CHAPTER THREE

Rational Design of Fluorophores for In Vivo Applications

Marcin Ptaszek
Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, Maryland, USA

Contents

1. Introduction 60
2. Cyanine and Related Fluorophores 62
   2.1 General characterization 62
   2.2 Bioconjugation 63
   2.3 Improvement of water solubility and chemical stability 67
3. Squaraines 71
4. Boron Dipyrrromethene and Related Fluorophores 74
   4.1 General characterization 74
   4.2 Energy-transfer dyads for increasing the Stokes shift of BDPs 77
   4.3 Related fluorophores 78
5. Porphyrins, Phthalocyanines, and Related Macrocycles 79
   5.1 General characterization 79
   5.2 Benzoporphyrins 80
   5.3 Strongly conjugated porphyrin arrays 81
   5.4 Chlorins and bacteriochlorins 81
   5.5 Hydroporphyrin arrays for increased Stokes shift and multicolor in vivo imaging 85
   5.6 Water solubility and aggregation 85
   5.7 Photocytotoxicity of tetrapyrrolic macrocycle 87
   5.8 Phthalocyanines 88
6. Special Types of Fluorophores 90
   6.1 Fluorophores for multicolor imaging 90
   6.2 Self-illuminating fluorophores 92
7. Conclusion 94
Acknowledgment 94
References 95

Abstract

Several classes of small organic molecules exhibit properties that make them suitable for fluorescence in vivo imaging. The most promising candidates are cyanines, squaraines, boron dipyrrromethenes, porphyrin derivatives, hydroporphyrins, and phthalocyanines.
The recent designing and synthetic efforts have been dedicated to improving their optical properties (shift the absorption and emission maxima toward longer wavelengths and increase the brightness) as well as increasing their stability and water solubility. The most notable advances include development of encapsulated cyanine dyes with increased stability and water solubility, squaraine rotaxanes with increased stability, long-wavelength-absorbing boron dipyrromethenes, long-wavelength-absorbing porphyrin and hydrophorphyrin derivatives, and water-soluble phthalocyanines. Recent advances in luminescence and bioluminescence have made self-illuminating fluorophores available for in vivo applications. Development of new types of hydrophosphyrin energy-transfer dyads gives the promise for further advances in in vivo multicolor imaging.

1. INTRODUCTION

Fluorescence spectroscopy, which has been proved to be an extremely powerful tool in analytical chemistry and cell biology,\(^1,2\) has reached the stage when it can be used to visualize biological processes and detect biologically relevant species in tissues and in whole living animals. It opens a particularly fascinating opportunity to noninvasively diagnose disease stages at the molecular level, which can revolutionize medicinal diagnosis. The progress in applications of fluorescence spectroscopy for in vivo imaging relies on both advances in excitation and detection technologies and development of molecular probes suitable for visualization of molecular processes in tissue or whole body. The fluorescent molecular probes must consist of two components: a reporter (fluorophore), that is, the unit that emits the light upon excitation, and a recognition unit, which can selectively recognize the given molecular process or species of interest and translate the recognition event in a well-defined manner into changes of the fluorescence properties of a reporter.\(^1,2\) Thus, fluorophore as a reporter is a centerpiece of every fluorescence molecular probe. Fluorophores for in vivo applications must fulfill a set of requirements as for their optical, chemical, and biological properties. The most critical properties are summarized below.\(^3–5\)

For in vivo applications, fluorophores must absorb and emit in the red and near-infrared (near-IR) spectral window, preferably in the range 650–900 nm. In this window, the tissue absorbance and autofluorescence, as well as light scattering, are diminished, while below 650 nm, tissue and cellular components strongly absorb the light and above 900 nm water absorbs (e.g., see discussion in Ref. 5). Fluorophores should possess high brightness, which is a product of the fluorophore excitation coefficient \(e\)
and the fluorescence quantum yield $\Phi_f$; thus preferably fluorophores should have both high absorbance and fluorescence quantum yield. They should possess a Stokes shift (i.e., spacing between excitation and emission wavelengths) large enough to avoid detection of scattered light from the excitation beam. They must retain their bright fluorescence in the biological milieu, so, ideally, fluorophores should be soluble in water and should not aggregate in aqueous solution, or at least one should be able to formulate them in a form in which they stay fluorescent in the biological environment. It is also important that fluorophores should not interact (or should not change their optical characteristic upon interaction) with biomolecules, particularly serum proteins. High chemotherapeutic and photostability and lack of (photo) toxicity are other important characteristics of fluorophores. In addition, fluorophores should be synthetically available, easy to functionalize, and able to be attached to biomolecules or targeting/recognition units.

For a chemist, development of fluorophores for \textit{in vivo} imaging is a particular challenge, as integration of all required attributes in one molecular framework is highly demanding, if at all possible. Rational design of optimal fluorophores starts from the selection of a molecular platform with suitable optical properties and then requires gaining a deep insight into their structure–property relationship in order to fine-tune their optical characteristics. The next step is usually optimization of their chemical properties, such as water solubility, bioconjugation, attaching targeting or recognition moiety, and necessary chemical modification to improve their performance \textit{in vivo} (such as proper blood circulation, cell permeability, accumulation in the target tissue, etc., depending on the desired application). The design of efficient methods for synthesis and chemical modification of the target systems is also a key part of fluorophore development. The lack of robust synthetic methods is often the limiting factor in determining the structure–property relationship and optimizing the properties of certain fluorophores. The whole process of fluorophore development requires deep insight into the electronic structure of the given molecules, robust synthetic methods for their preparation and functionalization, and understanding the biological processes that fluorophores undergo \textit{in vivo}, thus requiring expertise from various fields such as physics, chemistry, biochemistry, and biology.

There are several classes of fluorophores that have already been used or can be potentially applied, \textit{in vivo}, including small organic and inorganic molecules, fluorescent proteins, conjugated polymers, and inorganic nanoparticles. There are excellent up-to-date reviews covering fluorophores for biological applications,\cite{6,7} fluorescent molecular probes for biomedical
applications, \(^5\) fluorophores for medical \textit{in vivo} imaging, \(^3,4,8–11\) near-IR fluorophores, \(^12,13\) and fluorophores for labeling of biomolecules. \(^14\) This chapter is particularly focused on the recent progress in the small organic fluorophores for \textit{in vivo} imaging and specifically highlights the chemical approaches to achieve the properties of optimal fluorophores listed above. \textit{In vivo} application is defined here as an application in whole tissue or whole body, rather than in a single cell.

Several classes of organic fluorophores are discussed here, those being commonly used for \textit{in vivo} imaging (such as cyanines, squaraines) as well as the less commonly used ones but showing properties making them promising candidates for future use (such as boron dipyrromethenes (BDP) and tetrapyrrolic macrocycles). Finally, two special classes of fluorophores of growing importance for \textit{in vivo} applications are discussed: self-illuminating fluorophores and fluorophores for multicolor \textit{in vivo} imaging.

Small organic molecules comprise one of the major classes of fluorophores for \textit{in vivo} applications. They offer virtually unlimited diversity of structures, have a broad range of methods for modification of their physicochemical properties, and are relatively easily available, inexpensive, and usually nontoxic. \(^6\) On the other hand, small organic fluorophores suffer from serious imperfection of optical properties, such as low quantum yields (especially for near-IR fluorophores), moderate excitation coefficients, and often moderate photostability. \(^6\) For these reasons, intensive research effort has been made to find alternative fluorophores with improved optical properties and stability, which has led to the development of other classes of materials suitable for \textit{in vivo} imaging. These classes of fluorophores already investigated for \textit{in vivo} applications, such as near-IR fluorescent proteins, \(^15,16\) fluorescent polymer nanoparticles, \(^17\) quantum dots, \(^18–20\) carbon dots, \(^21\) dye-doped nanoparticles, \(^22\) and carbon nanotubes, \(^23,24\) as well as commercially available near-IR organic fluorophores, with unrevealed structures (such as Alexa Flours \(^25\)), are not discussed here. Readers interested in these topics are referred to the reviews and original articles listed above. This review covers the literature published approximately till the end of 2011.

2. CYANINE AND RELATED FLUOROPHORES

2.1. General characterization

Carbocyanine dyes (cyanines) remain the most prevalent fluorophores used for \textit{in vivo} imaging. The cyanine derivative indocyanine green (ICG, IR–125) is the only near-IR fluorophore approved by the Food and Drug
Administration for clinical use and is considered as a “gold standard” for fluorophores for in vivo applications.

Cyanines are formally compounds with two nitrogen atoms linked by an odd number of methene units. The nitrogen atoms are parts of the heterocyclic units (such as indole, benzoxazol, or benzothiazol). The structures and optical properties of representative cyanine dyes used for in vivo imaging are presented in Chart 3.1. Cyanines are characterized by long wavelength, tunable absorption and emission, very high extinction coefficient (up to 300,000 M⁻¹ cm⁻¹), good water solubility, and relatively straightforward synthesis. The wavelengths of absorption and emission in cyanines can be tuned and shifted toward longer wavelengths either by changing the number of carbon atoms in the polymethine chain or by expanding the aromatic part of the terminal heterocyclic units. The increase of polymethine chain by two carbon atoms shifts bathochromically the absorption band by ~100 nm, whereas fusing the benzo ring at the terminal indole moiety shifts the absorption band by about 30 nm (see data in Chart 3.1, as well as Refs. 29 and 30). The long-wavelength absorbing and emitting cyanine fluorophores suitable for in vivo applications are (a) pentamethine cyanines with an additional benzene ring fused to the terminal indole moieties (e.g., Cy5.5, Chart 3.1), with absorption and emission at ~675/695 nm; (b) heptamethine cyanines, with an indole terminal moiety, absorbing/emitting at 750–790 and 780–820 nm, respectively (e.g., Cy7, cybate, and NIR-820); and (c) heptamethine cyanines with benzoindole as terminal moieties absorbing/emitting around 780–822 and 810–847 nm (e.g., ICG, cypate, and CyTE-822). The main design and synthetic efforts have been recently dedicated to preparing derivatives suitable for further modifications (e.g., mono- and polyvalent bioconjugatable cyanines) and methods to improve water solubility and chemical and photochemical stability of cyanines.

2.2. Bioconjugation

In vivo application of any fluorophores often requires attaching a targeting group, or conjugation to biomolecules (such as antibodies), which ensures selective localization of fluorophores in the target cells, tissues, or organs. Therefore, availability of fluorophore derivatives with reactive functional groups that are suitable for bioconjugation is an important issue in designing new fluorophores. Bioconjugation of any molecule is usually achieved by attaching an amino-reactive N-hydroxysuccinimide ester (formed by
Chart 3.1 Structures and optical properties of representative red and near-IR emitting cyanines.
derivatization of carboxylic group), a thiol-reactive iodoacetyl substituent, or a thiol-reactive maleimide group. The available synthetic routes allow the introduction of a variety of substituents at both nitrogen atoms and the aromatic parts of terminal indole moieties (Fig. 3.1). The bioconjugatable polyvalent cyanine derivatives have been prepared by the introduction of suitable reactive groups as substituents either at the nitrogen atoms (R and R\textsuperscript{1} groups) or on the aromatic indole moiety (X and X\textsuperscript{1}, Fig. 3.1). Bioconjugatable Cy5.5\textsuperscript{29,30} and Cy7\textsuperscript{29} having hexanoic acid substituents (terminated by carboxylic acid group) at both indole nitrogen atoms have been prepared by Waggoner and coworkers. The polyvalent analog of ICG with propionic acid at both benzoindole nitrogen atoms (cypate),\textsuperscript{35,37,39,40} as well as the

![Figure 3.1](image_url) **Figure 3.1** Synthesis of symmetrical and nonsymmetrical cyanines (see text for references).
analogous derivatives of Cy7 (cybate), have been prepared and characterized by Achilefu and coworkers. The carboxylate groups in cypate have been subsequently used for attachment of a variety of functional moieties, such as polycarboxylic acids, glucosamine, peptides, and poly(ethylene oxide) dendrimers. Cyanine derivatives containing carboxylate groups at the benzo ring of each terminal indole moiety have been prepared by Licha and Tung (compound NIR-820, Chart 3.1) and used for attaching glucosaminid and transferrin, respectively.

The polyvalent symmetrical derivatives described above are equipped with two identical carboxylic groups. These groups can be selectively functionalized, so that only one is activated and derivatized; however, having two identical carboxylic groups complicates the derivatization and purification procedure because of the formation of the bis-derivatized ester as a side product. Therefore, monovalent derivatives (i.e., derivatives with one bioconjugatable group) have been developed. One strategy for the preparation of the monovalent cyanine derivatives entails the preparation of nonsymmetrical derivatives, where the functional derivatizable group is located only on the one indole moiety (either as a substituent on indole nitrogen or on the benzo ring, Fig. 3.1). A series of nonsymmetrical carboxylate derivatives of Cy5.5, Cy7, and benzoheptamethine cyanines have been prepared and conjugated with proteins, peptides, poly(ethylene) glycols, sugars, fluorescence quenchers, and other targeting agents. Tung and coworkers prepared the nonsymmetrical monovalent carboxylate-substituted hybrid cyanine with benzoindole moiety on one side and carboxylate-substituted indole on the other side of the heptamethine chain. This derivative has been subsequently conjugated to the PEGylated graft polymer (to make enzyme-activatable fluorescent probe) or to the folate residue (to prepare folate receptor expressing cancer cells).

Preparation of nonsymmetrical cyanine derivatives causes purification problems because of the inevitable formation of the corresponding symmetrical side product. Therefore, recently a new strategy for the preparation of monofunctional cyanine fluorophores has been pursued (Fig. 3.2). This strategy relies on the synthesis and derivatization of cycloheptamethine cyanines (Cl-cyclo-Cy) with chlorine atom on the cyclohexene ring. In these new cyanine derivatives, in which part of the polymethine chain is embedded in the cyclohexane ring, the chlorine substituent on the cyclohexane ring can be further derivatized by nucleophilic substitution, using phenolates (to form C—O bond), thiols (to form C—S bond), or amines (to form C—N

Marcin Ptaszek
Alternatively, palladium-catalyzed cross-coupling reaction has been employed to form the more robust C—C bond. Both approaches have been used for preparing a broad range of derivatives, including those equipped with bioconjugatable groups. Nucleophilic substitution of chlorine atom in cycloheptamethine and benzocycloheptamethine cyanines Cl-cyclo-Cy with oxygen, nitrogen, or sulfur nucleophiles has also been widely used for the attachment of groups to improve water solubility, prevent aggregation, and alter the cyanine affinity to albumin, as well as to target or recognize motifs such as peptides, glucosamine, photosensitizers, and zinc dipicolylamine group.

2.3. Improvement of water solubility and chemical stability

One of the main problems with cyanine fluorophores is their chemical and photochemical instability, especially under physiological conditions. Cyanines undergo many complex physicochemical transformations in solution,
which alter their optical properties. Cyanines in solution are prone to photobleaching, oxidation, solvatochromic effects, and nonspecific interactions with plasma proteins (see Refs. 27, 28, and introduction to Ref. 80 and references cited therein). Cyanine itself has a low fluorescence quantum yield because of the competitive internal conversion and photoisomerization.33 In addition, in aqueous solutions, cyanines undergo different types of aggregation,45 and in a biological environment, they nonspecifically interact with biomolecules.28 All of these effects cause diminishing of the fluorescence quantum yield and altering of the maxima of both absorption and emission bands of cyanines in vivo. Hence, much effort has been devoted to improving the chemical and photochemical stability of cyanines; minimizing the nonspecific interactions of cyanines with blood, plasma, or cellular components; diminishing their aggregation in aqueous solution; and increasing their quantum yields of fluorescence. Many of the reported methods collectively improve the optical properties, stability and water solubility, or aggregation behavior, so all of them will be discussed here in the same subchapter. Two general directions have been followed to improve the stability and fluorescence properties of cyanines: chemical modification of the cyanine structure and encapsulation of cyanines inside dendrimers, cyclodextrins, or nanoparticles.

2.3.1 Chemical modifications

Improvement of chemical stability and moderate increase in quantum yields of fluorescence have been observed for cyanines with a polymethine chain rigidified by the cyclohexene ring.31 However, cycloheptamethine derivatives substituted at cyclohexene ring with electron-donating amine groups exhibit significantly reduced stability.81,82 It has been subsequently demonstrated that installing an acetyl substituent on the nitrogen, which reduces electron density on the cyanine scaffold, significantly stabilizes the N-acyl-substituted derivatives.82,83

An another strategy to improve chemical stability has been pursued by Armitage and coworkers, who observed that polyfluorinated pentamethine cyanine having benzothiazolium units at both termini exhibits increased fluorescence quantum yield, reduced tendency to aggregate in aqueous solution, higher chemical stability, and greater resistance toward photobleaching compared to its nonfluorinated analog.84 The authors pointed out that fluorination might be a general strategy for the improvement of
properties of cyanines for imaging applications, but this strategy has not yet been tested for other cyanines or in vivo. Similarly, substitution of the merocyanine dye (i.e., cyanine analog having an oxygen atom at one of the terminal heterocycles instead of nitrogen) with an electron-withdrawing cyano group increases its chemo- and photostability.85

Contrary to the results described above, where chemo- and photostability have been improved by substitution of cyanines with electron-withdrawing groups, it has also been reported that substitution of cycloheptamethine with electron-donating groups at the nitrogen slightly inhibits the photobleaching of the resulting fluorophores.86

2.3.2 Encapsulation

Encapsulation of cyanines inside larger organic molecules, such as dendrimers or cyclodextrins, or inside nanoparticles would protect the fluorophore from interacting with external agents that may quench fluorescence or decompose the molecule, such as solvents, oxygen, degrading enzymes, proteins, etc. Moreover, encapsulation would prevent cyanines from aggregation and improve their water solubility and, in many cases, pharmacodynamic properties. As the simplest way of encapsulation is PEGylation, attachment of poly(ethylene glycol) chains can be considered. Brechbiel demonstrated that conjugation of the amine-reactive ICG analog with PEG of average molecular weight of 3400 g/mol improves the water solubility and prevents aggregation of cyanine, both in the free form as well as when the resulting PEGylated fluorophore is attached to the antibody panitumumab.55 PEGylation of cyanine does not affect the targeting ability of the cyanine–antibody conjugate; hence PEGylation seems to be a viable strategy to improve the performance of cyanine fluorophores for in vivo applications.

Properties of cyanines can be also improved by the attachment of large water-solubilizing dendrons. Fréchet and Achilefu have examined the cypate encapsulated within a covalently attached polyester dendrimer with poly(ethylene oxide) branches on its periphery.44 Such nanoencapsulated fluorophore exhibits higher water solubility, diminished aggregation, and higher quantum yield in aqueous solution than cypate itself. Moreover, it shows improved pharmacokinetics (longer plasma circulation, low accumulation in normal tissue) and better stability toward cytochrome P450–catalyzed oxidation compared to the clinically approved ICG. Weck studied the properties of cycloheptamethine cyanines substituted at the central carbon atoms with both short-chain substituents and large dendrons.74 The results indicate that the
aggregation behavior of resulting conjugates depends on the terminal groups present on the periphery of the dendrons.

Another strategy to improve the water solubility, stability, and optical properties of cyanines is encapsulation of fluorophore inside the cyclodextrin cavity. Complexes of cyclodextrins with pentamethine\(^\text{87,88}\) and heptamethine\(^\text{89}\) cyanines have been reported. The complexation generally reduces the fluorescence quantum yields but substantially increases photostability up to nine times compared to the uncomplexed cyanines, both in solution as well as in cells.\(^\text{87,88}\) Cyanine–cyclodextrin complexes have not been examined in vivo yet.

The encapsulation inside the nanostructures has been intensively studied recently as a method for improving stability as well as photochemical and pharmacodynamic properties of cyanines. For example, silica nanoparticles have received considerable attention as potential fluorophore stabilizers and nanocarriers in vivo because of the nontoxicity of silica and its optical transparency. ICG has been incorporated inside mesoporous silica nanoparticles, where dye molecules have been entrapped inside nanopores by electrostatic interaction between negatively charged sulfonic groups and tetraalkylammonium-modified silica.\(^\text{90}\) The resulting fluorescent nanoparticles with diameters of 50–100 nm are relatively stable under physiological conditions, without substantial fluorophore leakage, and show maximum fluorescence at 800 µg of ICG per gram of silica (whereas the maximum fluorescence of free ICG in solution is achieved at concentration of 2 µg/mL); thus encapsulation of ICG allows much brighter fluorescence. In vivo biodistribution of the resulting nanoparticles has been examined as well.\(^\text{90}\) The cyanine fluorophore Dy776 has been also encapsulated inside ultrafine (<20 nm diameter) organically modified silica nanoparticles, and their in vivo biodistribution has been examined.\(^\text{91}\) The use of organically modified silica as a nanocarrier offers an additional capability to attach the targeting agent.\(^\text{91}\)

ICG has also been encapsulated inside surface-modified calcium phosphate or calcium phosphosilicate composite nanoparticles.\(^\text{80,92}\) Phosphate buffered saline (PBS) suspension of ICG-doped calcium phosphate (average diameter of 16 nm) nanoparticles showed twofold higher quantum yield of fluorescence per molecule of ICG and five times longer fluorescence half-life than a solution of the free fluorophore. Both calcium phosphate and calcium phosphosilicate nanoparticles with encapsulated ICG have been used for in vivo cancer imaging.\(^\text{80,92}\)

The stability, water solubility, blood circulation time, and tumor accumulation of ICG (or its analogs) have also been improved by encapsulation in
nanoparticles made from organic polymers: poly(lactic-\(\gamma\)-glycolic acid)\(^{93,94}\) and poly-(\(\epsilon\)-lactic-\(\gamma\)-glycolic acid)--poly(vinyl alcohol) systems,\(^95\) lipid nanoparticles,\(^96\) self-assembled polyethylene glycol–phospholipid nanoparticles,\(^97\) self-assembled polyallylamine hydrochloride–dihydrogen phosphate nanoaggregates, \(^{98,99}\) poly-(\(\gamma\)-glutamic acid), \(^{100}\) low-density lipoprotein (LDL) nanoparticles, \(^{101}\) liposomes, \(^{102}\) frozen ionic liquid nanoparticles, \(^{103}\) perfluorocarbon nanoparticles, \(^{104}\) and peptosomes. \(^{105}\) Many of these systems have been examined \textit{in vivo}. Encapsulation of cyanine fluorophores in nanoparticles seems to be an excellent method for improving their chemical and optical properties, and given the significant progress in the formulation of a variety of self-assembled nanostructures, this direction is likely to be intensively investigated in the future.

**3. SQUARAINES**

Squaraines (squirillium dyes) are polymethine fluorophores containing a hydroxyoxocyclobutene core with electron-donating substituents on both sides.\(^{106,107}\) Squaraines possess a strong, narrow absorption band in the red and near-IR spectral region, and in many cases, they are intensely fluorescent. The structures and optical properties of the most representative squaraines are presented in Chart 3.2.\(^{108,109}\) The absorption maxima, extinction coefficients, and fluorescence quantum yields of squaraines depend on the nature of aromatic groups flanking the oxabutene core. Synthesis of symmetrical squaraines is relatively straightforward and entails one-step condensation of commercially

---

**Chart 3.2 Structures and optical properties of representative squaraine dyes.**

<table>
<thead>
<tr>
<th>Squarine</th>
<th>(\lambda_{\text{abs}}) (nm)</th>
<th>(\varepsilon) (M(^{-1}) cm(^{-1}))</th>
<th>(\lambda_{\text{em}}) (nm)</th>
<th>(\Phi_f)</th>
<th>Solvent</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQ1</td>
<td>637</td>
<td>257,000</td>
<td>660</td>
<td>0.45</td>
<td>THF/H(_2)O</td>
<td>109</td>
</tr>
<tr>
<td>SQ2</td>
<td>641</td>
<td>280,000</td>
<td>652</td>
<td>0.20</td>
<td>DMSO</td>
<td>108</td>
</tr>
</tbody>
</table>
available squaric acid with electron-rich aromatic or heteroaromatic compounds with simultaneous removal of the water formed during the reaction (Fig. 3.3).\textsuperscript{106,107} Two-step condensation affords nonsymmetrical squaraines containing two different (hetero)aromatic groups.\textsuperscript{106,107}

Despite the favorable optical properties of squaraines, their application for \textit{in vivo} imaging suffers from two major limitations. While squaraines exhibit often very high quantum yield of fluorescence in nonpolar solvents, they extensively aggregate in polar organic solvents or in water and the aggregation dramatically changes both absorption and emission properties: broadens their absorption spectra and reduces significantly their fluorescence quantum yields.\textsuperscript{110} The electron-deficient hydroxyoxocyclobutene core of squaraines is prone to reacting with nucleophiles, including those present in biological media (especially thiols). The nucleophilic addition breaks the electronic conjugation and the resulting product loses near-IR absorption and fluorescence.\textsuperscript{111}

The breakthrough that has overcome these problems and opened the door for the broad application of squaraines for \textit{in vivo} imaging was the discovery that the stability of squaraines can be significantly improved upon formation of rotaxanes (for a review of the early development of fluorescent

![Figure 3.3 General schemes for synthesis: (A) symmetrical squaraine, (B) nonsymmetrical squaraines, and (C) squaraine rotaxanes.\textsuperscript{109}](image-url)
squaraine rotaxanes, see Ref. 112). Rotaxanes are the supramolecular structures in which a rode-like molecule is threaded through the macrocyclic component, and the bulky groups located on both ends of the axle prevent de-threading.112 Squaraine rotaxanes have been assembled by the “clipping method,” that is, macrocyclic amide was synthesized from diamine and isophthalic chloride in situ in the presence of squaraines containing bulky terminal substituents (Fig. 3.3C).109,113–115 The macrocycle formed in situ “clips” the axle (squaraine) and the process is facilitated by the hydrogen bonding between amide N–H in the intermediate acyclic precursor of macrocycle and oxygen atoms from the oxabutadiene core of squaraine (template effect). In an alternative synthetic strategy (capping method), macrocyclic lactam is synthesized first and forms a reversible host–guest complex with squaraine (pseudo-rotaxane). In subsequent chemical reactions, bulky groups (stoppers) are installed at both ends of the threaded squaraine and lock rotaxane irreversibly.116

The resulting squaraine rotaxanes possess essentially the same optical properties as free squaraines (though they sometimes exhibit lower fluorescence quantum yields than the corresponding free squaraines), but they are substantially more inert toward nucleophiles in solution and less prone to aggregation in polar solvents.109 For example, both near-IR absorption and emission of the free squaraine SQI are reduced two times within 5 min in the presence of cysteine (due to the nucleophilic attack of thiol), whereas the corresponding rotaxanes react substantially more slowly.109 Similarly, whereas SQI exhibits a very broad absorption in a dimethylsulfoxide/water mixture (due to the aggregation), the corresponding rotaxanes show only minor broadening under similar conditions.109 Both effects, namely, reduced reactivity toward nucleophiles and diminished tendencies for aggregation in polar media, are attributed to steric protection of the macrocycle on the squaraine core.109 The macrocyclic teralactam surrounds the squaraine, makes it less accessible by nucleophiles, and prevents it from chromophore–chromophore interaction, leading to aggregation.

Squaraine rotaxanes have been converted into water-soluble derivatives by attaching multiple sulfonic, carboxylic, guanidine, and quarternary ammonium groups as terminal “capping” groups.117 In a similar fashion, multivalent bioconjugatable derivatives have been prepared.115 Squaraine rotaxanes have been utilized subsequently for intracellular118 and in vivo imaging.117,119

An increase in the application of squaraine can be expected in the future. There is a wealth of squaraine derivatives described in the literature with
absorption above 700 nm, and their relatively straightforward synthesis, recent advances in chemistry of squaraine rotaxanes, and recently discovered chemiluminescence properties of squaraine rotaxanes (see Section 6.2), all together make this class of fluorophores promising candidates for a broad variety of applications.

4. BORON DIPYRROMETHENE AND RELATED FLUOROPHORES

4.1. General characterization

Another class of organic fluorophores potentially useful for in vivo imaging are boron dipyrroethenes.120–122 BDP (4,4-difluoro-4-bora-3a4a-diaza-s-indacene, BODIPY) are a class of neutral organic fluorophores containing a conjugated system of two pyrrole rings linked by methine bridge and complexed by the difluoroboron moiety. Boron dipyrromethenes exhibit strong absorption and emission in the visible and near-IR spectral window, and their absorption and emission are relatively insensitive to the solvent polarity and pH. BDPs show also remarkable photo- and chemostability compared to cyanines. The rigid BDP molecular framework makes BDP derivatives less prone to nonradiative decay of the excited state; thus their fluorescence quantum yield is usually high. Synthesis of BDP typically entails an acid-catalyzed condensation between pyrrole and aldehyde, oxidation of resulting dipyrromethane, and finally, complexation of the resulting dipyrrin with boron trifluoride.120,122

BDPs with their strong absorption in visible region, high fluorescence quantum yields, relatively high chemical and photochemical stability, and the very rich chemistry that allows their versatile structural modification and enables further fine-tuning of their chemical and optical properties represent an excellent platform to develop fluorescence probes for in vitro and intracellular imaging (for the most recent review of the application of BDP as a fluorescent probe, see Ref. 121). However, there are fewer examples of in vivo applications of BDP derivatives.123–125

One of the major reasons is that simple BDPs exhibit rather short-wavelength absorption and emission bands (~500–600 nm), which are unsuitable for in vivo applications. Therefore, several strategies have been developed to shift the absorption and emission toward longer wavelengths. Representative examples of red- and near-IR-emitting BDP derivatives
that illustrate the design strategies to shift the absorption/emission bands toward longer wavelengths are given in Chart 3.3. Substitution on the pyrrole subunits with conjugated substituents, such as styryl or areylethynyl, with electron-donating groups shifts absorption/emission above 800 nm (e.g., compounds BDP-I, BDP-II, and BDP-III).\textsuperscript{126,132–134} BDP derivatives with a fused benzene or naphthalene ring at the pyrrole subunits exhibit absorption/emission bands around 700 nm (e.g., compounds BDP-IV and BDP-V).\textsuperscript{127,135,136} Replacement of meso carbon atom with nitrogen leads to the formation of aza-BDP derivatives whose absorption and emission bands are shifted toward longer wavelengths by about 150 nm compared to the corresponding BDP analogs.\textsuperscript{128} Aza-BDP derivatives substituted at the \(\alpha\)-pyrrolic position with aromatic substituents possessing an electron-donating group exhibit absorption/emission at \(\sim 688/715\) nm (aza-BDP-VI).\textsuperscript{128,137,138} An additional bathochromic shift of about 50 nm and an increase in extinction coefficient have been achieved by rigidifying the aryl substituents, either by embedding the aryl substituent into a cyclohexane ring (aza-BDP-VII)\textsuperscript{129} or by the formation of a boron–oxygen bond (aza-BDP-VIII).\textsuperscript{130} Fusing of the benzo ring on the pyrrole moieties allows shifting of the absorption/emission bands above 800 nm.\textsuperscript{139,140}

BDP derivatives with fused furan rings and \(p\)-methoxyphenyl substituents absorb and emit in the red or near-IR spectral window, depending on the substituent present at the meso position (BDP-IX and BDP-X).\textsuperscript{131} The latter derivatives exhibit exceptionally high extinction coefficients and high quantum yield of fluorescence, both of which make them probably the brightest near-IR organic fluorophores.\textsuperscript{131}

An inspection of the literature data, exemplified in Chart 3.3, indicates that the optical properties of BDP can be broadly tuned and optimized by careful molecular design, whereby derivatives with long absorption wavelength and high extinction coefficient and quantum yield of fluorescence can be obtained.

Another problem associated with use of BDPs for \textit{in vivo} imaging is their inherent hydrophobic character and the lack of water solubility of their simple derivatives. Water solubility, however, can be imparted by the attachment of hydrophilic groups to the BDP core. Several water-soluble, highly fluorescent BDP derivatives have been prepared containing hydrophilic groups, such as sulfonates,\textsuperscript{141,142} carboxylates,\textsuperscript{141,143,144} phosphonates,\textsuperscript{145} quarternary ammonium salts,\textsuperscript{141} di(hydroxyethyl) amine,\textsuperscript{146} oligoethylene glycol chains,\textsuperscript{134,147–150} sulfonated peptides,\textsuperscript{151}...
Chart 3.3 Structures and optical properties of representative red and near-IR BDP fluorophores.
nitrilotriacetic acid residue, nucleotides, and sugars. Most of them show similar optical properties in aqueous solutions as their water-insoluble counterparts in organic solvents. Despite the fact that most of the reported water-soluble BDPs are the ones absorbing at rather short wavelength (\(<650\) nm, with a few examples of water-soluble BDPs or aza-BDPs absorbing in red and near-IR), the water-solubilizing groups reported so far, in principle, can be used for long-wavelength absorbing derivatives. Monovalent bioconjugatable BDP and aza-BDP derivatives have been also prepared in straightforward manner.

4.2. Energy-transfer dyads for increasing the Stokes shift of BDPs

The inherent optical property of BDPs that potentially may hamper their application in vivo is their small Stokes shift (20–30 nm). This can be a potential problem, given the relatively narrow absorption bands in BDPs and aza-BDPs. Hence, to achieve efficient excitation, BDPs need to be excited very close to their absorption maxima. While modifications of BDP structures reported so far do not allow substantial increase of the Stokes shift, a potential solution can be the assembling of two different BDP derivatives in energy-transfer dyads. In an energy-transfer dyad, two chromophores are covalently connected by a nonconjugated bridge so that each chromophore retains the optical properties that it had as a monomer. The excitation of the chromophore absorbing at the shorter wavelength (donor) causes energy transfer to the chromophore with the longer wavelength of absorption (acceptor) and consequently emission of the acceptor. If the quantum efficiency of energy transfer is high and there are no other competitive processes (such as electron transfer), an energy-transfer dyad behaves as a single chromophore with the excitation wavelength of the donor and the emission wavelength of the acceptor (see Fig. 3.4).

The critical aspect in construction of energy-transfer dyads is the linker connecting the donor and acceptor, which determines the mechanism of energy transfer and thereby the choice of the donor and the acceptor. In dyads in which the donor and the acceptor are connected by a fully nonconjugated linker (such as an alkyl or a peptide chain), the dominant mechanism of energy transfer is through-space Förster resonance energy transfer (commonly referred to as a FRET). Efficient FRET requires a large spectral overlap, that is, overlap between the emission band of the donor and the absorption band of the acceptor, and thus limits the choice of both pairs of chromophores that fulfill this requirement. On the other hand, a conjugated linker that provides, to some extent,
electronic communication between the donor and the acceptor enables through-bond energy transfer.\textsuperscript{156,157} The linker-mediated through-bond energy transfer does not require spectral overlap between the donor and acceptor bands and hence offers a greater flexibility in choosing both molecules. Several energy-transfer arrays containing two different BDP, or aza-BDP subunits, have been reported, and many of them exhibit efficient energy transfer and large difference between the absorption maximum of the donor and the emission maximum of the acceptor (pseudo-Stokes shift).\textsuperscript{158–162} Most of the BDP dyads reported so far absorb and emit at shorter wavelength than is required for \textit{in vivo} imaging; however, in principle, energy-transfer dyads can be also constructed from red and near-IR absorbing/emitting derivatives.

Taken together, the excellent optical properties and the rich chemistry that allows synthesis of diverse derivatives and fine-tuning of their chemical and optical properties make BDP and aza-BDP likely candidates for broad \textit{in vivo} applications.

### 4.3. Related fluorophores

Besides BDP, there are classes of related boron complexes with excellent optical properties suitable for \textit{in vivo} applications. The boron complexes of pyrrolopyrrole cyanine dyes (Chart 3.4) exhibit absorption/emission wavelengths in the near-IR (up to 864 nm), a high extinction coefficient, narrow absorption and emission bands, and high fluorescence quantum yield.\textsuperscript{163–165} Pyrrolopyrrole cyanines are more chemo- and photostable than classical cyanine dyes. This class of compounds exhibits also relatively long
fluorescence lifetimes (2.5–3.8 ns, compared to 1.11 ns for ICG) and they have been examined as fluorescence lifetime probes for in vivo imaging.\textsuperscript{103,166}

5. PORPHYRINS, PHthalacyANINES, AND RELATED MACROCYCLES

5.1. General characterization

Porphyrins are a class of macrocyclic aromatic compounds composed of four pyrrole rings connected by methine bridges (Chart 3.5). Porphyrins are ubiquitous in nature, as a heme cofactor of hemoglobin, cytochromes, and other redox active enzymes, and, as more saturated analogs, in the photosynthetic apparatus in plants and bacteria. Tetrapyrrolic macrocycles have been widely examined for their unique optical and redox properties. In the biomedical field, tetrapyrrolic macrocycles have been mainly investigated as photosensitizers in photodynamic therapy.\textsuperscript{167–169} Applications of porphyrins and their

Chart 3.4 Structures and optical properties of pyrrolopyrrole cyanines utilized for in vivo imaging.
analogs for in vivo imaging have been less explored, though their optical properties make them also suitable for those applications. Porphyrins have a unique electronic structure that results in a complex absorption spectrum. Simple porphyrins (such as tetraphenylporphyrin) exhibit a very strong (with $\varepsilon \sim 500,000 \text{ M}^{-1} \text{ cm}^{-1}$) absorption band around 400 nm, a series of much weaker bands in the visible region (500–650 nm), and a very weak absorption band in red spectral window ($\sim 650$ nm). Porphyrins possess also moderate fluorescence quantum yields ($\sim 0.1$). Although simple porphyrins are not suitable for in vivo fluorescence imaging because of their weak absorption in red/near-IR spectral window and their rather moderate fluorescence quantum yields, several of their more elaborate derivatives exhibit strong absorption and intense fluorescence in the red and near-IR regions. Derivatives with optical properties most promising for in vivo applications are benzoporphyrins, strongly conjugated porphyrin arrays, and hydroporphyrins.

5.2. Benzoporphyrins

Extension of the aromatic systems in porphyrins by fusing the benzo (or naphtho) ring at the pyrrolic positions causes a bathochromic shift and substantial intensification of the long-wavelength absorption band (with the
absorption wavelength shifted above 700 nm and extinction coefficient up to 200,000 cm\(^{-1}\) M\(^{-1}\)), and significant increase in fluorescence quantum yield (up to 0.45 for naphthoporphyrin; see Chart 3.5).\(^{171}\) Anthraperoporphyrins (porphyrins with fused anthracene on the pyrrole units) absorb and emit above 800 nm.\(^{172}\) Benzo- and naphthoporphyrins upon complexation with palladium (II) and platinum (II) show also an intense and long-lived near-IR phosphorescence.\(^{171}\) Phosphorescent benzoporphyrins, because of the long lifetime of their phosphorescence, are prone to dynamic quenching by oxygen; thus, palladium and, therefore, platinum benzoporphyrins find applications in sensing oxygen \textit{in vivo}.\(^{173,174}\)

5.3. Strongly conjugated porphyrin arrays

An alternative strategy for increasing near-IR absorption and fluorescence of porphyrins is the extension of the porphyrin conjugation by assembling several porphyrin subunits into strongly coupled arrays, that is, arrays where the porphyrin subunits are connected by a linker that provides strong electronic coupling (e.g., acetylene linker,\(^{175,178}\) or butadiyne linker\(^{176,177}\)) between the subunits (Chart 3.6). Such arrays composed of two, three, or five zinc complexes of porphyrin connected by an acetylene linker exhibit a progressive bathochromic shift of long-wavelength absorption and emission bands (with emission up to 883 nm), increased extinction coefficient, and good quantum yield of fluorescence.\(^{178,179}\) The trimeric arrays have been examined for imaging of B16 melanoma cells\(^{180}\) and labeling of the dendritic cells,\(^{181}\) and their suitability for \textit{in vivo} imaging in living animals has also been demonstrated.\(^{182}\)

5.4. Chlorins and bacteriochlorins

The partial saturation of pyrrole units in porphyrins leads to formation of new types of tetrapyrrolic macrocycles with distinctive spectral properties: chlorins (with one partially saturated pyrrole ring), bacteriochlorins (two partially saturated pyrrole rings on opposite sites), and isobacteriochlorins (with two partially saturated pyrrole rings on the same site of macrocycle; see Chart 3.7). In contrast to porphyrins, chlorins exhibit a strong absorption in the red region (600–700 nm),\(^{183}\) whereas bacteriochlorins absorb strongly in the near-IR (700–800 nm).\(^{184}\) Both classes of macrocycles show also higher fluorescence quantum yields than the corresponding porphyrins (average of ~0.25 for chlorins\(^{185}\) and ~0.15 for bacteriochlorins\(^{184}\)). Iso-bacteriochlorins exhibit strong absorption and emission (with quantum yield
Chart 3.6 Structures and optical properties of representative strongly conjugated porphyrin arrays.
Chart 3.7 Structures and optical properties of representative natural and synthetic hydroporphyrin fluorophores.
of fluorescence up to 0.70) at somewhat shorter wavelengths (~600 nm). Both chlorins and bacteriochlorin derivatives have been used for in vivo imaging of cancer or, as conjugates with the fluorescence quencher, have been used in vivo to monitor phospholipase activity.

Naturally occurring chlorophylls and bacteriochlorophylls are attractive because of their availability, but their use imposes certain problems and limitations. First, naturally occurring derivatives have a full complement of substituents on the macrocycle periphery, and their chemical modification to tune their optical and chemical properties, although possible, is limited. Moreover, naturally occurring bacteriochlorophylls are rather unstable, and outside their natural environment, they undergo oxidation to the more conjugated derivatives with substantially different optical properties. Therefore, a chief effort has been dedicated to developing stable synthetic chlorin and bacteriochlorin analogs that would retain the optical properties of naturally occurring compounds and would be amenable for synthetic modifications and fine-tuning of their physicochemical properties.

The routes developed for fully synthetic hydroporphyrins entail either derivatization of porphyrins (which are usually much easier to prepare) or de novo synthesis of the hydroporphyrin macrocycle. The latter approach, though more synthetically challenging, is more versatile and enables full control of the position and numbers of substituents on the periphery of the macrocycle (thus allowing also the extensive tuning of the chemical and optical properties of hydroporphyrins). De novo routes enable also preparation of more stable hydroporphyrins resistant to oxidation to more conjugated porphyrins. Installation of geminal alkyl groups on the partially saturated pyrroline rings in hydroporphyrins prevents oxidation of chlorins and bacteriochlorins to more conjugated congeners.

Synthetic hydroporphyrins display a range of unique photochemical properties, which make them very attractive platforms to develop fluorophores for in vivo applications. Both synthetic chlorins and bacteriochlorins exhibit narrow and tunable absorption and emission bands. Their absorption and emission maxima can be broadly tuned by simple substitution on the periphery of the macrocycle, spanning the range of about 635–715 nm for chlorins and 715–823 nm for bacteriochlorins. The wavelengths of absorption and emission can be tuned virtually with
nanometer precision by relatively straightforward chemical modification so that one common precursor can be used for the synthesis of a range of derivatives with different emission bands.\textsuperscript{193,198} Moreover, chlorins and bacteriochlorins exhibit exceptionally narrow emission bands with full width at half maxima (FWHM) of $\sim 15$ nm for chlorins and $\sim 20$ nm for bacteriochlorins, which are probably the narrowest emissions among organic compounds.\textsuperscript{183–185,198} Most of the chlorin and bacteriochlorin derivatives exhibit also sufficiently high quantum yields of fluorescence and high fluorescence lifetimes: 8–10 ns for chlorins\textsuperscript{183} (which is of a magnitude higher than those for typical organic fluorophores) and 4–6 ns for bacteriochlorins.\textsuperscript{184} Fluorescence lifetime can be also tuned to some extent by substitution and metalation.\textsuperscript{183} Their narrow and tunable emission bands and long and tunable fluorescence lifetimes make them a superior choice for spectral and lifetime \textit{in vivo} multiplexing (see Section 6.1).

5.5. Hydroporphyrin arrays for increased Stokes shift and multicolor \textit{in vivo} imaging

The inherent spectroscopic property of hydroporphyrins, both chlorins and bacteriochlorins, is the small Stokes shift, which typically falls in the range 0–10 nm, regardless of the solvent, substitution pattern, and metalation state of the macrocycle.\textsuperscript{183,184} To overcome this limitation, which is critical for \textit{in vivo} imaging, hydroporphyrin energy-transfer dyads have been proposed. Holten and coworkers have demonstrated that, in dyads comprising chlorin and bacteriochlorin, efficient energy transfer from chlorin to bacteriochlorin moieties occurs, and dyads behave as a single chromophore with excitation wavelengths of chlorins (650 or 675 nm) and the emission wavelength of bacteriochlorin (760 nm) so that the effective Stokes shift can be increased up to 110 nm (Chart 3.8).\textsuperscript{201,202}

5.6. Water solubility and aggregation

The planar, aromatic, and highly hydrophobic structure of porphyrins and hydroporphyrins causes difficulties in aqueous solubility. Additionally, porphyrins tend to aggregate in aqueous solution.\textsuperscript{203} The aggregation behavior of porphyrins, which is mainly driven by $\pi-\pi$ stacking interactions and hydrophobic forces, is quite complex and depends on the substitution pattern. Introduction of the hydrophilic groups, such as carboxylates, sulfonic acids, quarternary pyridinium, or ammonium groups, only partially solves the problem because such porphyrins still tend to aggregate.\textsuperscript{203} The aggregation problem can be overcome by the introduction of a “swallow–tail”
solubilizing motif, that is, hydrophilic groups that are projected above and below a macrocyclic porphyrinic plane.\textsuperscript{204} Such a motif provides water solubility and prevents aggregation by sterically hindering the \( \pi \)-system. Thus, chlorins equipped with swallow-tail substituents with phosphonic acids have been reported, and they show good water solubility, no evidence of aggregation, and good fluorescence quantum yields in water.\textsuperscript{204} A similar approach has been developed by Vinogradov and coworkers, who prepared benzoporphyrins with dendritic substituents. Dendrons attached to the benzoporphyrin core possess peripheral hydrophilic groups (such as polyethylene glycol) and provide excellent water solubility, and crowded dendritic substituents protect porphyrin from self-aggregation and interactions with plasma proteins which may alter the optical properties of the fluorophore.\textsuperscript{173,174,205,206}

An alternative strategy that has been developed to overcome the lack of water solubility of porphyrinic compounds entails the encapsulation of hydrophobic tetrapyrrolic macrocycles inside nanostructured capsules. Therien and coworkers have embedded strongly coupled porphyrin arrays into the polymersome membranes by cooperative self-assembly of diblock

\begin{center}
\textbf{Chart 3.8} Structures and optical properties of chlorin–bacteriochlorin energy-transfer dyads with tunable apparent Stokes shift.
\end{center}
amphiphilic polymers and hydrophobic porphyrins. As a result, vehicles with diameters of 50 nm–50 μm are formed in which porphyrins are uniformly distributed in the vehicle membrane. As porphyrins stay in the hydrophobic environment of the vehicle membrane, they retain optical properties comparable to those in organic solvents. The resulting highly emissive polymersomes can be freely dispersed in water and have been used for in vivo imaging, labeling, and in vivo tracking of dendritic cells.

Porphyrin arrays have also been incorporated into the hydrophobic core of the LDL apo forms, forming nanoparticles highly emissive in water. The resulting nanoparticles show tumor specificity, as many tumor cells over-express the LDL receptor. Porphyrin-doped LDLs have been used for imaging B16 melanoma cells. Similarly, bacteriopheophytin a bisoleate has been incorporated into the hydrophobic core of high-density lipoprotein nanoparticles (size 12 nm) and used for in vivo imaging of tumor.

5.7. Photocytotoxicity of tetrapyrrolic macrocycle

The use of tetrapyrrolic macrocycles as fluorophores in vivo raises concern about their photocytotoxicity. Porphyrins, hydroporphyrins, and phthalocyanines upon excitation populate the corresponding triplet excited state, which reacts with ambient oxygen to produce highly cytotoxic singlet oxygen and other highly reactive oxygen species. This property is very useful in photodynamic therapy, where tetrapyrrolic macrocycles are widely used as photosensitizers, but can be potentially detrimental when one considers using them as fluorophores in living organisms. The photocytotoxicity depends on the many factors, such as the intrinsic photochemical properties of the photosensitizer (quantum yield and lifetime of the triplet state), localization of the photosensitizer inside the tissue, and the intensity of illumination. In general, even good photosensitizers require a higher dosage of light to induce photocytotoxicity than is typically used in fluorescence imaging experiments. Moreover, it is expected that tetrapyrrolic macrocycles with optical properties optimized for fluorescence imaging (i.e., with high quantum yield of fluorescence) would have a lower quantum yield of the triplet state, as these two processes compete with each other. For example, it has been reported that, in highly conjugated porphyrin arrays, the increase of fluorescence quantum yield is due to the accompanying decrease of triplet state formation, and thus highly conjugated porphyrin arrays are poor photosensitizers. Similarly, there are also
suggestions that, for hydroporphyrins, the fluorescence quantum yield increases at the expense of triplet state formation.\textsuperscript{185}

Vinogradov and coworkers studied the phototoxicity of phosphorescent dendritic benzoporphyrin used as \textit{in vivo} oxygen probes.\textsuperscript{208} In this case, there is a particular concern about phototoxicity of the probe, as the side product of phosphorescent oxygen sensing is a singlet oxygen. They found negligible phototoxicity of their probes, which they attributed to the inability of dendritic benzoporphyrins to penetrate cellular membranes and produce singlet oxygen inside the cellular organelles. Their studies pointed out the importance of probe localization on their toxicity and demonstrated that even a potentially highly photocytotoxic probe can be safely used \textit{in vivo}.

The elegant and general solution to overcome the problem of singlet oxygen generation by tetrapyrrolic fluorophores has been proposed by Moore and coworkers.\textsuperscript{209} They prepared the covalently linked carotene–porphyrin dyads and found that carotenoids effectively quenched the triplet state of porphyrins (and other tetrapyrrolic compounds) by energy transfer, thereby making them incapable of producing singlet oxygen (the same principle has been also used for the design of a quenched, protease-activatable, chlorophyll–based photosensitizer for anticancer therapy\textsuperscript{210}). This general approach seems to be a viable strategy when phototoxicity is a problem.

5.8. Phthalocyanines

Phthalocyanines are benzoporphyrin congeners having nitrogen rather than carbon bridging atoms. Phthalocyanines exhibit strong and sharp absorption in red spectral window and, in contrast to porphyrins, show high fluorescence quantum yields. Expansion of the aromatic system by fusing additional benzo ring gives naphthocyanines with absorption and emission above 700 nm.\textsuperscript{211–219} Phthalocyanines exhibit also excellent chemo- and photostability compared to the other near-IR fluorophores. Phthalocyanines have been used as fluorophores in polymerase chain reaction (PCR)\textsuperscript{211} and molecular beacons,\textsuperscript{212} and broadly examined as photosensitizers in photodynamic therapy,\textsuperscript{167} whereas their use as a fluorophore for \textit{in vivo} imaging has been rather neglected.

One of the reasons for this neglect is the fact that phthalocyanines, because of their planar, hydrophobic structure, are difficult to solubilize in water and tend to aggregate in aqueous solution. Their solubility in organic solvents is usually low as well, which makes their purification and handling rather difficult. The few available methods for synthesis and derivatization of phthalocyanines do not offer much flexibility in preparing functional derivatives, especially
monofunctional, bioconjugatable derivatives. Finally, similar to porphyrins and hydroporphyrins, phthalocyanines tend to be photocytotoxic.\textsuperscript{167} Recent progress in the chemistry of phthalocyanines has partially solved the above-mentioned issues. Nonaggregating, water-soluble phthalocyanines bearing neutral, cationic, or anionic hydrophilic groups have been recently reported by Vicente and coworkers\textsuperscript{214,215} and Ng and coworkers.\textsuperscript{216} The water solubility and suppressed aggregation have been achieved by installing bulky groups, containing poly(ethylene) glycols,\textsuperscript{216} quarternary pyridinium (cationic),\textsuperscript{215} or carboxylate (anionic)\textsuperscript{214} groups on the periphery of the macrocycle. Another notable approach to suppress aggregation and impose water solubility relies on the synthesis of silicon complexes of phthalocyanines and attaching hydrophilic groups as axial ligands to the central silicon atom (see Chart 3.9 for an example).\textsuperscript{213,217–219} As axial ligands are situated centrally above and below the phthalocyanine plane, they prevent aggregation by steric repulsion. PEG,\textsuperscript{217,219} sulfonic group-terminated alkyl amines,\textsuperscript{213} or polyamines\textsuperscript{218} have been used as axial hydrophilic groups.

Recently, notable progress has been made on the synthesis of monofunctional phthalocyanine derivatives. Hammer and coworkers reported

\begin{center}
\textbf{Chart 3.9} Structure and optical properties of representative phthalocyanine fluorophore.
\end{center}
the solid-phase synthesis of nonsymmetrically substituted phthalocya-
nines.\textsuperscript{220,221} This type of derivatives was previously prepared by statistical
condensation of different phthalonitriles,\textsuperscript{222} which led to the formation of
complex mixture of products, diminished the yield of desired derivative,
and required extensive purification. Solid-phase synthesis significantly
simplifies purification and improves the yield of nonsymmetrical
derivatives and enables an efficient synthesis of monofunctional,
biocojugatable derivatives.

6. SPECIAL TYPES OF FLUOROPHORES

6.1. Fluorophores for multicolor imaging

Multicolor imaging (spectral multiplexing) allows targeting simultaneously
multiple different markers, processes, physicochemical parameters, cells, or
organs. Multicolor imaging requires access to a set of fluorophores in which
each fluorophore has a distinctive spectral feature so that fluorescence from
each of them can be independently detected in the presence of other
fluorophores. Ideally, each fluorophore in such a set should be excited with
the same wavelength, and each should exhibit a narrow emission band cen-
tered at a different wavelength, without overlap with emission bands from
the other fluorophores. Alternatively, each fluorophore should exhibit a
narrow absorption band (so that each can be selectively excited at the dif-
ferent wavelengths) and emit at the same wavelength.

Multicolor fluorescence detection has been successfully used, for exam-
ple, in nucleic acid sequencing (where energy-transfer dyads with a com-
mon donor and different acceptors have been used)\textsuperscript{223,224} or in flow
cytometry.\textsuperscript{225} Application of multicolor imaging \textit{in vivo} has, however,
certain limitations. The simultaneous use of multiple organic fluorophores
\textit{in vivo} is limited because of their broad emission bands (typical FWHM
for most organic fluorophores is $>30$ nm) so that only a limited number
of fluorophores can be placed in the spectral window suitable for \textit{in vivo}
applications without strong overlap of their emission bands.\textsuperscript{6} Addi-
tionally, in the set of organic fluorophores with different emission
wavelengths, each fluorophore usually requires a different excitation
wavelength, which makes whole imaging process time consuming and
technically complex. Quantum dots, which have a tunable, narrow
emission band (FWHM $\sim 30$ nm) and broad absorption bands that allow
excitations of the whole set of different quantum dots with the common
wavelength are good candidates for spectral multiplexing.\textsuperscript{6} The use of the
quantum dots, however, raises concerns about their toxicity, as they often
contain toxic metals, such as cadmium and selenium. Recently, fluorescent proteins with large Stokes shifts, named Keima, have been developed, which, in combination with other fluorescent proteins, allow multicolor intracellular imaging with single excitation wavelengths (simultaneous imaging using six different fluorescent proteins has been demonstrated). The wavelengths of excitation and emission for Keima ($\lambda_{\text{abs}} = 440$ nm, $\lambda_{\text{em}} = 620$ nm for the longest emitting variant) make them less suitable for \textit{in vivo} applications.

So far, \textit{in vivo} multicolor imaging has been pursued using a cocktail of organic fluorophores (such as mixture of various rhodamines, coumarines, cyanine dyes, and/or Alexa Fluors), quantum dots (also together with organic fluorophores), or upconverting nanocrystals. Upconverting nanocrystals are a unique class of fluorophores that are composed of, for example, sodium yttrium fluoride and are doped with rare-earth metal cations and they can be excited in the near-IR region (e.g., 980 nm) and emit at a shorter wavelength. The wavelength of emission depends on their composition. Upconverting nanocrystals have been utilized \textit{in vivo} for multicolor imaging alone, or as an energy-transfer donor in conjugation with near-IR organic fluorophores (rhodamines).

Promising fluorophores for \textit{in vivo} multicolor applications are hydroporphyrin energy-transfer dyads (see Section 5.5 and Chart 3.8). The intrinsic properties of hydroporphyrins, that is, narrow and tunable absorption and emission bands, together with their tunable apparent Stokes shift, which can be achieved by assembling hydroporphyrins in energy-transfer dyads, make them well suited for multicolor imaging. Holten and coworkers studied pairs of model chlorin–bacteriochlorin dyads where each dyad had the same bacteriochlorin acceptor (thus the same emission wavelength) and different chlorin components (thus different excitation wavelengths). Because of the narrow absorption bands in chlorins, the 25-nm separation between the absorption maxima of both chlorins in the studied pair is sufficient for selective excitation one dyad in the presence of the other. This selectivity has been demonstrated also \textit{in vivo} and gives the promise for the development of a new class of fluorophores with either a common emission wavelength and different, well-resolved absorption bands, or a common excitation wavelength and different, well-resolved emission wavelengths.

An elegant strategy for fluorophores for multicolor imaging has been developed by Kool and coworkers. They assembled deoxyriboside monomers containing small fluorescent organic molecules (aromatic
hydrocarbons or small fluorescent heterocycles) in a DNA-like phosphodiester oligomer (oligodeoxyfluorosides). They found that several different interactions between the assembled fluorophores occur (such as excimer and exciplex formation, H-stacking and energy transfer), which result in different fluorescence characteristics for different combinations of the fluorophores. This enabled preparing the sets of short oligomers (containing 1–4 different fluorophores) each with a different fluorescence color, excitable at the common wavelength. The advantages of this approach are the ease of synthesis (as an automated DNA synthesizer can be utilized), water solubility (provided by phosphodiester backbone), and the ability to attach a single bioconjugatable group. The antibody–oligodeoxyfluoroside conjugates have been used for multicolor imaging of living cells and living zebrafish embryo.

6.2. Self-illuminating fluorophores

All the fluorophores described so far require photoexcitation prior to fluorescence, that is, the fluorophore must absorb a photon to be excited, which in turn requires illumination of the fluorophore by an external source of light. Such illumination causes excitation of the exogenous tissue or cell fluorophores and causes some background signal which reduces the signal-to-background ratio. Consequently, it diminishes both the sensitivity and the limit of detection, even when the excitation is done in the optimal spectral window.

One of the solutions to overcome this problem is to use fluorophores that do not require photoexcitation but can reach an excited state and subsequently emit fluorescence upon chemical reaction (chemiluminescence) or upon enzymatic reaction (bioluminescence). Chemi- or bioluminescence eliminates the need for an external light source for excitation and therefore eliminates almost completely autofluorescence from the tissue.

The prominent bioluminescent reaction is the luciferase-catalyzed oxidation of luciferin with the concomitant emission of light. Luciferin is a generic name given to the class of small organic molecules that can emit light upon oxidation catalyzed by various types of luciferases, among which firefly luciferin and coelenterazine are the most widely used in biotechnology and, recently, in in vivo imaging. The luciferin/luciferase pairs emit at relatively short wavelengths (for most luciferins between 480 and 560 nm); therefore, efforts have been made to obtain mutant luciferins or use alternative substrates to shift the resulting bioluminescence toward...
longer wavelengths.\textsuperscript{244,245} In an alternative strategy, the luciferase/luciferin pair is used as an energy-transfer donor in bioluminescence resonance energy transfer (BRET) and transfers the excitation energy to the acceptor emitting at longer wavelength. In one of the first BRET systems developed, luciferase was fused with green fluorescent proteins and this system was used to monitor intracellular molecular events.\textsuperscript{242,243} The luciferase–GFP (green fluorescent protein) pair has been also used \textit{in vivo},\textsuperscript{246} but the relatively short wavelength of emission of GFP prompted scientists to seek a BRET acceptor with a longer wavelength of emission.

Recently, a BRET system containing red fluorescent proteins emitting at 635 nm as acceptors has been designed and used for \textit{in vivo} imaging of protein–protein interactions.\textsuperscript{247} Alternatively, luciferase has been conjugated with organic near-IR–emitting fluorophores: Alexa Fluors (AF680 and AF750)\textsuperscript{245} or cyanine\textsuperscript{248} and both exhibit efficient BRET and emission in the red or the near-IR region.

Rao and coworkers have developed a self-illuminating BRET system suitable for \textit{in vivo} application, which consists of the luciferase/coelenterazine pair as donor and quantum dots as acceptors.\textsuperscript{249,250} In their system, quantum dots are conjugated to the eight copies of mutated \textit{Renilla reniformis} luciferase (called there Luc8) and, in the presence of coelenterazine, show quantum dot emission, due to the BRET, together with a much weaker emission at 480 from coelenterazine. This system has been examined \textit{in vitro} and \textit{in vivo} in mouse. Luc8 has been subsequently conjugated to quantum dots emitting at different wavelengths, 605, 655, 705, and 800 nm, respectively, and used for \textit{in vivo} spectral multiplexing.\textsuperscript{249} A BRET system utilizing the luciferase–quantum dot pair has been also used for \textit{in vivo} cancer detection.\textsuperscript{251}

Although chemiluminescence, that is, luminescence occurring upon a chemical, nonenzymatic reaction, is a well-known phenomenon,\textsuperscript{252} its application for \textit{in vivo} imaging has been neglected until recently.\textsuperscript{253,254} The main reasons are that most of the chemiluminescent reactions typically emit short-wavelength light, utilize unstable, highly reactive compounds (such as peroxides), or require reagents that are toxic or harmful (e.g., hydrogen peroxide).\textsuperscript{252} Chemiluminescent molecular probes suitable for \textit{in vivo} applications, where chemiluminescence is activated by temperature, have been developed by Smith and coworkers.\textsuperscript{254} They found that squaraine rotaxanes (see Section 3) having an anthracene core in their macrocyclic tetralactam components react with singlet oxygen in cycloaddition reaction to form an adduct, the so-called endoperoxide. This peroxide, upon warming up to the body
temperature, undergoes a cycloreversion reaction to produce singlet oxygen. The singlet oxygen decays to the ground-state triplet oxygen, exciting the encapsulated squaraine, which in turn fluoresces in the near-IR region. The exact mechanism of energy transfer from singlet oxygen to squaraine is not known. The system is very convenient, as rotaxane endoperoxide forms quantitatively upon irradiation of squaraine rotaxane solution in the presence of air; the resulting peroxides can be indefinitely stored at $-20 \, ^\circ C$ and near-IR fluorescence appears upon warming the sample to room temperature.

Scherman and coworkers developed organic nanoparticles with long-lasting (persistent) luminescence. Nanoparticles based on magnesium silicate doped with luminescent cations (Eu$^{2+}$, Dy$^{3+}$, and Mn$^{2+}$) emit upon irradiation red or near-IR luminescence for several hours after irradiation, which can be easily detected from the animal body several hours after injection.

7. CONCLUSION

Despite vigorous research efforts, the optimal fluorophore that fulfills all requirements for in vivo applications has not been created yet. Given the diverse applications of fluorescence in in vivo imaging, it is rather unlikely that a single universal molecular platform can be ever found. Searching for the new fluorescent materials, modification and improvement of the existing fluorophores, and conjugation of different fluorophores to combine their properties—these are the three areas that warrant further progress in the field of fluorophores. The latter approach seems to be particularly powerful for creating new systems with unique properties, as illustrated by some of the recent advances highlighted in this review, such as energy-transfer dyads with tunable Stokes shift, self-illuminating near-IR BRET systems, or near-IR luminescent squaraine rotaxanes. On the other hand, advances in related fields, namely, materials science, nanoscience, and biotechnology, will likely provide the new materials with currently unattainable properties and tools for improvements and expansion of the properties of currently existing fluorophores.

ACKNOWLEDGMENT

The author wishes to thank the University of Maryland, Baltimore County, for supporting this work (start-up funds and SRAIS award).
REFERENCES

1. Demchenko AP. Introduction to fluorescence sensing. Springer; 2009.


