Azo-Based Probes

Azo-Based Fluorogenic Probes for Biosensing and Bioimaging: Recent Advances and Upcoming Challenges

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DOI: 10.1002/asia.201700682
Abstract: The use of nonfluorescent azo dyes as dark quenchers in activatable optical bioprobes based on the Förster resonance energy transfer (FRET) mechanism and designed to target a wide range of enzymes has been established for over two decades. The key value of the azo moiety (–N=N–) to act as an efficient “ON-OFF” switch of fluorescence once introduced within the core structure of conventional organic-based fluorophores (mainly fluorescent aniline derivatives) has recently been exploited in the development of alternative reaction-based small-molecule probes based on the “profluorescence” concept. These unprecedented “azobenzene-caged” fluorophores are valuable tools for the detection of a wide range of reactive (bio)analytes. This review highlights the most recent and relevant advances made in the design and biosensing/bioimaging applications of azo-based fluorogenic probes. Emphasis is also placed on relevant achievements in the synthesis of bioconjugatable/biocompatible azo dyes used as starting building blocks in the rational and rapid construction of these fluorescent chemodosimeters. Finally, a brief glimpse of possible future biomedical applications (theranostics) of these “smart” azobenzene-based molecular systems is presented.

1. Introduction

Azobenzene (AB) derivatives (or azo compounds) are a well-known class of organic dye molecules that share the core AB structure, which is simply defined as the combination of two phenyl rings attached through a nitrogen–nitrogen double bond (–N=N–), with different substituents and/or further (hetero)cyclic rings fused to the benzene moieties. Such molecules exhibit unique large UV/Vis absorbance spectra, mainly owing to the significant number of vibronic states in each energy level, $S_0$. This mostly leads to high molar absorption coefficients in solution, with a wide range of colors relative to their absorbance maximum value. Thus, these molecules have been widely used as pigments since the 19th century.[1] This is all the more legitimate given their exceptional chemical stability under harsh conditions, except in reducing media.

The most important photophysical property of ABS is undeniably the prominence of the nonradiative deactivation of their electronic excited states $S_1$ and $S_2$ related to photochemical isomerization of the azo bridge, either through a rotation mechanism around the $–$N=N– double bond or an inversion mechanism, in which a planar variation of one of the $C$-$N$-$N$ angles can occur at the excited state (Figure 1).[2] Consequently, ABs are usually weakly fluorescent or nonfluorescent compounds. The preferred way to obtain fluorescent AB derivatives is to add a chemical functionality, such as an excited-state intramolecular proton-transfer (ESIPT)-active moiety[3] or a bis(pentafluorophenyl)boryl (or silyl) group to form an intramolecular N–B (or N–S) active bond to restrict rotation and isomerization.[4] Thus, associated with another emitting dye (fluorescent or luminescent) in a molecular system in which all prerequisites for the occurrence of an effective Förster resonance energy transfer (FRET) are fulfilled,[5] ABS can play the role of acceptor, which dissipates energy readily through nonradiative pathways. As a practical consequence, the fluorescent donor is turned “OFF”, as long as the FRET process can occur. Interestingly, other photophysical mechanisms, such as intramolecular photoinduced electron transfer (PeT) or internal charge transfer (ICT) have also been implemented for the rational design of nonemissive dyes (also known as quenchers) from fluorescent derivatives.[6,7] Among the different kinds of organic-based quenchers currently available, different members of the Black Hole Quencher family integrate within their backbone structure one or several azo groups to take advantage of their nonemissive de-excitation.[8,9] The successful use of these azo quenchers in enzymatically activatable FRET-based probes has been extensively described in the literature, particularly for the sensitive and selective detection of biologically relevant proteins, and comprehensive reviews on this topic have already been published.[10] Surprisingly, until five years ago, few research efforts were devoted to the synthetic chemistry of these azo-based quenchers with the aim of optimizing both their spectral and physicochemical properties (e.g., solubility in aqueous media) and introducing a bioconjugatable tether compatible with emerging and promising (bioorthogonal) reactions belonging to the repertoire of click chemistry.[11] We and other research groups have recently proposed innovative chemical solutions to develop unconventional azo dyes partic-
ularly useful for 1) convenient access to enzyme-sensitive (mainly, proteases, and reductases) FRET-based probes suitable for bioimaging experiments in living cells and/or in vivo, and 2) the preparation of unprecedented profluorophores\[^{12,13}\] based on protection–deprotection (i.e., reductive release of aniline-based fluorophores)\[^{13}\] or covalent-assembly probe design principles (i.e., in situ formation of a fluorescent scaffold)\[^{14}\] for sensing (bio)analytes in various contexts. This review focuses on these recent advances (both synthetic aspects and bioanalytical applications) related to the fluorescence quenching ability of ABs, but other important applications in the field of chemical biology, namely, molecular photoswitches for reversibly photocontrol of biological systems, related to photocromism of azo compounds are not covered herein.\[^{15}\]

2. Design and Synthesis of Novel Functionalized Azo-Based Quenchers with Enhanced Spectral Properties

The preparation of dark quenchers with one or two azo groups received substantial interest among (bio)organic chemists in the early 2000s, when Biosearch Technologies company (now part of LGC group) commercialized the first polyaromatic AB derivatives as bioconjugatable (activated N-hydroxysuccinimidyl (NHS) esters) quenchers named Black Hole Quenchers (BHQs) \[^{8,9,16,17}\]. Associated with DABCYL \[^{18}\] and Blackberry 650 (BBQ-650, recently developed and commercialized by Berry & Associates Co.),\[^{19}\] these molecules constitute the most popular set of azo-based quenchers currently used for designing activatable FRET-based probes and their clear usefulness has been proven (Figure 2). Carboxylic acid and some phosphoramidite derivatives are commercially available as reagents suitable for the labeling of peptides and oligonucleotides through solution or solid-phase methodologies (i.e., amidification reaction or phosphoramidite coupling followed by mild oxidation). However, these reagents are not suitable for biolabeling schemes/applications based on bioconjugation reactions developed during the past decade (i.e., those belonging to the repertoire of click chemistry and/or bioorthogonal reactions) and those that are fashionable (e.g., copper-catalyzed azide–alkyne cycloaddition (CuAAC), oxime ligation).\[^{11}\] Therefore, to expand the scope of these azo dyes in challenging bioconjugating applications, some academic research groups have developed novel azo-based quenchers with alternative bioconjugatable handles.

2.1. Synthesis of New Bioconjugatable Dark Quenchers

Even if many synthetic methods have been reported for the synthesis of ABs,\[^{20}\] the strategy currently used to prepare azo-based quenchers always relies on a conventional azo-coupling reaction between a diazonium salt (derived from one of the two aniline coupling partners) and an electron-rich N,N-dialkylaniline, the mechanism of which is closely related to that of a S\_2Ar reaction (Scheme 1). The use of an unsymmetrical mono-N-substituted tertiary aniline with a short alkyl chain ended by a protected or free functional group is the preferred synthetic method to functionalize azo dyes readily.

Arnoud Chevalier received his Ph.D. in bioorganic chemistry in 2014 from the University of Rouen under the co-supervision of Prof. Pierre-Yves Renard and Anthony Romieu. His research works focused on the development of new advanced chemical tools for applications in molecular imaging. As a postdoctoral fellow under the guidance of Prof. Sidney M. Hecht at Arizona State University (USA), he is carrying out chemical biology research to address some issues related to the biochemistry of mitochondria. He is co-author of more than 15 scientific papers and co-inventor of 3 patents.

Pierre-Yves Renard received his Ph.D. in organic chemistry in 1994 from the Université Paris-Sud/Ecole Polytechnique under the supervision of Prof. Jean-Yves Lallemand. After a nine year period (1994–2003) as French Department of Defense procurement agency staff (Délegation Générale pour l’Armement) at CEA Saclay, where he conducted research on bioconjugatable means to detoxify organophosphorus nerve agents under the supervision of Dr. Charles Mioskowski, he was appointed professor in 2003 at the University of Rouen (France), and junior member of the French University Institute (IFU) in 2006. He has, since then, led the bio-organic chemistry group, and his current research fields are the development of “smart” probes for the detection of bioanalytes and chemical tools for bioconjugation and medicinal chemistry. He is co-author of more than 125 research papers and co-inventor of 15 international patents; two of them have given rise to the ChemForAse start-up, which specializes in the development of purification methods for proteins of (bio)medical interest.

Anthony Romieu obtained his Ph.D. from the University Joseph Fourier (Grenoble, France) under the guidance of Drs. Jean Cadet and Didier Gasparutto (Laboratory of Nucleic Acids Damages, CEA-Grenoble) in 1999. After serving for two years as a CNRS study engineer in the laboratory of Prof. B.P. Roques at the University Paris 5, in the fields of medicinal chemistry and peptide synthesis, in June 2001 he joined a private biotech company, Manteia Predictive Medicine (a spin-off of the Serono group, Switzerland), as a senior scientist in bioorganic chemistry. The core business of this company was devoted to the development and commercialization of an original high-throughput DNA sequencing technology. Following a restructuring, leading to the acquisition of Manteia by Solexa Ltd. (this latter company became a wholly owned subsidiary of Illumina, Inc. in January 2007), he was appointed as a lecturer in bioorganic chemistry at the University of Rouen. From February 2004 to August 2013, he co-facilitated with Prof. Pierre-Yves Renard the bioorganic chemistry research team from the COBRA laboratory (UMR CNRS 6014). In September 2013, he was appointed as a full professor at the University of Burgundy and a junior member of the French University Institute (IFU). He also joined the ICMUB laboratory (UMR CNRS 6302) and his current research interests mainly focus on the development of advanced chemical tools (“smart” optical (bio)probes, novel fluorogenic reactions, and cross-linking reagents for multiple bioconjugation) for biosensing and bioimaging applications. He is co-author of more than 85 scientific papers and co-inventor of over a dozen patents; three of them are actively being pursued by the Illumina Company (genome analyzer DNA sequencing technology).
This synthetic strategy was primarily used by the groups of Burkart and Fossey for the preparation of a primary alcohol derivative of BHQ-2 and diol-appended methyl red analogues, respectively. The primary alcohol of BHQ-2 was converted into the activated para-nitrophenyl carbonate to achieve solid-phase labeling of ybbR peptide, used in combination with tetramethylrhodamine-coacetyl Ac onjugate to develop a high-throughput screening (HTS) assay for assessing inhibitors of the phosphopantetheinyl transferase Sfp. Diol-appended methyl red analogues were used in FRET-based probes with fluorescein as a reporter and suitable for monitoring the boronate ligation reaction. In 2012, the group of Kool developed a set of aldehyde-reactive fluorescence quenchers by introducing an α-nucleophile, such as oxymine and hydrazine moieties. Their great ability to quench the emission of fluorescein was assessed in the context of duplex DNA oligonucleotides made of two complementary strands labeled with an aminooxy/hydrazine quencher through oxime/hydrazone ligation and fluorescein, respectively. Interestingly, further functionalization of some of these BHQ-3 derivatives (e.g., azido derivative) can be readily achieved through postsynthetic click reactions (e.g., CuAAC), aimed at introducing 1) a novel valuable chemical function, the stability of which is not necessarily fully compatible with the azo-coupling reaction conditions; 2) neutral or ionic (charged) hydrophilic group(s) to impart water solubility and biocompatibility; or potentially 3) targeting moiety/balancing domain to improve in vivo performances, including specificity, biodistribution, and pharmacokinetics of NIR fluorescence imaging agents based on FRET pairs, including BHQ-3 as an acceptor. Such simple and reliable synthetic methodology was also applied to shorter wavelength dark quenchers, namely, BHQ-1 and BHQ-2, especially by us and the groups of Wagner and Klymchenko. The bioconjugation ability and quenching efficiency (QE) of some of these BHQ derivatives were demonstrated through the preparation and enzymatic activation (both in vitro and in living cells) of FRET-based fluorogenic substrates for urokinase plasminogen activator.
(uPA), a key protease in cancer invasion and metastasis.\cite{25,27,28}

Table 1 compiles all bioconjugatable BHQ derivatives published in the last six years. Depending on the biological vector to label and/or the conjugation site/reaction chosen, researchers may select the most appropriate BHQ within this valuable synthetic toolbox.

2.2. Synthesis of “Tuned” Dark Quenchers for Specific Applications

Although significant advances have been achieved to expand the nature of reactive groups and/or functionalities that provide specific properties to DABCYL, BHQ, or BBQ derivatives, synthetic efforts have exclusively focused on the side chains of these dye molecules (i.e., tertiary amine moiety) without altering their core structure and spectral characteristics. However, the development of more innovative “smart” fluorescent imaging agents and/or biosensing assays requires the availability of more sophisticated azo-based dark quenchers, the molecular scaffold of which is dramatically different from that of the parent chromophore, especially to obtain multivalent molecular platforms that allow access to unprecedented photoactive (bio)molecular systems, or to improve both spectrum width (full-width at half-maximum, $\Delta \lambda_{1/2}$) and QE parameters. In this context, in 2014, Kovaliov et al. reported a nucleoside–DABCYL analogue, namely dUDAB, which could be incorporated anywhere in an oligonucleotide sequence, in any number, and used as a quencher in different hybridization-sensitive probes (Figure 3a).\cite{36,37} The key step in the synthesis of a phosphoramidite synthon of dUDAB was a Suzuki palladium-catalyzed cross-coupling reaction between 5’-O-dimethoxytrityl-5-ido-2′-deoxuridine and the pinacol boronate ester of 4-((N,N-dimethylamino)azobenzene (DAB). In addition to its ability to quench fluorescein emission, photoisomerization of the dUDAB moiety upon irradiation at $\lambda = 365$ nm confirms its function as a photoswitch, which is particularly useful for the photocapture of DNA/RNA duplex formation. By analogy with multivalent bioconjugation platforms currently developed for applications in chemical biology and drug discovery,\cite{38} heteromultifunctional AB scaffolds that harness sequential click and/or bioorthogonal...
nal reactions should find significant utility, especially in the rapid construction of broad-spectrum dark quenchers and emerging multicomponent fluorogenic ensemble chemosensing systems, including dual-FRET-based fluorescence probes for the sequential detection of two distinct enzyme activities or probes installed with dual-reactive and -quenching groups for highly selective and sensitive sensing of a single analyte. The first examples of heteromultifunctional ABs have been developed by our research group and are shown in Figure 3b and c. Using two prefunctionalized primary and tertiary amines in the synthesis of the DABCYL core, we designed an azidocarboxylic acid azo-based quencher that was used for the preparation of a water-soluble, clickable DABCYL analogue through a postsynthetic sulfonation approach. At that time, this enabled us to fill a gap in the chemistry of azo-based quenchers, namely, the development of a versatile synthetic method to rapidly access highly polar derivatives without using harsh conditions (i.e., concentrated H$_2$SO$_4$ to perform aromatic sulfonation, see example of BHQ-10; Figure 3d) or a hydrophilic tertiary amine in azo coupling (e.g., N-alkyl-N-sulfopropylanilines), which sometimes exhibited poor reactivity/solubility, depending on the conditions used for this reaction. A clever extension to this preliminary work has also led to the first hetero-trifunctional thiol–alkyne–carboxylic acid azo-based dark quencher derived from a nitro-DABCYL core. The derivatization of this trivalent AB derivative with two other nonfluorescent azo dyes (BHQ-1 and BHQ-3), through effective thioalkylation and CuAAC reactions, has led to a universal dark quencher (UDQ) that is able to turn OFF an array of organic-based fluorophores covering the UV/NIR ($\lambda = 300–750$ nm) spectral range (see below).

2.3. Toward UDQs

The choice of dark quenchers as the donor part in FRET pairs is often limited by the required overlap between the absorbance spectrum of the donor and the emission spectrum of the acceptor (fluorophore). Despite some examples highlighting the possibility of effective quenching through the formation of a ground-state complex (intramolecular dimer between quencher and fluorophore) between two FRET partners not complementary from a spectral point of view, this static quenching approach, which is highly dependent on the chemical structures of donor and acceptor units, cannot be generalized. In this context, some recent studies have been devoted to the expansion of the quenching range (QR) of azo-based dark quenchers. The first achievements from our research group in this field were based on the covalent association of three spectrally distinct and complementary azo dyes within the same molecular architecture and by using the hetero-trifunctional nitro-DABCYL (structure shown in Figure 3c) as the central core. Essentially, the simultaneous quenching action of these three azo dyes provided a QE of up to 95% for organic-based fluorophores, the emission of which is centered in the range of $\lambda = 340–770$ nm (except for a sulfoindocyanine dye Cy 7.0, QE = 81%). The major drawback of this unusual azo-based quencher is clearly its considerable size, which could be a major limitation in biological applications through preventing its cell permeability or that of bioconjugates containing it. The issue of the extension of the QR with smaller molecules was first addressed in 2011, when the Kool and Crisalli had the ingenious idea to associate two donors to one acceptor within a small azo- or bis(azo)-based scaffold, with the aim of allowing multiplicity of conjugation pathways of varied length, leading to a positive effect of broadening the absorption spectrum of the resulting multipath quenchers (MPQs).
Figure 4. Structures and spectral absorption features of universalazo-based dark quenchers recently reported in the literature\textsuperscript{46,48} (nd = not determined). Notably, for MPQ-6, the absorption (Abs) maxima and QR were determined in the context of a 20-mer quencher–oligonucleotide conjugate.

Figure 4a) exhibits the most redshifted absorption maxima ($\lambda = 536$ and 656 nm) and a valuable spectrum width (full-width at half-maximum, $\Delta \lambda_{1/2\ max}$) of $\lambda = 270$ nm, ranging from $\lambda = 460$ to 730 nm, when conjugated to DNA oligonucleotides (15- or 20-mer). This quencher is able to turn OFF the emission of a wide range of fluorescent organic dyes, ranging from Alexa Fluor $350$ (7-aminocoumarin derivative) to Quasar 670 (cyanine Cy 5.0 derivative). The QE was determined in the context of DNA oligonucleotide duplexes placing the fluorophore and MPQ-6 within contact distance (to assess the efficiency of contact quenching, which was found in the range of 72–98%, depending on the fluorophore) or beyond this distance (to assess the efficiency of the mixed-contact FRET quenching mechanism and FRET quenching; found in the range of 65–95 and 50–78%, respectively). Historically, this work represents the first example of structural tuning of azo-based quenchers to improve their spectral properties.

By analogy with this promising approach, we have designed another class of broad-spectrum azo-based quenchers by using an anilinium moiety of unsymmetrical rhodamines as the acceptor group; these are named azo-sulforhodamine dyes (Figure 4b, SR101-Q-X).\textsuperscript{49} The use of an aniline-based fluorophore (e.g., 7-aminocoumarins, boron-dipyrromethene (BODIPY) derivatives with an amino group in the 2/6-position, pyronins, rhodamines, rhodols, N-substituted 4-amino-1,8-naphthalimides) as the primary aniline partner in azo-coupling reactions is also beneficial to easily bring valuable functionalities (e.g., targeting and/or water-solubilizing moieties) onto the resulting AB-caged fluorophore by taking advantage of the reactive groups already available on the starting fluorescent aniline (e.g., onto the meso-phenyl ring of xanthene dyes). The recent synthesis of unsymmetrical sulforhodamine SR101-110 achieved by our research group has enabled the practical implementation of this approach,\textsuperscript{48} which involves an azo-coupling reaction mediated by NOBF$_4$\textsuperscript{26} and leading to quenchers SR101-Q-X. By changing only the nitrogen substituent of the tertiary aniline partner, it was possible to introduce a wide range of biconjugatable handles (primary amine, terminal alkyne, azido, carboxylic acid, and maleimide) onto the SR101-Q scaffold, while retaining two sulfonate groups on the meso-phenyl ring, which is particularly beneficial for water solubility. The bioconjugation ability and excellent QE values (in the range of 93–99%, depending on the fluorescent donor) of these SR101-Q-X derivatives were demonstrated through the preparation and in vitro enzymatic activation of FRET-based fluorogenic substrates of uPA.

2.4. Future Prospects

All innovations in molecular design and synthetic chemistry of azo-based quenchers accomplished in the past five years have led to a wide array of valuable photoactive building blocks suitable to meet present and future challenges of biosensing/bioimaging involving the use of fluorescent chemodosimeters. Nevertheless, there is still an urgent need for broad-spectrum dark quenchers capable of completely switching OFF fluorophores emitting in the Cy 7.0/Cy 7.5 spectral region (i.e., $\lambda > 750$ nm, QE > 95%). In 2015, the group of Hanaoka has described silicon–rhodamine-based NIR dark quenchers (SiNQs), which show broad absorption covering this region.\textsuperscript{49} They are nonfluorescent, independent of solvent polarity and pH, probably owing to free rotation of the bond between the nitrogen atoms and the xanthene moiety (Figure 5a). A similar strategy was recently applied to a DHX–hemicyanine-fused scaffold\textsuperscript{50} (Figure 5b), but, in both cases, the synthetic access to these sophisticated molecules involves reactions more complicated than that of a conventional azo coupling. Thus, the development of effective azo-based NIR dark quenchers remains a major challenge in the field of chemistry of such azo dyes. With regard to biolabeling of peptides/proteins with an azo-based quencher, the recent development of bench-stable diazonium reagents and mild conditions to perform tyrosine-selective (or its genetically encoded 2-naphthol analogue) modifications of peptides/proteins through site-specific azo cou-
The first step leading to the radical anion intermediate is strongly dependent on oxygen concentration (i.e., oxygen partial pressure, pO₂) in the medium. Low levels of oxygen (hypoxia status, pO₂ = 0.1–5%) prevents the radical anion from reverting back to the neutral AB, and the radical is stabilized and further reduced to hydrazine, the –NH–NH– single bond of which is finally cleaved to give the two corresponding anilines.

From a biological standpoint, hypoxia is a feature of tumor tissue and a notable phenomenon in the occurrence and growth of solid tumors. In oncology, the formation of hypoxic tumors usually leads to a poor response to curative treatments and poor clinical outcome, owing to their resistance to radiation and chemotherapy (indeed, abnormal microvessels of

often, reductase or chemical reducing agent; approach 3). For the first approach, numerous FRET-based activatable probes containing an enzyme-cleavable substrate for the detection of biologically relevant hydrolytic enzymes (mainly proteases) in various biological contexts (i.e., extracellular fluids to cells, tissues, and various in vivo animal models) have been reported in the literature and already compiled in some comprehensive reviews. Herein, we focus only on a detailed description of the two other approaches to turn “ON” or ratiometric fluorescence detection (Table 2, entries 2 and 3) upon either reductive decomposition of FRET-based probes (approach 2) or conversion of an azo-based profluorophore into a fluorescent organic dye through reductive decomposition, elimination, or metal-catalyzed cyclization of the –N–N– double bond into a triazole (approach 3). Interestingly, alternative activation mechanisms that produce optical signal enhancement have also been implemented in azo-based chromo-/fluorogenic chemosensors for anion/cation detection, but they are not discussed herein.

### 3. Azo-Based Fluorogenic Probes

A survey of the literature on azo-based fluorogenic probes identified three main distinct probe design principles (summarized in Table 2). An azo dye can be either used as a dark quencher in the context of FRET-based probes (approaches 1 and 2) or as a profluorophore, which is defined as an AB-caged fluorophore, the activation of which is based on a “mild” chemical event induced by the targeted (bio)analyte (most

Figure 5. Structures of NIR dark quenchers recently reported in the literature (WS = water-solubilizing, DHX = dihydroxanthene). Please note: spectral absorption features of N-aryl DHX-hemicyanine hybrid was determined in EtOH.

Figure 6. Tyrosine-selective modification of peptides through site-specific azo coupling with the aim of creating a FRET pair (Ac = acetyl, Fluo = organic-based fluorophore, Tyr = l-tyrosine).
such tumors limit the perfusion of cytotoxic chemotherapeutic drugs). Thus, the assessment of the degree of tumor hypoxia is of great importance in predicting the efficacies of anticancer therapies. Among the different methods currently available to achieve this, those based on the fluorescence detection of hypoxia biomarkers (e.g., reductases and hypoxia inducible factor (HIF)) are widely explored. In this context, particular attention is paid to fluorogenic probes that are selectively activated by AzoRs. Interestingly, pioneering works from Biosearch Technologies have shown that some AzoRs (e.g., group II AzoR BTI1, working in tandem with NADPH) are able to readily cleave the azo bond of quenchers of fluorescence (i.e., BHQ-10 and Janus Green B (JGB), which is the non-bioconjugatable version of BHQ-3). As a natural extension of these findings, FRET-based probes (named QCys), involving the covalent association of a far-red fluorescent cyanine dye (Cy 5.0, Cy 5.25, or Cy 5.5 derivative) with the complementary azo-based quencher BHQ-3 through a carboxamide linkage, have been developed by the group of Nagano for in vivo imaging of hypoxia related to ischemia (Figure 7). The reductive cleavage of the azo bond of BHQ-3 induces a loss of quenching ability and, as a consequence, the restoration of the far-red emission of cyanine at \( \lambda = 670 \text{ nm} \) for high-level hypoxia (\( pO_2 = 1\% \) or less). This claimed activation mechanism of AzoR-sensitive fluorogenic QCy probes was demonstrated through an in vitro assay by using rat liver microsomes placed under normoxic and hypoxic conditions. Next, probe QCy5 was successfully applied for both visualizing the hypoxic status of MCF-7 breast cancer cells and in vivo imaging of ischemic organs (liver and kidney) in live mice. More recently, the group of Jayagopal published

Table 2. Overview of azo-based fluorogenic probes (FRET-based probes and profluorophores) currently used for reaction-based biosensing/bioimaging: schematic representation and principle for activation.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Probe design principle</th>
<th>Origin/function of azo moiety</th>
<th>Activation mechanism</th>
<th>Targeted analyte(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>quencher FRET acceptor</td>
<td>cleavage of the linkage between fluorophore and quencher, inducing the loss of FRET</td>
<td>hydrolytic enzymes, such as esterases, phosphatases, phospholipases, and proteases, or nucleophilic species, such as biothiols</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>quencher FRET acceptor</td>
<td>reduction of azo bridge, inducing the loss of quenching ability of azo dye</td>
<td>redox enzymes with AzoR activity</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>amine-masking group (left) reactive center for analyte (middle) reactive center for analyte (right)</td>
<td>reduction of azo bridge, inducing the release of a fluorescent aniline (left) nucleophilic displacement or oxidation of the azo bridge and subsequent release of fluorophore (middle) metal-catalyzed cyclization of the azo bridge to triazole moiety (right)</td>
<td>biothiols or hypochlorous acid (middle) copper(II) ions (right)</td>
<td></td>
</tr>
</tbody>
</table>

Scheme 2. Mechanism generally assumed for the AzoR-mediated reduction of azo dyes. The cofactor NADH or NADPH is omitted for clarity.
a similar azo-based FRET probe, namely, HYPOX-3, which involved a commercial monosulfonated derivative of Cy 5.25 as a fluorescent donor (named NIR-667-carboxylic acid, Abs/Em λ = 667/689 nm) and BHQ-3 as dark acceptor; both were linked through a carboxamide linkage.\(^{[60]}\) Similar results to those obtained with the QCy5 probe were observed for the fluorescence imaging of the hypoxic status of retinal cells (R28 cells). Further investigations demonstrated the potential utility of HYPOX-3 for early detection and timely treatment of retinal diseases (i.e., blinding retinal vascular diseases, including age-related macular degeneration, diabetic retinopathy, and retinopathy of prematurity) through in vivo retinal imaging of hypoxia.\(^{[63]}\) Furthermore, in the field of bioreductively activated FRET probes, the group of Qian reported an alternative donor–acceptor pair: rhodamine B (RhB) and a novel azo-based quencher derived from 8-N-substituted 4-amino-1,8-naphthalimide scaffold (named HPN) covalently associated through an ester linkage (Figure 8).\(^{[64]}\) This corresponding turn ON probe HP appeared to be a more sensitive chemodosimeter with detection observed from 10% \(p_{O_2}\), which proved that this novel FRET strategy was also a good way to detect low levels of hypoxia. The excellent oxygen sensitivity of HPN in living cells (HeLa cells) is linked to the reduction potential of its azo moiety, which is undoubtedly lower than that of the \(-N=\equiv N-\) double bond of BHQ-3. Nevertheless, we should express some reservations about in cellulo results because the ester linkage involved in the construction of this AzoR-sensitive FRET pair may be cleaved by esterases, to give the same fluorescence response (Em: \(\lambda = 570–600\) nm upon excitation at \(\lambda = 540–560\) nm) as that obtained after bioreduction of the azo bridge. In conclusion, all of these published results clearly show the usefulness of azo-based quenchers as reduction-responsive units in FRET probes for the detection of hypoxia.\(^{[65]}\) Furthermore, the same probe design principle was also applied to other applications, including 1) the tracking of microbial degradation of azo-based organic pollutants through green fluorescence unveiling of a nitrogen-substituted 4-piperidinyl-1,8-naphthalimide scaffold\(^{[23]}\) (Figure 9a), and 2) the validation of a new concept of chemically deactivatable quencher for FRET-based probes that turn ON their blue fluorescence (7-N,N-diethylaminocoumarin-3-carboxamide derivative as donor) through either enzymatic cleavage or the use of a chemical reagent (Na\(_2\)S\(_2\)O\(_4\)); both were applied to quantify caspase-3 activity in solution and visualize unreacted probes in apoptotic cells (Figure 9b).\(^{[22]}\) The only noticeable drawback associated with all bioreductively activated FRET probes is their large molecular size and structure, which may negatively influence cell penetration and the affinities of the involved redox enzymes to these fluorogenic two-component assemblies. A valuable alternative is the use of small-molecule activatable agents (also known as profluorophores) derived from common organic-based fluorophores (generally, fluorescent amines) and designed both to integrate an azo-based quenching unit within their core structure and to react selectively with their target (i.e., redox enzyme or reactive analyte).

3.2. Approach 3: Profluorophores Containing an Azo Moiety as a Quenching Unit

3.2.1. Fluorescence Activation through Reductive Cleavage of the Azo Bridge and Subsequent Release of Luminescent Aniline

Growing interest in alternative chemical structures of azo dyes required for high-tech applications (e.g., dye-sensitized solar cells, anion sensors) has recently led chemists to focus on the synthesis of hybrid structures based on the functionalization of organic-based fluorophores with one or several (di)aryl azo moieties. As partially illustrated in Figure 10, several fluorescent organic dyes belonging to different families, including BODIPYs,\(^{[66,67]}\) fluoresceins,\(^{[68]}\) 1,8-naphthalimides,\(^{[53,55]}\) porphyrins,\(^{[69]}\) perylenes,\(^{[70]}\) and pyrenes,\(^{[71]}\) have been converted into non-fluorescent azo dyes through either diazotization of their primary amino groups or covalent conjugation to a conventional bis(aryl) azo dye.

Based on this unconventional strategy for fluorophore quenching, we have considered the reversible masking of primary aniline(s) of rhodamines through the direct conjugation
of the \(-N-N-\) group(s). In addition to the ultrafast \(E/Z\) photoisomerization process of \(-N-N-\) bond(s), locking of the xanthenes platform in a nonconjugated spirolactone form may also contribute to a greater QE than that obtained with the FRET mechanism. Once again, the use of mild diazotization conditions previously mentioned (see Section 2) has enabled us to efficiently synthesize the first bioreductively activatable profluorophore derived from the green-emitting rhodamine 110 (Rho110) dye, and suitable for the detection of AzoR activity both in vitro and in the context of bacterial cultures.

Figure 8. Top: Structure and fluorescence mechanism of FRET-based probe HPN for hypoxia imaging, as reported by Qian et al.145 Bottom: Fluorescence and bright-field images of HeLa cells at different oxygen concentrations. The concentration of DMSO in the culture medium was 1%. The filter type was Texas-Red (excitation (Ex): \(\lambda = 540–560\) nm; emission (Em): \(\lambda = 570–600\) nm). Copyright 2015 Royal Society of Chemistry.

Figure 9. a) Structures of FRET-based probes reported by the group of Xu for the tracking of microbial degradation of azo-based organic pollutants.143 b) Structures of FRET-based probes reported by the group of Wagner for the quantification of caspase-3 activity in solution and visualization of unreacted probes in apoptotic cells.157 (CDQ = chemically deactivatable quencher; DEAC = 7-N,N-diethylamino coumarin; MR = methyl red, Asp = L-aspartic acid, Glu = L-glutamic acid, Gly = glycine, Val = L-valine).

Figure 10. Structures of nonfluorescent azo dyes derived from organic-based fluorophores (BODIPY166 (a), fluorescein168 (b), 1,8-naphthalimide155 (c), porphyrin160 (d), perylene170 (e) and pyrene171 (f)) already reported in the literature.
Asimilar approach was used by Li et al. to rationally design a turn ON reactive probe for the quantification of endogenous sulfides (i.e., predominant forms of gasotransmitter H$_2$S at physiological pH) in rat tissues. A set of AB-caged O-methyl rhodols were synthesized by using the Mills reaction (i.e., condensation between substituted nitrosobenzenes and O-methyl rhodol). The pentafluorophenyl derivative emerged as the best candidate, in light of its high reactivity towards the reducing analyte, resulting in high sensitivity (148-fold fluorescent switch-on response at $\lambda = 517$ nm, upon excitation at $\lambda = 468$ nm), good selectivity (22-fold more selective towards sulfides than other biothiols, such as cysteine, glutathione (GSH), and homocysteine) and low detection limit (500 nM; Figure 12). Further extension of this fluorogenic reduction process to other xanthene scaffolds belonging to the family of rhodamines (2-methyl rhodamine green (2Me RG) and its red-emitting silicon analogue (2Me SiR600)) was explored by the group of Nagano, to detect different levels of hypoxia (Figure 13, left-hand side). Promising small-molecule imaging agents, namely, MAR and MARS, were obtained, and MAR was successfully used to visualize retinal hypoxia in a rat model of retinal artery occlusion. For the dual purpose of redshifting the fluorescence and tuning the physicochemical properties (water solubility and cell permeability) of these hypoxia imaging agents without resorting to multistep de novo syntheses, AB-caged sulforhodamine dyes with either two methyl groups or two clickable azido groups were designed and prepared by our group (Figure 13, right-hand side). Post-synthetic derivatization with a positively charged or zwitterionic terminal alkyne and the CuAAC reaction led to compounds SR101-NaphthNH$_2$-Hyp-X ($X =$ sulfobetain or ammonium). These two fluorogenic probes were readily activated by reductases and NADPH under physiological conditions to give...
The A549 cells were incubated with SR101-NaphthNH$_2$-Hyp-diMe, MAR, or MASP (1 μM) containing DMSO (0.1 %) as a cosolvent at various oxygen concentrations (0.1, 1, and 20 %) for 6 h. Scale bar: 30 μm. The excitation wavelength and emission ranges of detection were λ = 488 and 515–553 nm, respectively, for MAR; λ = 590 and 600–670 nm, respectively, for SR101-NaphthNH$_2$-Hyp-diMe; and λ = 600 and 620–700 nm, respectively, for MASP. Copyright 2015 IOP Publishing Ltd.

SR101-NaphthNH$_2$, leading to 45- and 58-fold fluorescence emission enhancement at λ ≈ 625 nm within 1 h for sulfobetain and ammonium, respectively. However, their ability to image the hypoxic status in living A549 cells was a failure, possibly because their sizes were too large and/or they were too hydrophilic, which limited their cell penetration. Conversely and positively, fluorescence enhancement inside cells under severe hypoxia (0.1 % O$_2$) was obtained for non-postfunctionalized SR101-NaphthNH$_2$-Hyp-diMe (Figure 14). Likewise, Shin et al. recently conducted a systematic study on azo-rhodamines as fluorogenic substrates through the synthesis of a library of ten compounds, to establish a relationship between structure and reactivity towards AzoR. They found that azo-rhodamines with an electron-donating group (typically N,N-dimethylamino) underwent the two-electron reduction of –N=N– to the hydrazine by AzoR, followed by a nonenzymatic reaction, to afford the fluorescent product 2-Me RG, and appeared to be suitable fluorogenic substrates for an AzoR reporter system. With the aim of obtaining a turn ON response in the far-red or NIR range, the same AzoR sensing strategy was recently implemented to a dicyanomethylene-4H-chromene (DCMC) fluorophore, the emission of which was centered at λ = 650 nm (Figure 15).

The installation of an azo bridge between two 2,2'-bipyridyl (bpy) chelating units of a phosphorescent dinuclear ruthenium(II) or iridium(III) complex has also been considered by the group of Chao and reductively activatable probes suitable for sensing biothiols and endogenous (bis)ulfides (predominant forms of sulfur dioxide), respectively, were obtained (Figure 16a–c and 17a) . The main advantages of these phosphorogenic probes, relative to conventional aniline-based phosphorophores previously discussed, are red emission (λ ≈ 600 nm), large Stokes shifts (typically λ = 165–200 nm), and the opportunity for time-resolved fluorescence measurements, even if this last feature was not used in these studies. As a highly relevant application, azo-based iridium(III) complexes (IrX) have been used as multicolor phosphorescent probes to detect hypoxia in both adherent living cells and oxygen-gradi- ent 3D multicellular spheroids at depths of over 100 μm, which have many similarities to the in vivo environment (Figure 16d). More recently, the group of Choi showed that an azo-based binuclear ruthenium(II) complex, Ru–azo, could also be used as a smart photosensitizer in two-photon photodynamic anticancer therapy (Figure 17b). Indeed, this complex showed high accumulation in the mitochondria of cancer cells and produced efficient photocytotoxicity upon bioreductive activation by GSH and two-photon excitation at λ = 810 nm.

In addition to these reaction-based probes, in which the analyte-responsive azo unit is always directly connected to aniline-based luminophore, it is worth mentioning the unusual ratiometric azo-probes reported by the group of Freeman and for which the azo moiety acts as a conformational locking group to promote the formation of a fluorescent dimer/excimer (Figure 18). Bacterial reduction of the azo group leads to a conformational change of the cyclohexane ring and subsequent dimer/excimer disruption revealed by a blueshifted fluorescence emission.

3.2.2. Fluorescence Activation through Elimination of the (Di)Aryl Azo Moiety

As for the examples discussed above, this second class of azo-based profluorophores were developed based on the quenching ability of the AB moiety either fused to a fluorescent core or covalently conjugated to an organic-based fluorophore. However, the activation mechanism does not necessarily involve the reductive cleavage of the azo bridge, but also its displacement through nucleophilic substitution or oxidation reactions mediated by the target (bio)analyte. The first example il-

![Figure 14](image-url) Fluorescence confocal microscopy images of SR101-NaphthNH$_2$-Hyp-diMe, MAR, or MASP in live A549 cells. The A549 cells were incubated with SR101-NaphthNH$_2$-Hyp-diMe, MAR, or MASP (1 μM) containing DMSO (0.1 %) as a cosolvent at various oxygen concentrations (0.1, 1, and 20 %) for 6 h. Scale bar: 30 μm. The excitation wavelength and emission ranges of detection were λ = 488 and 515–553 nm, respectively, for MAR; λ = 590 and 600–670 nm, respectively, for SR101-NaphthNH$_2$-Hyp-diMe; and λ = 600 and 620–700 nm, respectively, for MASP. Copyright 2015 IOP Publishing Ltd.

![Figure 15](image-url) Structure and fluorescence mechanism (reductive cleavage of the azo bridge and subsequent release of fluorescent aniline) of far-red turn ON fluorescent probe AZO-DCM (Em: λ$_{\text{ex}}$ = 650 nm) applied in cytochrome P450 reductase (AzoR activity) detection and hypoxia imaging in tumor cells.
Illustrating these alternative reaction-based strategies was published in 2014 by Lim et al., and focused on the development of an NIR fluorescent probe, MitoGP, based on an azo-heptamethine cyanine fused chromophore, for the selective detection of mitochondrial GSH over cysteine and homocysteine. A dramatic fluorescence turn ON response at $\lambda = 810$ nm was obtained upon removal of the PeT-active 4'-nitro-4-hydroxyazo-benzene moiety through an addition–elimination process induced by GSH. Furthermore, the lipophilic cation unit available within the core structure of the cyanine dye (i.e., indole unit of Cy 7.0; N-quaternarized with propionic acid butyl ester) acted as an effective mitochondria-targeting moiety (Figure 19). Another nonemissive fluorophore–azo dye conjugate, namely, Hyp-Ly, was recently reported by Luo et al. that relied on the covalent association between 4-(N,N-dimethylamino)-4'-methylazobenzene and a 3-N-piperazinyl-BODIPY through a carbamate of mitochondrial GSH over cysteine and homocysteine. A dramatic fluorescence turn ON response at $\lambda = 810$ nm was obtained upon removal of the PeT-active 4'-nitro-4-hydroxyazo-benzene moiety through an addition–elimination process induced by GSH. Furthermore, the lipophilic cation unit available within the core structure of the cyanine dye (i.e., indole unit of Cy 7.0; N-quaternarized with propionic acid butyl ester) acted as an effective mitochondria-targeting moiety (Figure 19). Another nonemissive fluorophore–azo dye conjugate, namely, Hyp-Ly, was recently reported by Luo et al. that relied on the covalent association between 4-(N,N-dimethylamino)-4'-methylazobenzene and a 3-N-piperazinyl-BODIPY through a carbamate.
Analogous to the azo-based profluorophores described in Section 3.2.1, this fluorogenic compound can be readily activated by reductive enzymes (typically AzoR activity), but the formed aniline (a para-aminobenzyl derivative) is able to undergo further 1,6-elimination (self-immolation process) to release the green–yellow fluorescent BODIPY BOD-Ly. This piperazine-based BODIPY can be protonated in an acidic environment and trapped within cellular organelles such as lysosomes (pH 4.5–5.5), which are vital machines in mediating a variety of physiological processes, including protein degradation and restoration, intracellular transport, and plasma membrane repair. Live-cell fluorescence microscopy experiments with human liver cancer cell line HepG2 have shown the potential of this azo-based profluorophore to visualize lysosomes in hypoxic tumor cells (Figure 21).

In addition to these studies, an unprecedented activation mechanism of azo-based profluorophores through the oxidation of the −N=N− double bond by hypochlorous acid (HClO) was recently independently reported by two Chinese research groups (Figure 22). The nonfluorescent probe YDN was readily obtained through a conventional azo coupling between meta-phenylenediamine and 5-amino-fluorescein. Green fluorescence of the parent fluorescein (centered at λ = 516 nm) was unveiled upon reaction with HClO under simulated physiological conditions (i.e., phosphate-buffered saline (PBS), 10 mM, pH 7.4). Relatively stable and good performances in the fluorescence turn ON response were obtained for pH 5–9. High selectivity of YDN for HClO was demonstrated through competitive experiments with other reactive oxygen species (ROS)/reactive nitrogen species (RNS). Finally, the potential of YDN for imaging HClO was evaluated in both living cells (HeLa cells) and live mice. The same probe design principle was also applied to an ESIPT fluorophore, namely, HBT, the fluorescence emission maximum of which was in the blue region (centered at λ = 466 nm). The corresponding probe PHC2 featured a high degree of selectivity and sensitivity for HClO with a low detection limit (13.2 nM) under simulated physiological conditions (i.e., PBS, 10 mM, pH 7.4 + 1% EtOH). However, contrary to YDN, no fluorescence imaging experiments in cells or more complex biological systems were performed; no doubt because the blue fluorescence emitted by the released HBT is only really fully compatible with in vitro measurements.

These recent examples contribute to make AB leaving groups valuable assets in the design of fluorogenic turn ON probes for the detection of a wide range of reactive (bio)analytes, especially nucleophilic species. Considering the large number of nucleophilic biomolecules present in living cells (e.g., proteins/peptides, thiols, gaseous signaling molecules, ROS/RNS), which would be useful to detect, this way of designing reaction-based fluorescent probes will probably be developed in the coming years, provided that the probe presents a high selectivity for the targeted biological nucleophile.
3.2.3. Fluorescence Activation through Metal-Catalyzed Cyclization of the Azo Bridge to the Triazole Moiety

The last fluorescence activation mechanism implemented in the context of azo-based profluorophores relies on the inhibition of $\text{N} = \text{N}$ isomerization and subsequent oxidative cyclization to a triazole moiety catalyzed by the target analyte. With the aim of developing a turn ON reactive probe for fluorescence detection of copper(II) ions in neutral aqueous solutions, Jo et al. synthesized a water-soluble and nonemissive azoaniline dye (A; i.e., AB derived from meta-phenylenediamine; Figure 23). Coordination of the copper(II) ion favors a cascade of electron- and proton-transfer events, leading to the in situ formation of a highly fluorescent 2-phenylbenzotriazole derivative (B; Figure 23). This chemistry can be readily effected by micromolar-level concentrations of copper(II) ions in water (pH 6–8) at room temperature to elicit >80-fold enhancement in the green emission at $\lambda = 530$ nm. Remarkably, this azo-based probe showed excellent sensitivity to and selectivity for copper(II) over other metal ions (Figure 23). More recently, the same fluorescence turn ON signaling mechanism was implemented to more sophisticated anilines with the dual aim of widening the working pH range and improving the sensitivity of this unusual class of copper(II)-sensitive probes (Figure 24a). This was achieved with coumarin–azo dye C, which exhibited excellent sensitivity within a wide range of pH (3–10).

Figure 22. Structures and fluorescence mechanism (oxidation of the azo bridge and subsequent release of fluorophore) of azo-based profluorophores developed for the detection of HClO (HBT = 2-(2-hydroxyphenyl)benzothiazole).
mimic, and copper(II) ions (i.e., 63 nm bathochromic shift of absorbance maximum, as a result of the phenol phosphorylation reaction and turn ON emission at \( \lambda = 455 \text{ nm} \) induced by \( \text{Cu}^{2+} \)). Some other organometallic reaction approaches leading to the conversion of the azo quenching unit into substituents promoting greater \( \pi \) conjugation are likely to be considered to monitor other biologically relevant metals (especially those implicated in diseases) in a variety of oxidation states.

4. Summary and Outlook

Major advances over the past 15 years in the design and synthesis of site-specifically functionalized azo dyes with enhanced spectral properties were presented. The main goal of these studies was to provide effective fluorescence quenchers that were fully compatible with challenging analytical applications in various biological systems. The practical implementation of these advanced chemical tools in the context of analyte-sensitive fluorogenic probes constituted the second part of this literature survey. Thus, representative examples of
small-molecule fluorescent probes, the activation mechanism of which was based on chemical/enzymatic deactivation of the (di)aryl azo unit, were discussed. Except for some specific and rare examples, a turn ON emission increase was preferred and obtained to dramatically improve the signal-to-noise ratio and maximize spatial resolution in bioimaging experiments. The most popular FRET-based probes, in which the azo dye acts as an effective dark quencher, are mainly used for the detection of hydrolytic enzyme activities. However, the recent identification of biocompatible chemical reactions triggered by relevant (bio)analytes and known to selectively affect the chemical integrity of the (di)aryl azo moiety opens up new promising perspectives in this field of bioimaging. The use of azo-based quenchers as sonophores for optoacoustic imaging, recently demonstrated by a first proof-of-concept reported by Grimm et al.,[96] is certainly a complementary path that needs to be explored. Indeed, BHQ-1 exhibited a strong and concentration-dependent optoacoustic signal, and its conjugation to a cyclic RGD-peptide led to a targeted contrast agent, which was effective for detecting α₅β₃-integrin-expressing glioblastoma tumors in vivo. In addition to these innovations related to diagnostics, some research groups had the idea to take advantage of the hypoxia-dependent bioreductive metabolism of azo compounds in the prodrug design of anticaner agents. Some self-immolative prodrugs have been designed to generate the corresponding active drug upon reductive activation by AzoR and subsequent domino processes often involving cyclization reactions.[97] Further implementation of this concept of hypoxia-activated prodrugs to nanocarriers, such as polymer–drug conjugates (through a covalent strategy), micelles, nanoparticles, and polymersomes (through encapsulation strategies), has already been extensively studied for cancer therapy.[62, 98, 99] The possible synthetic access to unsymmetrical AB derivatives acting as both an azo-based profluorophore and a prodrug should promote advances in small-molecule theranostics (Figure 25a).[100] Indeed, these compact and biocompatible (bio)molecular conjugates are attractive candidates for both diagnosis and therapy owing to their unique features, such as targeted delivery, imaging abilities, facile modification, reliability, and real-time information on drug release and pharmacokinetics. The first practical example of this dual imaging/therapy strategy was recently published by Verwilst et al. and concerned the design and construction of a theranostic drug-delivery system (DDS) for cancer therapy (Figure 25b).[101] It was based on an AB scaffold that acted as both a fluorescence quencher (i.e., nonfluorescent azo-RhoB hybrid) and deactivator of an alkylating anticancer drug (i.e., nitrogen mustard N,N-bis(2-chloroethyl)- para-phenylenediamine), and it also contained a TPP group that combined excellent targeting of the hyperpolarized mitochondria of cancer cells in solid tumors with a convenient means for evading drug resistance. As claimed by the authors and supported by preliminary in vivo therapeutic experiments, this bioreductively activated theranostic DDS could result in the selective eradication of hypoxic regions within solid tumors, providing better treatment options for this challenging fraction of cells within solid tumors. Without a doubt, this first spectacular achievement will boost further innovations in this field and practical biomedical applications.

Acknowledgements

We acknowledge INSA Rouen, Rouen University, CNRS, Labex SynOrg (ANR-11-LABX-0029), région Haute-Normandie (CRUNCH network), and FEDER (TRIPODE, no. 33883, for a Ph.D. grant to A.C. (October 2011–September 2014)) for their financial support. A.R. also thanks the Institut Universitaire de France (IUF), the Burgundy region (“FABER” program, PARI Action 6, SSTIC 6 “Imagerie, instrumentation, chimie et applications biomédicales”), the French Embassy in Singapore (PHC Merlion grant 2015, 5.04.15), and Iris Biotech GmbH company to fund his research activities at ICMUB (since September 2013).

Keywords: azo compounds · biosensors · fluorescence · FRET · imaging agents

Cytochrome and uridine azo dye analogues derived from 6-aminouracil (named 6-aminoaracil-containing nucleobases), which form good FRET pairs with 2-amino-purine (2AP) and pyrrolo-lysozyme (PyC), have been recently described, see: N. S. Freeman, C. E. Moore, L. M. Wilmshurst, Y. Tor, J. Org. Chem. 2016, 81, 4530–4539.


H. E. Johansson, M. K. Johansson, A. C. Wong, E. S. Armstrong, E. J. Pe-...

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Manuscript received: May 7, 2017
Accepted manuscript online: June 8, 2017
Version of record online: July 21, 2017
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