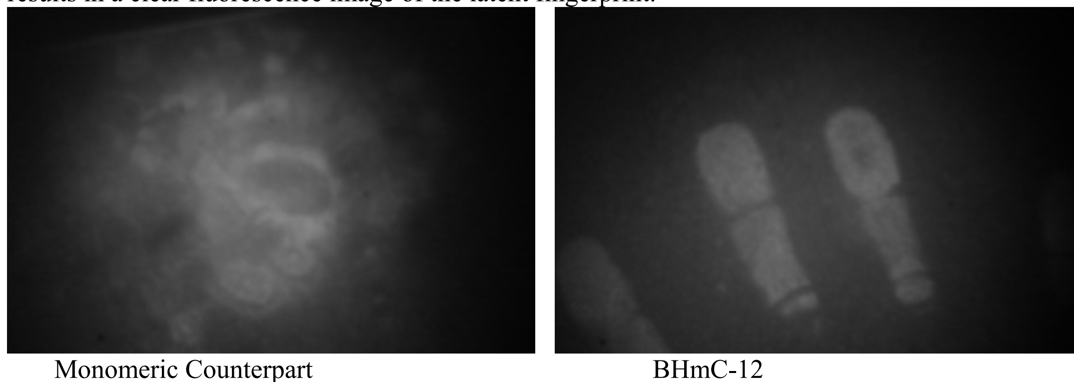


Figure 3. Separation of dye-HSA complex by CE-NIR-LIF. 1 uM of BHmC-12 is used in different solvents. HSA was prepared in 20 mM phosphate buffer at pH 7.2. Instrument conditions: 5 second pressure injection (0.5 psi), 23 kV applied voltage, 75 um x 57 cm capillary; 20 mM borate running buffer at pH 9.3.

Another interesting application of BHmC-12 reflects its ability to bind lower molecular weight biologically or medically important molecules, such as fatty acids. One possible use of this phenomenon is latent fingerprint detection. As can be seen in Figure 4, the use of monomeric counterpart shows no discernable interaction to latent fingerprints. However, BHmC-12 exhibits strongly enhanced fluorescence upon binding to fatty acids that are present in latent fingerprints and results in a clear fluorescence image of the latent fingerprint.



Monomeric Counterpart BHmC-12

Figure 4. Fingerprint detection for forensic purpose. 100 uM dye solutions (monomeric on left, dimeric on right) in MeOH sprayed on a surface containing the latent fingerprint. Visualization: night vision goggles attached to digital camera (after 2 min drying).

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