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Synthesis and characterization of membrane stable bis(arylimino)isoindole dyes and their potential application in nano-biotechnology

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Abstract

A synthetic methodology of preparing novel membrane stable, responsive dyes is revealed in this manuscript. 1,3-bis(arylimino)isoindole dyes were synthesized and their properties to undergo intramolecular hydrogen bonding was studied with fluorescence spectroscopy in varying solvent polarities. Based on the functional moieties, compound that is capable of hydrogen donor and acceptor interactions produces predominant photoexcitation in comparison to the responsive dyes that lack these functionalities. These dyes, by the virtue of the presence of long chain acyl groups could be incorporated stably within the phospholipids membrane of core-shell nanoparticles. Nanoparticle was 'cracked' to release the dye from a hydrophobic to a hydrophilic environment, A significant change in fluorescence intensity was then observed, indicating the direct change in effect of intramolecular hydrogen bonding based on solvent polarity changes. This unique study provided implications of many further applications towards nanomedicine and nano-biotechnology.

Keywords

Fluorescence dye; Bis(imino)isoindole; Responsive dyes; Membrane stable; Nano-biotechnology

Molecules that are able to undergo intra molecular hydrogen bonding are significant contributors in diverse applications e.g. high-energy radiation detectors,^{1–3} ultra violet photostabilizers,^{4–5} fluorescent probes,^{6–7} and laser dyes.^{8–9} These molecules are well known for their ability to facilitate proton migration from a donor atom to a proximal acceptor atom usually within a proximity of $<2\text{\AA}$.^{10–13} For obvious reasons, they have prominent effects on molecular structures and properties in terms of lipophilicity, membrane permeability, water solubility and rationales for drug design.¹⁴ Many factors can play in affecting the spectrum of a solute e.g. static factors (interactions between the solvent and the solute), permanent dipoles, dynamic factors (i.e. London Dispersion forces and dielectric relaxation), and specific interactions including hydrogen bonding between the solute and the solvent. Florescent probes that undergo intra molecular hydrogen bonding are found to be

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Supplementary Material

Experimental details are available online.

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highly sensitive to solvent polarity. Subsequently, they provide valuable information about the environment in heterogeneous media.¹⁵ Thus changes in solvent composition induce shifts of the absorption and emission peaks of molecules that have the ability to cause hydrogen bond formation with its solvents.¹⁶ Consequently, such fluorescent excitation of the molecules triggers a shift to higher emission wavelengths with changing solvent polarities which is a result of introducing a specific site for hydrogen bonding to occur within the solvent media or within the molecule itself.¹⁷ Thus the intra molecular hydrogen bonding is strongly influenced by the polarity of the solvent or the environment that it is exposed to. Thereby it regulates the intensities and shifts the wavelengths of the excited-state intra molecular hydrogen bonding emissions.^{18–19} A probe that could differentiate between aqueous and non-aqueous environments will be widely used in applications such as *in vitro* diagnostics, drug delivery, clinical pathology and numerous other nano-biotechnology applications. Depending on their sensitivity, these molecules may potentially be used *in vitro* and *in vivo* in conjunction with phospholipids coated nanoparticle systems.^{20–23} We hypothesize that these probes can be designed based on their ability to form intra molecular hydrogen bonds (Figure 1). Alternatively, these probes may comprise functional moieties that are capable of undergoing hydrogen bonding with solvents. Towards this aim, we developed novel molecules as membrane stable, ‘responsive’ dyes centered on its ability to intuit changes in cellular environments.

Our design of responsive dyes use 1,3-bis(imino)isoindole as a central moiety. 1,3-bis(imino)isoindole derivatives are known to undergo intra molecular hydrogen bonding through their extended conjugation by benzannulation of aromatic rings and its pronounced fluorescence excitation properties.²⁴ Platinum coupled 1,3-bis(imino)isoindole derivatives exhibit great photophysical and photoemission properties.²⁵ In the present work, the design of the dyes utilized a 1,3-bis(aryl imino)isoindole (aryl: compound **1**= 2-aminopyridine; compound **3**= 4-butyl aniline) moiety and a strategically placed acyl fatty acid chain (R= –C₈H₁₇) to provide membrane stability (Figure 1; compounds **1**, **3**). The aryl amines were selected based on their potential ability to form intra molecular hydrogen bonds within the molecule itself. We hypothesize that the benzannulation of pyridyl and isoindole rings in **1** will be influenced by the solvent polarity. A similar compound, **3**, which lacks the extended conjugation by benzannulation of pyridyl and isoindole rings, was synthesized as a control compound. In order to investigate the compounds’ ability to undergo intra molecular hydrogen bonding in changing environments, dyes will be encapsulated within a phospholipids-coated nanoparticle and studied *in vitro*.

Our initial attempt to produce **1** and **3** by a solventless microwave assisted method was unsuccessful. Briefly, 3,6-Dioctyloxy-1,2-benzenedicarbonitrile (**2**) was added to the aryl amine (for compound **1**: 2-aminopyridine; for compound **3**: 4-butyl aniline) and calcium chloride followed by heating the mixture in a domestic microwave for 1–3 mins (Figure 1a–b). The formation of compound **1** was identified by ¹H-NMR spectroscopy of the crude mixture. However, we were unsuccessful to isolate the desired compound from a complex mixture of side products. In case of **3**, the product was never identified by the microwave route. In an alternative pathway, **1** and **3** were synthesized based on the nucleophilic addition of aryl amines to 1,2-benzenedicarbonitrile to form 1,3-bis(arylimino)isoindole derivatives (Figure 1c–d) following a modified method.²⁶ Briefly, 3,6-Dioctyloxy-1,2-benzenedicarbonitrile (**2**, 0.65 mmol), 2-aminopyridine (1.62 mmol) and 25 mg of CaCl₂ was refluxed in 1-butanol under argon for 15h to produce **1** in 70% yield. In a similar fashion, compound **3** was synthesized from 4-butyl-aniline in 81% overall yield. The compounds were purified by repeated recrystallization to obtain fine needle-shaped orange crystals and characterized *via* ¹H-NMR and Mass Spectrometric analyses. The synthesized compounds were preserved at 4°C protecting from light and moisture. Interestingly, upon exposure to moisture, the 1,3-bis(arylimino)isoindole derivatives were found to undergo

slow hydrolytic degradation as indicated by the appearance of O-phthalamide derivatives in $^1\text{H-NMR}$ studies. (Figure 1f)

To further assess the ability of the dyes for environmentally sensitive photo-excitability *in vitro*, a phospholipids-polysorbate micellar nanoparticle was synthesized. Our design of the nanoparticle involved a co-self assembly of lecithin-PC and polyoxyethylene (80) sorbitan monooleate in water. Sorbitan monooleate was strategically chosen as a co-surfactant to divulge stability to the micelles and to restrict the particle diameter within 20 nm with low polydispersity. A simple, two step procedure was followed to incorporate bis(arylimino)isoindole derivatives, which was facilitated by the use of the co-surfactant. In a typical procedure, phosphatidylcholine (100 mg) was weighed in a long neck test tube and dissolved in 2 mL chloroform. To this, a solution of **1** (5 mg) in chloroform was added. The solvent was evaporated gently to produce the lipid film and dried for 30 minutes in vacuum at 40°C. To this, nanopure water (0.2 μM , 10ml) was added with polyoxyethylene (20) sorbitan monooleate and was probe-sonicated at 25°C to form a transparent light yellow suspension. The suspension was dialyzed using 10kDa MW cut-off cellulosic membrane against infinite sink of nanopure water to remove any unbound dye. Multiple analytical techniques were employed to characterize these particles. Dynamic light scattering (DLS) measurements revealed the particle hydrodynamic diameter as 19 ± 7 nm with low polydispersity (PDI= 0.039). Electrophoretic potential values ($\zeta = -12 \pm 7$ mV) were measured as negative, which is an indirect measure of the colloidal stability of the system and further confirms a successful encapsulation with phospholipids.

Fluorescence emission spectra of the dyes (ca. 1.8 mM) were studied in solvents with varying polarity.³² Solvent polarity dependent variations in absorption and emission spectra were observed for both **1** and **3**. However, for **1** a significant change in the fluorescence intensity and intrinsic optical properties were observed. As shown in Figure 2a, the emission from compound **1** is red-shifted ($\lambda_{\text{em}} = 356$ to 408 nm) with decrease in solvent polarity. In THF, λ_{max} was observable at 408 nm. This indicated the activation and presence of an excited state intra molecular hydrogen bond shift that allowed the photo-excitation to occur. From the fluorescence experimental data of compound **1**, a general trend can be brought out. As the solvent changes to a more polar environment from dichloromethane to methanol, the shift in λ_{max} and its emission increases. As shown, compound **1** exhibited the lowest emission λ_{max} in dichloromethane, and as it shifted its environment towards more polar, e.g. methanol and tetrahydrofuran, its emission λ_{max} increased. However, the effect of tetrahydrofuran and methanol are similar and thus indicates a threshold for the compound undergoing intra molecular hydrogen bonding according to solvent changes. The solvent dependence optical property of **1** is expected and can be attributed to the structural aspects of the amine functionalities. The intra molecular hydrogen bond in **1** is stabilized through a six-membered ring transition state, which involves nitrogen N^b to N^a to undergo a temporary resonance stabilized configuration. This further enhances the transition state's stability and feasibility. Such effect is enhanced as solvent polarity is increased, enabling the relay of hydrogen 'hopping' towards its N^a acceptor site.²⁷ The nitrogen atom from the pyridine ring (N^a) has a higher affinity than the nitrogen atom in the five membered ring (N^b) from the 1,3-bis(imino)isoindole due to the trigonal planar configuration of the sp^2 hybridized N^a coordination. Such geometry alignment with the hydrogen from N^b allows it to have a higher proton affinity, which is also due to the assistance of the increased electron density of the pyridine ring. Thus the $[-\text{N}^a\text{-H}\cdots\text{N}^b]$ valence bond (VB) is more favorable than the $[-\text{N}^a\cdots\text{H-N}^b]$ VB, which further assists in the formation of the intra molecular hydrogen bonding within the neighboring nitrogen moieties to further drive the compound towards a stable transition state ring formation. Consequently, the two tautomeric forms become energetically close to each other due to the counterbalance between the stability gained through the six-membered ring type.²⁸⁻³¹ Such naphthalene ring is also complemented by

the conjugated 1,3-bis(imino)isoindole structure, which further supports the transfer of electron density around the four adjacent ring systems and thus, ultimately favors intra molecular hydrogen bond to occur and exhibits the shift in λ_{max} . Compound **3** was prepared as a control molecule, which lacks donor or acceptor atom crucial for rendering potential intra molecular hydrogen bonding capability. Compound **3** (ca. 1.8 mM) in dichloromethane exhibited its absorption maximum at 348 nm. Although, Figure 2b indicated a change in the fluorescence intensity with the polarity of the changing solvents, it is also suggestive that rather than intra molecular hydrogen bonding occurring within the internal mode of the structure, the lone hydrogen is forming weak bonds with the solvents. This is causing a lower shift in wavelength and causing a change in fluorescence properties due to the quick shift in hydrogen position. The lower shift in λ_{max} could also indicate that the conjugation of the system just with the solvent interaction is not as prominent as that of when an intra molecular hydrogen bond occurs forming a higher level of conjugated ring structures, thus a lower emission results. Such absence of the capacity to intra molecularly hydrogen bond with proximal acceptor atoms validates that a shift in hydrogen bonds cannot occur without a donor-acceptor interaction, thus shows minimal variation in shifts in wavelengths with solvent polarity.

Compound **1** was successfully incorporated within the nanoparticles as described before. (Figure 2c) The successful encapsulation of the dye as a component of phospholipids surfactants indicated that it was membrane stable *via* hydrophobic interactions of the acyl chains ($-\text{C}_8\text{H}_{17}$) on the dye with the internal lipid coating and was exposed to a hydrophobic environment. In order to expose the dye into a less hydrophobic and more polar environment, the nanoparticles were cracked with isopropanol and were transferred to methanol. Figure 2d exhibits a significant change in the fluorescence intensity and properties as the dye gradually encounter a polar environment with increasing amount of isopropanol. As shown, the emission wavelength before exposing compound **1** to a polar environment (methanol) is similar to the emission properties displayed by **1** alone in dichloromethane. This indicates that the **1** is localized stably at the hydrophobic acyl phospholipids chains. Once the dye is exposed to the polar environment, a significant shift in intensity and excitation maxima was observed. With the addition of more isopropanol, the effect is more prominent, which altogether suggests a greater degree of intra molecular hydrogen bonding of the released dye in a polar environment. This experiment further supports the hypothesis that the ability to form intra molecular hydrogen bonding is strongly dependent on the polarity of the solvent, which in turn regulates the intensities and wavelength shifts.

In summary, we have successfully designed and synthesized novel, membrane stable responsive dyes. Fluorescence spectroscopic study revealed that the optical properties are highly dependent on the solvent polarity as reflected its ability to cause photoexcitation and a shift in emission wavelengths. The fluorescence properties of the control compound, which lack the ability to form intra molecular hydrogen bonding only showed a variation presumably due to hydrogen bonding of the compound with the solvent. Finally, the potential application of these membrane stable dyes was successfully examined in a phospholipids-based nanoparticle platform, which clearly indicates the implications of many further applications towards nanomedicine and nano-biotechnology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a–b) Microwave assisted syntheses of **1** and **3** in presence of CaCl_2 ; (c) 2-aminopyridine, CaCl_2 , 1-butanol, reflux, 15h; (d) 4-butyl aniline, CaCl_2 , 1-butanol, reflux, 15h; (e) self-assembly of lipid-nanoparticle and encapsulation of dye **1**: preparation of phospholipids thin films composed of egg lecithin PC and **1**; resuspension of the thin film in water (0.2 μM), polysorbate, probe sonication at room temperature, 1 min, dialysis (cellulosic membrane, MWCO 10K); (f) moisture sensitivity of the responsive dyes as confirmed by $^1\text{H-NMR}$; (g) schematic representation of the proposed responsive dye comprising of moieties for giving membrane stability and probability to undergo intra molecular H-bonding affected by the solvent polarity.

Figure 2.

Fluorescence spectroscopic study: Compound **1** (a) and compound **2** (b) show variation in their fluorescence properties in solvents of different polarities; (c) a cartoon illustrating the encapsulation of **1** distributed within the hydrophobic acyl chains of the phospholipids coated nanoparticles; dye molecules are exposed to aqueous environment once the nanoparticles are 'cracked'; (d) fluorescence spectrum of dye-loaded nanoparticles showing similar spectral profile as the free drug in non polar solvent (dichloromethane, (i)); after the nanoparticle cracking (ii–iii), the variation in the fluorescence intensity and emission maxima are predominant. (iii) corresponds to the addition of more isopropanol. Solvents were chosen based on their polarity difference. Relative polarity of methanol: 0.762, 1-butanol: 0.586, THF: 0.207, isopropanol: 0.546, toluene: 0.099, dichloromethane: 0.309.³²