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Functional Near-Infrared Fluorescent Probes

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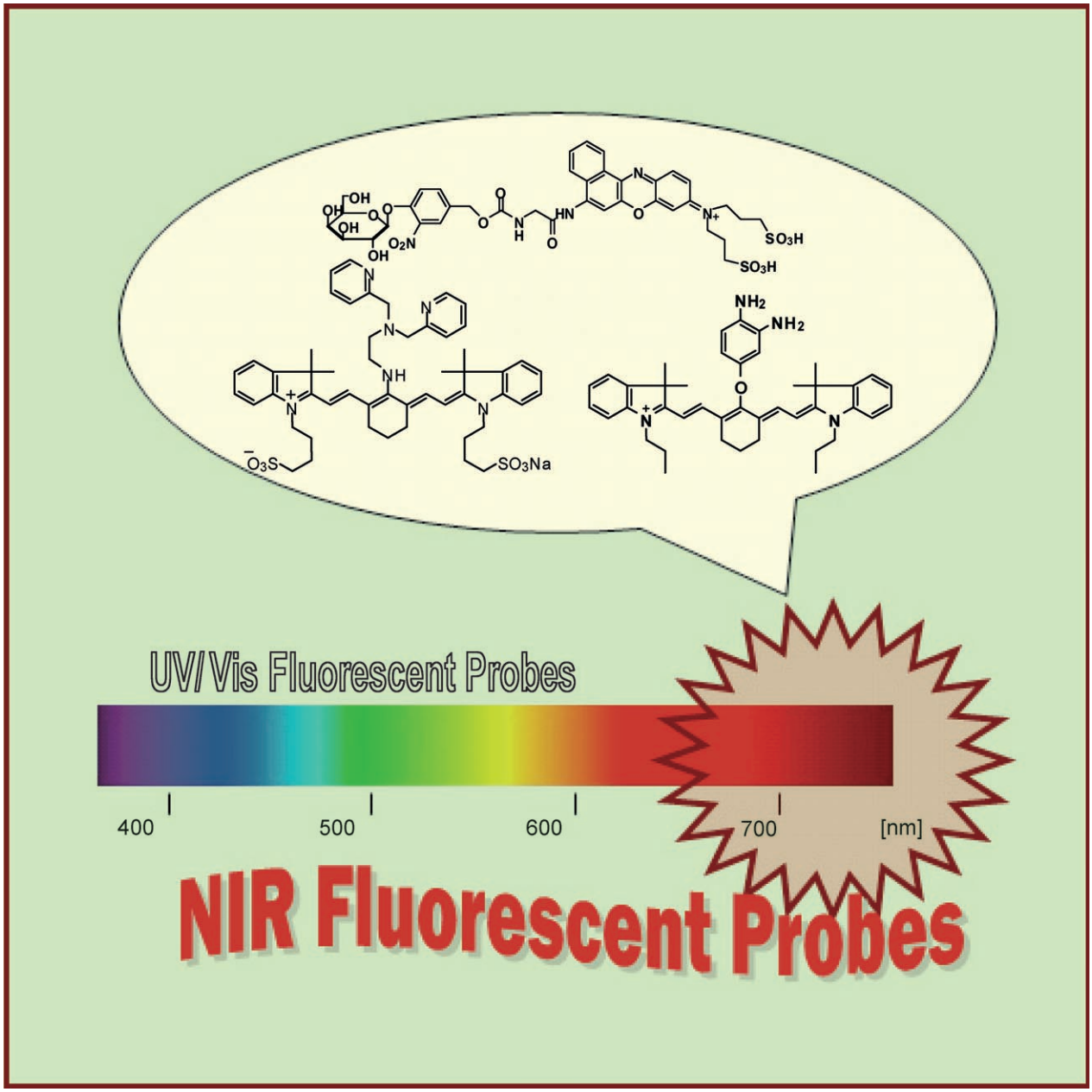

Abstract: Near-infrared (NIR) fluorescent probes have attracted much attention, but despite the availability of various NIR fluorophores, only a few functional NIR probes, that is, probes whose absorption and/or fluorescence spectra change upon specific reaction with biomolecules, have been developed. However, functional probes operating in the NIR range that can be targeted to protons, metal ions, nitric oxide, β-galactosidase, and cellular stress markers are expected to be effective for fluorescence imaging in vivo. This Focus Review concentrates on these functional NIR probes themselves, not their applications.

Keywords: biosensors · electron transfer · fluorescent probes · functional probes · ratiometric probes

1. Introduction

Fluorescence imaging is widely used in various research fields, because it has fine temporal and spatial resolution and can be safely performed with simple instruments and facilities. Molecular imaging, including fluorescence imaging, has proved useful for clinical diagnosis[1] and drug discovery,[2] and contrast agents such as Gd^{3+} complex[4] or fluorodeoxyglucose[3] are used in MRI (magnetic resonance imaging) or PET (positron emission tomography) for clinical diagnosis.[5] However, fluorescent probes are hardly used for clinical diagnosis. This is because most conventional fluorescent probes for bioimaging are based on chromophores with peaks in the UV/Vis region, in which absorption by biomolecules is high. Light in this wavelength region is well-absorbed by biomolecules, for example, water and hemoglobin. Furthermore, visible light is easily scattered and cannot penetrate deeply into tissues. There is also strong autofluorescence in this region, which causes high background noise. These drawbacks result in low signal-to-noise ratios, and thus conventional dyes are unsuitable for in vivo imaging.[6]

On the other hand, light in the near-infrared (NIR) region (650–900 nm) is relatively poorly absorbed by biomolecules and can penetrate deeply into tissues. There is also less autofluorescence in this region.[6,7] Thus, much higher signal-to-noise ratios can be obtained, and probes that absorb and emit in the NIR are expected to be suitable for in vivo imaging. Recently, many NIR imaging agents, such as fluorophore–biomolecule conjugates, have been developed and applied to vascular mapping, evaluation of tissue perfusion, detection of inflammation and atherosclerosis, and so on.[7] Some NIR fluorophore–biomolecule conjugates are already commercially available for in vivo imaging.[8] However, because they are always fluorescent, high background noise, which causes low signal-to-noise ratios, is often a problem. Therefore, fluorescent probes whose fluorescence intensity changes upon specific reaction with biomolecules are required for in vivo imaging. Herein, we review recent developments relating to such functional NIR fluorescent probes.

2. NIR Fluorescent Probes Activated by Proteases

Generally, when two fluorophores are in close proximity to each other, they become nonfluorescent because of self-quenching. Therefore, changing the distance between two fluorophores by means of enzymatic or other reactions can provide a means of controlling fluorescence intensity. Recently, Weissleder et al. developed a method of imaging tumor-associated lysosomal protease activity in a xenograft mouse model in vivo by using NIR fluorescent probes based on the above mechanism.[9] Probes were bound to a graft copolymer of poly-L-lysine and methoxypolyethylene glycol succinate (Scheme 1). Following intravenous injection into nude mice, the NIR fluorescent probe carrier accumulated in solid tumors, and an intratumoral NIR fluorescence signal was generated through cleavage of the macromolecule by lysosomal proteases in tumor cells, thereby releasing the quenching. In vivo imaging showed a 12-fold increase in the NIR fluorescence signal, thus allowing the detection of submillimeter-sized tumors.

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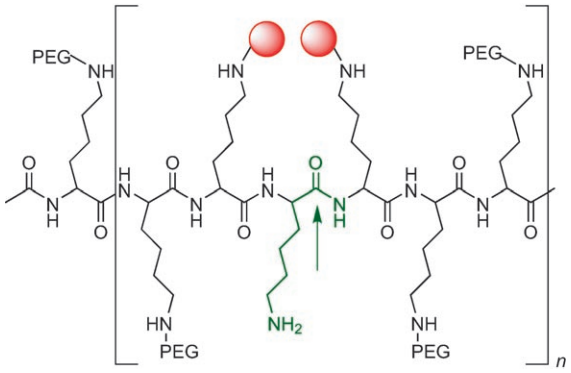
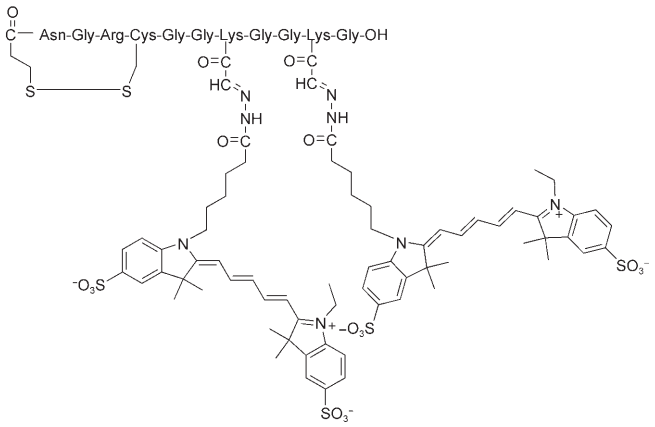


Scheme 1. Chemical structure of segment of repeating graft copolymer indicating quenching of Cy and site of enzymatic degradation (green arrow).



Scheme 2. Structure of self-quenching pH probe.

Weissleder and co-workers then developed some NIR protease probes based on the same strategy.[10] Some are commercially available for imaging in animals.

3. NIR Fluorescent Probes for Detecting pH Changes

Intracellular pH changes in response to diverse physiological and pathological processes; thus, quantitative measurement of pH is useful for cellular research or diagnosis. An acidic environment may be related to inflammation or tumor.[11] Consequently, many pH probes have been developed, and some are commercially available.[12] However, most of them have fluorophores that absorb and emit in the visible region, and there are few NIR pH probes.

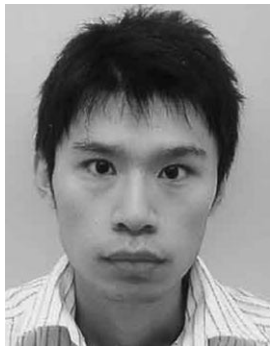
3.1. Self-Quenching Fluorescent pH Probes

Weissleder and co-workers found that acid-catalyzed hydrolysis can be used as the basis for designing a new type of fluorescent probe for detecting acidic environments.[13] They combined two dicarbocyanines and a peptide with an acid-labile hydrazone linker (Scheme 2). At the physiological pH of 7.4, this is weakly fluorescent owing to self-quenching. At pH 4.5, the hydrazone linker is cleaved, and the dicarbocyanines are released from the peptide, thus resulting in a more-than-threefold fluorescence enhancement.

The probe can be applied to the monitoring of tumor angiogenesis and the probing of the acidic environment of the

Abstract in Japanese:

近年、近赤外蛍光プローブが注目を集めている。しかしながら、近赤外蛍光色素の有用性とは裏腹に、例えばターゲットと反応して蛍光特性が変化するような機能を有するプローブは驚くほど少ない。その一方で、機能性蛍光プローブが臨床診断や薬効評価への応用等、医学薬学領域において大きな可能性を秘めているのもまた事実である。本稿では、そのような機能性プローブに焦点を絞り、概説する。



Kazuki Kiyose *was born in 1982. He obtained his MSc in Pharmaceutical Sciences (2007) from the University of Tokyo. Currently, he is a PhD student working with Dr. T. Nagano. He is also a Research Fellow of the Japanese Society for the Promotion of Science (JSPS). His research interest is in the development and application of NIR fluorescent probes.*

Hirotatsu Kojima *is currently an Associate Professor at the Chemical Biology Research Initiative at the University of Tokyo. His main research interests are in the design, synthesis, and application of fluorescent probes for molecular imaging in vivo. He has developed several nitric oxide probes, such as diaminofluoresceins (DAFs) and diaminorhodamines (DARs). He is also working on the development of a chemical library, a collection of small organic molecules that can be used as chemical probes to regulate protein function.*

Tetsuo Nagano *is a Full Professor at the Lab. of Chemistry and Biology, Graduate School of Pharmaceutical Sciences, the Univ. of Tokyo and Director General of its Chemical Biology Research Initiative. His research interests are in the molecular design, synthesis, and applications of novel bioimaging probes for physiologically active species such as NO, Zn^{2+}, singlet and reactive oxygen, and caspase. He has developed more than 40 bioimaging fluorescent probes through rational molecular designs based on different mechanisms, of which 12 are commercially available and used by biologists worldwide. He has also received many prizes.*

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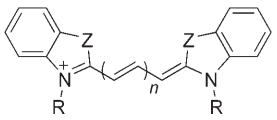
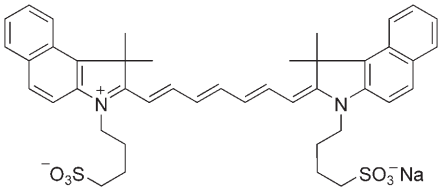
tumor interstitium, and it also provides a tool for in vivo imaging of the pharmacokinetics of hydrazone-linked prodrugs.

3.2. Norcyanine-Based NIR Fluorescent pH Probes

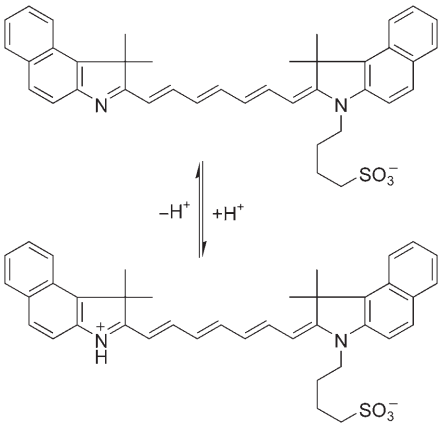
The general structure of cyanine dye is shown in Scheme 3. Dyes with R = H, so-called norcyanines, are pH-sensitive, because they have a protonatable amine group within the fluorophore core. The nordicarbocyanines ($n=2$) and nortricarbocyanines ($n=3$) show absorption and emission peaks in the NIR region and can work as pH probes. Alonso and coworkers synthesized nortricarbocyanine with various substituents on the indole ring and reported the relation between substituent and pK_a.[14] Zhang and Achilefu focused on pH in the physiologically relevant range.[15] They modified the structure of indocyanine green (ICG) (Scheme 4) and developed a novel NIR pH probe, H-ICG (Scheme 5), which has strong NIR absorption and fluorescence emission at pH < 4.



Scheme 3. General structure of cyanine dyes.



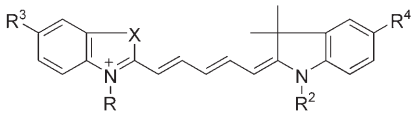
Scheme 4. Structure of indocyanine green (ICG).



Scheme 5. Mechanism of pH response by protonation and deprotonation of H-ICG.

The simple structure of H-ICG and its ease of synthesis, solubility in aqueous media, and pK_a of 7.2 are advantageous for biomedical research.

Cooper and co-workers synthesized pH probes based on nordicarbocyanines.[16] The photochemical properties of their probes are similar to those of H-ICG, but their probes contain a pendant carboxylic acid that allows rapid conversion into the NHS ester, thus facilitating the labeling of biological molecules such as proteins or antibodies. This feature is expected to be widely useful. A series of these pH probes was synthesized (Scheme 6), which led to CypHer5, which has a carboxy group and a pK_a of 6.1 (Table 1);[17] this pK_a



I: R = H, $R^2 = (CH_2)_4SO_3H$, $R^3 = SO_3H$, $R^4 = CH_2CO_2H$, X = $C(CH_3)_2$
II: R = H, $R^2 = CH_2CH_3$, $R^3 = SO_3H$, $R^4 = SO_3H$, X = $C(CH_3)_2$
III: R = $(CH_2)_5CO_2H$, R^2 = H, R^3 = H, $R^4 = SO_3H$, X = S
IV: R = H, $R^2 = (CH_2)_5CO_2H$, $R^3 = SO_3H$, $R^4 = SO_3H$, X = $C(CH_3)_2$
V: R = H, R^2 = H, $R^3 = CO_2H$, $R^4 = SO_3H$, X = $C(CH_3)_2$

Scheme 6. Range of Cy5 pH-sensitive fluorescent probes.

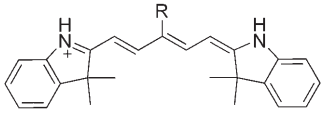
Table 1. Spectral properties of Cy5 pH-sensitive probes.

Dye	λ_{max} (abs) [nm]		λ_{max} (em) [nm]	pKa
	pH 4.67	pH 9	(ex=630 nm)	
I	645	484	665	7.5
II	650	515	665	6.4
III	640	531	670	6.85
IV	653	501	660	7.5
V (CypHer5)	655	500	665	6.1

value is excellent for biological applications. At pH > 7, CypHer5 is > 95% deprotonated, and this causes a change in the absorption spectrum such that the fluorophore cannot be excited at 633 nm.

By N-terminally tagging GPCR (G-protein-coupled receptor) with an epitope and labeling an antibody to that epitope with CypHer5, the internalization of GPCR from the membrane (pH 7.4) to the endosomal pathway (pH 4–5) can be monitored. This was the first report of the application of an NIR pH probe.[17]

Hilderbrand and Weissleder subsequently developed novel NIR pH probes with lower pK_a values than CypHer (Scheme 7 and Table 2).[18] These probes are almost nonfluorescent at pH 7.4, but emit strong fluorescence at lower pH. They showed that the Cl-substituted dye can distinguish between samples of mouse urine (pH 6.78) and blood (pH 7.69) by fluorescence reflectance imaging. They also



R = H, Cl, Br

Scheme 7. Structure of novel NIR pH probes.

Table 2. Optical properties of the novel pH probes in Scheme 7 in aqueous media.

R in dye	λ_{abs}[a] [nm]	λ_{abs}[a] [nm]	ε[a] [$M^{-1}cm^{-1}$]	Θ[a] [%]	pK_a[b]
H	638	664	141000	12.9	6.3
Cl	642	663	167000	6.4	5.7
Br	639	659	146000	3.5	5.7

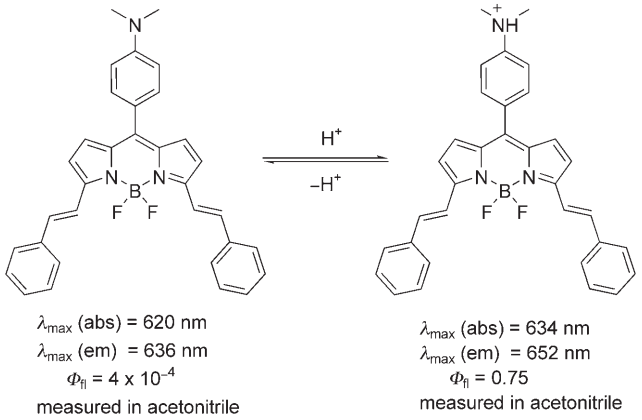
[a] Measured in 50 mM glycine buffer at pH 3.0. [b] Determined by pH titration in 50 mM phosphate/citric acid buffer.

showed that the pK_a values of the dyes can be modulated by variation of the heteroatom substituents on the polymethine-conjugated system.

3.3. Difluoroboradiaza-*s*-indacene-Based NIR Fluorescent pH Probes

4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) is a well-known fluorophore with a high molar extinction coefficient and high fluorescence quantum efficiency (Φ_{fl}) that has found widespread application in biological research.[19,20] BODIPYs that emit in the NIR region have recently been used to obtain functional probes.[21]

Daub and co-workers developed a fluorescent pH probe based on distyryl-BODIPY (Scheme 8).[22] This is essentially



Scheme 8. Structure and photochemical properties of NIR pH probe based on distyryl-BODIPY.

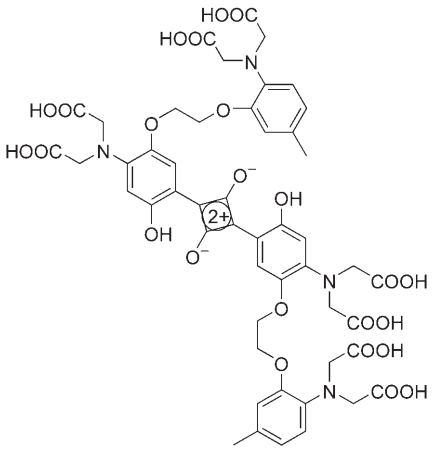
nonfluorescent in a highly polar environment, but becomes strongly fluorescent in the presence of protons. The pK_a value was determined to be 2.32 in ethanol/water (1:1 *v*/*v*). This probe is unsuitable for biological research because of its low pK_a value and low solubility in water, but its quantum efficiency is much higher than that of norcyanine-based probes. Structural modification to improve the pK_a value and solubility in water may be feasible.

4. NIR Fluorescent Probes for Metal Ions

4.1. Calcium Probes

Calcium is an important secondary messenger. Many biological processes are regulated by temporal and spatial fluctuations in calcium concentration. Some excellent fluorescent probes for calcium have been developed.[19]

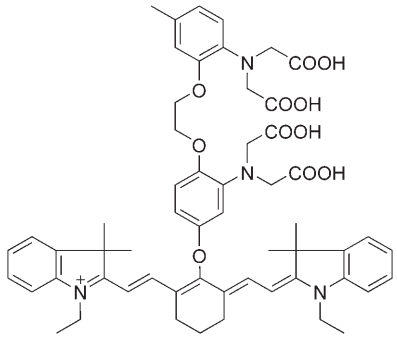
Akkaya and Turkyilmaz developed a squaraine-based probe for calcium (Scheme 9).[23]



Scheme 9. Structure of NIR probe for Ca^{2+} based on squaraine.

In pH 7.2 aqueous buffer, the fluorescence intensity decreased in the presence of Ca^{2+}, whereas a large excess of Mg^{2+} ions had no effect on either the absorption or the emission spectrum. The dissociation constant was determined to be 3.4 μM, which seems suitable for biological studies, for example, of large ion spikes near the cellular membrane.

Ozmen and Akkaya also developed a tricarbocyanine-based probe for Ca^{2+} (Scheme 10).[24] The absorption and



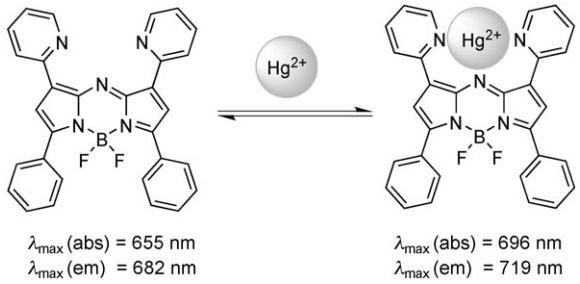
Scheme 10. Structure of NIR probe for Ca^{2+} based on tricarbocyanine.

emission maxima are at 766 and 782 nm, respectively, which is suitable for fluorescence imaging in vivo. The fluorescence is modulated through the mechanism of photoinduced electron transfer (PeT). The quantum efficiency was found to be

0.05 for the free chelator and 0.12 for the probe–Ca^{2+} complex. Thus, their work demonstrates that effective PeT-generated fluorescence signaling is possible in the NIR range. The dissociation constant was determined to be 240 nM, which is close to the resting concentration of intracellular Ca^{2+}, so the dynamic range of this probe is practical for biological applications.

4.2. Mercury Probe

Akkaya and co-workers also developed an NIR fluorescent probe for Hg^{2+}.[25] They synthesized bis(2-pyridiyl)-substituted boratriazaindacene (AzaBODIPY) and found that the 2-pyridyl substituents at the 1- and 7-positions create a well-defined pocket for metal-ion binding. At a sufficiently high concentration of Hg^{2+}, a 35-nm red shift of the absorption maximum and a 37-nm red shift of the emission maximum were observed, which indicates that this probe could work as a ratiometric probe for Hg^{2+} (Scheme 11). The dissociation constant was determined to be 5.4×10^{-6} M, and the quantum efficiencies in acetonitrile were 0.19 for the probe and 0.17 for the probe–Hg^{2+} complex. This probe shows outstanding metal-ion selectivity because of the rigid nature of the ligand and the selectivity imposed by the type of donor atom. This is the first AzaBODIPY analogue acting as a probe for metal ions, and its mechanism of metal-ion sensing is unique. However, its solubility in water is very poor, so improvement will be necessary for application in biomedical imaging.



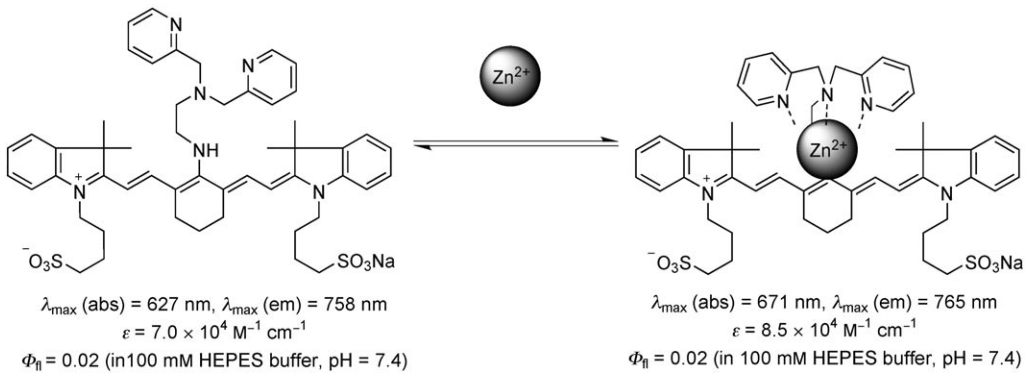
Scheme 11. Structure and photochemical properties of NIR Hg^{2+} probe based on AzaBODIPY.

4.3. Zinc Probes

Zinc is the second-most-abundant transition-metal ion in the human body, where it has multiple roles in both intra- and extracellular functions.[26] Fluorescent sensor molecules offer useful information about chelatable Zn^{2+} in cellular systems, because we can study the concentration or distribution of Zn^{2+} in real time, and fluorescence imaging of Zn^{2+} has become a widely used technique.[19,27] Many probes for Zn^{2+} have been developed; however, we were the first to develop a zinc ion probe that operates in the NIR.[28] A series of tricarbocyanine derivatives were synthesized to examine the relationship between the nature of the amine substituent and the photochemical properties. It was found that the lower the electron density of the amine, the longer the wavelength of the absorption maximum, with little change in the emission maximum.

Thus, we synthesized dipicolylcyanine (DIPCY; Scheme 12), which has an amine-substituted tricarbocyanine as the NIR fluorophore. It has a high extinction coefficient of 7.0×10^4 M^{-1} cm^{-1} and a large Stokes shift, and the dipicolylethylenediamine (DPEN) moiety acts as a metal chelator. When the dipicolylamine coordinates to a metal ion, the electron density of the DPEN moiety is decreased, thus lowering the electron-donating ability of the amine in the fluorophore.

At a sufficiently high concentration of Zn^{2+}, a 44-nm red shift of the absorption maximum was observed, which indicates that DIPCY could work as a ratiometric probe for Zn^{2+} (Scheme 12). The apparent dissociation constant (K_d) of (98 ± 0.9) nM at pH 7.4 in HEPES buffer for Zn^{2+} was determined by plotting the fluorescence ratio at 760 nm for 627- and 671-nm excitation, and it was confirmed by plotting the absorption ratio at 627 and 671 nm. The metal selectivity of DIPCY was investigated. Various metal ions other than Co^{2+} and Cu^{2+}, whose concentrations in biological materials are extremely low, had little influence on the Zn^{2+}-induced red shift of the absorption maximum. This result indicates that DIPCY is Zn^{2+}-selective (Figure 1).



Scheme 12. Structure and photochemical properties of NIR Zn^{2+} probe.

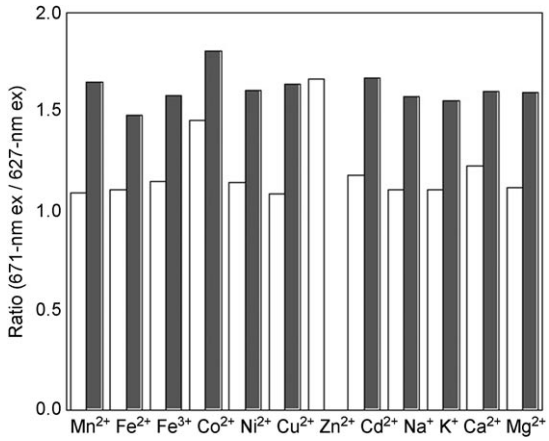
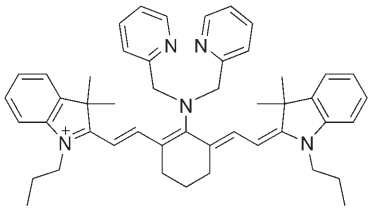


Figure 1. Metal-ion selectivity of DIPCY. Bars indicate the fluorescence ratio (671/627-nm excitation, 760-nm emission). DIPCY (1 μM) was added to heavy metals (1 μM) and other agents (5 mM). All samples were measured in 100 mM HEPES buffer, pH 7.4, $I=0.1$ ($NaNO_3$). White bars: the respective cation was added. Dark bars: the respective cation and zinc ion were added.

This fluorescence modulation of amine-substituted tricarbocyanines should be applicable to dual-wavelength measurements of various biomolecules or enzyme activities. Studies along this line are in progress, and novel ratiometric probes should be reported shortly.

Just after we reported DIPCY, Tang et al. developed a fluorescent probe for Zn^{2+}, DPA-Cy, which utilizes a PeT mechanism (Scheme 13).[29] They chose a tricarbocyanine with two propyl groups, which can readily penetrate cellular



Scheme 13. Structure of DPA-Cy.

membranes, as the NIR fluorophore, and 2,2-dipicolylamine (DPA) to chelate zinc. Inhibition of PeT by coordination of d^{10} transition metals or protons to amines is a commonly observed mechanism for fluorescence enhancement. DPA-Cy gave a 20-fold turn-on response for detecting Zn^{2+}. Tang et al. applied DPA-Cy with a Zn^{2+}-selective ionophore to macrophage cells (Raw264.7 cells) to examine whether it worked in biological systems, and they showed that DPA-Cy is cell-permeable and can respond quickly to Zn^{2+}.

5. NIR Fluorescent Probes for Nitric Oxide

Nitric oxide (NO), which is synthesized through conversion of L-arginine into L-citrulline by NO synthase in vivo, is an important signaling molecule involved in the regulation of a wide range of physiological and pathophysiological mechanisms, and many disorders related to the impairment of NO signaling have been reported.[30] Therefore, methods for visualizing NO would be powerful tools for examining in detail the mechanisms of NO signaling[31] and might eventually be useful for diagnosis as well.

Recently, we developed several fluorescent probes for NO, whose fluorescence is modulated through a PeT mechanism, and these probes have been widely used in biological applications.

Figure 2A shows the increase in fluorescence intensity of DAC-P (an NIR NO probe; see Scheme 14) in rat kidney upon administration of NOC13, one of the NO donors. The

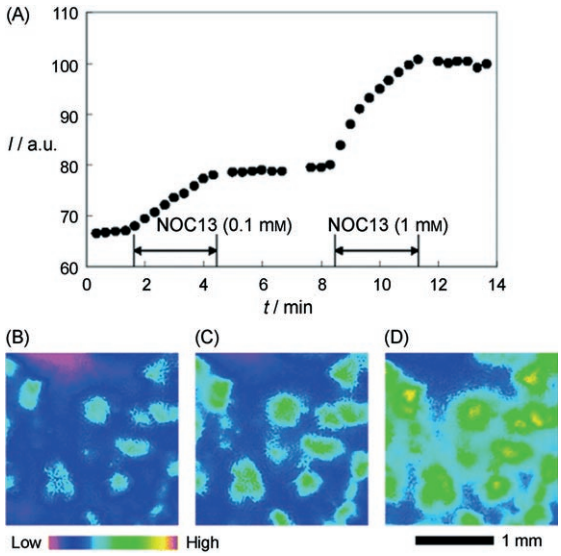
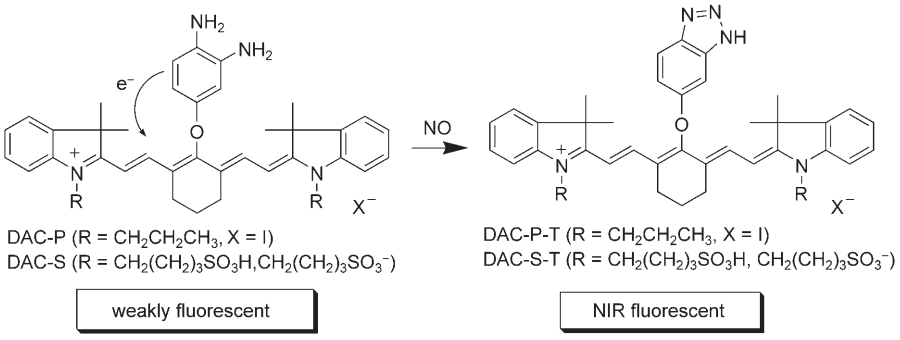


Figure 2. A) Increase in fluorescence intensity of DAC-P in rat kidney upon administration of NOC13. The fluorescence intensity is an average value calculated from an entire picture plane. The rat kidney was perfused with Krebs–Henseleit buffer at 5 mL min^{-1}. After loading of DAC-P (5 μM) for 4 min, NOC13 (0.1 or 1 mM) was administered for 3 min (indicated by arrows). B) Captured NIR fluorescence image of a part of a rat kidney after the loading of DAC-P. C) Image after the administration of NOC13 (0.1 mM). D) Image after the administration of NOC13 (1 mM). All images are reproduced in pseudocolor.

fluorescence intensity is an average value calculated from the entire picture plane. The rat kidney was perfused with Krebs–Henseleit buffer at 5 mL min^{-1}. After loading of DAC-P (5 μM) for 4 min, NOC13 (0.1 or 1 mM) was administered for 3 min (indicated by the arrows). Figure 2B shows the captured NIR fluorescence image of a part of the rat kidney after loading of DAC-P. Figure 2C and D shows the same kidney after administration of 0.1 mM and 1 mM NOC13, respectively. All the images are reproduced in pseudocolor.

Our NIR fluorescent probes for NO (DAC-S, DAC-P)[33] are based on the change in the electron-donating ability of *o*-phenylenediamine upon selective NO-mediated transformation of diamine into triazole under aerobic conditions (we named the corresponding triazole compounds DAC-Ts) (Scheme 14). The NIR fluorescence intensity of the DACs greatly increased in an NO-concentration-dependent



Scheme 14. Structure and photochemical properties of NIR NO probes.

manner, and the quantum efficiency of the DAC-Ts was 14-fold higher than that of the DACs. We confirmed that DAC could be employed in isolated rat kidneys (Figure 2).

Because the reaction of DACs with NO is fast and the observation of their NIR fluorescence is less subject to interference by biological substances, our NO-detecting probes are expected to be applicable not only to cellular, but also to in vivo NO imaging.

6. NIR Fluorescent Probes for β-Galactosidase

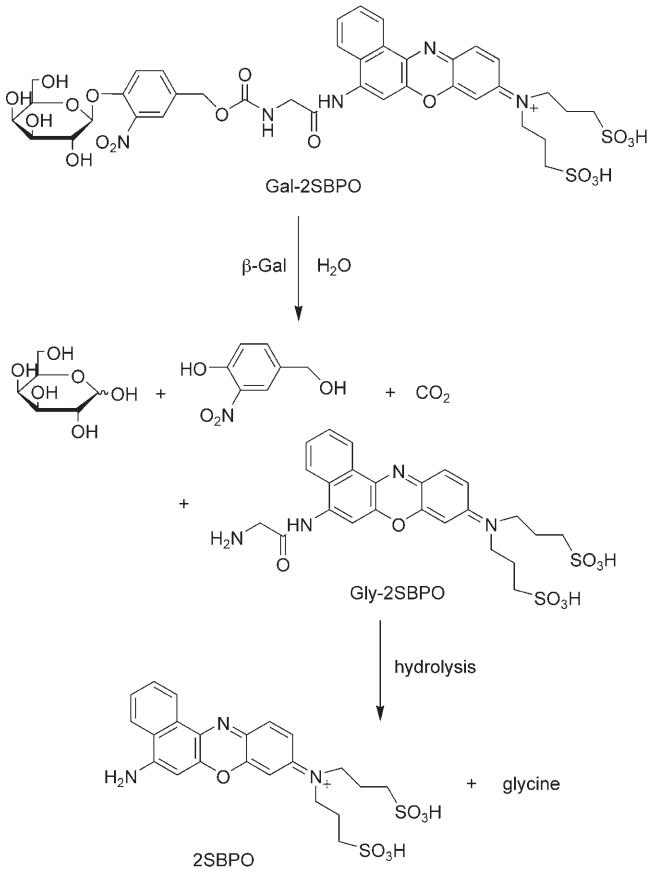
Escherichia coli β-galactosidase (β-gal) is well-characterized and extensively used as a marker enzyme in enzyme-linked immunosorbent assays (ELISA), in situ hybridizations, the classification of mycobacteria, and gene-expression studies. A wide variety of fluorescent probes for β-gal have been developed and are in widespread use.[34] However, they have various limitations with regard to in vivo applications.

Recently, Weissleder and co-workers developed an NIR fluorescent probe for β-gal sensing.[35] The probe is composed of three moieties: galactopyranoside as a β-gal substrate, 4-hydroxy-3-nitrobenzyl alcohol connected to a glycine residue through a carbamate linkage as a spacer, and disulfonated benzo[a]phenoxazine (2SBPO)[36] as a fluorophore. The spacer and 2SBPO were released through a cascade of spontaneous hydrolytic steps following enzymatic cleavage of the O-glycoside bond (Scheme 15). First, 4-hydroxy-3-nitrobenzyl alcohol was liberated, followed by the formation of Gly-2SBPO. The unstable Gly-2SBPO was further hydrolyzed to release glycine and free 2SBPO at neutral pH. A sevenfold increase in fluorescence was obtained. The kinetic parameters indicate that the catalytic efficiency of β-gal for the substrate is higher than for a non-self-immolative substrate. Because the substrate emits in the NIR region, it can be used as a reporter molecule for in vivo imaging of β-gal activity.

7. Lanthanide-Based NIR Fluorescent Probes for Cellular Stress Markers

Luminescent lanthanide complexes (Tb^{3+}, Eu^{3+}, etc.) have excellent properties for biological applications, including extraordinarily long lifetimes and large Stokes shifts.[37] However, there have been few reports of lanthanide-based functional probes, because of the difficulty in designing suitable complexes with a luminescence on/off switch.

Recently, we developed a rational design for luminescence probes and synthesized a long-lived protease probe in which

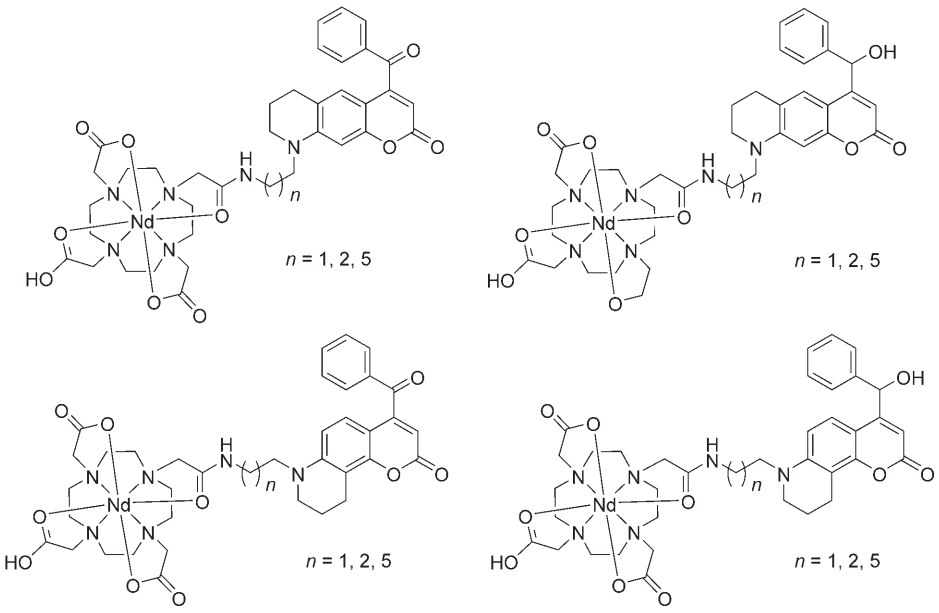


Scheme 15. Proposed mechanism of Gal-2SBPO activation.

luminescence is modulated through the PeT mechanism.[38] Furthermore, we confirmed the usefulness of the probe by applying it in clinical diagnosis. However, it is not suitable for in vivo imaging because the excitation is in the UV region.

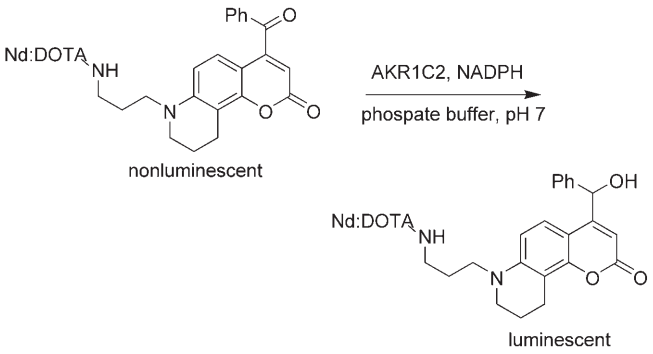
Sames and co-workers focused on an Nd^{3+} complex, which emits in the NIR range.[39] They developed an NIR luminescence redox probe, based on an Nd^{3+} complex, that serves as a reporter substrate for human aldoketoreductase (AKR1C2).

They investigated several aminocoumarin-Nd:DOTA pairs (Scheme 16; DOTA = 1,4,7,10-tetraazacyclododecane-



Scheme 16. The various compounds synthesized to examine the effect of linker length and mode of attachment of the Nd:DOTA complex on luminescence switching and enzyme–substrate properties.

N,*N*′,*N*″,*N*‴-tetraacetic acid) and found that alcohols all displayed characteristic Nd^{3+} luminescence with the emission maximum at around 1060 nm, whereas ketones had negligible emission in this region. Thus, the ketone/alcohol pair is a robust luminogenic switch in the reduction direction. They



Scheme 17. Enzymatic reduction of reporter substrates. NADPH = nicotinamide adenine dinucleotide phosphate, reduced form.

found that the compound shown in Scheme 17 is an excellent substrate for AKR1C2 with kinetic parameters comparable with those of the parent coumarin probe and the physiological steroid substrates. This substrate represents a promising NIR imaging probe for the cellular stress marker AKR1C2. Although the excitation wavelength is too short for use in vivo, two-photon excitation operating in the 800/1060-nm regime should be suitable for in vivo imaging.

8. Conclusions and Perspectives

Functional NIR fluorescent probes are attracting increasing attention, although relatively few biomolecule-targeting NIR probes have been developed. Poor solubility in water, low quantum efficiency, or cytotoxicity are issues in some cases, and at present fluorophore–biomolecule conjugates are much more widely used. However, compared with the long history of functional fluorescent probes working in the UV/Vis region, studies of NIR fluorescent probes have only just begun, and the scope for developing truly practical NIR fluorescent probes is enormous. This Focus Review has summarized briefly the progress to date.

[1] M. Rudin, R. Weissleder, *Nat. Rev. Drug Discovery* **2003**, 2, 123–131.

[2] N. Shah, A. Cerussi, C. Eker, J. Espinoza, J. Butler, J. Fishkin, R. Hornung, B. Tromberg, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 4420–4425.

[3] a) M. Botta, *Eur. J. Inorg. Chem.* **2000**, 399–407; b) H. Schmitt-Willich, M. Brehm, C. L. J. Ewers, G. Michl, A. Muller-Fahrnow, O. Petrov, J. Platzek, B. Raduchel, D. Sulzle, *Inorg. Chem.* **1999**, *38*, 1134–1144; c) R. J. Maxwell, J. Wilson, V. E. Prise, B. Vojnovic, G. J. Rustin, M. A. Lodge, G. M. Tozer, *NMR Biomed.* **2002**, *15*, 89–98; d) G. M. Nicolle, E. Toth, H. Schmitt-Willich, B. Raduchel, A. E. Merbach, *Chem. Eur. J.* **2002**, *8*, 1040–1048.

[4] a) P. Rigo, P. Paulus, B. J. Kaschten, R. Hustinx, T. Bury, G. Jerusalem, T. Benoit, J. Foidart-Willems, *Eur. J. Nucl. Med.* **1996**, 23, 1641–1674; b) S. S. Gambhir, C. K. Hoh, M E. Phelps, I. Madar, J. Maddahi, *J. Nucl. Med.* **1996**, *37*, 1428–1436; c) H. Young, R. Baum, U. Cremerius, K. Herholz, O. Hoekstra, A. A. Lammertsma, J. Pruim, P. Price, *Eur. Polym. J.* **1999**, *35*, 1773–1782; d) A. F. Shields, J. R. Grierson, B. M. Dohmen, H. J. Machulla, J. C. Stayanoff, J. M. Lawhorn-Crews, J. E. Obradovich, O. Muzik, T. J. Mangner, *Nat. Med.* **1998**, *4*, 1334–1336.

[5] J. H. Lee, Y. M. Huh, Y. Jun, J. Seo, J. Jang, H. T. Song, S. Kim, E. J. Cho, H. G. Yoon, J. S. Suh, J. Cheon, *Nat. Med.* **2007**, *13*, 95–99.

[6] a) R. Weissleder, V. Ntziachristos, *Nat. Med.* **2003**, *9*, 123–128; b) R. Weissleder, *Nat. Biotechnol.* **2001**, 19, 316–317.

[7] J. V. Frangioni, *Curr. Opin. Chem. Biol.* **2003**, *7*, 626–634.

[8] a) W. K. Moon, Y. H. Lin, T. O'Loughlin, Y. Tang, D. E. Kim, R. Weissleder, C. H. Tung, *Bioconjugate Chem.* **2003**, *14*, 539–545; b) A. Becker, C. Hessenius, K. Licha, B. Ebert, U. Sukowski, W. Semmler, B. Wiedenmann, C. Grotzinger, *Nat. Biotechnol.* **2001**, *19*, 327–331; c) G. Zheng, H. Li, K. Yang, D. Blessington, K. Licha, S. Lund-Katz, B. Chance, J. D. Glickson, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1485–1488; d) X. Y. Chen, P. S. Conti, R. A. Moats, *Cancer Res.* **2004**, *64*, 8009–8014; e) Z. Cheng, Y. Wu, Z. M. Xiong, S. S. Gambhir, X. Y. Chen, *Bioconjugate Chem.* **2005**, *16*, 1433–1441; f) S. Ke, X. X. Wen, M. Gurfinkel, C. Charnsangavej, S. Wallace, E. M. Sevick-Muraca, C. Li, *Cancer Res.* **2003**, *63*, 7870–7875; g) A.

Zaheer, R. E. Lenkinski, A. Mahmood, A. G. Jones, L. C. Cantley, J. V. Frangioni, *Nat. Biotechnol.* **2001**, *19*, 1148–1154.
[9] R. Weissleder, C. H. Tung, U. Mahmood, A. Bogdanov, *Nat. Biotechnol.* **1999**, 17, 375–378.
[10] a) U. Mahmood, C. H. Tung, A. Bogdanov, R. Weissleder, *Radiology* **1999**, 213, 866–870; b) C. H. Tung, S. Bredow, U. Mahmood, R. Weissleder, *Bioconjugate Chem.* **1999**, *10*, 892–896; c) C. Bremer, C. H. Tung, R. Weissleder, *Nat. Med.* **2001**, *7*, 743–748; d) C. H. Tung, R. E. Gerszten, F. A. Jaffer, R. Weissleder, *ChemBioChem* **2002**, *3*, 207–211.
[11] P. Montcourrier, P. H. Mangeat, C. Valembois, G. Salazar, A. Sahuquet, C. Duperray, H. Rochefort, *J. Cell Sci.* **1994**, 107, 2381–2391.
[12] a) M. A. Cousin, D. G. Nicholls, *J. Neurochem.* **1997**, *69*, 1927–1935; b) W. C. Sun, K. R. Gee, D. H. Klaubert, R. P. Haugland, *J. Org. Chem.* **1997**, *62*, 6469–6475; c) C. C. Overly, K. D. Lee, E. Berthiaume, P. J. Hollenbeck, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 3156–3160; d) Z. J. Diwu, C. S. Chen, C. L. Zhang, D. H. Klaubert, R. P. Haugland, *Chem. Biol.* **1999**, *6*, 411–418.
[13] A. K. Galande, R. Weissleder, C. H. Tung, *Bioconjugate Chem.* **2006**, *17*, 255–257.
[14] a) S. Miltsov, C. Encinas, J. Alonso, *Tetrahedron Lett.* **1998**, *39*, 9253–9254; b) C. Encinas, S. Miltsov, E. Otazo, L. Rivera, M. Puyol, J. Alonso, *Dyes Pigm.* **2006**, 71, 28–36.
[15] Z. R. Zhang, S. Achilefu, *Chem. Commun.* **2005**, 47, 5887–5889.
[16] M. S. Briggs, D. D. Burns, M. E. Cooper, S. J. Gregory, *Chem. Commun.* **2000**, 23, 2323–2324.
[17] M. E. Cooper, S. Gregory, E. Adie, S. Kalinka, *J. Fluoresc.* **2002**, *12*, 425–429.
[18] S. A. Hilderbrand, R. Weissleder, *Chem. Commun.* **2007**, 28, 2747–2749.
[19] R. P. Haugland, *The Handbook A Guide to Fluorescent Probes and Labeling Technologies*, *10th ed.*, Invitrogen, Carlsbad, **2005**.
[20] a) G. Ulrich, R. Ziessel, *J. Org. Chem.* **2004**, *69*, 2070–2083; b) Y. Gabe, Y. Urano, K. Kikuchi, H. Kojima, T. Nagano, *J. Am. Chem. Soc.* **2004**, *126*, 3357–3367.
[21] a) Y. H. Yu, A. B. Descalzo, Z. Shen, H. Rohr, Q. Liu, Y. W. Wang, M. Spieles, Y. Z. Li, K. Rurack, X. Z. You, *Chem. Asian J.* **2006**, *1*, 176–187; b) T. Rohand, W. W. Qin, N. Boens, W. Dehaen, *Eur. J. Org. Chem.* **2006**, 4658–4663; c) Y. J. Mei, P. A. Bentley, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3131–3134; d) Y. J. Mei, P. A. Bentley, W. Wang, *Tetrahedron Lett.* **2006**, *47*, 2447–2449; e) S. Atilgan, Z. Ekmekci, A. L. Dogan, D. Guc, E. U. Akkaya, *Chem. Commun.* **2006**, 42, 4398–4400; f) A. Coskun, E. Deniz, E. U. Akkaya, *Org. Lett.* **2005**, *7*, 5187–5189.
[22] K. Rurack, M. Kollmannsberger, J. Daub, *New J. Chem.* **2001**, *25*, 289–292.
[23] E. U. Akkaya, S. Turkyilmaz, *Tetrahedron Lett.* **1997**, *38*, 4513–4516.
[24] B. Ozmen, E. U. Akkaya, *Tetrahedron Lett.* **2000**, *41*, 9185–9188.
[25] A. Coskun, M. D. Yilmaz, E. U. Akkaya, *Org. Lett.* **2007**, *9*, 607–609.
[26] a) B. L. Vallee, K. H. Falchuk, *Physiol. Rev.* **1993**, *73*, 79–118; b) J. M. Berg, Y. G Shi, *Science* **1996**, *271*, 1081–1085.
[27] a) T. Hirano, K. Kikuchi, Y. Urano, T. Higuchi, T. Nagano, *J. Am. Chem. Soc.* **2000**, *122*, 12399–12400; b) G. K. Walkup, S. C. Burdette, S. J. Lippard, R. Y. Tsien, *J. Am. Chem. Soc.* **2000**, *122*, 5644–5645; c) T. Hirano, K. Kikuchi, Y. Urano, T. Higuchi, T. Nagano, *Angew. Chem.* **2000**, *112*, 1094–1096; *Angew. Chem. Int. Ed.* **2000**, *39*, 1052–1054; d) S. C. Burdette, G. K. Walkup, B. Spingler, R. Y. Tsien, S. J. Lippard, *J. Am. Chem. Soc.* **2001**, *123*, 7831 –7841.
[28] K. Kiyose, H. Kojima, Y. Urano, T. Nagano, *J. Am. Chem. Soc.* **2006**, *128*, 6548–6549.
[29] B. Tang, H. Huang, K. Xu, L. Tong, G. Yang, X. Liu, L. An, *Chem. Commun.* **2006**, 34, 3609–3611.
[30] a) H. Y. Yun, V. L. Dawson, T. M. Dawson, *Mol. Psychiatry* **1997**, *2*, 300–310; b) C. Napoli, L. J. Ignarro, *Nitric Oxide* **2001**, *5*, 88–97.
[31] T. Nagano, T. Yoshimura, *Chem. Rev.* **2002**, *102*, 1235–1269.
[32] H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, *Anal. Chem.* **1998**, *70*, 2446–2453.
[33] E. Sasaki, H. Kojima, H. Nishimatsu, Y. Urano, K. Kikuchi, Y. Hirata, T. Nagano, *J. Am. Chem. Soc.* **2005**, *127*, 3684–3685.
[34] M. Kamiya, H. Kobayashi, Y. Hama, Y. Koyama, M. Bernardo, T. Nagano, P. L. Choyke, Y. Urano, *J. Am. Chem. Soc.* **2007**, *129*, 3918–3929.
[35] N. H. Ho, R. Weissleder, C. H. Tung, *ChemBioChem* **2007**, *8*, 560–566.
[36] N. H. Ho, R. Weissleder, C. H. Tung, *Bioorg. Med. Chem. Lett.* **2006**, *16*,2599–2602.
[37] a) I. Hemmila, V. M. Mukkala, *Crit. Re*V. *Clin. Lab. Sci.* **2001**, 38, 441–519; b) W. Lei, A. Dürkop, Z. Lin, M. Wu, O. S. Wolfbeis, *Microchim. Acta* **2003**, 143, 269–274.
[38] T. Terai, K. Kikuchi, S. Y. Iwasawa, T. Kawabe, Y. Hirata, Y. Urano, T. Nagano, *J. Am. Chem. Soc.* **2006**, *128*, 6938–6946.
[39] M. Halim, M. S. Tremblay, S. Jockusch, N. J. Turro, D. Sames, *J. Am. Chem. Soc.* **2007**, *129*, 7704–7705.

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