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Highlight Article

Small molecule-based ratiometric fluorescence probes for cations, anions, and biomolecules

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Abstract
Quantitative determination of specific analytes is essential for a variety of applications ranging from life sciences to environmental monitoring. Optical sensing allows non-invasive measurements within biological milieus, parallel monitoring of multiple samples, and less invasive imaging. Among the optical sensing methods currently being explored, ratiometric fluorescence sensing has received particular attention as a technique with the potential to provide precise and quantitative analyses. Among its advantages are high sensitivity and inherent reliability, which reflect the self-calibration provided by monitoring two (or more) emissions. A wide variety of ratiometric sensing probes using small fluorescent molecules have been developed for sensing, imaging, and biomedical applications. In this research highlight, we provide an overview of the design principles underlying small fluorescent probes that have been applied to the ratiometric detection of various analytes, including cations, anions, and biomolecules in solution and in biological samples. This highlight is designed to be illustrative, not comprehensive.

Introduction
Developing sensor systems that allow for the effective and rapid detection of analytes constitutes a major focus in supramolecular and biological chemistry. A specific subset of sensors, fluorescent probes have attracted attention for both analytical sensing and optical imaging because of their high sensitivity, fast response time, and technical simplicity.[1-10] Ideally, fluorescence sensing systems are able to provide a reliable fluorescent response under the conditions of analysis associated with the targeted analyte, be those chemical, environmental, or biological. Fluorescence sensing typically requires the synthesis of small molecules that permit the quantitative determination of a given analyte with a high selectivity. However, quantification of a target analyte using fluorescent probes that provide only a single emission feature can be fraught with difficulty, since interference from a variety of analyte-independent factors, such as instrumental parameters, the microenvironment around the probe molecule, the local concentration of the probe molecule, and photobleaching, etc. can all interfere with the analysis.[1-10] One way of overcoming these problems and insuring reliability is to take advantage of the so-called ratiometric approach. Ratiometric fluorescent sensors rely on analyte-induced changes in the intensity of two or more emission bands. The result is an effective internal referencing that greatly increases sensitivity and improves quantification.

In the context of ratiometric fluorescence sensing, a number of strategies, including internal charge transfer (ICT),[1,2] excited-state intramolecular proton transfer (ESIPT),[13,14] fluorescence resonance energy transfer (FRET),[1,2,11,12] through-bond energy transfer (TBET),[11] and monomer-excimer formation,[1,2] have long been the subject of study. In fact, to date, ratiometric fluorescent probes have permitted the quantitative determination of target analytes, as well as the fluorescence imaging of toxic substances associated with various human diseases. In this highlight, we summarize some recent advances made in the area of ratiometric fluorescent sensor design. Particular emphasis is placed on the chemical features of successful probes and their use in biological applications where appropriate.

ICT-based ratiometric fluorescent probes
Internal charge transfer (ICT) based probes are characterized by an electron-donating unit conjugated to an electron-accepting unit within one molecule that give rise to a "push-pull" π-electron system in the excited state. They have been extensively used for cation sensing. Upon interaction of the electron-donating part with a cation, the electron-donating character of the probe decreases. This leads to a blue shift in the absorption spectrum. Conversely, when a cation interacts with the electron-accepting part, an apparent red shift is observed in the absorption spectrum due to the ICT becoming more developed. In addition, changes in the fluorescence quantum yields and lifetimes are observed (Figure 1a). The ICT strategy for cation sensing was introduced early on by Valeur.[15] Since then, a wide variety of ICT-based fluorescent molecules based on ratiometric detection have been used for the imaging of target analytes. For instance, Tsien *et al.* reported ICT based probes that permit the physiological monitoring of H^+, Na^+, and Mg^{2+}, and Ca^{2+} ions.[1,16] Nagano *et al.* designed ratiometric Zn^{2+} probes, termed ZnAF-R1, ZnAF-R2, ZnIC, and DIPCY.[17-19] The cell membrane permeable probes, ZnAF-R2 and ZnIC, were also used to monitor Zn^{2+} concentrations in living cells. Taki *et al.* developed a ratiometric Cd^{2+} sensor, CadMQ, which exhibited an excellent Cd^{2+} selectivity relative to other heavy and transition metal ions, including Zn^{2+} in aqueous media. This probe could be used effectively for the ratiometric sensing of Cd^{2+} in living cells.[20] In seminal work, the Shinkai and James groups have prepared saccharide sensing ICT probes that show promise for use in real world applications.[21,22]

Disulfide-carbamate based naphthalimide derivatives have been widely exploited as ratiometric fluorescent probes for biological thiol detection.[23-28] In the presence of thiols, the disulfide bond is readily cleaved to release 4-aminonaphthalimide. This bond scission results in a red shifted emission and an obvious colour change from colourless to yellow. Using this basic approach, biological thiols, e.g., glutathione, cysteine, and thioredoxin could be quantified both in aqueous solution and in living cells. Detection proved possible at the

subcellular level, including in the mitochondria (**Probe 1**) or membrane plasma (**Probe 2**). Organ-specific sensing (**Probe 3**) also proved possible (e.g., in the liver).[25-27] A related bond cleavage approach was used to construct an elaborated drug delivery system that provides both a cancer targeted-therapeutic effect and drug uptake-related imaging at the subcellular level (Figure 1b).[28] Separately, a *p*-nitrobenzylcarbamate containing a naphthalimide subunit was synthesized for the ratiometric detection of endogenous H_2S in living cells and in mouse hippocampus.[29] Yoon *et al.* reported a naphthalimide-based Zn^{2+} probe (**Probe 4**) applicable to Zn^{2+} sensing in living zebrafish embryos.[30] Kumar *et al.* also reported a naphthalimide-based ensemble that could detect pyrophosphate (ppi) and H_2O_2 *via* a Zn^{2+} ion displacement strategy.[31]

Two-photon microscopy (TPM) has emerged as a useful technique for the ratiometric imaging of species in live cells and tissues. TPM, which employs two near-IR photons as the excitation source, provides several advantages over other optical-based sensing methods, including a relatively deep penetration depth (> 500 μm), a localized excitation, and longer observation times. Cho *et al.* designed several two-photon probes (e.g., **Probe 5**) based on aminonaphthalene derivatives for the ratiometric detection of H^+ and thiols.[32,33] These probes could be used to estimate the pH value and thiol levels in cells and tissues, including mouse brain.

A hybrid fluorophore (**Probe 6**) constructed by combining coumarin and merocyanine was developed for the ratiometric sensing of H_2S.[34] This probe shows a strong absorption band due to its ICT character. It displays two well-resolved emission bands arising from the coumarin and merocyanine subunits, respectively. In the presence of H_2S, the intensity of the merocyanine emission decreases while that of the coumarin increases. When dissolved in phosphate-buffered saline (PBS, pH 7.4), the solution color was found to change from dark blue to very pale blue upon exposure to H_2S. The detection limit for H_2S was determined by the intensity ratio of the two emission bands and was found to be ca. 1 μM. In addition, this particular probe is preferentially localized in mitochondria and allows a real-time imaging of intracellular H_2S.

Cyanine dyes have been also widely used in bioimaging studies. These dyes have emission and absorption maxima that fall in the near-IR region (i.e., 650-900 nm). Such photophysical features are advantageous *in vitro* and *in vivo* imaging applications since biological samples display weak auto-fluorescence in the NIR region. Kircher *et al.* reported the NIR fluorescence ratio imaging of a protease by mapping its activity *in vivo*.[35] In addition, Peng *et al.*[36] suggested that an amine-substituted tricarbocyanine would be more suitable than common cyanine dyes as a ratiometric fluorescence probe.[36] Recently, an aminocyanine moiety attached to nano beads was exploited as a ratiometric fluorescent pH sensor (**Probe 7**) by Nagano *et al.*[37]

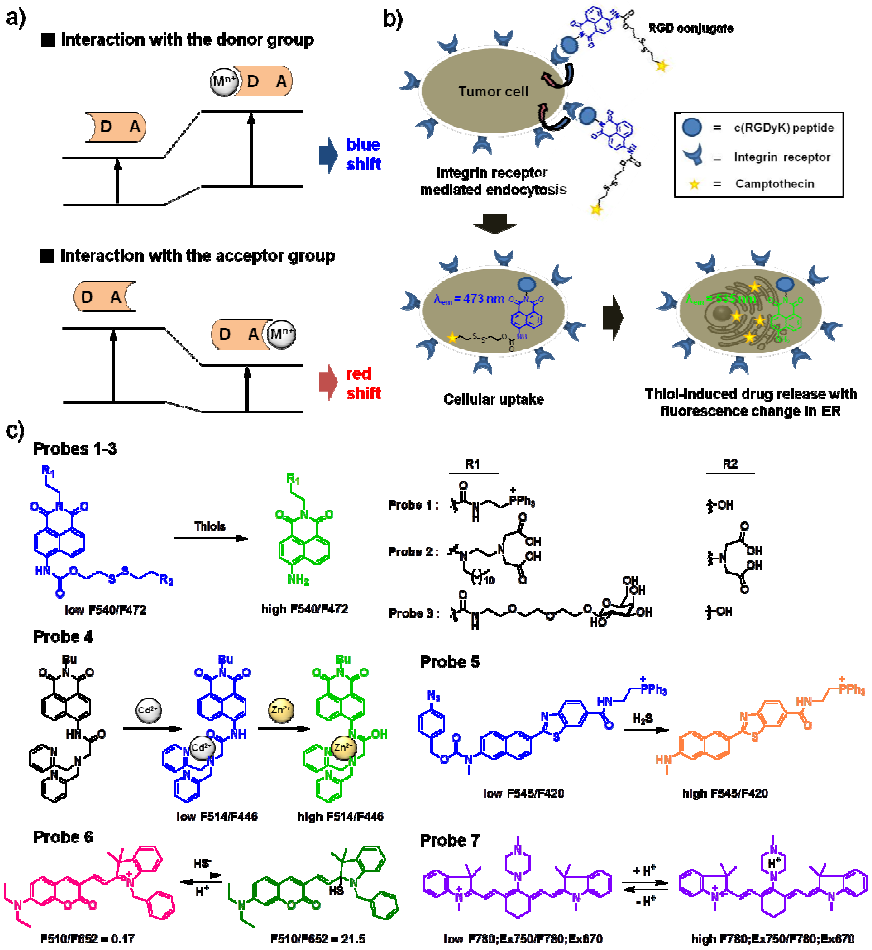

Fig. 1 (a) Schematic representation of the spectral shifts expected for ICT-based sensors as the result of cation binding to the constituent electron donor and electron acceptor groups. (b) A naphthalimide-based ICT molecule that functions as a theragnostic agent. (c) Structures of the ICT-based molecules and corresponding ratiometric fluorescence responses, **Probes 1-7**. The illustration in Figure (b) is reproduced with permission of the American Chemical Society from ref. 28. Copyright © 2012.

ESIPT-based ratiometric fluorescent probes

Excited-state intramolecular proton transfer (ESIPT) typically involves a fast proton transfer from a hydroxyl (or amino) unit to a carbonyl oxygen (or imine nitrogen) atom in the excited state of a fluorophore. For example, 2-(2′-hydroxyphenyl)benzoxazole (HBO) exists preferentially in the *enol* form (E) and is stabilized by an intramolecular six-membered ring H-bonding motif in the ground state. Upon excitation at 320 nm, the excited *enol* form (E*) is converted into the excited *keto* tautomer (K*) as a result of ESIPT. This process gives rise to an emission band (~ 500 nm) with a large Stokes shift. After decaying to the ground state, the *keto* form (K) reverts back to the *enol* form through a reverse proton transfer. Other excited *enol* molecules that do not undergo ESIPT typically display emission bands at higher energies (e.g., ~ 430 nm). Under many conditions, ESIPT fluorophores show two emission bands originating from the *enol* and *keto* forms. Variations in the relative intensities of these bands can provide the basis for ratiometric sensing systems (Figure 2a).[13,14] Perturbations to the intramolecular arrangement through intermolecular H-bonding interactions or changes in solvent polarity have been extensively studied.[38] Moreover, environment-dependent modulations in the optical features provide the background for several applications of ESIPT dyes, including as polarity-sensitive probes in analytical chemistry, molecular logic gates, luminescent materials, as readout elements in polymer science, markers in colloidal chemistry, and biochemical markers.[39]

Several other ESIPT dyes, including 2-(20-hydroxyphenyl)-benzoxazole (HBO), 2-(20-hydroxyphenyl)benzothiazole (HBT), *N*-(3-(benzo[d]thiazol-2-yl)-4-(hydroxyphenyl)benzamide (3-BTHPB) and 3-hydroxyflavone (3HF) have been developed as ratiometric fluorescent probes. For example, Demchenko and Mely reported 3HF-based ESIPT probes that serve as fluorescent biomembrane probes that permit the ratiometric detection of apopotosis.[40] Peng *et al.* reported HBO-based ESIPT probes (**Probe 8**) for the detection of fluoride and acetate ions.[41] Metal ion binding can also trigger an ESIPT effect. For instance, Pang *et al.* reported a HBO-based fluorescent probe (**Probe 9**) that produces an ESIPT emission upon Zn^{2+} ion complexation.[42] In other studies, Kim *et al.* reported an HBT-based probe (**Probe 10**) that produces an *enol*-derived emission feature as the result of inhibiting ESIPT *via* phosphorylation of the HBT hydroxyl group.[43] Upon selective enzymatic (MKP-6; a protein tyrosine phosphatase) hydrolysis, however, ESIPT occurs to give a *keto* tautomer-based emission. Similarly, the probe (**BTTPB**), where the hydroxyl group was protected by *tert*-butyldiphenylchlorosilane, was found effective for fluoride anion detection in micellar mixtures (Figure 2c). Sensitivities at the ppb level were observed and extensions to easy-to prepare test papers proved easy to effect.[44] Unfortunately, ESIPT-based ratiometric detection has proved limited in certain instances since simple protonation of the probe in aqueous media can give rise to unexpected and non-analyte dependent fluorescence changes.

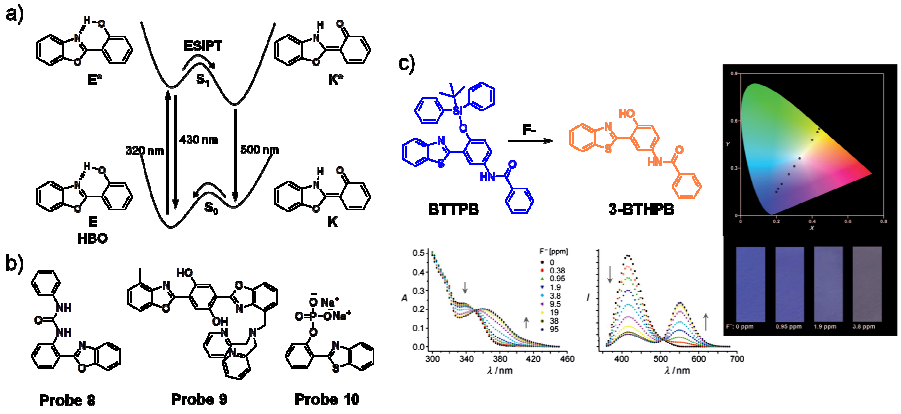

Fig. 2 (a) An illustration of ESIPT showing the photo- and thermal driven changes that occur upon photoexcitation of 2-(20-hydroxyphenyl)-benzoxazole (HBO). (b) Chemical structures of ESIPT-based molecules, **Probes 8-10**. (c) Structure of **BTTPB**, a probe fluoride ions and spectral traces show the analyte-induced absorption and emission changes observed upon exposure to F^- in micellar suspensions in water. CIE 1931 (x,y) chromaticity diagram of test papers for the detection of NaF that are based on 3-BTHPB as derived from fluorescence spectra recorded at different analyte concentrations. The absorption and emission spectra and the CIE chromaticity diagram are reproduced with permission of the Wiley from ref. 44. Copyright © 2010.

FRET/TBET-based ratiometric fluorescent probes

Fluorescence resonance energy transfer (FRET) and through-bond energy transfer (TBET) mechanisms involve energy transfer between a pair of fluorophores that act as energy donors and acceptors, respectively.[1,2,11,12] As applied in sensing applications, the emission of the donor at relative short wavelength serves to activate emission of the acceptor at longer wavelength with the ratio of these two emissions modulated by the target analytes. Substantial spectral overlap between the donor emission and the acceptor absorption bands is generally required to show a high FRET efficiency.[11,12] As a consequence of this photophysical requirement, FRET-based dyads are typically linked by a nonconjugated spacer with energy transfer occurring through space (Figure 3a). Since Stryer and Haugland exploited FRET as a "spectroscopic ruler",[45] FRET has emerged as a critical tool for the analysis of DNA structures, nucleic acid regulation, protein structure, function analysis, and immunoassays.

In the case of TBET-based dyads, the donor and acceptor are linked by an electronically conjugated bond. This prevents the donor and acceptor fragments from becoming planar. Energy transfer can thus occur through a linking bond or bonds without the need for spectral overlap (Figure 3b).[11] Early on, a systematic study of TBET was undertaken by Verhoeven and co-workers.[46]

Subsequent to these pioneering efforts, many FRET/TBET-based fluorescent probes have been put forward for the ratiometric detection of metal cations. Metal ions, such as Hg^{2+}, Pb^{2+}, Ag^{+}, and Cu^{2+}, generally act as excited state quenchers *via* electron transfer or heavy metal ion effects. As a result, the fluorescence of the probe is quenched. In early work, Akkaya *et al.* designed several FRET-based "cassettes" having π-extended BODIPY derivatives and demonstrated their use in Hg^{2+} ion sensing (**Probes 11** and **12**).[47] The energy transfer efficiency is enhanced in the presence of Hg^{2+} ions. This increase produces a correlated ratiometric change in the fluorescent emission features. Burgess *et al.* reported a design concept for TBET cassettes that could be used in many molecular biology and biotechnology applications.[48] In one proof-of-concept study, involving a series of TBET molecules bearing a BOPIPY-based donor and a cyanine acceptor (**Probes 13-15**),[49] the Burgess group demonstrated how upon excitation of the BODIPY moiety the excitation energy is transferred to the cyanine dye acceptor through an acetylene bridge. The emission wavelength was found to depend on the structure of the cyanine acceptor and could be varied from approximately from 600 to 800 nm. Hence, this provides a large spectral window for imaging and was considered to be a useful feature. This set of probes could be encapsulated in calcium phosphate/silicate nanoparticles, which could be dispersed freely in water and applied to intracellular imaging.

Rhodamine has a time-honoured role in sensing. Its use dates to the early days when Czarnik *et al.* reported the application of a rhodamine derivative for Cu(II) sensing.[50] Rhodamine is generally colourless and non-fluorescent in its spiro-ring closed form. In contrast, the ring-opened form of rhodamine is red and characterized by a strong fluorescence. The rhodamine ring-opening process can be activated by metal complexation, analyte binding, substrate-induced reactions, or exposure to acidic pH.[51] In a typical FRET system, ring-opening of rhodamine is used to control an Off-On signal by inducing a spectral overlap between the emission of the donor dye and the absorption of the rhodamine acceptor. To date, a number of the rhodamine-based FRET sensors have been reported. These include rhodamine-dansyl dyads for Cu^{2+} sensing, rhodamine-coumarin dyads for nitric oxide and HOCl detection (**Probe 16**), rhodamine-BODIPY dyads for Hg^{2+} sensing or for the analysis of thiol-containing analytes, such as cysteine, etc. Some of these systems have been used to effect the ratiometric imaging of certain analytes in living cells.[11,52] When used in a TBET system, the rhodamine ring-opening process activates a through bond energy transfer event.[11] This has been exploited by Tan *et al.* who developed a rhodamine-based two-photon TBET probe (**Probe 17**) that allowed for the two-photon imaging of living cells and tissues. This system permitted high resolution ratiometric imaging with good tissue penetration (up to 180 μm).[53]

In another study, FRET-based probes having two Zn^{2+} binding sites and containing coumarin as an energy donor and xanthene as an energy acceptor were used for the ratiometric detection of nucleoside polyphosphates (Figure 3d).[54] In the absence of Zn^{2+} ions, the FRET process occurred and two emission bands, corresponding to the coumarin and xanthene moieties, respectively, were observed. However, addition of Zn^{2+} ions induced an increase in the coumarin fluorescence followed by a near-complete quenching of the xanthene fluorescence, providing for a FRET-Off effect. The binuclear Zn^{2+} complexes were also used for the fluorescent ratiometric sensing and imaging of nucleoside polyphosphates in HEPES (pH 7.4) buffer and in living cells.

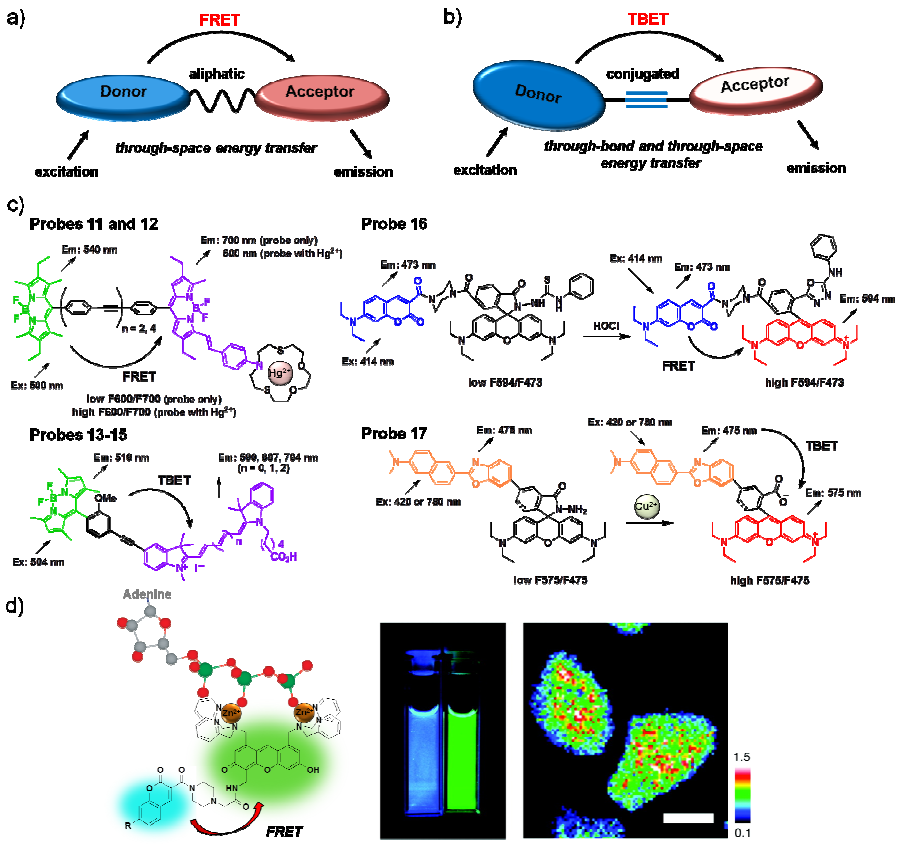

Fig. 3 (a) Through-space and (b) through-bond energy transfer cassettes. (c) Structures of the FRET/TBET-based molecules, **Probes 11-17**. (d) Schematic illustration of FRET-based probes used for the dual emission sensing of ATP and its ratiometric analysis in living cells. The fluorescence images of obtained in solution and in cells are reproduced from ref. 54 with permission of the American Chemical Society. Copyright © 2010.

Monomer-excimer based ratiometric sensing

An excimer is a complex formed by the π-orbital interaction of a fluorophore in the excited state with a fluorophore of the same structure in the ground state.[1,2] Excimer formation is driven by excitation and also by the local concentration of the components. The emission features of an excimer are distinct from those of the monomer emission (uncomplexed fluorophore) and are typically characterized by a red-shifted wavelength and a lack of vibrational structure (leading to a broad emission band). Based on these differences, analyte-driven changes in the separation and relative orientation of the excimer can lead to ratiometric differences in the monomer and excimer emissions. The well studied fluorophore, pyrene, is regarded as one of the most useful sensing molecules in the context of excimer-based analyte detection. It is characterized by a monomer emission at 370-380 nm and an excimer emission at 460-480 nm. To date, many research groups have reported pyrene-based probes for metal ions,[2] phosphates[55] or phosphate-containing biomolecules, such as bacterial alarmone (p)ppGpp[56] and adenosine-5′-triphosphate (ATP).[57]

Several pyrene-functionalized oligonucleotides, locked nucleic acids, and molecular beacons (MBs) have been exploited for the ratiometric sensing of nucleic acids.[58,59] For example, Schmuck *et al.* reported a pyrene-based peptide beacon (**Probe 18**) that interacts effectively with double-stranded DNA (Figure 4).[60] In this case, the folded form of the pyrene-based peptide beacon shows a typical pyrene excimer emission. Upon the binding of double-stranded DNA, the pyrene-based peptide beacon undergoes a conformational change from a folded to an extended form. This switches the fluorescence from excimer (490 nm) to monomer emission (406 nm). Thus, monitoring the relative intensities of the two emissions (F406/F490) allowed the targeted nucleic acids to be detected in a ratiometric manner.

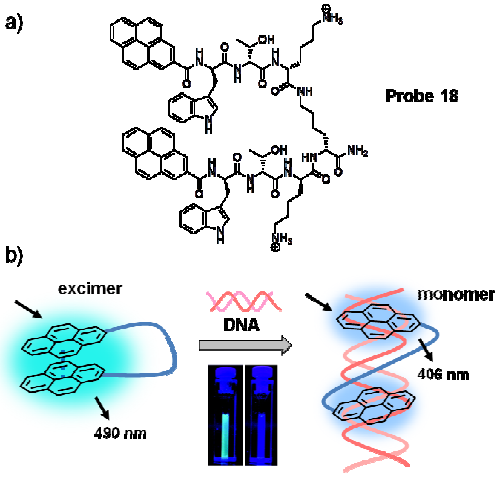

Fig. 4 (a) Molecular structure of **probe 18**. (b) Schematic illustration of **probe 18** and its display of (right) pyrene monomer emission and (left) excimer formation and emission as applied to ratiometric DNA sensing. The fluorescence image of the solution is reproduced from ref. 60 with permission from the American Chemical Society. Copyright © 2012.

Summary and outlook

Fluorescent probes have received considerable attention in recent years due to their simplicity, convenience, and potential for use in a wide range of chemical, biological, and environmental applications. Among the probes currently being explored, ratiometric fluorescent probes are of particular interest since they may permit greater precision under conditions of quantitative analysis. In this Highlight, we have illustrated several strategies whereby a ratiometric fluorescence signal can be produced using appropriately designed small molecules. We have also presented classic molecular motifs that have been used to create ratiometric fluorescent probes and shown how these probes may prove useful in addressing a variety of sensing and imaging challenges. At present, internal charge transfer (ICT), excited-state intramolecular proton transfer (ESIPT), fluorescence resonance energy transfer (FRET), through-bond energy transfer (TBET), and monomer-excimer formation are among the determinants most useful for creating small molecule-based ratiometric probes. Exploiting these mechanistic modalities in conjunction with appropriate fluorophores, analyte-specific receptors, or reactive sites, has allowed for the creation of fluorescent probes that permit the ratiometric sensing and imaging of toxic substances and essential cellular components, including small chemical species correlated with various human diseases. It is our hope that this Highlight will provide an overview of seminal and current research efforts and provide a mechanistic framework for the creation of new ratiometric fluorescent probes. This highlight may also stimulate work that leads to the development of new sensing approaches, such as, perhaps, the use of mixed organic-inorganic constructs, thus giving rise to more effective and economically attractive sensor systems.

Acknowledgements

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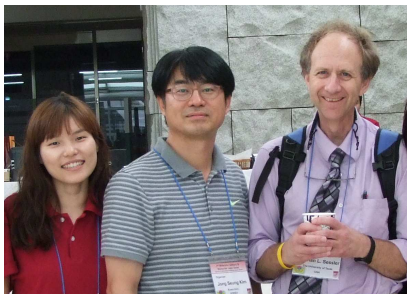
[Biography]



From left to right: Dr. Min Hee Lee, Prof. Jong Seung Kim, and Prof. Jonathan L Sessler

Jonathan L Sessler

Prof. Jonathan L. Sessler received a BS degree (with Highest Honours) in chemistry in 1977 from the University of California, Berkeley and a PhD in organic chemistry from Stanford University in 1982 (supervisor: Professor James P. Collman). After completing NSF-CNRS and NSF-NATO Postdoctoral Fellowships with Professor Jean-Marie Lehn at L'Universite´ Louis Pasteur de Strasbourg, France, he was a JSPS Visiting Scientist in Professor Tabushi's group in Kyoto, Japan. In September 1984, he accepted a position as an Assistant Professor of Chemistry at the University of Texas at Austin, where he is currently the Roland K. Pettit Chair. To date, Dr Sessler has authored over 600 research publications, written two books, and been an inventor of record on almost 80 issued US Patents. Dr Sessler is a co-founder of Pharmacyclics, Inc., a publicly traded company (pcyc; NASDQ) dedicated to developing new cancer therapies.

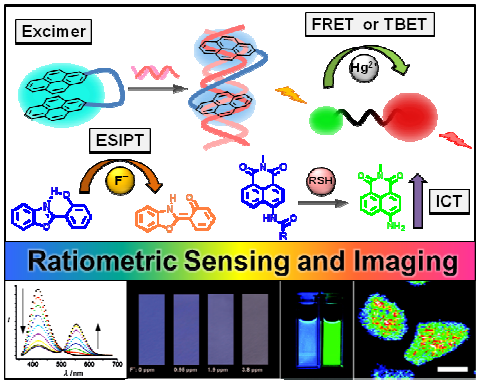
Jong Seung Kim

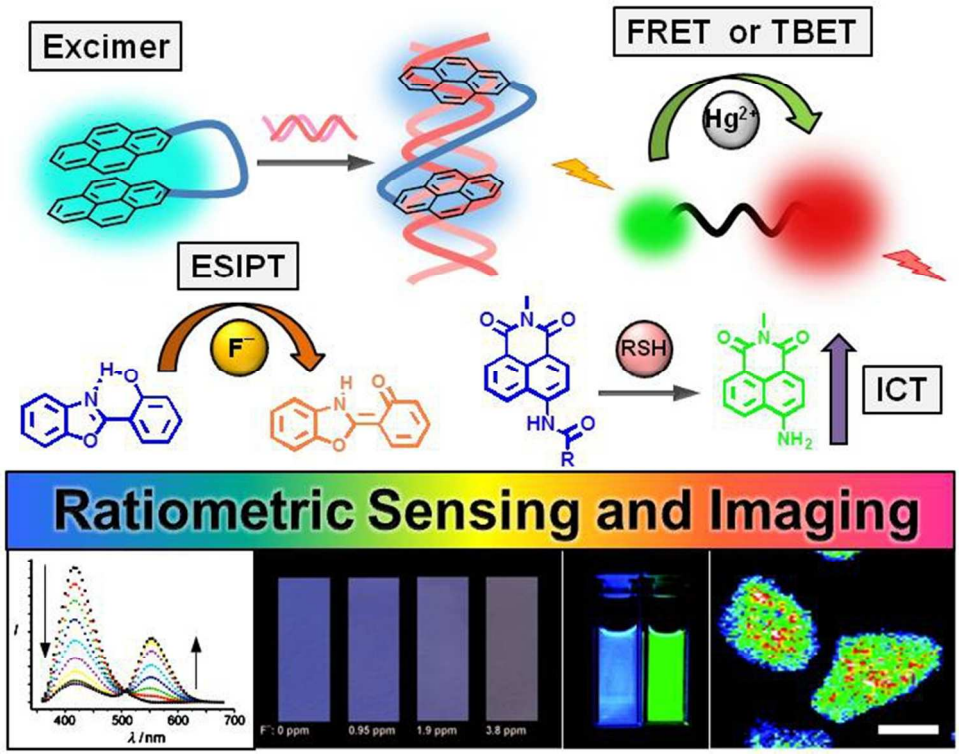
Prof. Jong Seung Kim received a Ph. D. from the Department of Chemistry and Biochemistry at Texas Tech University under the supervision of Prof. Richard Bartsch. After one-year as a postdoctoral fellow at University of Houston under the guidance of J. Kochi, he joined the faculty at Konyang University in 1994. He moved to Dankook University in 2003. In 2007, he accepted a professorship in the Department of Chemistry at Korea University in Seoul. To date, Dr. Kim has published over 330 scientific publications. He is a listed inventor on over 50 Korean and international patents.

Min Hee Lee

Dr. Min Hee Lee received a B.S. (summa cum laude) in chemistry in 2006 and a MS degree in organic chemistry in 2008 from Dankook University, Seoul, and a PhD in organic chemistry from Korea University, Seoul, in 2012 (supervisor: Prof. Jong Seung Kim). She has been working as a postdoctoral fellowship with Prof. Jonathan L. Sessler at The University of Texas at Austin since 2012. To date, Dr. Lee has published 37 peer reviewed papers and been a listed inventor on 6 patents.

[Graphical abstract]





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