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NIR Dyes for Bioimaging Applications

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Summary of recent advances

Fluorescent dyes based on small organic molecules that function in the near infra red (NIR) region are of great current interest in chemical biology. They allow for imaging with minimal autofluorescence from biological samples, reduced light scattering and high tissue penetration. Herein, examples of ongoing NIR fluorophore design strategies as well as their properties and anticipated applications relevant to the bioimaging are presented.

Introduction

Dyes active in the near infrared (NIR) region have attracted ongoing attention due to their diverse applications in biomedical, materials and related fields. Advantages include minimal interfering absorption and fluorescence from biological samples, inexpensive laser diode excitation, reduced scattering and enhanced tissue penetration depth. However, there are only relatively few classes of NIR dyes that are readily available. These include the phthalocyanines, cyanine dyes and squaraine dyes.

Aqueous insolubility and ease of aggregate formation are problems often encountered with phthalocyanine and squaraine dyes in biological systems. Squaraine dyes are also highly chemically reactive. Cyanine dyes are excellent NIR dyes that have high molar absorptivity, strong fluorescence, and good photostability. However, their intrinsically small Stokes shifts may produce excitation and scattered light interferences.

Great effort has gone into improving the photophysical and photochemical properties of existing NIR dyes. For example, various hydrophilic groups such as sulfonate, pyridinium, glycol and carboxylate, have been appended to increase aqueous solubility. Moreover, the addition of charged functional groups and increased sterics has aided in reducing aggregation. Cyanine dyes have also been functionalized in order to increase their Stokes shifts.

Although the modification of existing dye skeletons with appropriate functional groups has much improved their physicochemical properties, it has also led to new issues. Increasing the molecular weight of the dyes can lead to interference with the functioning of biomolecules or precipitation, apart from synthetic challenges. Large molecular weight dyes cannot be readily used for *in vivo* amyloid labeling since such studies require blood brain barrier penetration, and can cause increases in the serum pharmacokinetics of drug-dye conjugates.

The development of simple and novel low molecular weight NIR platforms that can be further modified is thus of great interest to the biomedical imaging community. The visualization of

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tumors and plaques as well as vascular mapping of the heart and brain are aspects of basic biomedical research and disease diagnostics that can continue to benefit substantially from the creation of improved NIR dyes. The development of new and improved fluorophores for trafficking studies, particularly the trafficking of tagged nutrients or drugs, should dramatically increase the number of live cell imaging and *in vivo*, real-time advanced imaging studies for fundamental research and translational applications.

This review highlights very recent progress (since 2008) towards the synthesis and evaluation of new NIR-active dyes with enhanced optical and physical properties for potential bioimaging applications. In this relatively short period of time research groups from around the world, spanning several disciplines, have created new NIR fluorophores that creatively address a broad scope of significant current challenges. The following sections summarize these ongoing studies.

Novel Cyanine Dyes

The cyanines are a unique class of charged chromophores with an odd number of carbons in a conjugated polymethine framework that has no significant bond alternation. Pyrrolopyrrole cyanine (PPCy) dyes are a new type of NIR fluorophore synthesized via the reaction of diketopyrrolpyrrole with heteroarylacetonitriles. They exhibit narrow and intense absorptions as well as quantum yields ranging from 0.32–0.69 ($CHCl_3$). Imaging applications for these promising materials have been proposed. Aqueous soluble derivatives have not yet been reported [1].

Tang and co-workers appended a terpyridine moiety to a tricarbocyanine dye to afford a water soluble fluorophore (**1**) that can respond to minor fluctuations in pH (6.70–7.90) via a photoinduced electron transfer (PET) mechanism. Real time imaging and pH detection pH detection was achieved in studies involving live HepG2 and HL-7702 cells [2]. Pham and co-workers modified the cyanine dye NIR820 with four sulfonates to increase water solubility (compound **2**). They additionally substituted the chlorine atom at the methine bridge to increase the Stokes shift. Their molecule 4-Sulfonir exhibited a molar extinction coefficient of 1.5 × 105 $M^{-1}cm^{-1}$, a quantum yield of 0.37 and a Stokes shift of 140 nm. Its potential utility in biological media is under investigation [3]. The Smith group had previously derivatized a carbocyanine in a similar fashion with a zinc (II) dipicolylamine (Zn-DPA) moiety to detect *Staphylococcus aureus* infection in mice [4,5**]. This embodies a unique example of a non-genetic reporter molecule that specifically targets bacteria *in vivo* via its inherent affinity for anionic cell surfaces. Similar NIR-mercury ion [6] and NIR cancer [7] probes also have been synthesized.

The Achilefu group has recently reported several studies of interest involving new functional cyanines. A new pyrimidine-fused pH-sensitive NIR active fluorescent probe was discovered via an attempted substitution of the chlorine atom at the cyanine methine moiety with barbituric acid. The product (**3**) was formed via an unusual debenzoindolation mechanism [8]. In another study, unique cyanine dye-based FRET pairs were developed and conjugated to peptides to monitor caspase activity [9]. The fluorescence lifetimes of a series of cyanines were investigated as a function of solvent and structural features. Decreases in structural rigidity were correlated to losses of excited state energy via non-radiative pathways [10].

Strekowski and co-workers reported the synthesis of a number of benzo[*c*,*d*]indolium-derived cyanines (for example, compound **4**). Using readily available 1,8-naphtholactam as a substrate they were able to obtain both hydrophobic and water soluble fluorophores. These molecules are expected to be more stable and exhibit enhanced bathochromic shifts as compared to other benzoindolium-derived cyanine congeners [11].

Borohydride-reduced cyanines (“hydrocyanines”) are weakly fluorescent until oxidized. They are thus activated by reactive oxygen species (ROS) including superoxide. Murthy and co-workers successfully measured ROS generation at nanomolar levels and *in vivo* using hydrocyanines. The hydrocyanines are less prone to autofluorescence as compared to comparable reagents [12].

Iodoacetamide-functionalized cyanines were synthesized by Bruschi et al. They are useful for labeling cysteine residues as alternative dyes for the analysis of plasma proteins via 2-D DIGE (differential display electrophoresis). The authors also report their potential utility in evaluating protein redox status [13].

Commercially available cyanines were linked to radionucleotide-binding moieties and conjugated to tumor-targeting antibodies. This afforded a dual modality positron emission tomography-fluorescence imaging material that exhibited strong binding to HER2-expressing cancer cell lines. This design strategy should be applicable to the generation of libraries of antibody-based multimodal probes [14].

Novel Phthalocyanines and Porphyrin Derivatives

Porphyrins and phthalocyanines and their metal complexes embody some of the most intensively studied NIR-active dyes. A series of conjugated porphyrin dimers with intense absorptions ranging from 650–800 nm and fluorescence emission from 700–800 nm have been used in imaging studies [15]. The authors noted greatly improved photostability as compared to their monomeric counterparts and outstanding potential for photodynamic therapy. These molecules disaggregated and fluoresced upon binding albumin.

Synthetic chlorin and bacteriochlorin macrocycle dyads (“C-B dyads” such as compound **5**) were created that exhibit large Stoke’s shifts (85 – 110 nm). Water soluble analogs are planned. The absorption and emission bandwithds of each constituent of each dyad exhibited very narrow spectral widths (< 20 nm). This feature should enable efficient multicolor imaging applications [16].

Hammer and co-workers have solved many long-standing classical challenges involving the difficult synthesis and purification of discreet phthalocyanine isomers by employing innovative solid-phase synthesis methods [17]. Their asymmetrically-substituted phthalocyanines and their oligonucleotide conjugates have found immediate utility in PCR applications [18] and as molecular beacons [19].

Vicente has synthesized and evaluated cationic water soluble Zn and Si pyridyloxy phthalocyanines [20]. Amphiphilic Si phthalocyanines localized preferentially within the lysosomes and exhibited high phototoxicity towards human Hep2 cells with an IC50 of 2.2 μM at a 1 J cm^{-2} light dose.

Rurak and colleagues have designed an expanded porphyrin based on a rubyrin core functionalized with sulfur atoms and polycylic aromatic units fused to the pyrrole rings. This hexaphyrin demonstrated preferable detection of Hg^{2+} over other metal cations [21]. The extended conjugation resulted in exceptional bathochromic shifts and good molar absorptivity at common laser lines from 322–780 nm.

Furota and Osuka synthesized triangular shaped triply-*N*-confused expanded porphyrins. One of these (**6**) displays a broad band at 475 nm in dichloromethane, Q-bands at 835, 947 and 1087 nm, and changes the color upon protonation from blue to green. A study of how related shape changes influence fundamental properties is ongoing [22*].

A benzotexaphyrin with an extensively delocalized π-electron system was synthesized and demonstrated a distinctive red-shifted Q-like absorption band in the near-IR region (λ_{max} 730 nm and λ_{ex} 825 nm). It possesses low triplet excited-state energy, high triplet quantum yield and efficient singlet oxygen generation. The synthesis of derivatives suitable for bioimaging studies is planned [23].

Expanded pentafluorphenyl porphyrins were prepared by the condensation of meso-pentafluorofipyrromethane and pentafluorobenzaldehyde. Two of the compounds obtained exhibited emission bands at 939 and 953 nm [24]. The acid-catalyzed condensation of sterically hindered 3,5-bis(trifluoromethyl)benzaldehyde and pyrrole afforded fluorinated hexa- and heptaphyrins with near-IR optical activity [25]. A near IR fluorescent chemodosimeter for Ag^+ cation based on an expanded fluorine-containing porphyrin was synthesized [26*]. The authors reported intense fluorescence with λ_{ex} = 514 nm and λ_{em} = 1050 nm in MeOH.

Squaraine derivatives

The squaraines are dyes with an electron deficient central four-membered ring and a resonance stabilized zwitterionic structure. The central ring is typically appended with donor moieties to afford a donor-acceptor-donor motif. Suzuki and co-workers [27] introduced multiple water-solubilizing sulfonate moeities into a squaraine framework. A bovine serum albumin (BSA) conjugate containing a squaraine tetrasulfonate exhibited absorption maxima at 787 nm, excitation maxima at 760 nm, emission maxima at 812 nm and a quantum yield of 0.08. The authors envision these dyes to be useful in protein detection, as covalent labeling probes, and as contrast agents for in vivo imaging.

The Smith group has reported recent progress [28,29**] on their pioneering studies of the squaraine rotaxanes [30–32] in which a macrocycle encapsulates the highly electrophilic squaraine thereby shielding it from nucleophilic attack as well as from self-aggregation. In the latest embodiments, highly soluble rotaxane stopper groups were shown to possess excellent stability and solubility in aqueous media [28] and new capping chemistry was developed, allowing for tunable fluorescence properties [29**].

Nucleophilic sulfur generally reacts readily with unprotected squaraines. This property had been previously used to develop chemsodosimeters for biological thiols. Ajayaghosh and co-workers report that they have significantly improved upon previous squaraines used in the detection of cysteine and homocysteine. They developed a ratiometric probe which loses its extended conjugation and NIR fluorescence upon reaction with a thiol while developing a very strongly emissive band in the visible region [33].

Patil et al. have created tetrahydroquinoxaline-based squaraines (e.g., **7**). The rationale for incorporating a quinoxaline was to use its strong electron donor properties to afford enhanced bathochromic shifts. The squaraine quinoxalines were additionally shown to function as effective chemical sensors for copper ions [34].

BODIPY analogs

The BODIPY (borondipyrromethane) dyes typically have relatively shorter wavelength emission maxima and smaller extinction coefficients as compared to the cyanines and other NIR active fluorophores. Suzuki found that by fusing π-excessive aromatic rings to the BODIPY core (e.g., compound **8**) significant increases in quantum yield, higher extinction coefficients, improvements in photostability and NIR optical activity could be achieved [35, 36].

The O'Shea group has synthesized tetraarylazadipyrromethenes which contain aryl groups bonded to the chromophore resulting in emission in the range of 650–789 nm [37]. Judicious

positioning of receptors on the aryl rings has afforded photoinduced electron transfer (PET) or internal charge transfer (ICT) chemosensors [38,39]. The tetraarylazadipyrromethenes exhibit an interesting intramolecular oxygen-fluorine displacement reaction [40] which has recently been used as a key step in a facile covalent immobilization strategy [41].

Benzo[*c*]heterocycles

Swager and co-workers [42**] have recently reported eight unique push-pull-type near-infrared dyes (**9**) that contain isobenzofuran or isothianaphthene moieties. The isobenzofuran fluorophores exhibit red-shifted absorptions relative to the isothianaphthenes. This was attributed to isobenzofuran's relatively greater pro-quinoidal character. These dyes may be of great promise for imaging applications, particularly for the visualization of β-amyloid plaque [43,44]. To date, their photophysical properties have been studied in $CHCl_3$ and MeOH. Strong emission in the NIR and large (>200 nm) Stokes shifts have been achieved.

Xanthenes

The xanthene dyes include some of the most common fluorophores such as fluorescein and rhodamine; however, they are typically not active in the NIR region. A series of seminaphthofluorone xanthene dye regioisomers, synthesized by the authors' group, exhibits dual excitation and emission from fluorescent neutral and anion forms. Systematic alterations to the angle of benzannulation and the placement of ionizable moieties afforded deep-red to NIR emission from the dyes' anionic states. Unusually large Stokes shifts for xanthene dyes (up to ~200 nm in aqueous buffer, compound **10**) was observed. allowing for NIR emission upon excitation in the blue/green wavelength region. These fluorophores embody minimalist structures and are thus potentially highly useful templates for further functionalization and optimization [45].

Summary

Outstanding, rapid progress of great scope has been made in designing new NIR-active probes. These studies promote collaborative efforts from scientists specializing in divergent fields. Despite the major ongoing efforts, significant opportunities remain. For example, despite the widely accepted utility of NIR small molecule probes, apparently the only clinically-approved material to date is indocyanine green (ICG). ICG has a quantum yield of only 0.01 in aqueous solution, and there have been reports of poor stability, rapid clearance form the liver and cytotoxicity [46]. In the near future improved NIR fluorophores will continue to be discovered that address issues such as improved molar absorptivity, spectral bandwidths, quantum yield, Stokes shift, lifetime, photostability, solubility, molecular targeting, systematic clearance, low toxicity, synthesis, purification and ease of functionalization and conjugation.

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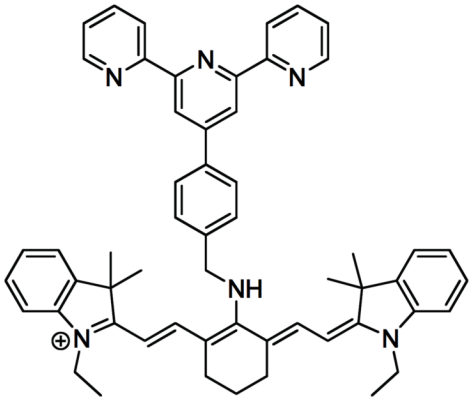

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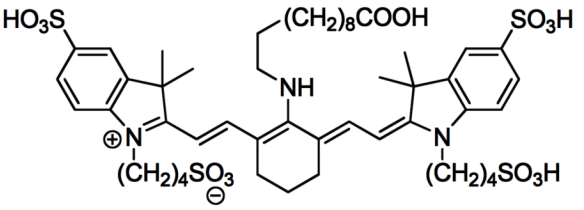

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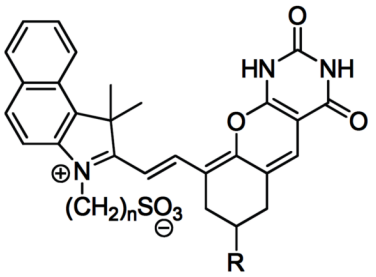

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R = COOH, H

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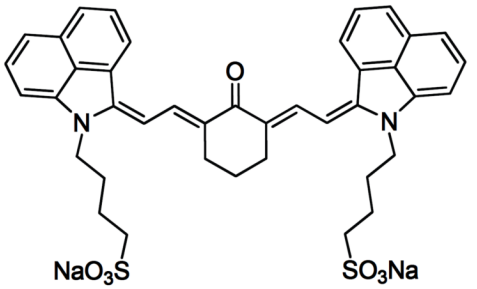

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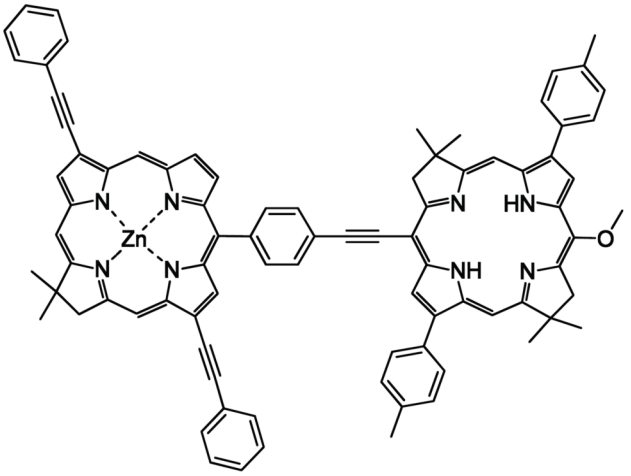

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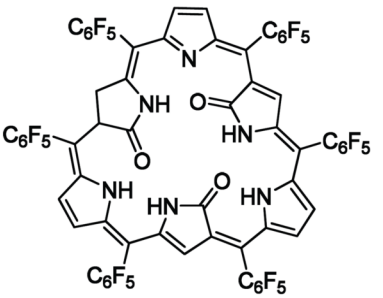

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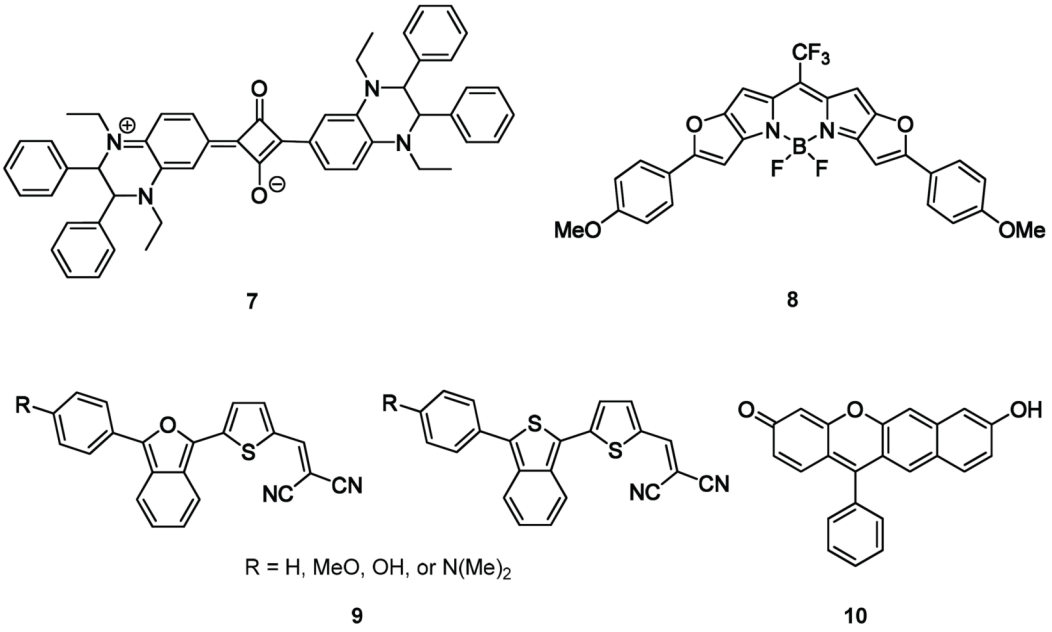

Figure.

Table 1

Summary of features in representative compounds.

Compound	Spectral Properties	Application	Reference
1	Excitation wavelength: 648 nm; emission: 750 nm.	Rapid monitoring of minor pH fluctuations and live cell imaging (HepG2 and HL-7702)	2
2	Stokes shift of 140 nm	Multichannel imaging	3
3	Absorption maxima: 690 nm (neutral pH), 605 nm (acid pH); excitation maxima: 605 nm; emission maxima: 690 nm.	pH monitoring	8
4	Absorption maxima in MeOH, 645 nm (ε = 50800 $M^{-1}cm^{-1}$); Absorption maxima in acidified MeOH (pH < 2), 914 nm (ε= 142000 $M^{-1}cm^{-1}$)	pH monitoring	11
5	Absorption maxima for the chlorin component of the dyad: 650 nm (ε= 60000 $M^{-1}cm^{-1}$); emission maxima: 760 nm. Large Stokes shift (110 nm). Solvent: toluene.	Potential application in NIR imaging upon further modifications to create water-soluble bioconjugatable chlorin-bacteriochlorin (B–C) dyads.	16
6	Absorption maximas: 475, 591. 835, 947 and 1087 nm (free base), 638, 809, 888 and 1016 nm (protonated form); excitation maxima: 600 nm; emission maxima: 1094 nm (free base), 1044 nm (protonated). Solvent: CH2Cl2.	No specific application is mentioned.	22
7	Absorption maxima: 717 nm (ε= 104400 $M^{-1}cm^{-1}$); emission maxima: 774 nm. Solvent: $CHCl_3$.	Affinity for copper	34
8	Absorption maxima: 723 nm (ε= 253000 $M^{-1}cm^{-1}$); emission maxima: 738 nm. Solvent: $CHCl_3$.	High-resolution multicolor bioanalysis and bioimaging.	35,36
9	In the isothianaphthene family, for the compound with $R=N(Me)_2$ the absorption maxima is 579 nm and the emission maxima is 785 nm. Stokes shift = 206 nm	Under testing as NIR contrast agents for biomedical applications.	42
10	Absorption maxima: 536 nm; emission maxima: 733 nm. Stokes shift: 197 nm. Solvent: Aqueous phosphate buffer with 1% DMSO.	Live cell imaging	45