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On the Design of Fluorescent Ratiometric Nanosensors

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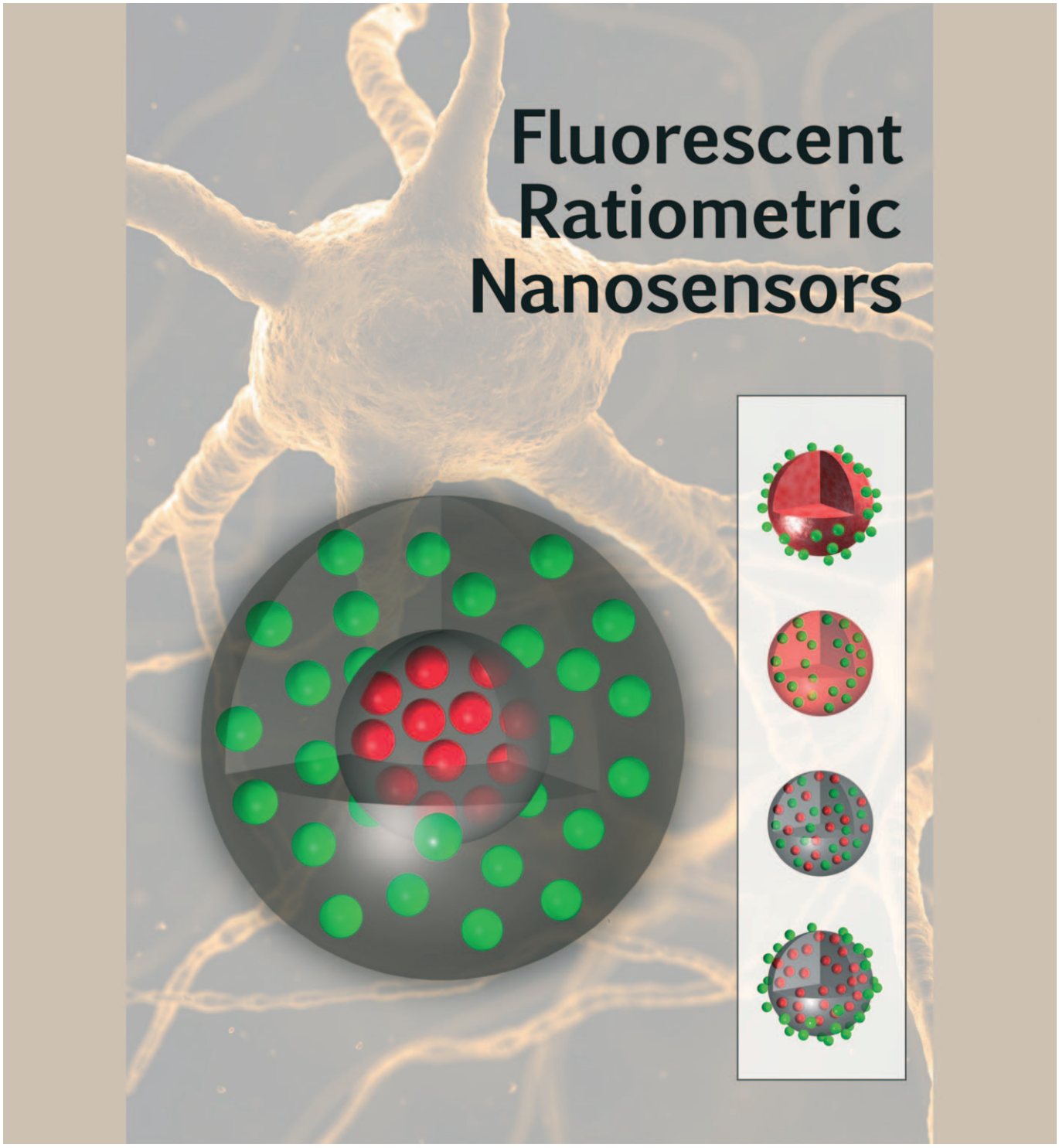

Abstract: Advances in nanoparticle technology have recently offered new tools to the bioanalytical field of research. In particular, new nanoparticle-based sensors have appeared able to give quantitative information about different species (ions, metabolites, biomolecules) in biosamples through ratiometric measurements. This article describes the methodologies developed so far in the design of such nanosensors. In particular, the different approaches to immobilize fluorescent chemosensor dyes to nanoparticles are presented. Concept designs of ratiometric nanosensors in terms of composition and architecture are also described and illustrated with examples taken from the literature.

Keywords: analytical methods · dyes/pigments · fluorescence · nanoparticles · nanosensors

Introduction

Imaging techniques are essential to visualize and monitor physiological parameters and biological functions, such as analyte concentration (ions, metabolites, biomolecules) or tissue properties in biological samples. Consequently, they are employed for medical diagnostics or for monitoring the efficiency of an applied therapy.[1] Recent revolutionary advances in the discovery of new biomarkers and probes as well as in the development of the technological aspects (e.g., instrumentation, image analysis) provided new tools for this purpose. In particular, fluorescence microscopy has taken advantages of major developments in the design of functional luminescent probes, or in the technology of ultrafast pulsed lasers.[2] These optical techniques of imaging allow biologists to carry out a precise structural and functional examination of biological samples at high spatial and temporal resolution in a non-invasive manner.

The development of analyte-sensitive fluorescent probes during the last twenty years resulted in new powerful tools to investigate intracellular processes or cell viability.[3] Designing of fluorescent sensors for biological applications has even become a wider field of research with materials ranging from the molecular level to the micro- and nanoscale.[4] Basic requirements for fluorescent sensors are stability, brightness (high signal-to-noise ratio), and both selectivity and sensitivity towards the targeted analyte. For applications in biosamples, other issues of crucial importance have to be taken into account such as the biocompatibility of the sensors and the quantitative determination of the targeted analyte in real-time (reversibility and response time). The quantitative determination of the targeted analyte in real-time can be achieved with fluorescent molecular probes that allow ratiometric measurements, but they are difficult to design.[5] Furthermore, at the molecular level, proper derivatization or bioconjugation of the probes is often necessary to reach the required biocompatibility. Time-resolved imaging techniques or phase-sensitive detection would be alternatives to obtain quantitative information from nonratiometric molecular probes, but they are technologically more demanding. Moreover, these probes usually suffer from photobleaching, aggregation, and a number of interferences in biological samples such as local pH changes or unspecific cross-reactivity to biomolecules causing undesirable changes in fluorescence. Finally, their possible toxicity is also a relevant key issue.

Nanotechnology, and in particular the recent development of nanoparticles, has provided a significant contribution to the bioanalytical field of research.[6] Nanoparticles are objects with dimensions less than 1 μm possessing controlled size and shape. They can be intrinsically fluorescent (e.g., quantum dots) or functionalized with one or more fluorescent dyes. Interestingly, nanoparticles often show enhanced stability and brightness in comparison with fluorescent molecular probes. Another major advantage is that they are highly engineerable platforms in terms of composition and architecture. For instance, the co-immobilization of a reference fluorophore providing an analyte-independent signal and the surface functionalization with biomolecules are only a few examples. Consequently, very efficient non-invasive ratiometric nanosensors can be designed that are tailormade to the desired application.

In the first part, this paper focuses on fluorescent chemosensor dyes (also named indicator dyes) for pH, biologically relevant ions, and small molecules that are further implemented in the design of advanced ratiometric nanosensors. A particular emphasis will be laid on principles that rule the immobilization of the indicator dyes to the nanoparticles. Basics on chemosensor dye–analyte interactions will be also mentioned here. The second part deals with the actual design of the nanosensors and their input to sensing technology. The summary and outlook will sum up this article.

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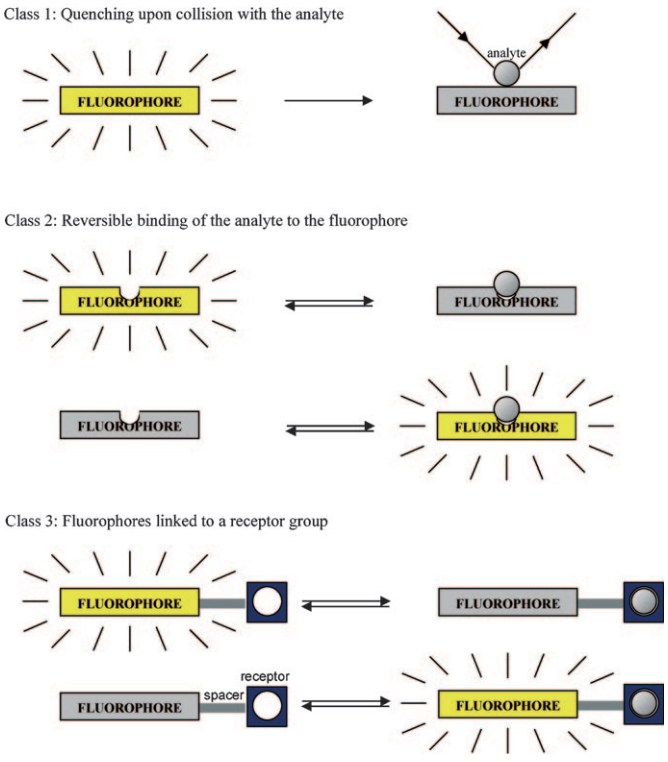
Design of Chemosensor Dyes to be Implemented into Nanosensors

Fluorescent chemosensor dyes (or indicator dyes) for pH, ions, or other biologically relevant small molecules, such as dioxygen or hydrogen peroxide, are commonly used in a "direct" sensing way. This means that the reaction that occurs between the probe and the analyte leads to changes in the optical properties of the chemosensor dye. For satisfactory performance, the probe–analyte interaction has to be sufficiently sensitive (in the relevant analytical range), selective, fast, and reversible. The dominating concept that governs these sensors is based on "OFF–ON" switching of the fluorescence. The schematic designs of the different types of chemosensor dyes are depicted in Scheme 1 adapted from Valeur.[7]



Scheme 1. Classification of chemosensor dyes (adapted from reference [7]).

The chemosensor dyes can be classified into:

1) Fluorophores that are quenched upon collision with the analyte (e.g., O_2, Cl^-).
2) Fluorophores reversibly binding to the analyte with subsequent fluorescence quenching or enhancement.
3) Fluorophores that are linked to a receptor group by means of a spacer unit and the fluorescence is either quenched or enhanced upon analyte recognition at the receptor due to photoinduced processes such as electron transfer, charge transfer, and energy transfer.

The resulting three different sensing mechanisms will only be discussed very briefly, since these processes are described in detail by Valeur[7] or Lakowicz.[8]

The first class of chemosensor dyes includes both static and dynamic quenching, which are reversible processes and occur upon collision of an excited fluorophore (M*) with heavy ions like Br^- and Cl^- or with paramagnetic species (e.g., O_2, NO•). From the analytical point of view, dynamic quenching is considered to be the most important mechanism. The rate constant for deactivation by quenching is k_q and the rate constant k_M for radiative deactivation of M* is determined by the fluorescence lifetime of M* in absence of the quencher molecule Q (τ_0). According to this Scheme the fluorescence lifetime and therefore also the fluorescence intensity of M* depends on the concentration of Q. The relation is described by the Stern–Volmer equation [Eq. (1)], which is commonly used for quantitative optical determination of anions like chloride, bromide and iodide and in which F_0 and τ_0 are the fluorescence intensity and lifetime of the fluorophore in absence, and F and τ represent the fluorescence intensity and lifetime in presence of the quenching species, respectively.

$$\frac{\tau_0}{\tau} = \frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (1)$$

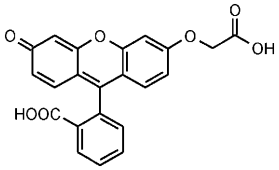
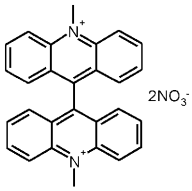
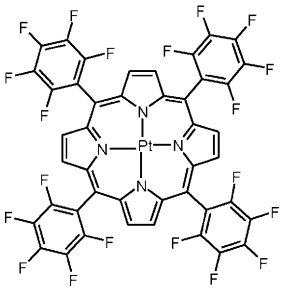
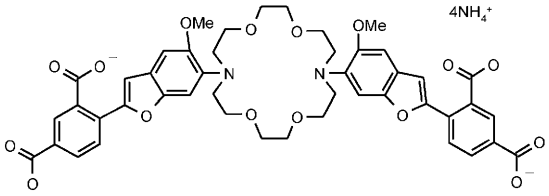
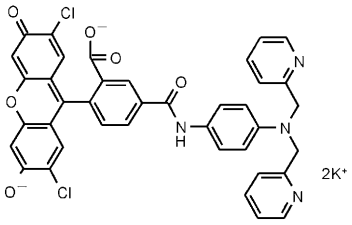
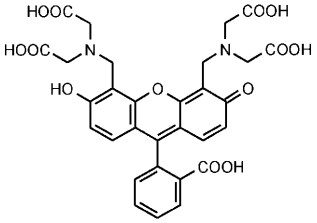
Plotting the ratio of F_0/F against the quencher concentration gives the Stern–Volmer quenching constant K_{SV}. The higher the quenching constant the more sensitive is the fluorophore to the quencher. The second class of chemosensor dyes includes many pH-sensitive fluorophores, such as fluorescein, pyranine, or hydroxycoumarins, but also metal-chelating fluorophores. Two types of pH-sensitive fluorophores can be distinguished: those subject to photoinduced proton transfer (pyranine, hydroxycoumarins), and those not subject to photoinduced proton transfer (fluorescein, eosin Y). The occurrence of this photoinduced phenomenon leads to differences in the measurable transduction signal (for details see references [7,8]). Belonging as well to the second class of chemosensor dyes, metal-chelating fluorophores are usually subject of photoinduced charge transfer (PCT). The metal interacts with an electron-donating or electron-withdrawing moiety of the fluorophore causing changes in the photophysical properties (absorption, fluorescence, Stokes' shift, fluorescence lifetime). The third class of chemosensor dyes is usually characterized by the presence of a spacer linking the fluorophore and the analyte–receptor units. The sensing mechanism is based on the interaction between the analyte and the receptor group with following signal transduction to the fluorophore. A transduction process that is widely used in the design of such indicator dyes is photoinduced electron transfer (PET). PET describes processes that prevent the relaxation of the excited state electron of a fluorophore (LUMO) to the ground state (HOMO) of the fluorophore. The fluorescence that commonly follows the relaxation is therefore prevented as well. A good example is

N,N-dimethylaminomethyl anthracene, in which the intramolecular transfer of an electron occurs from the electron-rich part of the molecule (dimethylamino moiety, donor) to the aromatic system (anthracene, acceptor). In presence of protons, the lone pair of the dimethylamino moiety is used to bind the protons and is no longer available to transfer the electron to the HOMO of the excited molecule. Thus, strong fluorescence becomes visible.

The use of these chemosensor dyes in more advanced devices, such as nanosensors, requires their entrapment in or their linkage to a matrix or a surface. This can be achieved through physical adsorption, encapsulation or covalent binding.

Noncovalent immobilization strategies: Noncovalent strategies might appear to be most simple to handle, since no derivatization of the molecular sensor is needed. However, a matched combination between the matrix and the dye in terms of hydrophilicity or electronic charge has to be reached. In addition, relatively elaborate nanosensor preparation procedures might be needed to yield stable and sensitive sensors. Table 1 shows a non-exhaustive list of several

Table 1. Non-exhaustive list of indicator dyes involved in the design of ratiometric nanosensors through noncovalent immobilization strategies.

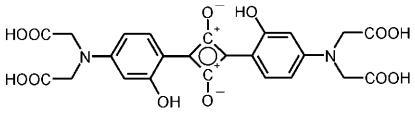
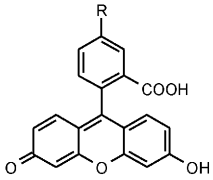
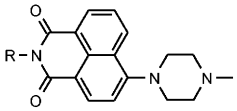
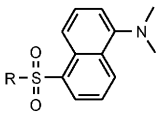
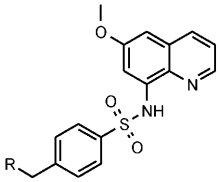
Dye	Role	Matrix loading method	Class	References
	pH indicator	encapsulation	2	[9b]
	Cl^- indicator	encapsulation	1	[10]
	O_2 indicator	encapsulation, hydrophobic interactions	1	[11]
	K^+ indicator	layer-by-layer, electrostatic interactions	3	[13]
	Zn^{2+} indicator	encapsulation	3	[9c]
	Mg^{2+} indicator	encapsulation	3	[9d]

indicator dyes embedded in nanoparticles using noncovalent approaches.

Kopelman and co-workers are pioneers in the preparation of ratiometric nanosensors to investigate biosamples.[9] Their main strategy was to physically encapsulate hydrophilic commercially available chemosensor dyes for H^+, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, or Fe^{3+} within poly(acrylamide) nanoparticles during a reverse water-in-oil microemulsion polymerization. The microemulsion approach yielded well-defined and homogeneous nanoparticles in terms of size and shape. This also allowed the sequestration of the indicator dyes within the water droplets prior to the initiation of the polymerization process within the same droplets. After polymerization and recovering of the nanoparticles, fluorescent nanosensors were obtained in which indicator dyes were physically entrapped. In a similar approach, Graefe and co-workers designed chloride nanosensors physically entrapping the chloride-sensitive Lucigenin dye.[10] However, leaching of the dye from the matrix cannot be fully avoided during the analysis, thus possibly affecting the reliability of the measurements. Through an oil-in-water microemulsion procedure, Cywinski and co-workers described the preparation of fluorescent polystyrene nanobeads for measuring dissolved dioxygen.[11] Here, lipophilic Pt^{II}–*meso*-tetra(pentafluorophenyl)porphine was sequestered within the oil droplets, while the polymerization started. In this case, the obtained nanosensors took benefits from the strong hydrophobic interaction between the dye and the matrix. Klimant and co-workers also developed dioxygen nanosensors embedding, in particular, cyclometallated iridium(III)–coumarin complexes within polystyrene beads.[12] Unfortunately, such beads tend to aggregate in biosamples, limiting their applicability. McShane and co-workers used the layer-by-layer approach to physically entrap the commercially available potassium-sensitive benzofuran isophthalate dye within the shell of their nanosensors.[13] Here, electrostatic interactions between polycationic polymer chains and the negatively charged dye maintained the latter within the shell. Again, leaching of the dye over time cannot be fully precluded.

Covalent immobilization strategies: To reach a larger stability of the sensor materials and to guarantee better reproducibility in preparation, the dye has to be covalently linked to the particle matrix. In principle, this approach also provides higher shelf and operational lifetime to the nanosensors. Table 2 shows selected examples taken from the literature.

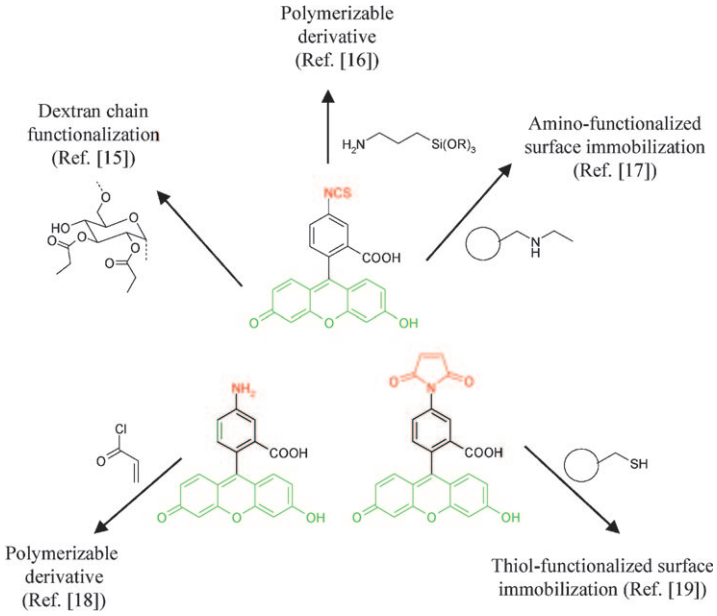
Table 2. Selected chemosensor dyes that can be covalently immobilized to a matrix. R stands for functional group, which allows these indicator dyes to be covalently immobilized (see details in text).

Dye	Role	Class	References
	pH indicator	2	[14]
	pH indicator	2	[15–19]
	pH indicator	3	[20a,b]
	Pb^{2+} indicator	1	[21a]
	Zn^{2+} indicator	3	[21b]

Bawendi and co-workers used the well-known conjugation technique between amine (or hydroxyl) and carboxylic acid functions via the formation of a reactive ester intermediate.[14] A pH-sensitive squaraine dye bearing carboxylic acid functions is activated by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and is subsequently reacted with ω-hydroxyl function supported on CdSe/ZnS nanocrystals.

The probably most well-known pH-sensitive dye is fluorescein. Furthermore, fluoresceins bearing reactive chemical functions that allow their covalent coupling are commercially available. From examples taken in the literature, Scheme 2 illustrates how fluorescein can be used in the design of nanosensors.

The isothiocyanate derivative of fluorescein was used to functionalize dextran with a subsequent shaping of nanosensors by means of a precipitation process.[15] Furthermore, the isothiocyanate was involved in the preparation of a polymerizable derivative by reaction with an aminosilane (usually the 3-(aminopropyl)triethoxysilane).[16] This allowed the fluorescent indicator dye to be co-polymerized with other molecular silica precursors (such as tetraethoxyorthosilicate, TEOS) to form a hybrid matrix. The same reactive fluores-



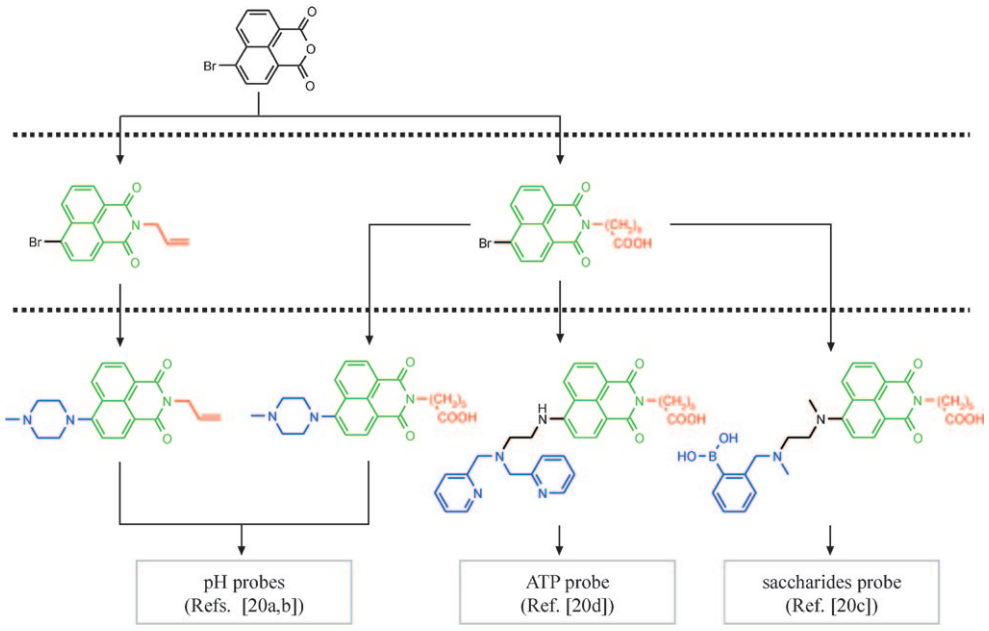
Scheme 2. Covalent immobilization strategies of fluorescein to nanoparticles.

cein was successfully used by Larpent and co-workers to immobilize the pH sensor on amino-functionalized polystyrene nanoparticles.[17] Almdal and co-workers prepared the *N*-acryloyl-5-aminofluorescein derivative by reaction of 5-aminofluorescein with acryloyl chloride.[18] The resulting derivative was then involved in the polymerization process to design the nanosensor. Suzuki and co-workers used the maleimide derivative of fluorescein to immobilize it on the outer surface of amino-functionalized quantum dots (QDs) previously treated with Traut's reagent.[19] The approach of covalent attachment through the prior preparation of a copolymerizable derivatives offers several advantages over surface immobilization: 1) higher dye loading is usually obtained, 2) cross-reactivity with other components of the biological medium is largely prevented because large species such as proteins do not enter the particle, and 3) to some extent, the composition and the architecture of the nanosensor can be tuned.

Another class of well-known fluorophores besides fluoresceins are the naphthalimides. They exhibit several desirable properties such as a large Stokes' shift, high quantum yield, and photostability. Naphthalimides, as shown in Scheme 3, can be built up in only few steps starting from 1,8-naphthalic anhydride. In this way, an appropriate receptor unit for the respective analyte (blue) as well as an anchor moiety used for immobilization (red) can be introduced. The modular construction leads to a highly flexible fluorescent chemosensor dye, which is tailor-made for the desired analyte and matrix.[20] Schulz and co-workers synthesized a pH-sensitive naphthalimide fluorophore possessing a polymerizable vinyl group to develop poly(acrylamide) nanosensors.[20a] In a similar way, Doussineau and co-workers prepared a pH-sensor with a carboxyl moiety for covalent attachment to amino-functionalized silica nanoparticles.[20b]

The same carboxyl anchor moiety was used by Trupp and co-workers to develop nanosensors for saccharides as well as by Moro and co-workers for designing ATP-nanosensors based on silica nanoparticles.[20c,d]

The chemical modification of commercially available indicator dyes with a trialkoxysilyl moiety and the subsequent immobilization to silica nanoparticles has been demonstrated by Mancin and co-workers.[21] For instance, a dansyl de-



Scheme 3. Synthetic pathways for designing naphthalimide-based indicator dyes bearing a chemical function allowing their subsequent immobilization to nanoparticles. In green: fluorophore ; in blue: receptor unit ; in red: chemical function allowing subsequent immobilization to nanoparticles.

rivative bearing a silica polymerizable moiety was prepared in one step by reaction between dansyl-chloride and 3-(aminopropyl)triethoxysilane. This derivative was then co-condensed with molecular silica precursors to yield a lead(II) nanosensor.[21a] A polymerizable derivative of the known zinc(II)-sensitive 6-methoxy-8-(p-toluenesulfoamido)-quinoline (TSQ) was synthesized in four steps allowing the design of the silica-based zinc(II) nanosensor.[21b]

In addition to the enhanced chemical stability of nanosensors, the covalent immobilization of indicator dyes offers the possibility to tune these nanosensors by controlling the amount and the location of the dyes. Furthermore, the preparation of nanosensors through the co-polymerization of chemosensor dyes with monomers as well as through post-functionalization of nanoparticles is feasible and reproduceible.

Design of Fluorescent Ratiometric Nanosensors

The design of fluorescent ratiometric sensors for biosamples aims at the monitoring of analytes in real-time. Considering intensity-based signal acquisition, this can be achieved using dual-emissive fluorescent materials.[5] Basically, two signals have to be detected from each sensor entity: one signalling the analyte binding, and the other allowing this primary signal to be scaled. At the molecular level, ratiometric sensors can be designed by subsequent addition of an analyte-insensitive dye to the sensor structure[22] or by the use of structures exhibiting analyte-binding-driven phenomena, such as Förster resonance energy transfer (FRET),[23] excimer formation,[24] or intramolecular charge transfer (ICT).[25] However, such fluorescent molecular probes are difficult to prepare and still suffer from photobleaching and interferences. The versatile composition and architecture of nanoparticles enables the preparation of ratiometric nanosensors that overcome, to some extent, these problems.

As shown in Figure 1, one can currently distinguish two types of nanosensors: those based on FRET in which the reference signal changes in correlation with the signal of the indicator (A), and those that exhibit an analyte-independent reference signal (B). FRET-based nanosensors are usually built up around an intrinsically fluorescent nano-object (A1 and A2). The sensing dye is either immobilized onto the surface (A1) or embedded into the matrix of this nanoobject (A2). Type B nanosensors are mainly made from an optically transparent nanomaterial that bears both the indicator dye and an analyte-independent reference dye. In terms of architecture, the reference dye can be located within the core of the nanoparticle while the indicator dye is either immobilized onto the outer surface (B1) or embedded in a thick shell (B2). A type AB3 nanosensor consists of an optically transparent nanoparticle in which both indicator and reference dyes are randomly distributed in the matrix. The reference dye can be used for FRET-based sensing ("A3") or to give an analyte-independent signal ("B3").

Quantum dots (QDs) are spherical semiconductor light-emitting nanoparticles with size-tunable light emission, high brightness, and resistance to photobleaching.[26] Moreover, the relative versatility of surface engineering of QDs allows them to be further functionalized with fluorescent chemosensor dyes. Bawendi and co-workers designed a type A1 ratiometric and reversible pH-nanosensor conjugating a squaraine dye to the surface of CdSe@ZnS nanocrystals.[14] In this nanosensor, the ratiometric measurements were based on the pH-dependent modulation of the FRET efficiency between the QD (donor) and the conjugated pH-sensitive squaraine dye (acceptor). The dye absorption band was suppressed under basic conditions. Consequently, energy transfer from the QD to the dye became inefficient, and the emission spectrum was dominated by the QD at 613 nm. With decreasing pH, the absorption cross section of the dye increased, and FRET from the QD to the dye became more efficient. Therefore, emission from the QD–dye conjugate became dominated by the signal of the dye at 650 nm. Inter-

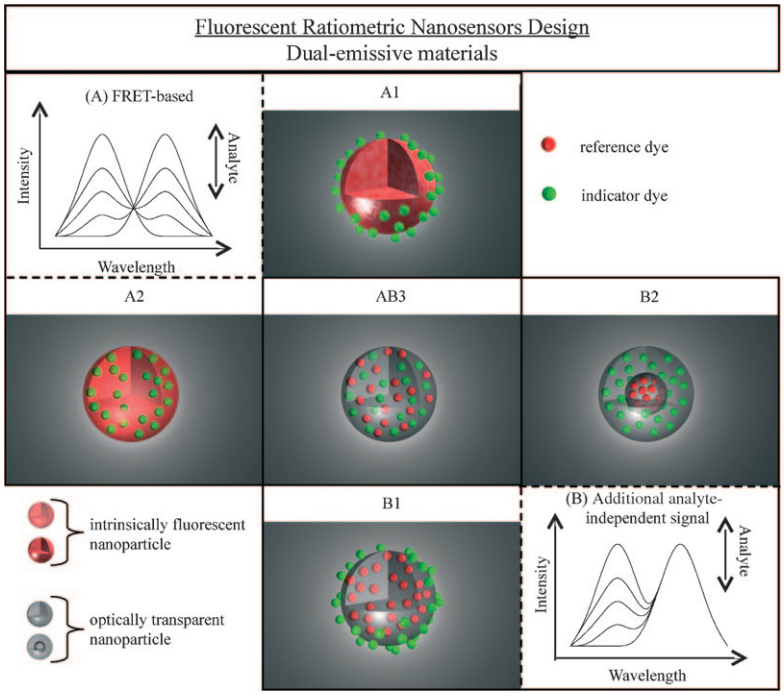


Figure 1. Concept designs - composition and architecture - of ratiometric nanosensors. A) Nanosensors based on FRET mechanism and B) nanosensors based on an additional analyte-independent signal.

estingly, due to the broad absorption spectrum of the QD FRET-donor, the resulting emission was observed to be independent of the excitation wavelength (380 nm, 450 nm or 520 nm). However, the apparent pK_a was found to be around 8.5, which is not of relevance for biological applications. Suzuki and co-workers also designed an A1-type pH nanosensor through the conjugation of fluorescein to QDs.[19] The QD donor emitted at 490 nm perfectly overlapping the absorption spectrum of the pH-sensitive acceptor dye. The apparent pK_a of 6.4 in solution was more appropriate for pH sensing in biological media. Unfortunately, the authors did not report on the possibility for ratiometric measurements. Nevertheless, using the same principle, the authors described the preparation of nanobiosensors to probe enzymatic reactions occurring at the surface of the QDs. For instance, a green fluorescent protein (GFP) variant was immobilized on the surface of QDs allowing FRET to occur. An inserted sequence of the GFP was recognized by trypsin, leading to its cleavage from the surface. The occurrence of the reaction was monitored by the fluorescence enhancement coming from the QDs. Similarly, the preparation of highly sensitive QD-based nanobiosensors has been described in the literature based on the labeling of one of the elements of the biological recognition system, that is, the probe or the target.[27] The occurrence, or not, of the biological recognition event at the surface of the QDs affects the FRET efficiency allowing the ratiometric determination of the target biomolecule. Hall and co-workers described a chloride QD-based sensor in which the chloride-sensitive dication Lucigenin was adsorbed through electrostatic interactions on the negatively charged surface of QDs (capped with mercaptopropionic acid).[28] The authors demonstrated that Stern–Volmer quenching of Lucigenin (caused by chloride anions) competed with other short-range quenching mechanisms of the QD luminescence that occur at the interface upon radiation (spin-orbit coupling, charge transfer). As a result of this sensing event, the luminescence of the QDs was switched on. Finally, another advantage of QD-based nano(bio)sensors is the possibility for multiplexed detection of the targeted analytes or biological processes taking advantage of single wavelength excitation of these nanoassemblies. However, inherent drawbacks of QD-based sensors such as toxicity, relatively poor stability in biosamples, or tendency to blink slow down their widespread use in sensing applications.

Intrinsically fluorescent nanomaterials are not the exclusive privilege of QDs. McNeill and co-workers recently described the preparation of dye-doped π-conjugated polymer nanoparticles to yield A2-type ratiometric O_2 nanosensors.[29] The 25 nm-sized fluorescent host nanoparticles were made from polyfluorene derivatives and prepared by precipitation. The O_2-sensitive platinum(II) octaethylporphine was physically entrapped within the luminescent material during the precipitation. FRET occurring between the fluorescent polymer nanoparticles (donor) and O_2-sensitive dye (acceptor) allowed the authors to perform imaging of local molecular dioxygen concentration in living cells.

Another type of ratiometric nanosensor design that uses FRET (AB3-type) was described by Larpent and co-workers.[30] In this case, two fluorophores were physically entrapped in 16 nm latex nanoparticles, namely 9,10-diphenyl-anthracene acting as a FRET donor and pyrromethene PM567 acting as the acceptor. FRET efficiencies up to 80% between the two dyes were reached. In addition, these nanoparticles were post-functionalized with a cyclam ligand selective towards copper(II) ions. Interestingly, the emission of the acceptor was efficiently quenched by the cyclam copper(II) complex, whereas the emission of the donor was much less affected. Ratiometric measurements of copper(II) ions over a wide range of concentration (20 nM to 8.5 mM) were possible upon excitation at a single wavelength (of the donor). Consequently, even using FRET, the fluorescence signal of the donor acted as "stable" reference signal in these nanosensors.

The embedding of two dyes within optically transparent nanoparticles (type AB3) constitutes the most common design for ratiometric nanosensors. One of the two dyes provides an analyte-independent reference signal to which the sensing signal is scaled.[9–12,15,16b,18,21b] These reference dyes are immobilized within the optically transparent matrix following the same principles described for the sensor dyes, that is, either covalently or noncovalently. In most of the studies, rhodamine derivatives were used since they exhibit very convenient optical properties (high extinction coefficients and high quantum yields). Furthermore, they are inert towards most of the targeted analytes as well as towards changes in pH (the most common interfering species). The group of Kopelman has significantly contributed to this type of nanosensors designing two-dye-doped nanoparticles mostly made from poly(acrylamide).[9] For example, a Texas Red/dextran derivative as a reference and the magnesium(II)-sensitive C343 dye were physically entrapped within the particles during the microemulsion polymerization.[9d] The authors reported on the resulting ratiometric nanosensors to be characterized by a dynamic range of 1 to 30 mM with a response time < 4 s. They were able to monitor in vitro Mg^{2+} concentration in C6 glioma cells, which remained viable after gene gun injection, showing the biocompatibility of the particles. However, since both dyes are randomly distributed within the nanosensors, one can not fully exclude that energy transfer between them may occur. Schulz and co-workers demonstrated that energy transfer actually took place in fluorescein–rhodamine co-doped dextran pH-nanosensors, but no significant effect on the performance was observed.[31]

Core-shell architectures (types B1 and B2) in which the core is doped with the reference fluorophore and the shell with the indicator dye allow the spatial separation that is supposed to minimize undesired energy transfer.[13,16a,-c,17,20,21a] Type B1 ratiometric nanosensors are based on the surface post-functionalization of reference nanocores through reaction of, for example, surface-supported amino groups with carboxyl derivatives of the indicator dyes.[20b–d] For example, Doussineau and co-workers immobilized a

pH-sensitive naphthalimide derivative to the surface of amino-functionalized rhodamine-doped silica nanoparticles.[20b] Prior to the surface functionalization an undoped silica shell was coated around the cores to prevent any energy transfer to occur between the reference and the indicator dyes. The resulting ratiometric pH-nanosensors showed satisfactory sensitivity and were characterized by an apparent pK_a value of 6.5. An analogous approach was used to immobilize a saccharide-sensitive naphthalimide derivative.[20c] Selectivity towards fructose was observed as well as a comparable sensitivity with the free chemosensor dye in solution. Similarly, Moro and co-workers covalently linked a Zn^{II}-complexed naphthalimide derivative.[20d] The resulting ratiometric nanosensors showed selectivity towards adenosine triphosphate (ATP) in model solutions. Such a design allows the direct environment of the particles to be probed but, as in the case of free indicator dyes, this may lead to undesired interactions with other components in the biological medium. B2-type nanosensors can overcome this problem. They consist of the fluorescent indicator dye embedded within a thick shell made from the same material as the core[16a,20a,21a] or from another type of matrix yielding composite or hybrid structures.[13,16c] As an example, Doussineau and co-workers developed a pH-nanosensor using fluorescent zeolite-beta nanoparticles (reference), which were coated, in a secondary growth procedure, with a fluorescein-containing amorphous silica shell.[16c] Ratiometric measurements of pH were reported with an apparent pK_a value of 6.4. Mancin and co-workers exploited the versatile chemistry offered by such silica-based nanoparticles by preparing two-dye-doped core-shell silica nanoparticles further post-functionalized with thiol ligands.[21a] The surface-supported thiol groups acted as Pb^{II} scavengers triggering the quenching of the dye-doped shell. However, one must take into consideration that the use of such thick sensing shell may, to some extent, limit the diffusion of the analyte through the matrix or prevent the sensing event to occur properly.

Summary and Outlook

Nanoparticles are promising platforms to design smart and efficient ratiometric sensors for real-time monitoring of analytes in biosamples. Nanosensors can be designed in various compositions and architectures based on different signal transduction strategies. In this highly active field of research, few current advances of these nanosystems are of high prospect. For instance, the possibility to design nanosensors with near-infrared optical properties would provide a better contrast when applied to biosamples.[32] Similarly, ratiometric nanosensors excitable with a two-photon laser beam as already described by Bawendi and co-workers are of high interest.[33] The development of nanosensors based on upconverting luminescent materials also appears to be a promising alternative.[34] Furthermore, other nanosensor designs of interest have recently been described. For example, Li and co-workers prepared naphthalene–thiourea–thiadiazole fluorescent organic nanoparticles that showed selectivity towards cysteine.[35] Upon interaction with the analyte, a 74 nm red-shifted emission band appeared allowing the ratiometric determination of cysteine in the micromolar concentration range. Fraser and co-workers designed dual-emissive nanoparticles based on iodide-substituted difluoroboron dibenzoylmethane/poly(lactic acid).[36] These nanoparticles exhibiting balanced fluorescence and phosphorescence intensities were successfully applied as ratiometric tumour hypoxia imaging agents. Murthy and co-workers described the preparation of dyed peroxalate nanoparticles highly selective towards hydrogen peroxide.[37] Here, the sensing event is based on a three-component chemiluminescent reaction involving peroxalate polymer material. However, no ratiometric measurements were reported in this study.

Moreover, owing to the high engineerability of nanoparticles, multifunctional nanodevices can be designed. For instance, nanosensors for simultaneous multiple-analyte ratiometric measurements, targetable to specific locations of the biosample, stealthy (for in vivo intraveneous administration), allowing a subsequent drug release (theranostics), or bimodal imaging (e.g., with a co-embedded MRI contrast agent) are conceivable. For the design of such smart nanodevices for biological applications, silicate-based materials appear most promising. Their inherent biocompatibility, and ease of preparation coupled with the high versatility of silica chemistry (particle- and pore-size tunability, inorganic–organic coupling) foster the production of tailor-made nanodevices. As a relevant example of the current developments made on this type of material, Wolfbeis and co-workers recently reported on the attachment of fluorescent dyes to the surface of silica nanoparticles using the so-called "click chemistry".[38] This efficient organic–inorganic coupling approach could provide new experimental tools to design such nanosensors.

While promising, the currently developed ratiometric nanosensors—and in a general point of view, all types of nanoparticles—still lack a comprehensive investigation with respect to toxicity, biocompatibility, resorption, or transfection into cells. This is a major task for future nanosensor design and characterization.

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[1] R. Kherlopian, T. Song, Q. Duan, M. A. Neimark, M. J. Po, J. K. Gohagan, A. F. Laine, *BMC Syst. Biol.* **2008**, *2*, 74.

[2] a) D. J. Stephens, V. J. Allan, *Science* **2003**, *300*, 82–86; b) R. Yuste, *Nat. Methods* **2005**, *2*, 902–904.
[3] a) J. F. Callan, A. P. de Silva, D. C. Magri, *Tetrahedron* **2005**, *61*, 8551–8588; b) D. W. Domaille, E. L. Que, C. J. Chang, *Nat. Chem. Biol.* **2008**, *4*, 168–175; c) T. Terai, T. Nagano, *Curr. Opin. Chem. Biol.* **2008**, *12*, 515–521.
[4] L. Basabe-Desmonts, D. N. Reinhoudt, M. Crego-Calama, *Chem. Soc. Rev.* **2007**, *36*, 993–1017.
[5] A. P. Demchenko, *Lab Chip* **2005**, *5*, 1210–1223.
[6] a) P. Sharma, S. Brown, G. Walter, S. Santra, B. Moudgil, *Adv. Colloid Interface Sci.* **2006**, *123–126*, 471–485; b) Y.-E. K. Lee, R. Kopelman, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2009**, *1*, 98–110; c) S. M. Borisov, I. Klimant, *Analyst* **2008**, *133*, 1302–1307.
[7] B. Valeur, *Molecular Fluorescence-Principles and Applications*, Wiley-VCH, Weinheim, **2002**.
[8] J. R. Lakowicz, *Principles of fluorescence spectroscopy*, 3rd ed., Springer, Heidelberg, **2006**.
[9] a) S. M. Buck, H. Xu, M. Brasuel, M. A. Philbert, R. Kopelman, *Talanta* **2004**, *63*, 41–59; b) H. A. Clark, M. Hoyer, M. A. Philbert, R. Kopelman, *Anal. Chem.* **1999**, *71*, 4831–4836; c) J. P. Sumner, J. W. Aylott, E. Monson, R. Kopelman, *Analyst* **2002**, *127*, 11–16; d) E. J. Park, M. Brasuel, C. Behrend, M. A. Philbert, R. Kopelman, *Anal. Chem.* **2003**, *75*, 3784–3791.
[10] A. Graefe, S. E. Stanca, S. Nietzsche, L. Kubicova, R. Beckert, C. Biskup, G. J. Mohr, *Anal. Chem.* **2008**, *80*, 6526–6531.
[11] P. J. Cywinski, A. J. Moro, S. E. Stanca, C. Biskup, G. J. Mohr, *Sens. Actuators B* **2009**, *135*, 472–477.
[12] S. M. Borisov, I. Klimant, *Microchim. Acta* **2009**, *164*, 7–15.
[13] J. Q. Brown, M. J. McShane, *IEEE Sens. J.* **2005**, *5*, 1197–1205.
[14] P. T. Snee, R. C. Somers, G. Nair, J. P. Zimmer, M. G. Bawendi, D. G. Nocera, *J. Am. Chem. Soc.* **2006**, *128*, 13320–13321.
[15] S. Hornig, C. Biskup, A. Graefe, J. Wotschadlo, T. Liebert, G. J. Mohr, T. Heinze, *Soft Matter* **2008**, *4*, 1169–1172.
[16] a) A. Burns, P. Sengupta, T. Zedayko, B. Baird, U. Wiesner, *Small* **2006**, *2*, 723–726; b) J. Peng, X. He, K. Wang, W. Tan, Y. Wang, Y. Liu, *Anal. Bioanal. Chem.* **2007**, *388*, 645–654; c) T. Doussineau, M. Smaïhi, G. J. Mohr, *Adv. Funct. Mater.* **2009**, *19*, 117–122.
[17] E. Allart, C. Larpent, *J. Polym. Sci. Polym. Chem. Ed.* **2008**, *46*, 6206–6213.
[18] H. Sun, A. M. Scharff-Poulsen, H. Gu, K. Almdal, *Chem. Mater.* **2006**, *18*, 3381–3384.
[19] M. Suzuki, Y. Husimi, H. Komatsu, K. Suzuki, K. T. Douglas, *J. Am. Chem. Soc.* **2008**, *130*, 5720–5725.
[20] a) A. Schulz, J. Wotschadlo, T. Heinze, G. J. Mohr, *J. Mater. Chem.* **2010**, *20*, 1475–1482; b) T. Doussineau, S. Trupp, G. J. Mohr, *J. Colloid Interface Sci.* **2009**, *339*, 266–270; c) S. Trupp, unpublished results; d) A. Moro, T. Doussineau, A. Lapresta-Fernandez, G. J. Mohr, unpublished results.
[21] a) M. Arduini, F. Mancin, P. Tecilla, U. Tonellato, *Langmuir* **2007**, *23*, 8632–8636; b) P. Teolato, E. Rampazzo, M. Arduini, F. Mancin, P. Tecilla, U. Tonellato, *Chem. Eur. J.* **2007**, *13*, 2238–2245.
[22] S. Jayaraman, J. Bawersi, A. S. Verkman, *Am. J. Physiol.* **1999**, *276*, 747–757.
[23] K. Kikuchi, H. Takakusa, T. Nagano, *TrAC Trends Anal. Chem.* **2004**, *23*, 407–415.
[24] J.-S. Yang, C.-S. Lin, C.-Y. Hwang, *Org. Lett.* **2004**, *6*, 889–892.
[25] G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **1985**, *260*, 3440–3450.
[26] A. M. Smith, H. Duan, A. M. Mohs, S. Nie, *Adv. Drug Delivery Rev.* **2008**, *60*, 1226–1240.
[27] a) I. L. Medintz, A. R. Clapp, H. Mattoussi, E. R. Goldman, B. Fischer, J. M. Mauro, *Nat. Mater.* **2003**, *2*, 630–638; b) C.-Y. Zhang, H.-C. Yeh, M. T. Kuroki, T.-H. Wang, *Nat. Mater.* **2005**, *4*, 826–831.
[28] M. J. Ruedas-Rama, E. A. H. Hall, *Analyst* **2008**, *133*, 1556–1566.
[29] C. Wu, B. Bull, K. Christensen, J. McNeill, *Angew. Chem.* **2009**, *121*, 2779–2783; *Angew. Chem. Int. Ed.* **2009**, *48*, 2741–2745.
[30] M. Frigoli, K. Ouadahi, C. Larpent, *Chem. Eur. J.* **2009**, *15*, 8319–8330.
[31] A. Schulz, S. Hornig, T. Liebert, E. Birckner, T. Heinze, G. J. Mohr, *Org. Biomol. Chem.* **2009**, *7*, 1884–1889.
[32] R. Weissleder, *Nat. Biotechnol.* **2001**, *19*, 316–317.
[33] E. J. McLaurin, A. B. Greytak, M. G. Bawendi, D. G. Nocera, *J. Am. Chem. Soc.* **2009**, *131*, 12994–13001.
[34] L.-N. Sun, H. Peng, M. I. J. Stich, D. Achatz, O. S. Wolfbeis, *Chem. Commun.* **2009**, 5000–5002.
[35] H. Li, J. Xu, H. Yan, *Sens. Actuators B* **2009**, *139*, 483–487.
[36] G. Zhang, G. M. Palmer, M. W. Dewhirst, C. L. Fraser, *Nat. Mater.* **2009**, *8*, 747–751.
[37] D. Lee, S. Khaja, J. C. Velasquez-Castano, M. Dasari, C. Sun, J. Petros, W. R. Taylor, N. Murthy, *Nat. Mater.* **2007**, *6*, 765–769.
[38] H. Mader, X. Li, S. Saleh, M. Link, P. Kele, O. S. Wolfbeis, *Ann. N. Y. Acad. Sci.* **2008**, *1130*, 218–223.

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