



Fluorescent Probes for Applications in Bioimaging

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Abstract. Optical bioimaging has played a central role in fundamental research and clinical practice. The signals emitted by biological tissues can provide molecular information about various physiological and pathophysiological processes. NIR light (650–1700 nm) can penetrate the blood and biological tissues more profoundly and effectively because, at longer wavelengths, less light is diffused and absorbed. Therefore, many probes have been developed for bioimaging in the NIR window for real-time, high-sensitivity deep tissue imaging. The library of optical probes has been expanded in recent years to include a wide range of probes with emission in the Red-NIR window. The emergence of these new contrast media has provided an essential alternative for realizing the full potential of bioimaging. The most recent advances in small molecule potential probes for detection and imaging in biological systems are examined below.

Keywords: Fluorescence probes · NIR · Optical bioimaging · Small-molecule

1 Introduction

Biological imaging (bioimaging) is a powerful tool in biological research nowadays because it offers a unique approach to visualize the morphological details of cells.

Living organisms include several cells, which in turn, contain a tremendous variety of biomolecules [1]. Very often, in biological systems, a small difference between remarkably similar biomolecules can lead to different functions [2, 3]. For example, cysteine (Cys), homocysteine (Hcy), and N-acetyl-L-cysteine (NAC), three analog amino acids with the only difference of one methylene or acetyl group in their residues, have been shown to play a different role in cellular processes. Some diseases, such as loss of leukocytes and psoriasis, are associated with Cys deficiency, while excess Hcy is a risk for cardiovascular disease and Alzheimer's. As a result, it is of crucial importance to probe Cys, Hcy, and NAC separately. However, their structural similarity makes it difficult to discriminate one from the other [4]. To this end, several

technologies have been developed over the years to study the interactions of these molecules within the cells. The technologies used must respond to several conditions: they must be sensitive enough to visualize biological compounds and physiological concentrations (usually from nanomolar to micromolar), they must have a sufficient spatial-temporal resolution to analyze dynamic cell processes, not invasive and above all not expensive. Among the technologies used over the years, radioisotope labeling, magnetic resonance imaging, electrochemical detection and fluorescence bioimaging, fluorescence-based technique has proven to be the most powerful as it allows a highly sensitive, convenient, non-invasive and safe detection [3, 5]. This technique requires the use of “fluorescent probes” that, reacting specifically with biological molecules, present changes in their photochemical properties (fluorescence intensity, excitation, and emission wavelength). In the last years, there has been an increasing development of new probes, and some of them are already used in *in vivo* imaging as probes based on fluorescent proteins, inorganic nanoparticles and conjugated polymers [6–9].

The basic principle of fluorescence imaging is similar to that used in fluorescence microscopy techniques, which is based on the spectral range of visible light between 400–650 nm. However, when we consider biological tissue imaging, we must consider that microscopic components such as sugars, amino acids, nucleotides, and macromolecules such as proteins, phospholipids, and polysaccharides can absorb light and can limit light penetration to a few hundred microns in the tissue. Also, the autofluorescence of some of these molecules is located in the visible area. Higher depths can be achieved by using light in the far-red or near-infrared (NIR) range (660–1700 nm). In this spectral region, the absorption by the cellular components is lower and provides penetration through several centimeters of tissue [10]. Therefore, bio-imaging in the window of the red/NIR required the development of a considerable number of NIR fluorescent probes, such as those based on small organic molecules, designed to detect various biologically essential species, including ROS/RNS, metal ions, anions, enzymes and other related species, as well as intracellular pH variations [11, 12].

Typically, fluorescent probes are exploited to label the target with specific chemical structures and thus to generate fluorescent signals during the fluorescence-based bioimaging. Also, nanostructure-based detection platforms can provide many advantages over traditional approaches in terms of sensitivity, signal stability, and multiplexing capability so that growing interest has been shown recently in the design of different fluorescent nanostructured probes for bioimaging. The emergence of these new contrast agents has provided a valuable alternative to realize the full potential of NIR bioimaging.

The optical properties of probes can be modulated through structural modifications, for example, the absorption and emission wavelengths of small-molecule probes can be extended from the visible-light region to the NIR region by enhancing intramolecular charge transfer and expanding the π - conjugated system. This allows the design of probes that present significant changes in their spectroscopic properties as a result of interaction with specific biomolecules or even that can be anchored to specific sites based on modifications with target groups [13, 14]. In the last decade, a variety of probes have been developed, exploiting one of the mechanisms of modulation of the fluorescence properties listed below: Photoinduced electron Transfer (PET), Förster Resonance Energy Transfer (FRET), Intramolecular Charge Transfer (ICT) [9, 15–21].

This flexibility enables the achievement of long-term and in situ bioimaging and raises the possibility of generating small-molecule NIR probes for deep-tissue and high-contrast bioimaging.

In this mini-review, we will focus on recent advances in small-molecule probes in selected categories based on fluorescent nuclei. For each category, we will see how, depending on small changes in chemical structure, probe applications may vary from metal ion recognition to pH changes.

Finally, in the last paragraphs, a selection of active molecules in the NIR II region is reported, and some promising solid-phase fluorescent probes.

2 Organic Small Molecule Fluorescent Probes

To reach red emission, dye molecules generally have large planar rings with extended conjugation or strong π -conjugated electron-donating and accepting groups. Although these red fluorophores are strongly emissive when dissolved in solution, the emission signals are often weakened or even annihilated in aggregates, due to what is known as the aggregation-caused quenching (ACQ) effect. Conventional organic dyes tend to aggregate, which is highly dependent on their intrinsic hydrophobicity, and the excited state often decays via non-radiative channels, which reduces the brightness and sensitivity in biological applications. Many chemical and physical approaches have been employed to prevent fluorophore aggregation, but they have met with only limited success.

Interestingly, some propeller-like organic dyes show unique fluorescence phenomena with aggregation-induced emission (AIE) characteristics. These fluorogens generally contain rotor structures and exhibit low-frequency vibration motions in dilute solutions. However, in aggregated states, instead of quenching, AIEgens emit efficiently due to the restriction of intramolecular rotations (RIR) and the lack of energy dissipation via non-radiative channels. Since the first discoveries by Oelkrug and Hanack in 1998 [22] and then by Tang and coworkers in 2001 [23], luminescent materials with AIE features have attracted extensive research interest for applications such as electroluminescent devices, bio-sensing, and cell imaging. Until now, a wide variety of AIE dyes have been designed and synthesized based on the mechanism of RIR. Even so, synthesizing AIE-active probes with better biocompatibility, biodegradability, and red/near-infrared emission is essential for bioimaging applications.

Among traditional probes, cyanines, fluorescein, rhodamine, coumarins, and BODIPY (basic nuclei reported in Fig. 1) have shown to be the molecules most subject to structural modifications because they have favorable optical properties, a high absorption coefficient, biocompatibility, and low toxicity and are a valid choice for biomedical applications.

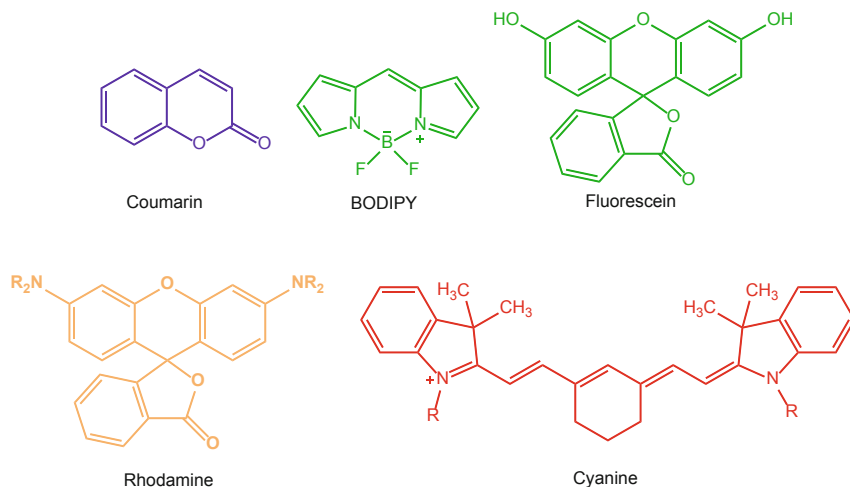


Fig. 1. A representative set of conventional dyes that can be transformed into fluorescent probes for bioimaging, sorted by structure and emission color. [2]

2.1 Cyanine-Derivates Probes

The development of NIR fluorescent probes for the study of metal ions has had a great focus on the role that ions have in biological environments [24].

In this context, probes based on modified cyanines are widely used for the detection of metal ions and [25–27]. For example, the introduction of a sulfur-based receptor, reported by Tasuku Hirayama et al. [28], shows selectivity for the Cu⁺ ion in living cells. Copper is an essential element, and the maintenance of its correct homeostasis is essential for the growth and development of living organisms; consequently, the loss of copper homeostasis has severe consequences due to its redox activity. This fact results in diseases such as cancer, cardiovascular disease or Alzheimer's. The probe, Copper sensor CS790AM (Fig. 2), combines an infrared cyanine dye with a sulfur-rich receptor to provide a selective response to copper. The probe can detect increases in the level of copper in the case of Wilson's disease, a genetic disease characterized by the accumulation of copper. The probe also has no toxicity and is eliminated in a few days.

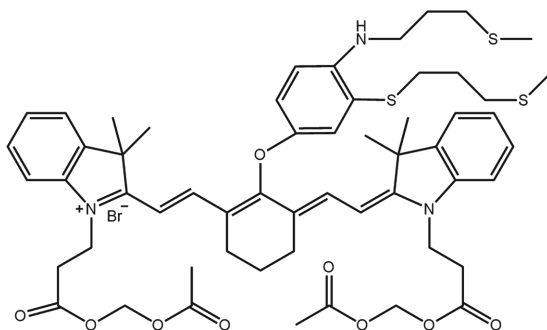


Fig. 2. Chemical structure of CS790AM copper-selective probe [28].

Similarly, selective probes have been designed for other ions such as Cu^{2+} , Ca^{2+} , Li^+ , Zn^{2+} , Hg^{2+} , Cd^{2+} , and Ag^+ , combining cyanine fluorophore with a selective metal ion receptor [27, 29, 30]. Tang group [31] developed the probe Cy-Cu, reported in Fig. 3, composed of tricarbocyanine (Cy) and a receptor for the Cu^{2+} ion, 2,2'-azanediyil bis(N-hydroxyacetamide). The probe is based on the photoinduced electronic transfer mechanism. When the Cu^{2+} ion is coordinated and binds to the receptor, PET is blocked, and an increase in fluorescence is observed (Fig. 3).

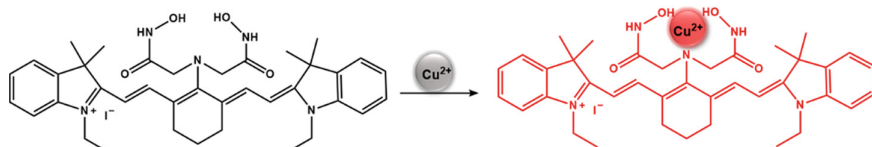


Fig. 3. Proposed binding mechanism of the probe Cy-Cu to Cu^{2+} [31].

This was the first NIR imaging probe for Cu^{2+} *in vitro* and *in vivo*. Fluorophores 2 and 3 show selectivity for Cd^{2+} by coordination of the tetramide receptor with the ion [32]. Alternatively, the incorporation of adenine as a substitute for the fluorescent nuclei of cyanine, is an active linker for Ag^+ (probe 4 in Fig. 4) [33].

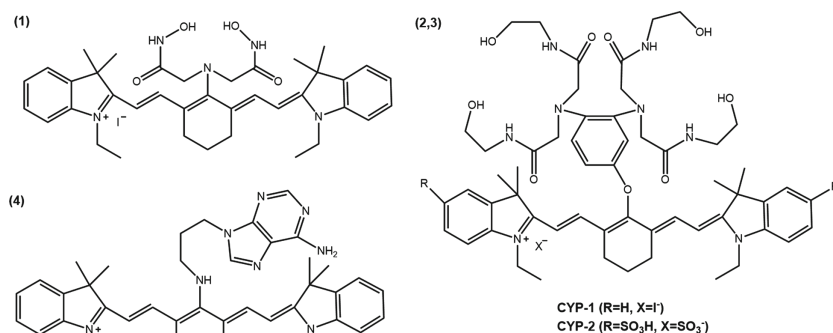


Fig. 4. Chemical structure of some cyanine probes selective for 1) Cu^{2+} [31]; 2,3) Cd^{2+} [32] e 4) Ag^+ [33].

Many other cyanine-based probes have been developed to detect intracellular pH variation [34–36]. Intracellular pH changes lead to abnormal cell growth and division, resulting in inflammation or disease such as cancer or Alzheimer's disease. The pH-sensitive cyanine-derivate probes can be classified into two types: probes characterized by non-N-alkylated indolic group whose protonation or deprotonation depending on the protonic concentration; the second type of pH-sensitive probes are the tricarbocyanines that are based on the classical mechanisms ICT and FRET [37, 38]. In the first type of cyanine probes, the non-N-alkylated indole in the acid environment can be

protonated inducing a strong fluorescence, in basic environment on the contrary, the deprotonation of the nitrogen atoms of the indole groups shows no fluorescence. Nagano and co-workers have designed several pH-sensitive probes based on tricarboanines that have a diamino group. These probes have shown high stability under acidic conditions and a drastic shift in absorption towards red when the nitrogen is protonated. This behavior is due to the decrease of the electron-donor nature of the amine [39].

Some pH-sensitive tricarboanicyanine probes structures are shown in Fig. 5.

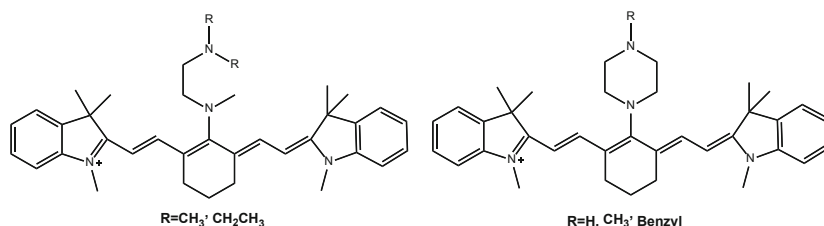


Fig. 5. Chemical structure of pH-sensitive tricarboanicyanine probes [39].

2.2 Coumarin-Derivates Probes

Coumarin (2H-cromen-2-one) is a chemical compound of the benzopyrone class found in many natural species. Coumarin has a variety of biological activities and unique photophysical properties; among these, the fluorescent property has recently received attention because of its high quantum yield, high stability and biological compatibility [40–42]. Derivatives of benzocoumarin are classified based on the position of the aromatic ring fused to the nuclei, as shown in Fig. 6.

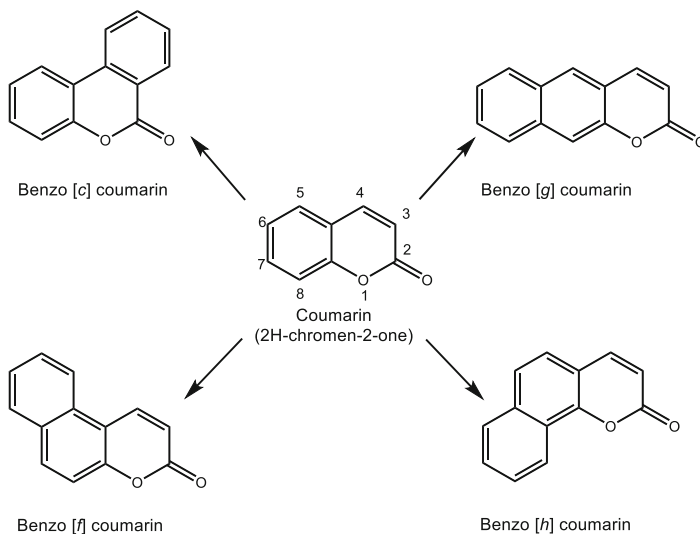


Fig. 6. Chemical structure of coumarin and benzo-derivates compounds [40–42].

Among the derivatives, benzo[g]coumarin exhibits better photophysical properties in bioimaging applications than other derivatives. Besides, a high two-photon absorption capacity, high photostability, and high chemical stability are the beneficial characteristics of benzo[g]coumarin derivatives. The photophysical properties of the analogs of benzocoumarin can also be predicted depending on the type and position of the substituents: it has been observed that the combination of electron-withdrawing and electron-donating substituents causes a significant shift of both absorption and emission spectra at longer wavelengths (red and NIR region) [42]. Cho et al. [43] reported a benzo[g]coumarin-derivative fluorescent probe for Cu^{2+} and quantitatively estimated ion concentrations in human tissues by two-photon microscopic imaging (Fig. 7). The probe, amide- and dimethylamino-substituted, to which is linked a benzo[h]coumarin analog as internal reference (sensitive to the environment or to the substrates, which maintains constant the fluorescence intensity) presents a decrease of the fluorescence intensity in the red, following chelation of the copper ion. It was thus possible to study the concentration of Cu^{2+} ions in live cells, rat brain tissue and human colon tissue samples using two-photon microscopy.

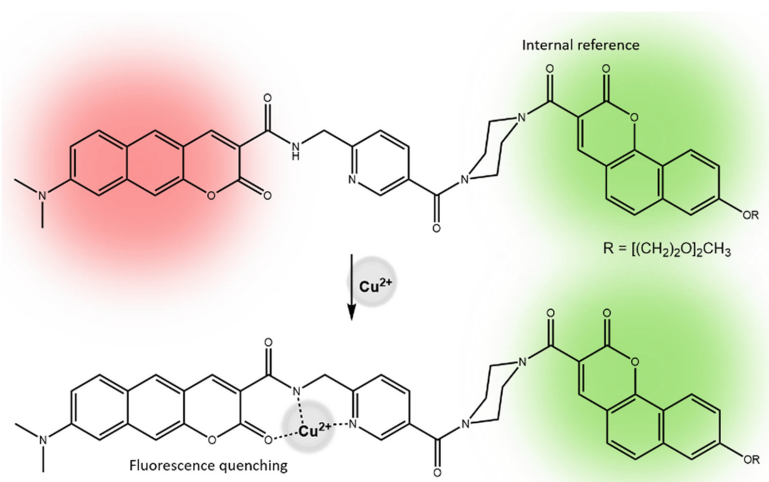


Fig. 7. Benzo[g]coumarin- and benzo[h]coumarin-based fluorescent probe for Cu^{2+} ions [43]

In 2014, a derivative of benzo[g]coumarin was reported by Kim et al. [44] for monitoring mitochondria, the malfunction of which could be related to many diseases (Fig. 8). The probe had a mitochondrial attack site consisting of the triphenylphosphonium salt bound to benzo[g]coumarin through an amide bond. The resulting probe showed maximum emission in red, without pH-sensitive variations. Mitochondria tracking ability has been verified on the T98G cell line with MitoTracker Green (MTG) as a known mitochondrial marker.

Subsequently, structural modifications resulted in a new probe that was sensitive to pH changes, through the introduction of an electron-donor hydroxyl group that can be protonated or deprotonated to pK_a near 8.0 (mitochondria pH). The shift of the absorption and emission peaks as a function of the pH variation indicates the capacity of the selective imaging probe for mitochondria [42, 45].

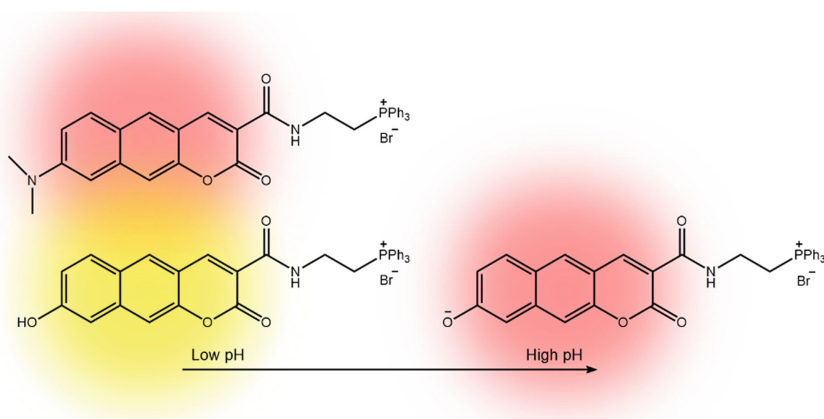


Fig. 8 Triphenylphosphonium salt-linked Benzo[g]coumarin probe for mitochondria tracking and for measuring mitochondrial pH values [44].

2.3 BODIPY-Derivates Probes

The family of 4,4-difluoro, 4-bora-3a, 4a-diaza-s-indacene (called BODIPY) is widely used among the various fluorescent dyes. These probes have excellent properties for biological applications: high fluorescence quantum yield, excellent photostability, high solubility. As for cyanine derivatives, also this class of probe is used in bio-imaging because they have red emission [46]. In particular, one of the uses of BODIPY derivatives concerns the recognition of metal ions in cells such as Cd^{2+} , Ca^{2+} , Hg^{2+} , and Zn^{2+} [47, 48].

Akkaya and co-workers have developed a fluorescent NIR probe for Hg^{2+} , the bis(2-pyridyl)-replaced boratriazaindacene probe (AzaBODIPY) and found that the 2-pyridyl substituents create a metal ion pocket due to the rigid nature of the ligand and the donor's selectivity [49]. At a sufficiently high concentration of Hg^{2+} , there is a shift at higher wavelengths in the absorption and emission spectra (Fig. 9).

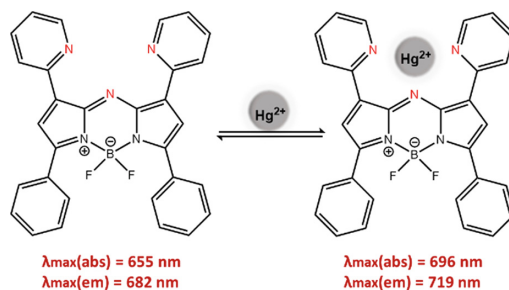


Fig. 9 Coordination mechanism of the Hg^{2+} to the AzaBODIPY probe [49].

An interesting pH-sensitive probe based on BODIPY has been developed by O'Shea et al. [50]. In the probe, two amine substituents act as pH modulators leading to three-channel fluorescence (Fig. 10). Depending on whether the molecule was mono or di-protonate, a color change from red to purple, up to blue corresponding to the three forms: the non-protonate, mono-protonate, and di-protonate form in response to pH-induced change in the ICT (intramolecular charge transfer) properties of the system is observed.

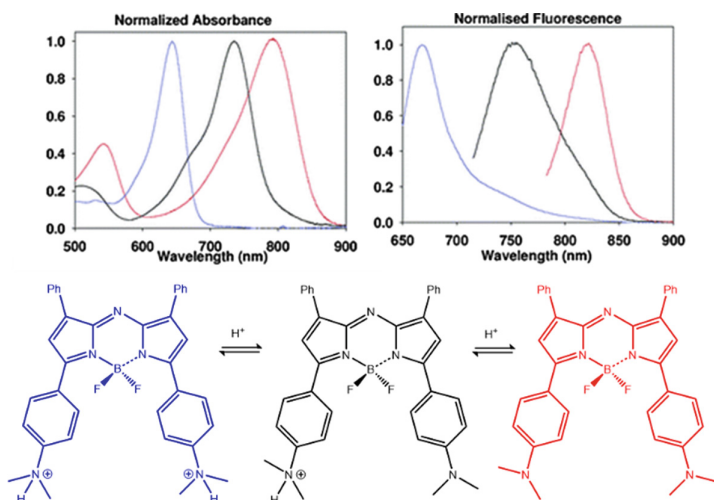


Fig. 10 Protonation and deprotonation of the pH-sensitive probe and related emission spectra [50].

As mentioned above, very often structurally similar biomolecules can have different functions, as in the case of cysteine, homocysteine, and N-acetyl-L-cysteine [4]. It is therefore essential to design probes that are selective for these biomolecules. Ravikanth et al. [51] have developed a red fluorescent probe for the specific detection of cysteine (Cys) and homocysteine (Hcy) in living cells based on BODIPY 3,5-bis(acrylaldehyde)

(see Fig. 11). The probe, following cyclization of the aldehyde group with thiol, shows the formation of a hexaindro-1,4-thiazepine derivative, which results in a change in fluorescence properties (cells pretreated with Cys followed by incubation with the probe show bright yellow-green intracellular fluorescence).

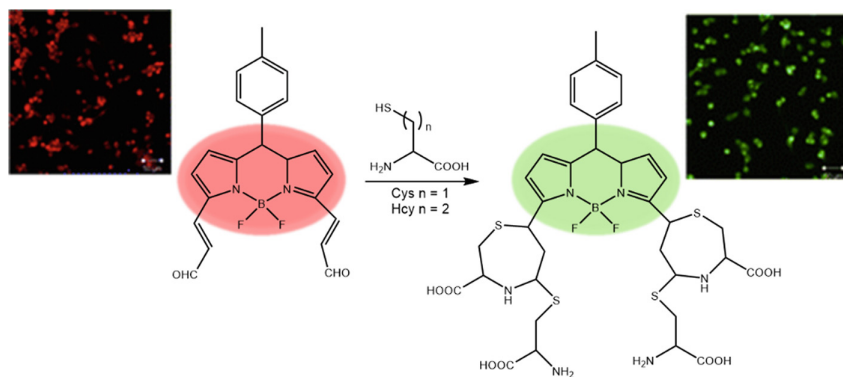


Fig. 11 The schematic reaction of BODIPY based probe with Cys or Hcy [51]

However, the NIR-I window (650–900 nm), in which almost all the above probes emissions fall, can present the problem of tissue autofluorescence, producing a considerable background noise [52, 53].

3 Fluorescent Probes in the NIR-II Region

Recently, new fluorescent probes have been attempted to operate at longer wavelengths, particularly in the NIR-II window (1000–1700 nm), in order to offer better penetration and overcome autofluorescence problems [54]. They meet these probes requirements based on Donor-Acceptor-Donor (D-A-D) systems that show emissions in the NIR-II zone as widely reported in the literature [55, 56]. In the work of L. Antaris et al. [17] a new probe based on the D-A-D system with bis-thiadiazole nuclei, called CH1055, is described. The reported probe has been modified with carboxylic acid groups to improve the solubility of the molecule; the carboxylic acid groups can be subjected to PEGylation to increase their solubility further, or even be conjugated to target ligands (Fig. 12).

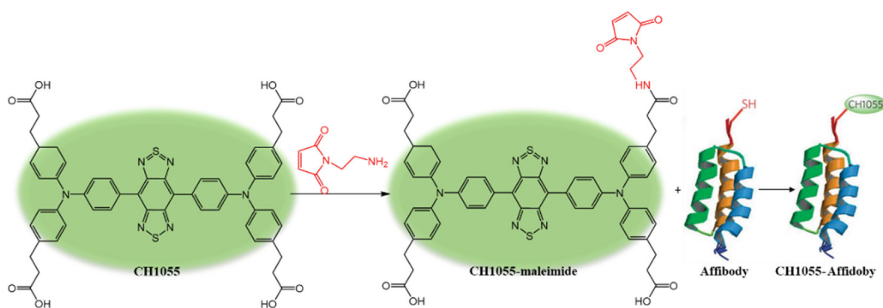


Fig. 12 A simplified reaction schematic illustrating the synthesis of the CH1055-a-body [17].

The CH1055-PEG probe has proven effective in mapping sentinel lymph nodes (SLN) and identifying brain tumors *in vivo*. It was noted that after intradermal injections of the CH1055-PEG probe used for imaging lymphatic vessels and lymph nodes, strong tumor fluorescence was observed, starting 57 h after injection.

The conjugation of the fluorescent molecule to a small protein such as anti-EGFR affibody (EGFR: receptor of epidermal growth factor) allows rapid and economical detection of early cancers and therefore represents a valuable aid for the removal of the imaging-guided tumor.

The probe showed rapid renal excretion (about 90% in 24 h) compared to other NIR-II probes, and PEGylation has become an accepted practice in the pharmaceutical industry as it offers many advantages such as longer probe circulation time, higher stability and protection against degradation. The probe also allowed the first guided NIR-II export surgery, simultaneously displaying the tumor excision under white light and the NIR-II fluorescence system.

4 Solid-State Fluorescent Probes

In the context of organic-core chromophores with emission properties in the near-infrared region, fluorophores using the effect of aggregation-induced emission have recently been of interest for their multiple applications such as dopants in the fabrication of OLED devices, in optical probes for bioimaging, and biomedical applications [57]. Unlike classic NIR fluorophores, which tend to form aggregates in highly concentrated solutions or solid-state, resulting in quenching (ACQ effect) of emission properties due to strong dipole-dipole interactions, these new types of fluorophores exploit the effect of emission induced by AIE aggregation. These molecules show weak emissions in solutions and strong emission in the aggregated state, high photoluminescence quantum efficiency, significant Stokes shifts (for eliminating self-absorption), excellent chemical and thermal stability, good solubility and processability [22, 58, 59]. In literature, there is a limited number of organic molecules with solid-state fluorescence.

Our research group has conducted several studies on the solid-state emission properties of chromophores, such as those of Phenylenevinylene (PV) derivatives based on a donor-acceptor system. In addition, the introduction of an electron-withdrawing group such as the cyan group leads to an increase in the energy of the

π -occupied and π^* -unoccupied states and causes a red shift in emission maxima compared to non-substituted phenylenevinylene systems [60–62] (see Fig. 13).

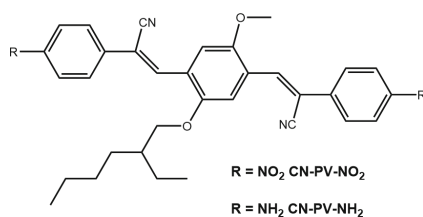


Fig. 13 Chemical structures of PV-based dyes [60]

All compounds show intense red photoluminescence with a pronounced shift of Stoke larger in the solid state than in solution.

This can be attributed to the presence of the cyano substitute that limits molecular movements and stabilizes the twisted conformation of the solid. This conformation may lead to intense aggregation-induced emission effect (AIE). Based on the above compounds, to improve photophysical performance, recently the same research group has synthesized new symmetric dyes with a modified skeleton phenylenevinylene (PV) and azobenzene (AB) adding groups such as N-N'-bis (salicylidene)ethylenediamine (salen) that would reduce molecular movements and improve their solid-state properties [63]. To this should be added the ESIPT process to which salen substitutes are subjected, which could potentially still improve the performance of the dyes. In the area of solid-state fluorescence, the same research group reported a study on the regulation of solid-state fluorescence properties of polymorphic compounds. In fact, polymorphism (different arrangements and conformations of the same molecule) can be used to produce materials with emissive properties in which thermal or mechanical stimuli can regulate the transition. The work [58] reports a derivative of N-salicylidene aniline containing the 2-methylbenzotriazole (BTz) showing solid-state ESIPT fluorescence, as shown in Fig. 14.

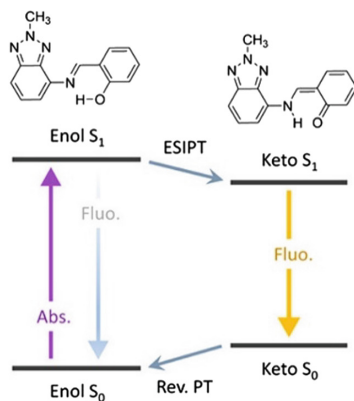


Fig. 14 ESIPT process in 2-methylbenzotriazole (BTz) chromophores [58].

The compound shows three phases: yellow (called 1-Y), orange (1-O), and red (1-R). The transformation from one shape to another is accompanied by a significant change in fluorescence intensity and color, as shown in Fig. 15.

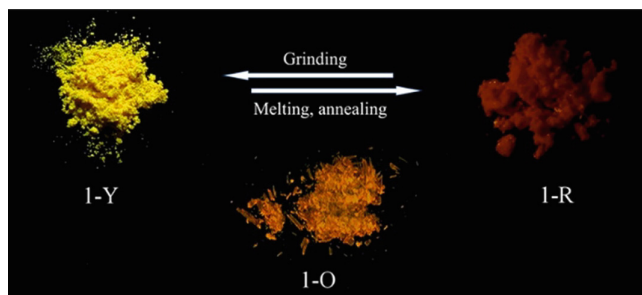


Fig. 15 Photograph of 1-Y, 1-O, and 1-R powders under a UV lamp and scheme of mechano-responsive behavior [58].

The 1-R form can be converted to 1-Y by grinding and returned to 1-R by melting and annealing, resulting in mechanical-responsive luminescence. The different photo-physical properties have been explained by changes in molecular conformation in the three polymorphs. These findings offer an advantageous possibility to control the emission fluorescence for stimuli-response applications. The unique photochemical properties of these compounds make them potentially suitable for their encapsulation in nanoparticles (NPs) and use in bio-imaging. In bioimaging applications, AIE fluorophores can be immune to the limitations arising from the concentration of the dyes loaded into the nanoparticles (NPs), and AIE NPs are supposed to be more emissive, as well as more resistant to photobleaching [64].

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