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Hemicyanine dyes: Synthesis, considerations, solutions and photophysical properties

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Abstract
Hemicyanine dyes are widely used in various fields, including fluorescence imaging, optical data storage, and photodynamic therapy. The activatable fluorescent probes for diverse biological analytes presents a challenge due to chemical instability and photoinstability. This is exemplified by naphthalene-based hemicyanines incorporating the common hemicyanine moiety, 2-indolium. However, these challenges can be effectively addressed by employing a 4-pyridinium derivative, which also exhibits excellent two-photon imaging capabilities.

Keywords: Fluorescent, hemicyanine dyes, naphthalene, indolium, pyridinium derivative

Introduction
Fluorescence imaging of target molecules in cell and tissue is essential in studying biological systems. Accordingly, fluorescent molecules with desirable features for bioimaging application have attracted continuing research interest. Cyanine dyes composed of heterocycles conjugated with polymethine units constitute an important class of near-infrared (NIR) emitting dyes, which are useful for noninvasive biomedical imaging [1]. Recently, hemicyanine dyes, asymmetric analogues of cyanine dyes, have received much attention in the development of fluorescent probes for their diverse structural features and functionalities [2]. Typical cyanine dyes contain an indolium moiety, while other heterocycles such as benzothiazolium [3], benzooxazolium [4], or benzoimidazolium[5] moieties are also available (Fig. 1).

To target a specific analyte in biological systems, various reaction-based fluorescent probes based on hemicyanine dyes have been explored recently [6]. Notable classes of the hemicyanine dyes used for the probe development are the NIR- emitting hemicyanine dyes 2 and 3, which are synthesized from the heptamethine cyanine dyes [7]. In the course of our journey toward the development of the reaction-based fluorescent probes [8], we have been frequently confronted with hemicyanine-based probes in literature that respond to different biological analytes through chemical reactions at the heterocyclic moieties. For example, the coumarin-derived hemicyanine probe 4 or its analogues respond to several "reactive" biological analytes such as hydrogen sulfide [9], peroxynitrite [10], bisulfites [11], or hypochlorite [12]. Several other (hemi) cyanine dyes are also known to display reactivity toward those biological analytes (Table 1, ESI). These reports, on the contrary, raise a critical issue on the chemical stability of the cyanine and hemicyanine dyes in bioimaging application, in particular, where those analytes are potential competitors. We should be aware of the chemical stability issue in the development of a reaction-based, (hemi) cyanine probe for a biological target that competes with those "reactive" analytes. To evaluate the chemical stability of the typical heterocyclic moieties found in (hemi) cyanine dyes and to develop chemically robust NIR-emitting hemicyanine dyes, we have investigated an electron push-pull type of hemicyanine dyes containing a naphthalene core. The results reveal that the typical heterocyclic moieties found in (hemi) cyanine dyes are not chemically stable to those reactive biological analytes. To resolve this issue, that is, to avoid the undersired interference from those analytes, it is thus necessary to develop robust hemicyanine dyes. We reveal that the hemicyanine dye based on the 4-pyridium moiety meets the necessity. In addition, the new dye system, due to its dipolar nature, is promising for two- photon bioimaging, an important but lacking property in the case of cyanine dyes.

We chose three naphthalene-based hemicyanine dyes containing the typical heterocyclic electron-acceptor, 2-indolium, along with 2-benzothiazolium and 4-pyridinium moieties, which are denoted as NVId, NVBt, and NVPy, respectively (Scheme 1). These dyes, at this stage, are designed to have minimal structural and functional features, but we can consider different amine donors (-NH_2 or -NHR) and also the salt components for the development of activatable probes.

The new dyes were readily prepared by the Knoevenagel condensation between 6-(pyrrolidine-1-yl) naphthaldehyde [13] and the corresponding 2- or 4-methyl-heterocyclic salt in moderate to good (20–70%) yields. They were fully characterized by NMR and HRMS analysis (ESI).

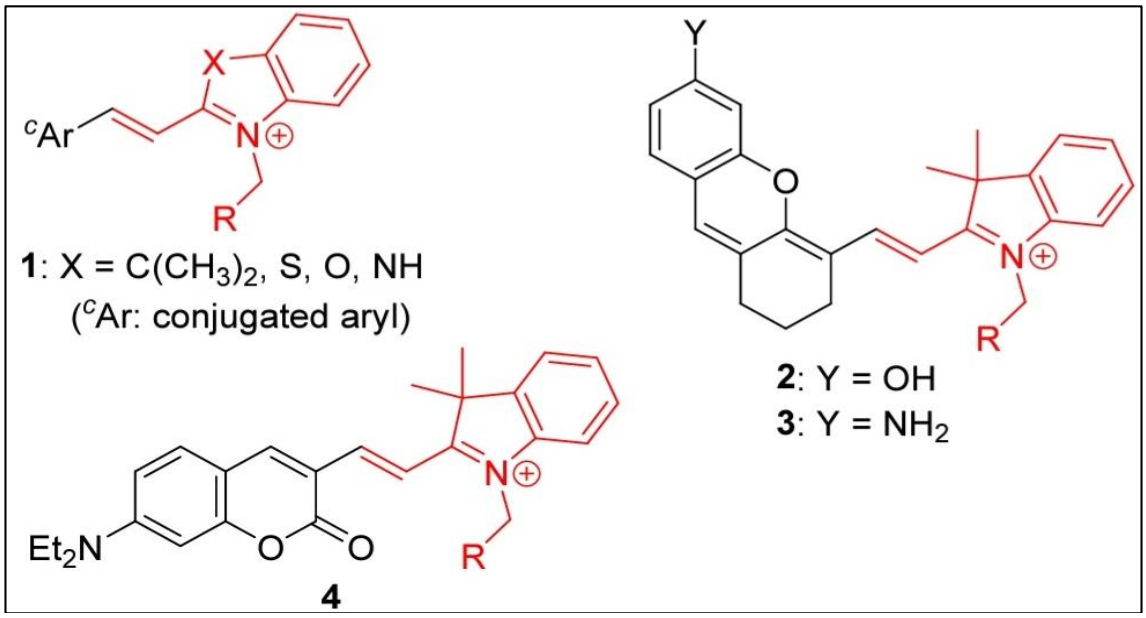
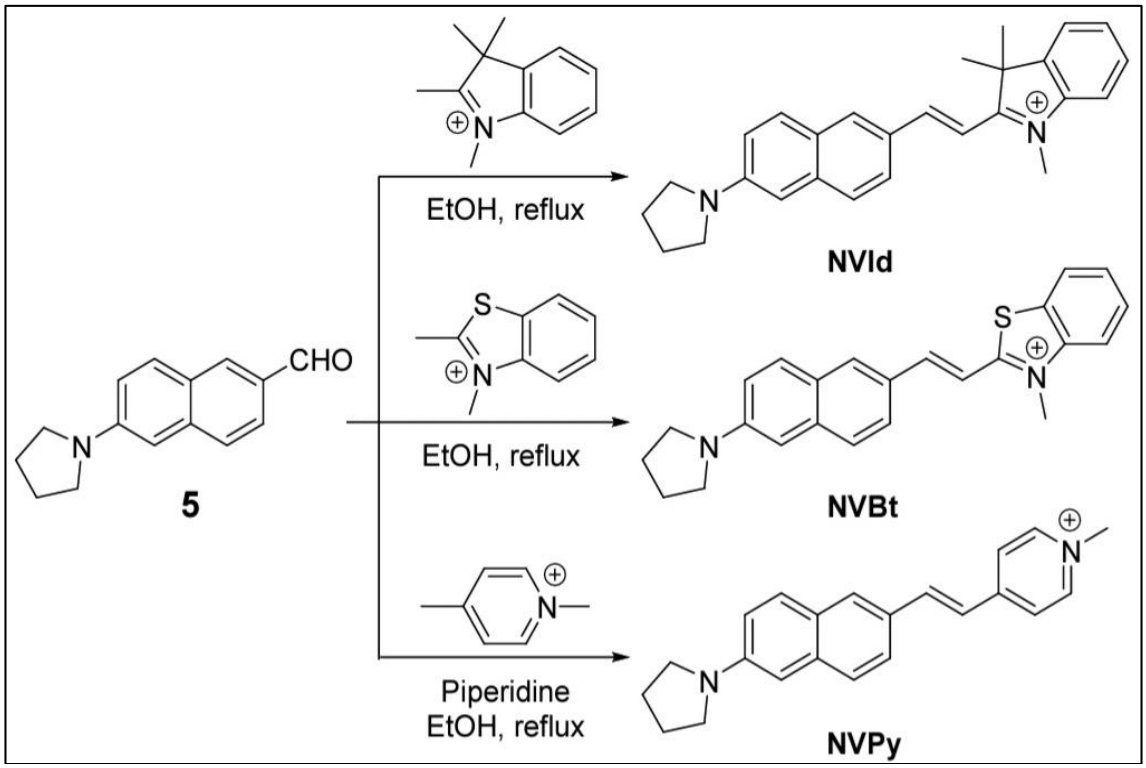


Fig 1: Hemicyanine components and a few selected hemicyanine dyes.



Scheme 1: Synthesis of the hemicyanine dyes.

The dyes emit in the NIR wavelength region (λ_{em} = ~700 nm, EtOH), albeit they have different absorption maxima (Fig. 2). NVPy absorbs at a rather shorter wavelength than those of the others (λ_{max} = 497 nm vs. 552 and 583 nm in EtOH), showing the largest Stokes shift ($\Delta\lambda$ = 195 nm) (Table 1). The hemicyanine dyes 2 and 3 have rather smaller Stokes shifts ($\Delta\lambda$ = ~20 nm). Emission bands of newly synthesized dyes undergo substantial red-shifts with increasing medium polarity, plausibly through intramolecular charge-transfer (ICT) excited states (Fig. 1a–c, ESI). Their absorption bands, however, are blue-shifted with increasing medium polarity (Fig. 2a–c, ESI). This is most likely due to their ionic nature: they have polar ground states but seem to have relatively less polar excited states

owing to the ICT. With increasing medium polarity, the more polar ground states will be more stabilized than the less polar excited states, leading to the blue-shifts. They emit strongly in viscous (glycerol) (Fig. 3, ESI) and less polar (chloroform) media, but weakly in relatively polar acetonitrile (Fig. 3a–c) [14] and poorly in aqueous media plausibly owing to solvent-mediated nonradiative decay processes [15]. These medium-dependent absorption and emission behaviour can be understood by the ionic and dipolar nature of the dyes. Interestingly, NVPy behaves somewhat differently from NVId and NVBt: the former dye emits strongly in less polar chloroform (Φ_F = 0.04) but the latter two emit rather weakly in the same solvent (Φ_F = 0.024 and 0.03, respectively) (Table 2, ESI).

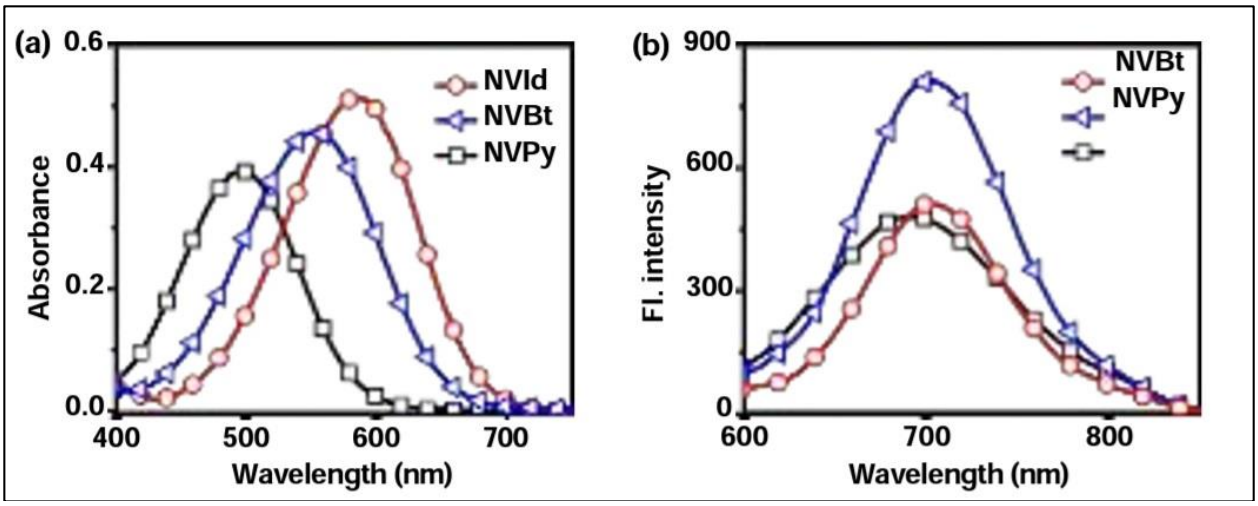


Fig 2: (a) UV–vis absorption and (b) emission spectra of NVId, NVBt and NVPy (each at 10 μM in EtOH), measured at 25 °C under excitation at λ_{max} of each compound.

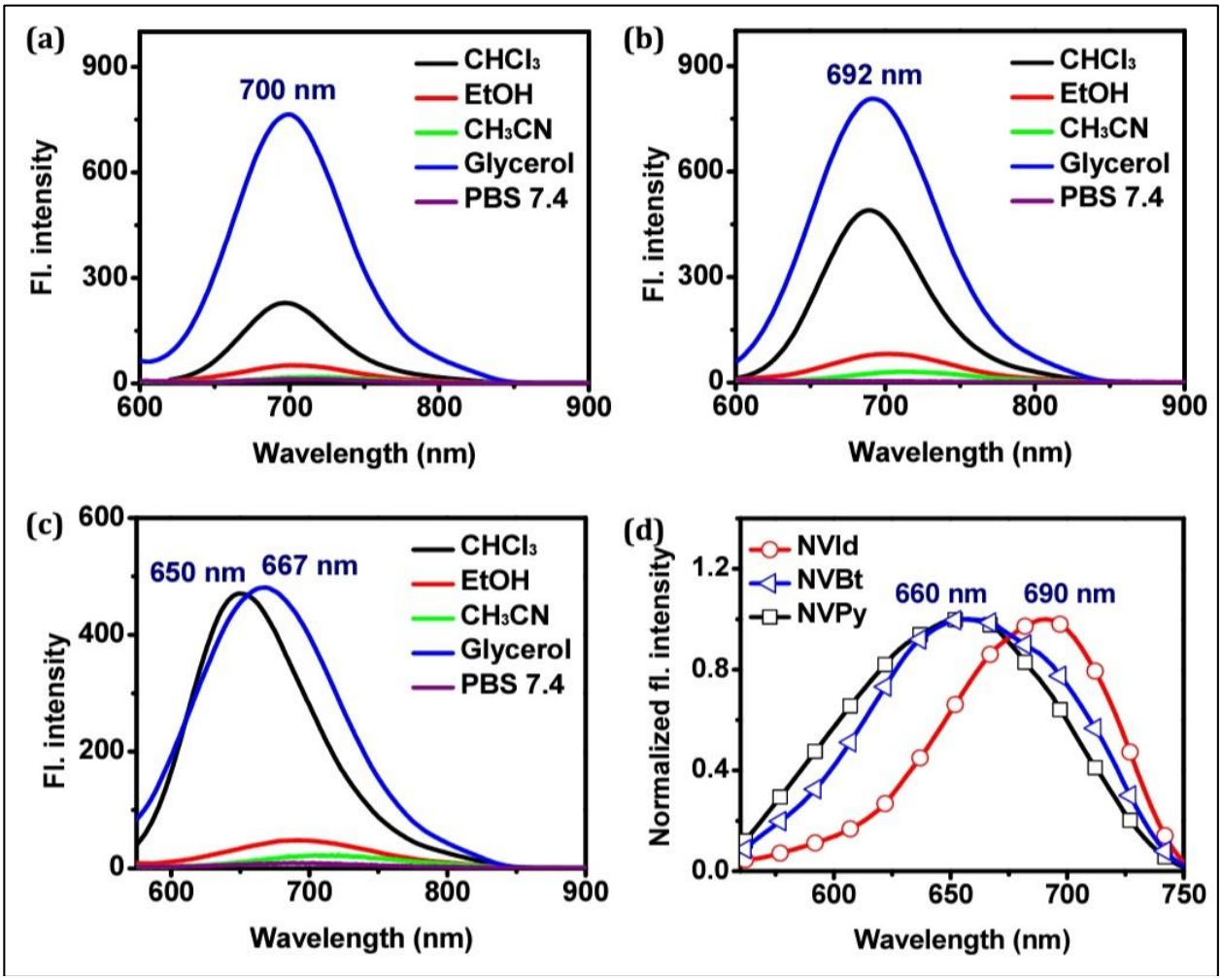


Fig 3: Fluorescence spectra of (a) NVId, (b) NVBt and (c) NVPy (each at 10 μM) in different solvents (dielectric constant) $CHCl_3$ (4.81), EtOH (24.5), CH_3CN (37.5), glycerol (46.5), and PBS 7.4 (80.1). The spectra were obtained by irradiating at λ_{max} of each compound at 25 °C. (d) Normalized fluorescence spectra of NVId, NVBt, and NVPy in HeLa cells incubated with each dye at 10 μM for 30 min, obtained under excitation at 561 nm and collection of emissions from 580–800 nm.

Also, compared to the others, NVPy exhibits significant blue- shifts as the medium polarity decreases (Fig. 4, ESI). These results suggest that NVPy has higher ICT in the excited state than the others, as evident from its significantly higher dipole moment (12.2 D) than those of NVId (2.5 D) and NVBt (3.6 D). DFT calculations in vacuum show that the HOMO is mainly distributed close to the amino donor side whereas the LUMO is distributed close to the heterocyclic acceptor side, which supports the ICT character of the dyes (Fig. 5, ESI).

Next, we compared the dyes' cellular emission behaviour with that in solution. The "in solution" emission peak of the dyes shifted significantly in the cellular environment (Fig. 3d). In HeLa cells, the emission peak of NVId shifts little but that of NVBt blue-shifts significantly and appears close to that of NVPy. Their cellular emission spectra are broad; that of NVPy has a band width of $\Delta\lambda$ = 85 nm (from 615–700 nm) at the half intensity maximum.

Importantly, they allow bioimaging above 630 nm where the autofluorescence interference from innate biomolecules is minimal under two-photon microscopic imaging.[16] Both NVPy and NVId possess two-photon imaging capability (see below). As noted before, the reaction-based probes containing the hemicyanine [17] moiety have been widely used to sense bioanalytes such as bisulfite, hydrogen sulfide, and hypochlorous acid (Table 1, ESI). This fact conversely suggests that the hemicyanine moiety may not be chemically stable toward the reactive analytes. To figure out this concern, we have evaluated the chemical stability of NVId, NVBt, and NVPy toward selected analytes including those reactive ones by a fluorescence assay (Fig. 4a–c).

Table 1. Photophysical properties of **NVId**, **NVBt**, and **NVPy**.

Dye[a]	λ_{abs} (nm)	ε[b]	λem (nm)[c]	Stokes shift	Φf[d]
NVId	582	51,400	702	120	0.010
NVBt	551	45,700	701	150	0.015
NVPy	496	39,400	691	195	0.013

[a] All the measurements were conducted for each dye at 10 μM in EtOH at 25 °C. [b] Unit = L mol^{-1} cm^{-1}. [c] Measured under excitation at the absorption maximum of each dye. [d] Fluorescence quantum yields determined using Rhodamine 6G as a reference dye (Φ_F = 0.95 in EtOH).

The results show that, to our surprise, NVId that contains the typical hemicyanine moiety is highly sensitive to bisulfite and hydrogen sulfide, exhibiting dramatic fluorescence[18] quenching. The fluorescence quenching is likely owing to the conjugate addition of these nucleophilic species to the hemicyanine moiety, causing deconjugation in the fluorophore. Similar sensing mechanisms were reported in the various reaction-based probes (Table 1).

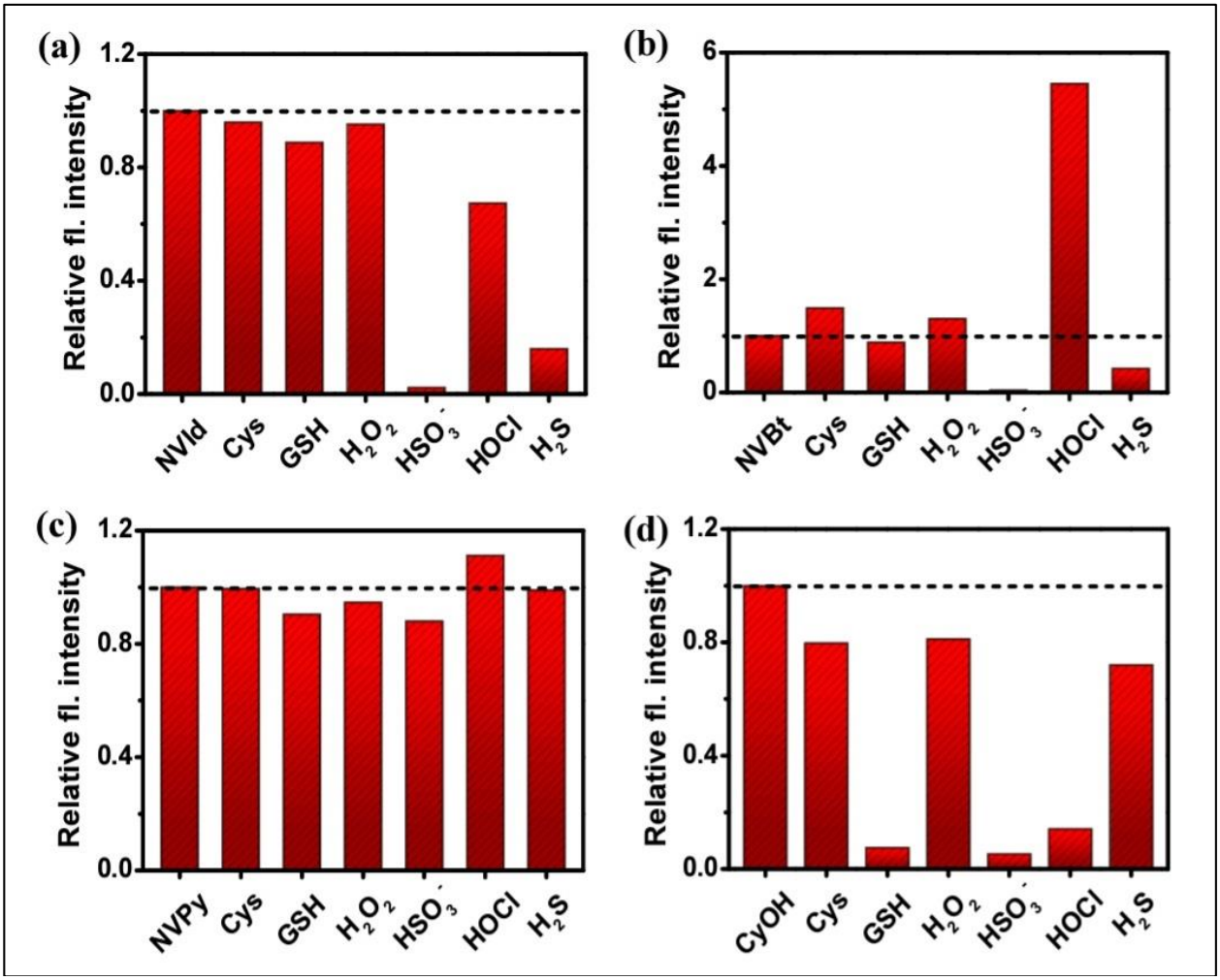


Fig 4: Fluorescence response of (a) NVId, (b) NVBt (ɕ) NVPy and (d) CyOH upon treatment with Cys (200 μM), GSH (10 mM), H_2O_2 (200 μM), HSO^- (200 μM), OCl^- (200 μM), and HS^- (200 μM), each for 30 min in PBS (10 mM, pH 7.4) at 37 °C. The excitation wavelength was 460 nm for a–c. CyOH was excited at 670 nm. The emission peak at 700 nm was used for the analysis.

The vinyl-indolium moiety also responds to hypochlorous acid to some extent. NVBt also responds to bisulfite and hydrogen sulfide, with substantial fluorescence decreases. Interestingly, NVBt responds to hypochlorous acid with a marked fluorescence enhancement, probably owing to the oxidation of the sulfur atom to sulfonyl: The conversion will enhance the ICT and thus fluorescence [19]. Fortunately, NVPy is rather insensitive, displaying marginal intensity variation toward the selected analytes. The negligible reactivity is likely caused by the higher chemical stability of the aromatic pyridinium moiety that is resistant to de-aromatization by possible nucleophilic addition.

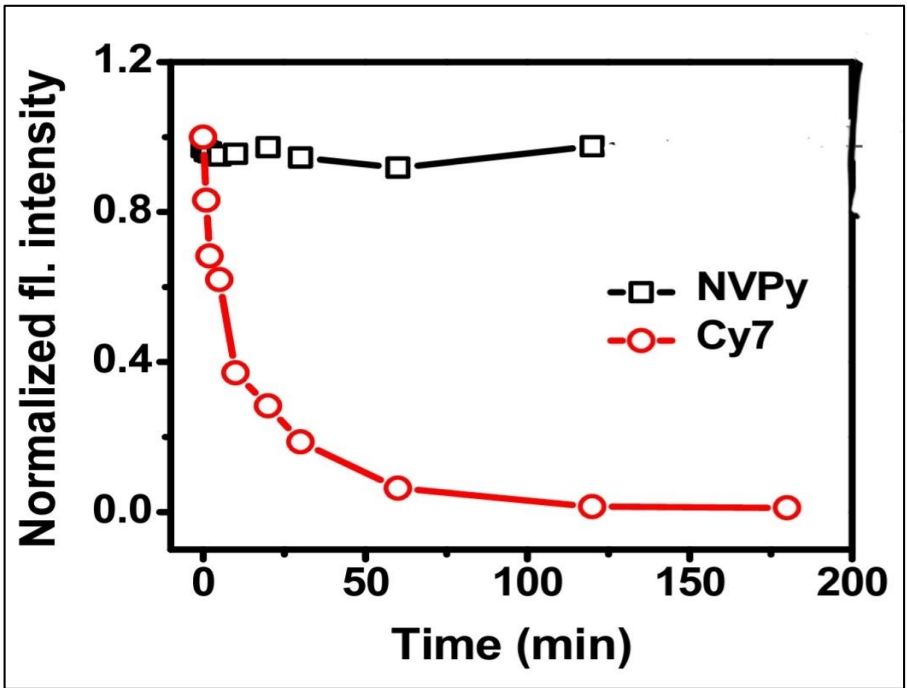


Fig 5: Fluorescence intensity changes of NVPy and Cy7 (IR-786) (each at 10 μM in PBS pH 7.4), under irradiation with 365 nm UV light. The emission peak at 692 nm for NVPy and at 790 nm for Cy7 was followed.

Whereas, the nucleophilic addition to NVId or, NVBt, either to the vinyl group conjugated with the heterocyclic ring or directly to the iminium bond of the heterocycle, does not affect the heterocyclic aromaticity and thus would be more facile. NVPy maintains its emission intensity in cell lysate or in serum (FBS) when monitored for 1 h, and also it is not sensitive to other biologically relevant metal cations, anions, and reactive oxygen species, showing little or small signal enhancement (Fig. 6, ESI). LC-MS analysis for the dyes treated with the reactive analytes indicate that both NVId and NVBt react with bisulfite and hypochlorous acid. Interestingly, in both cases, the dye peak remained in the presence of hydrogen sulfide, which caused fluorescence quenching. Probably, the reversibility of the addition reaction seems to intervene. In contrast, NVPy seems to be chemically inert to bisulfite and hydrogen sulfide, but a small shoulder peak (less than 25%) appeared in the presence of hypochlorous acid (Fig. 7, ESI). At this stage, it is difficult to reason what species it is and how it causes the signal enhancement.

We also checked the chemical stability of the hemicyanine dye 2 (Y = OH; known as CyOH), under similar conditions (Fig. 4d). The dye is highly sensitive to bisulfite, glutathione (GSH), and hypochlorous acid, but a little sensitive to hydrogen sulfide that caused substantial signal change to NVId. The high reactivity of CyOH toward GSH, rather than toward Cys, is quite surprising. The results warn us to take care of such interference in interpreting the sensing results obtained from the hemicyanine- based probes.

Given that NVPy is chemically most stable toward the reactive analytes among the hemicyanine dyes, we evaluated its photostability. The results reveal that NVPy maintains its fluorescence intensity for 3 h, in contrast to a reference cyanine dye, Cy7 (IR-786), which photo-degrades quickly (Fig. 5). NVPy is composed of aromatic rings conjugated through only one "flexible" carbon–carbon double, which feature may explain its high photo-stability. In addition, NVPy is highly soluble in aqueous media and maintains the linear concentration- dependent absorbance up to 50 μM evaluated (Fig. 8, ESI).

Next, we evaluated cellular imaging capability of the three dyes. NVPy provided the brightest cellular image followed by NVId and NVBt, under normal imaging conditions (30 min-incubation at 5.0 μM of the dye; with 3% laser power). The observed chemical response that involves fluorescence quenching might cause the less bright cellular images in the case of NVId and NVBt (Fig. 6a). NVPy is also capable of two-photon imaging, providing bright cellular image under two-photon excitation at 950 nm (Fig. 6b): It has a high two-photon action cross-section value (TPACS: $\Phi_F\sigma_2$ = 217 GM; two-photon excitation at 990 nm) suitable for deep tissue imaging (Fig. 6c).

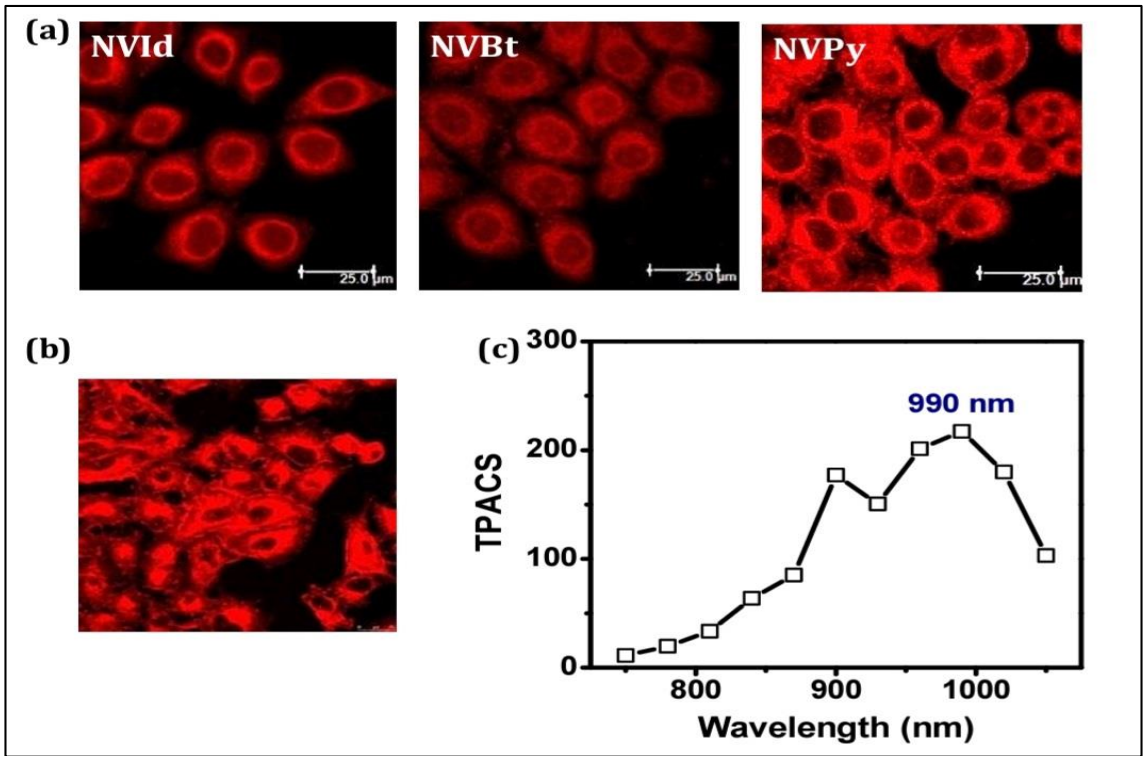


Fig 6: (a) CLSM images of HeLa cells incubated with NVId, NVBt and NVPy (5.0 µM in pH 7.4 PBS) for 30 min, obtained by exciting at 561 nm for NVId and NVBt and at 488 nm for NVPy with 3% laser power and collecting emissions from 600–800 nm in all cases. (b) Two-photon microscopic images of A549 cells incubated with NVPy (5.0 µM) for 30 min, obtained under two-photon excitation at 950 nm and collection of emissions from 415– 665 nm. (c) Two-photon action cross-section (TPACS) spectrum of NVPy (100 µM in dioxane), obtained using rhodamine B (100 µM in MeOH) as a reference dye.

Conclusion

In conclusion, hemicyanine dyes that provide diverse structural and functional features are increasingly used in the reaction- based fluorescent probes. The hemicyanine-based probes react with several biological analytes such as bisulfite, hydrogen sulfide, and hypochlorous acid, raising a chemical stability issue. To evaluate and address the chemical stability issue, we have investigated three hemicyanine dyes that contain the 2- indolium, 2-benzothiazolium, and 4-pydridinum heterocycle, respectively. These hemicyanine dyes exhibited medium-dependent absorption and emission behaviour when evaluated in solution and in cellulo. A fluorescence assay toward selected biological analytes including the reactive ones reveals that the hemicyanine dyes containing the typical hemicyanine moiety, indolium, and the benzothiazolium moiety, are chemically reactive, particularly toward the reactive analytes, whereas the pyridinium derivative is quite inert. The chemical stability issue is further confirmed with a well-known hemicyanine dye, which warrants careful interpretation of the sensing results obtained by the hemicyanine-based probes. The pyridinium-based hemicyanine dye is also photochemically stable and provides bright cellular fluorescence images in the deep-red wavelength region. Furthermore, it has a high two-photon action cross- section value, enabling two-photon microscopic imaging.

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