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Recent Development of Two-Photon Fluorescent Probes for Bioimaging

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5 **Abstract.**

Fluorescent probes are essential tools for studying biological systems. The last decade has witnessed a particular interest in the development of two-photon excitable probes, owing to their advantageous features in tissue imaging compared to the 10 corresponding one-photon probes. This review summarizes various types of two-photon probes that have been applied to bioimaging, categorized by the principles in the probe design and the target analytes, which would provide a basis for the future development of novel two-photon probes to tackle important 15 biological issues.

Introduction

Understanding the molecular interactions in biological systems is of fundamental importance. Various types of assay and imaging tools have been developed for studying diverse biological 20 processes. Among these tools, fluorescence methods have received great attention as they enable sensitive *in vivo* detection and imaging by relatively simple operations.[1] Fluorescent probes with desirable sensing properties (selectivity, sensitivity, bioimaging capability, etc) are essential for the investigation of 25 molecular interactions and thus have been widely used as diagnostic, monitoring, and analytical tools in biochemical, medical, and environmental research areas as well as in industry.[2] Development of microscopy technology combined with various fluorescent probes has kept up with the bioscience growth. Along 30 with conventional one-photon confocal microscopy, two-photon microscopy (TPM) based on the two-photon excitation combined with laser scanning technology has received increasing interest in recent years for its several advantageous features in tissue imaging where scattering of light becomes a serious issue.[3]

35 Two-photons that arrive simultaneously at a spot combine their energies to excite the molecules there; therefore, two-photon excitation probability diminishes dramatically outside the focal point. This "localization of excitation" allows excitation to a tiny focal volume (~1 femtoliter), which is a distinct feature of two- 40 photon excitation microscopy compared with one-photon excitation microscopy. The focal point is scanned throughout a desired region of the sample to offer very high resolution with 3D images.[4] The use of two-photon excitation at near-infrared wavelength of 700-1100 nm, so called the biological optical 45 window, also enables tissue imaging at increased depths (down to 1 mm). Furthermore, as TPM uses NIR light with focussed

excitation capability, it causes less photo-damage and photo-bleaching to tissues than the corresponding OPM. These promising features of TPM for live tissue imaging in turn have 50 inspired a quest for fluorescent molecules (fluorophores) with enhanced two-photon absorption property.

The efficiency of two-photon absorption property is expressed by two-photon absorption cross-section (TPACS, σ) in units of GM (1 GM = 10^{-50} cm^4 seconds) in recognition of Göppert-Mayer 55 who theoretically predicted the two-photon excitation process.[5] The brightness of a two-photon fluorophore is represented by the product of the fluorescence quantum yield (Φ_F) and TPACS value, so-called the two-photon action cross-section value ($\sigma\Phi_F$). Fluorophores with very low action cross-sections can still be used 60 for two-photon imaging.[6] Two-photon excitable dyes with high action cross-sections, however, allow use of a lower laser power to reduce the probe photobleaching and to enhance cellular viability.[3d] Most common fluorescent dyes have $\sigma\Phi_F$ values in the range of 1–300 GM. Generally, a large $\sigma\Phi_F$ value can be obtained 65 by conjugating electron donating (D) and accepting (A) substituents through π-bridges (-π-).[7] Most two-photon excitable fluorophores can be classified into several types depending on the spatial arrangement of D and A groups:[8] dipolar, quadrupolar, and octupolar types. All of these dyes are designed to maximize 70 the degree of intramolecular charge transfer upon excitation and the transition dipole moment.[9]

In recent years many two-photon excitable fluorescent probes have been developed by introducing an analyte-specific binding or a reaction site to a two-photon excitable dye in such a way that 75 the binding interaction can cause fluorescence signal change. Quadrupolar and octupolar two-photon dyes can show large TPACS values; however, they have relatively large molecular size which can make them less soluble in water.[10] Thus, in most cases, additional functionalization is necessary to enhance the 80 solubility. Also, owing to symmetrical nature of the quadrupolar and octupolar dyes, the corresponding probes have more than two binding sites. In this case, a multiple equilibrium result upon binding more than one analyte, which can complicate the quantification process based on the fluorescence change that is 85 dependent on the binding event as well as the structural change. A variety of two-photon probes have been developed, which contain small aromatic cores (naphthalene,[11] fluorene,[12] stylene,[13] porphyrin,[14] coumarin,[15] fluorescein,[16] rhodamine,[17] naphthylimide,[18] quinoline,[19]), metal complexes (boron 90 complex,[20] platinum complex,[21] and lanthanide complexes[22]), and hydrocarbon diradical species.[23] Two-photon probes based on

even larger platforms such as quantum dots,[24] dendrimers,[25] and polymers[26] are known. Some of these two-photon excitable dyes have been used for immunoassay,[27] cell staining,[28] *in vivo* blood vessel staining,[29] brain tissue staining,[30] and cancer imaging.[31] In this feature article, we focus on dipolar dye-based two-photon probes that have been applied to bioimaging.

Known two-photon probes can be classified into three groups based on the signalling mode: (1) "always-on", (2) "turn-on" or "turn-off", and (3) "ratiometric". The always-on probes are suitable for staining purposes such as cell staining, protein labelling, and histology analysis. The definition of turn-on is arbitrary in scientific community; however, in general sense it is acceptable to use the terminology when the signal enhancement is more than ten times. The turn-on probes are preferred over the turn-off probes in terms of sensitivity and resolution. The ratiometric probes give fluorescence change in a ratiometric manner, which allows quantification free from environmental parameters such as pH, polarity, temperature, emission collection efficiency, effective cell thickness in the optical beam, and changes in the excitation intensity.[32] The ratiometric fluorescent change of probes may be obtained two ways, either by inducing the intramolecular charge transfer (ICT) or by the fluorescence resonance energy transfer (FRET) through molecular interactions with analytes.

Most of two-photon probes can be also classified into two categories according to their sensing mechanism: (1) reaction-based and (2) coordination-based probes. The reaction-based or reactive probes[33] undergo chemical conversion upon interaction with analytes and produce fluorescence change. Although in most cases it involves an irreversible process, the reaction-based approach complements the conventional supramolecular approach in that it can provide a complete selectivity toward the target analyte by employing an analyte-specific chemical conversion. Also, through the reaction-based approach we can realize turn-on sensing of certain analytes that apt to quench fluorescence (such as amine, carboxylate, and paramagnetic metal ions) in the conventional supra-molecular approach. The coordination-based two-photon probes are well developed for metal cations by introducing the metal-binding ligands to two-photon excitable dyes.
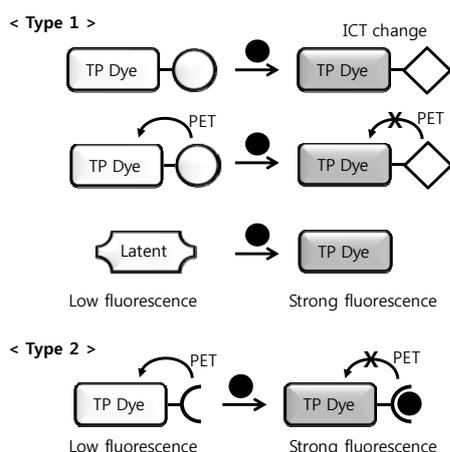
Scheme 1. Category of fluorescence probes: reaction-based (type 1) and coordination-based (type 2) probes. Black circle indicate an analyte.

It is necessary to note typical sensing principles that are widely used to develop two-photon dyes. Three approaches are used to induce fluorescence change in the reaction-based approach (Scheme 1).

The first one is to modulate the ICT state in dipolar dyes by chemical conversion; dipolar dyes have ICT excited states that are sensitive to the electronic perturbation.[34] The second one is to alleviate the photo-induced electron transfer (PET) process that quenches fluorescence of the dye, by chemical conversion, which restores or light up fluorescence.[35] The third one is to transform a latent fluorescent molecule into an fluorescent one, which usually involves a significant structural change. In all cases, the key issue is how to install a reactive site to a two-photon dye in such a way that the target analyte can selectively induce the desired chemical conversion.[36] Various ingenious approaches have been disclosed in the last decade, as seen in the following examples of two-photon probes. In the case of coordination-based approach, it is common to modulate the PET quenching process from a heteroatom-based (mostly nitrogen) ligand by metal coordination, which, in principle, is impossible to extend to sense anions.

In this feature article, we introduce selected examples of various two-photon fluorescent probes with bio-imaging applications, categorized by the two sensing modes.

1. Probing analytes through chemical conversion (reactive probes)

Various chemical probes have been developed based on the receptor–analyte noncovalent interactions such as hydrogen bonding, π–π, hydrophobic and electrostatic interactions.[37] Also, chemical probes based on the metal-coordination bonding have been developed for securing effective binding affinity in aqueous media. However, it is only in recent years to arrest a surge of attention toward the reaction-based fluorescence probes, which involve the use of highly selective and usually irreversible chemical reactions coupled with signal transduction.[38] Such probes in most cases provide higher selectivity with larger spectroscopic change than the chemical probes based on the non-covalent interactions, owing to the large structural changes from the chemical conversion.[39]

In this section, the reaction-based two-photon probes for biothiols, a few gaseous molecules, enzymes and metal ions are described.

H$_2$S (Hydrogen sulfide). Hydrogen sulfide (pK_a = 6.9 in 0.01–0.1 M at 18 °C) exists with its hydrosulfide anion (HS$^-$) under physiological conditions. H$_2$S has emerged as the third gasotransmitter along with NO and CO. H$_2$S appears to be involved in diverse physiological processes including modulation of neuronal activity, regulation of insulin release, smooth muscle relaxation, induction of angiogenesis, suppression of inflammation, protection of cells against oxidative stress, and reduction of the metabolic rate.[40] To understand its role in physiology, it is crucial to monitor the H$_2$S level in living systems. For that purpose, a variety of fluorescent probes have been developed in recent years, mainly based on the reaction-based approach by utilizing the strong nucleophilicity and tandem reaction capability of H$_2$S. Representative one-photon excitation probes are aryl azides (Scheme 2),[41] copper complexes of azamacrocycles,[42] and H$_2$S-specific Michael acceptors.[43]

Following the azidofluorescein probe of Chang and co-workers,[44] Cho and Peng groups introduced two-photon excitable aryl azide probes **1** and **2** based on the fluorene and benzopyran dyes, respectively. [45] Nonfluorescent azidofluorene **1** underwent reduction by H_2S to produce the corresponding arylamine that is strongly fluorescent (turn-on; $\lambda_{ex/TP}$ = 750 nm, λ_{em} = 550 nm, σ = 302 GM; HEPES buffer). Probe **1** was used to image H_2S in the living tissues at 90–190 μm depth under biologically relevant conditions. In a similar manner, probe **2** also reacted with H_2S to give the corresponding amine that emitted fluorescence (turn-on; λ_{em} = 670 nm, $\lambda_{ex/TP}$ = 820 nm, σ = 50 GM; DMSO) (Scheme. 2). Cho and co-workers later reported a related sensing system, probe **3**, which was used to reveal reduction in mitochondrial H_2S production in Parkinson's disease gene knockout astrocytes (ratiometric; $\lambda_{ex/TP}$ = 750 nm, $\lambda_{em/probe}$ = 420 nm, $\lambda_{em/product}$ = 500 nm; σ = 14 GM (probe), 63 GM (product); HEPES buffer).[46] Such aryl azide type probes can show a good level of selectivity to H_2S over competing biothiols (GSH in particular) under biologically relevant concentrations, but by sacrificing reactivity (> 1 h for signal saturation).
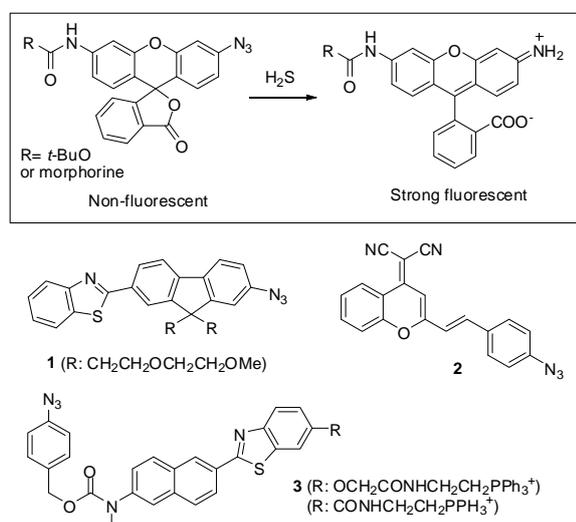
Scheme 2. Turn-on sensing of H_2S through reduction of aryl azides (in the box: the original sensing scheme), and two-photon probes.

NO (Nitric oxide). Nitric oxide (NO) is an important cellular signalling molecule associated with a variety of physiological and pathological processes in living organisms.[47] NO is involved in the vascular smooth muscle relaxation and thus acts as a powerful vasodilator. Also, NO plays a key role in synaptic activity in the brain and host immune system defense.[48] Dysfunction in the NO production pathways can cause various diseases such as ischemia, inflammation, cancer and neurodegenerative disorders.[49] In order to understand its roles in biology, it is crucial to monitor NO in intact tissues. Accordingly, many fluorescent probes have been developed mainly based on or by mimicking the NO-promoted chemical conversion of *o*-diaminobenzene to the corresponding benzotriazole, a sensing scheme originally developed by T. Nagano and co-workers (Scheme 3).[50]

Cho and co-workers introduced *o*-diaminobenzene moiety onto two-photon excitable acedan dye to generate probe **4**.[51] Probe **4** is nonfluorescent because of PET quenching from the *o*-diamino groups. Upon treatment with NO, probe **4** produced the

corresponding benzotriazole with strong fluorescence restored (turn-on; $\lambda_{ex/TP}$ = 750 nm, λ_{em} = 502 nm, σ = 170 GM; PBS buffer). With probe **4**, they were able to selectively detect NO over other competing reactive nitrogen and oxygen species (RNS, ROS) including nitroxyl (HNO). The probe was able to detect NO down to 5 nM level in a wide pH range. Furthermore, probe **4** enabled real-time monitoring of NO in living tissues at 100–180 μm depth for a long period (1200 s).

Jin and co-workers reported a naphthalene derivative **5** containing both an *o*-diaminobenzene moiety and a lysosome-targetable morpholine group.[52] Upon treatment with NO, probe **5** produced the corresponding benzotriazole that emitted strong fluorescence (turn-on; $\lambda_{ex/TP}$ = 840 nm, λ_{em} = 550 nm, σ = 210 GM; PBS buffer). Again, *o*-diaminobenzene **5** showed high selectivity to NO over other ROS and RNS species, with lysosomal NO targeting property. For the first time, probe **5** was used to detect endogenous NO that was captured in lysosomes of macrophage cells.

Recently, Liu and co-workers reported a quinolone-based two-photon fluorescent probe **6**, which emitted at a longer wavelength compared with that of the acedan based probes (turn-on; $\lambda_{ex/TP}$ = 810 nm, λ_{em} = 535 nm, σ = 52 GM; PBS buffer).[53]
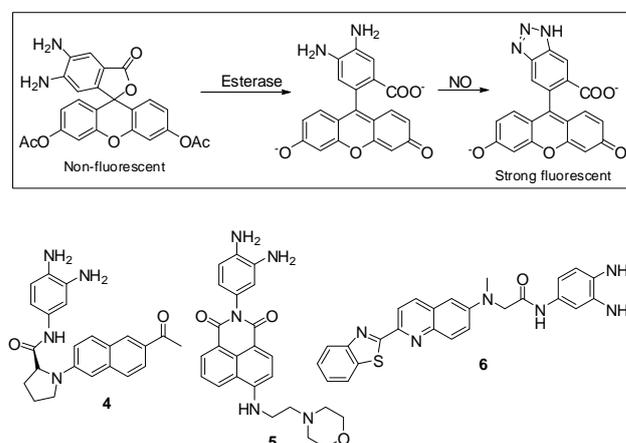
Scheme 3. Turn-on sensing of NO through formation of benzotriazoles (in the box: the original sensing scheme), and two-photon probes.

H_2O_2 (Hydrogen peroxide). Hydrogen peroxide is a prominent member of the reactive oxygen species (ROS) family, and plays important roles in physiology, aging, and disease in living organisms.[54] While unregulated increase in cellular H_2O_2 levels have been linked to DNA damage, mutation, and genetic instability, controlled bursts of H_2O_2 are utilized for cell signaling.[55] Therefore, detection of H_2O_2 in living systems is prerequisite for studying its diverse biological roles. Consequently, various fluorescent probes have been developed so far, in most cases based on the oxidative cleavage of aryl boronates by H_2O_2, a reaction-based sensing scheme developed by Chang and co-workers (Scheme 4).[56] The majority of known probes belong to one-photon excitable probes. Recently, Cho and co-workers reported acedan-based two-photon probe **7,** which contained an aryl boronate reaction site.[57] H_2O_2 oxidatively cleaved the arylboronates in **7** to give the corresponding phenol that was unstable and underwent a sequential bond cleavage to generate 4-methylenecyclohexa-2,5-dienone, carbon dioxide, and *N*-methyl-acedan. This conversion results in a ratiometric fluorescence change because acedan in its carbamate form also emits fluorescence with a hypsochromic shift from that of the

acedan (ratiometric; $\lambda_{ex/TP}$ = 750 nm, $\lambda_{em/probe}$ = 450 nm, $\lambda_{em/product}$ = 500 nm; σ = 12 GM (probe), 45 GM (product); HEPES buffer). Probe **7** detected endogenous H_2O_2 in a brain tissue. In a further study, they developed probe **8** that contained mitochondria-localizing triphenylphosphonium moiety.[58]
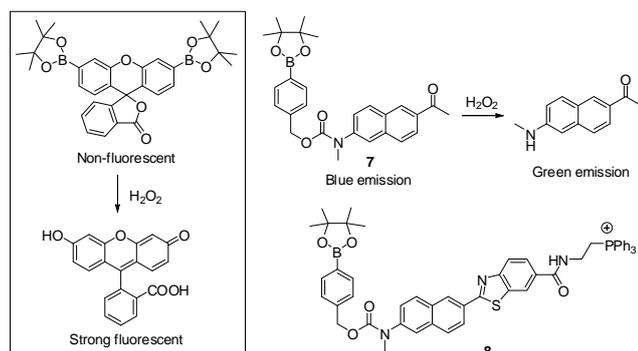
Scheme 4. Fluorescent sensing of H_2O_2 through oxidative cleavage of an aryl boronate (in the box: the original sensing scheme), and ratiometric two-photon probes.

Biothiols. Biological thiols or biothiols such as glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and hydrogen sulfide (H_2S) play key roles in biological systems.[59] These thiol compounds are known to be associated with infectious hepatitis, cancers, Alzheimer's and Parkinson's diseases, and HIV/AIDs.[60] The development of fluorescent probes for biothiols have been pursued mainly through the reaction-based approach by exploring thiol's unique chemical reactivity:[61] (1) conjugate (Michael) addition to α,β-unsaturated carbonyl compounds,[62] (2) nucleophilic substitution of activated sulfonates,[63] sulfon-amides,[64] *N*-selenides,[65] and electron deficient heterocycles,[66] (3) disulfide exchange,[67] (4) tandem aldehyde and enone addition,[68] (5) demetallation of copper complexes.[69]

Cho and co-workers developed acedan derivative **9** as a two-photon excitable probe for biothiols, by adopting the thiol–disulfide exchange reaction.[70] Upon treatment of biothiols, probe **9** underwent the thiol-disulfide exchange reaction to generate the acedan (2-mercaptoethyl)carbamate, which further underwent a fragmentation step to generate *N*-methyl-acedan. The two-photon action spectra of **9** showed fluorescence enhancement in the presence of thiols (turn-on; $\lambda_{ex/TP}$ = 780 nm, λ_{em} = 500 nm, σ = 113 GM; MOPS buffer). Probe **9** detected thiols in live cells and tissues at 90–180 μm depth, without noticeable interference from other biologically relevant species (Scheme 5).

The formation of thiazolines from aryl aldehydes was also used to develop two-photon probes for biothiols. Zhang et al. and Yang et al. developed aryl aldehydes **10** and **11** as two-photon probes for cysteine, respectively (Scheme 6).[71] The chemical conversion resulted in turn-on fluorescence change, owing to ICT change.

Ahn and co-workers evaluated an arylsulfonyl azide system as two-photon probes for biothiols.[72] Although related arylsulfonyl azides were reported as fast-reacting probes for H_2S, it was found that those arylsulfonyl azides do not discriminate H_2S from other biothiols under biologically relevant concentrations (turn-on; $\lambda_{ex/TP}$ = 750 nm, λ_{em} = 445 nm; PBS buffer). A phosphonium containing probe **12** derived from 6-(methylamino)naphthalene-2-sulfonyl azide was found to be biocompatible, hydrolytically stable, water soluble, fast-responsive, and capable of two-photon
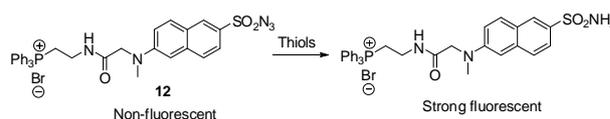
imaging of biothiols in mitochondria (Scheme 7). The reduction of the azido group by biothiols resulted in turn-on fluorescence change, plausibly as the PET process from the azido group was disabled.
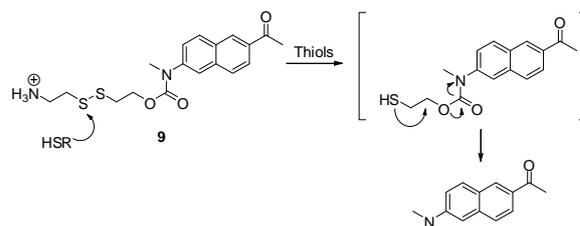
Scheme 5. A two-photon probe that senses biothiols through the thiol–disulfide exchange.
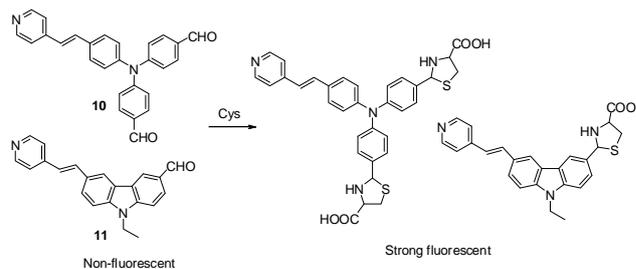
Scheme 6. Two-photon probes that sense Cys/Hcy through thiazoline formation.

Scheme 7. A two-photon probe that senses Cys/Hcy through reduction of arylsulfonyl azide.

Monoamine oxidase (MAO). MAO A and MAO B are two isoforms of MAO. MAOs play a crucial role in regulating tissue levels of amine neurotransmitters and dietary amines. MAO inhibitors have been developed for the treatment of neurological disorders, such as Parkinson`s disease and schizophrenia.[73] Known fluorescent sensing systems for MAO include luciferin–luciferase,[74] resorufin[75] and quinazolinone[76] derivatives, which are based on one-photon fluorophores.

Ahn and co-workers developed compounds **13** (R = H, Me) as two-photon probes for MAOs for the first time, by applying the known reaction-based sensing scheme.[77] The amine functionality in **13** underwent enzymatic oxidation to the iminium ion, which underwent hydrolysis followed by β-elimination to generate the phenol intermediate which, in turn, underwent fast cyclization to form the π-extended iminocoumarin (Scheme 8). It is notable that the π-extended iminocoumarin showed improved two-photon properties compared to acedan: a higher quantum yield, a longer absorption wavelength maximum and a larger TPACS value (Φ_F = 0.63, λ_{em} = 585 nm, λ_{abs} = 446 nm, $\lambda_{ex/TP}$ = 900 nm, σ = 180 GM; EtOH) (acedan: Φ_F = 0.52, λ_{abs} = 365 nm, σ = 151 GM; EtOH). The improved photophysical properties of the π-extended iminocoumarin and its analogues are promising for two-photon imaging of tissues where autofluorescence become significant in addition to the shallow penetration issue when excited at a shorter wavelength.

Probes **13** were used to two-photon fluorescent imaging of MAOs

in C6 glioma cells (MAOs not expressed) and chromaffin cells (MAOs expressed), in the presence and absence of MAO inhibitors (Moclobemide for MAO A, Pargyline for MAO B).

Yao and co-workers reported a MAO B-selective two-photon probe **14** and its use to fluorescently image MAO B activities in mammalian cells, flies, mice and patients during Parkinson's disease development. Furthermore, they assessed MAO-B activities in proteome lysates prepared from B lymphocytes and also from fibroblasts, both derived from PD patients. Importantly, they found elevated MAO B activities only in B lymphocytes, not in fibroblasts. They concluded that the difference in MAO-B activity profiles between control and PD patients are cell-type-specific. This finding hints that the detection of MAO B activity in blood with such MAO probes may provide a rapid diagnosis of PD.[78]
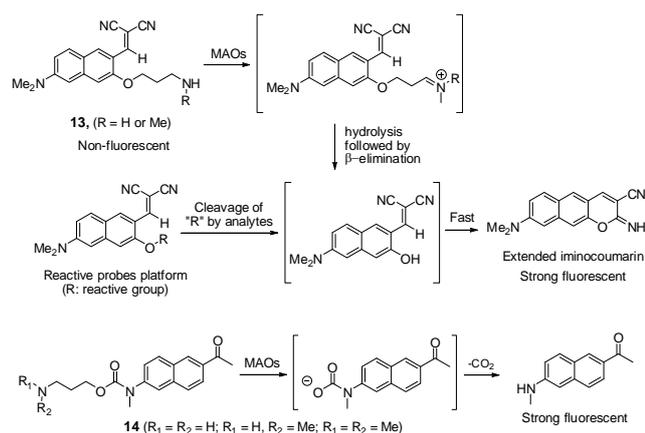
Scheme 8. Two-photon probes that sense MAOs by enzymatic oxidative cleavage of acedan-derived aminopropyl ethers (**13**) and aminopropyl carbamates (**14**).

Tyrosinase. Zhou and co-workers reported an acedan derived 3-(4-hydroxyphenyl)-1-methylurea as the first two-photon probe for tyrosinase.[79] Tyrosinase is a copper-containing enzyme that catalyzes the hydroxylation of phenol to catechol and also the conversion of catechol to the corresponding *o*-quinone which eventually forms melanin. Thus, upon treatment with tyrosinase probe **15** was converted to the corresponding *o*-quinone, which further underwent hydrolysis to generate the *N*-methyl-acedan fluorophore (Scheme 9) with turn-on fluorescence change ($\lambda_{ex/TP}$ = 770 nm, λ_{em} = 503 nm; PBS buffer). This probe was used to image tyrosinase activity in living cells by TPM.
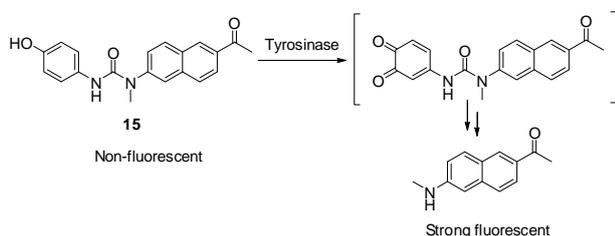
Scheme 9. A two-photon probe that senses tyrosinase through oxidative cleavage.

Phosphatase. Yao and co-workers reported fluorescently quenched activity-based probes for enzymes. One of the probes contained a photo-caged substrate for tyrosine phosphatase (PTP), a two-photon excitable fluorophore, and a quencher (probe **16**).

UV irradiation of the probe (photo-uncaging) could expose the phenyl phosphate moiety to PTPs, causing hydrolysis and subsequent fragmentation into the quinodimethane and the quencher moieties. A phosphatase removes a phosphate group from its substrate, a reverse process of kinase activity; both activities play key roles in many signal transduction pathways.[80] The quinodimethane intermediate could be further captured by bionucleophiles, to afford fluorophore-tagged products (Scheme 10).[81] This sensing scheme was demonstrated by *in vitro* fluorescence assay using purified four PTPs or PTP-expressing cell lysates. Upon uncaging and enzymatic treatment of PTPB, one of the phosphatases, in HEPES buffer, two-photon action cross-sections ($\sigma\Phi_F$) of 140 was obtained at 800 nm excitation. Furthermore, the probe was used to image endogenous PTP activities in HeLa cells, the results of which indicated most endogenous PTP activities in HeLa cells were cytosolic.
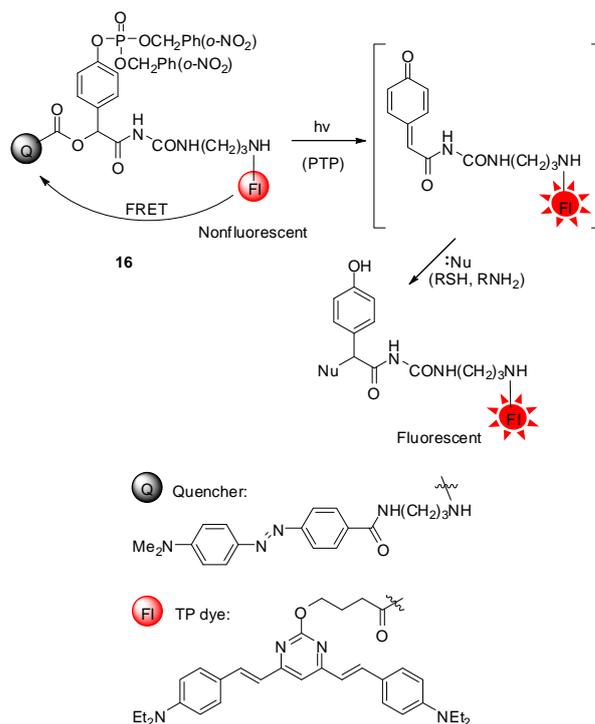
Scheme 10. An activatable two-photon probe for tyrosine phosphatase.

The same group extended the fluorescently quenched activity-based labelling approach to the SNAP-tag technology,[82] this time employing a two-photon excitable acedan dye.[83]

Yao and co-workers further devised aryl (*o*-nitrobenzyl) phosphate **17** as a two-photon probe for phosphatase. The probe enabled them to imaging of phosphatase activities in both live mammalian cells and Drosophila brains.[84] Probe **17** containing cell-penetrating peptides (CPPs) (Scheme 11) was used to organelle- and tumour cell-specific imaging of phosphatase activities in cells and, furthermore, in Drosophila brain with >100 µm detection depth by TPM. This result shows an advantageous feature of two-photon probes over the one-photon probe.[85]
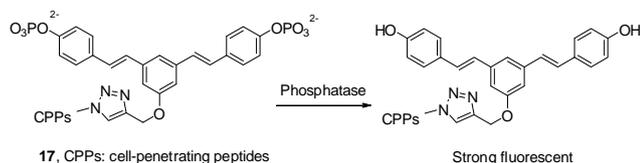
Scheme 11. A two-photon probe that senses phosphatase through the enzymatic dephosphorylation.

Cathepsin L. Cathepsins are proteases that degrade proteins, mostly in lysosomes. There are a dozen members of cathepsins. Over-expressions of these enzymes are associated with a number of pathological conditions including cancers.[86] Yao and co-workers also developed two-photon probes for cathepsin L, based on the modular approach of quencher–enzyme substrate–fluorophore conjugation (Scheme 12). The TP dye and a quencher were deliberately conjugated to the enzyme inhibitors that were identified through a microarray screening.[87] As-prepared probes **18a** and **18b** were used to sense cysteinyl cathepsins in mammalian lysates form HepG2 cells; gradual increase in the fluorescence intensity was observed, as the quencher part was enzymatically cleaved. The probes were further used to live cell imaging to detect endogenous cysteinyl cathepsin activities in endolysosomal compartments of the HepG2 cells by TPM.
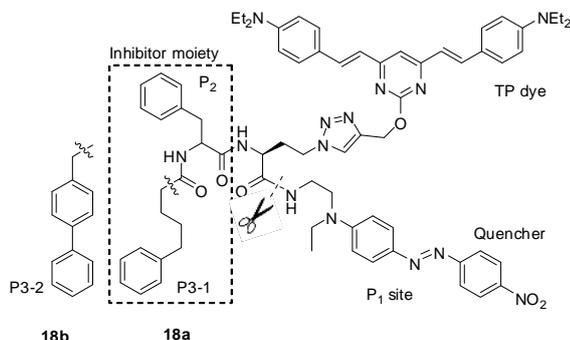
Scheme 12. Ativatable two-photon probes for cathepsin L.

**Mercury ions.** Mercury ions are highly poisonous and widespread pollutant. Mercury ions can damage DNA, impair mitosis, and disrupt the central nervous and endocrine systems.[88] Accordingly, a great number of methods for the detection of mercury ions have been developed. The reaction-based approach that utilizes mercury ion-specific reactions has emerged as a powerful approach to the development of highly selective and sensitive fluorescent probes.[89]

As mentioned in the introduction, two-photon probes based on dipolar D-π-A dyes can give fluorescence change when the ICT excited states are perturbed. Thus, structural change in the donor and acceptor functional groups that can induce significant fluorescence signal change has become a useful sensing scheme for the development of two-photon probes for metal species which can interact with the functional groups.[90] On the basis of this strategy, thioacetals **19** and **20** were concurrently developed as two-photon probes for mercury ions by Zhou and Ahn groups, respectively (Scheme 13).[91] Deprotection of the thioacetals promoted by mercury ions generated acedan. Interestingly, Zhou's cyclic acetal **19** responded to Hg^{2+} very slowly with turn-on fluorescence change, whereas Ahn's acyclic thioacetal **20**

showed very fast response (within a few minutes) with a ratiometric fluorescence change. Thus, probe **20** itself showed strong fluorescence at 428 nm in pH 7.4 HEPES buffer containing 1% acetonitrile; this peak gradually decreased upon addition of Hg^{2+} with simultaneous increase of a fluorescence band at 525 nm (under excitation at 330 nm). Two-photon fluorescence imaging of probe **20** in the absence and presence of Hg^{2+} ions in B16F10 mouse skin cancer cells were successfully carried out using emission filters (430–480 nm for the probe and 500–550 nm for the presence of Hg^{2+}) under excitation at 730 nm.
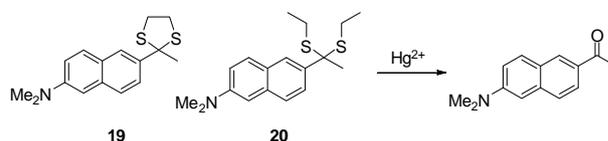
Scheme 13. Two-photon probes that sense Hg^{2+} through hydrolysis of dithioacetals.

Fluoride. Fluoride is added to drinking water to promote dental health in some countries (fluorination), which, however, may cause dental fluorosis. An acute intake of fluoride can cause gastric and kidney disorders, skeletal fluorosis, and urolithiasis in humans.[92] Many fluorescent probes for fluoride have been developed, and most of them are based on hydrogen bonding interactions between fluoride and NH hydrogen, boron–fluoride complex formation, and fluoride-mediated desilylation.[93] Among them, the desilylation strategy seems to be suitable for the development of fluoride probes for biological applications, because silyl ethers are generally not cytotoxic, stable, and inert in cellular environments. The fluoride mediated desilylation approach was originally devised by Swager and co-workers.[94] The widespread use of fluoride and its biological effects obviously demand a fluorescent probe with bioimaging capability. Kim and co-workers developed a silyl enol ether containing a naphthalimide dye as a fluoride probe, which showed a ratiometric response to fluoride along with two-photon excitable property.[95] However, owing to very slow response (3–8 h in 8:2 CH_3CN–HEPES buffer) and poor solubility in aqueous media, the probe was not applied to two-photon bioimaging.

Ahn and co-workers developed a fast-reacting two-photon probe for fluoride, probe **21**, which provided the π-extended iminocoumarin (Scheme 8) through the fluoride-specific desilylation reaction (Scheme 14).[96] As demonstrated in other probes, the desilylation sensing scheme was highly selective, and hence various other anions and biothiols such as cysteine caused little inference. Probe **21** was used to fluorescently image fluoride in B16F10 cells (turn-on; $\lambda_{ex/TP} = 880$ nm, $\lambda_{em} = 600$ nm; HEPES buffer). Furthermore, the probe enabled two-photon imaging of fluoride in a whole body of zebrafish with spatial and time-dependent manner for the first time.
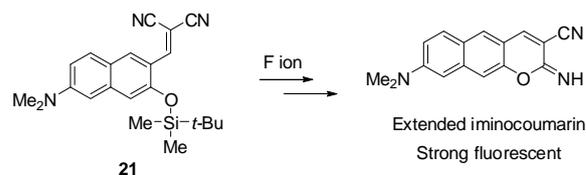
Scheme 14. A two-photon probe that senses F^- through desilylation followed by cyclization.

2. Probing of analytes through coordination or binding

Along with the reaction-based approach, the coordination-based sensing approach has also been enjoyed by chemists particularly for the development of fluorescent probes for metal cations, because metal–ligand binding interactions provide strong affinity even in aqueous media. The most popular sensing mechanism used in sensing metal ions is the modulation of PET process by employing heteroatom-containing ligands (amine, sulphur, and carboxylate). In this case, probes are usually at a quenched state owing to the PET process from the heteroatom lone pair but become fluorescent upon binding metal ions, which process blocks the PET quenching (Scheme 1).

Among various target metal ions, biologically essential metal ions such as Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^+ have received much attention in search of fluorescent two-photon probes. Less commonly, heavy metal species such as Ni^{2+}, Hg^{2+}, and Cd^{2+} have been targets of two-photon probes.

In contrast to metal ions, two-photon probes for biologically important anionic species such as ATP, DNA, and RNA are very limited at present, plausibly because it is much more challenging to selectively recognize these negatively charged and complex species in aqueous media in the fluorescence turn-on mode than the case of metal ions.

Metal ions

The development of two-photon probes for the biologically essential metal ions has been pioneered by Cho group.[97] By introducing the known metal binding ligands to two-photon excitable acedan dye and its derivatives, Cho group has developed two-photon probes for the detection and bioimaging of Ca^{2+}, Mg^{2+}, Na^+, and Zn^{2+}. The listed probes structures show that all the ligands contain heteroatoms that can quench fluorescence of the dye through the PET process; upon binding metal ions, this PET process is blocked and thus fluorescence signals are restored, establishing turn-on type sensing in all the cases. As excellent review articles on two-photon metal probes have been reported by Cho group,[98] in this feature article we will briefly overview some of notable features observed in two-photon bioimaging.

Calcium ion (Ca^{2+}). Calcium ion, one of the most widespread second messenger, plays an important role in signal transduction pathways associated with neurotransmitter release, muscle contraction, and fertilization. Calcium ion levels in mammals are tightly regulated by controlled release from bone and intracellular organelles (mitochondria and endoplasmic reticulum), at roughly 10 nM level of intracellular concentration but with 10- to 100-fold dramatic increase during cellular functions.[99]

Fluorescent probes for calcium ion are developed by combining a metal chelate and a dye indicator. A typical chelate used for calcium ion is BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N, N, N', N'*-tetraacetic acid),[100] which has high selectivity over competing magnesium ion. Typical one-photon probes for calcium ion include Fura-2, Indo-1, Fluo-3, Fluo-4, and Calcium Green-1.[101] To take advantages of two-photon dyes in bioimaging, Cho and co-workers developed a series of acedan–BAPTA compounds **22–24** as two-photon probes for calcium ion (Scheme 15).[102]
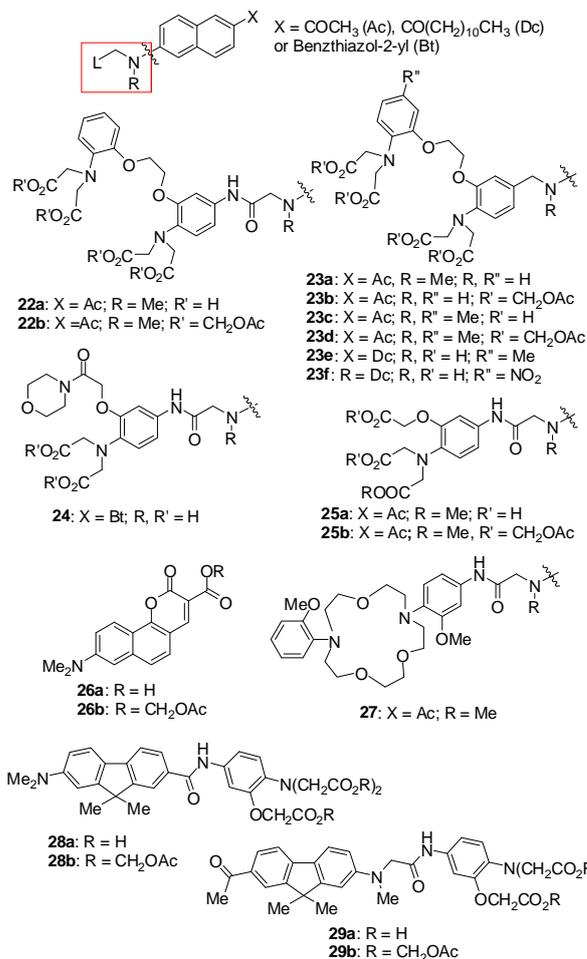
Scheme 15. Two-photon probes for Ca^{2+} (**22**, **24**), Mg^{2+} (**25**, **26**, **28**, and **29**), and Na^+ (**27**).

These probes selectively responded to calcium ion in the fluorescence turn-on mode, as the metal binding to BAPTA blocks the PET quenching process. Probes **22** and **23** showed $\Phi\delta_{max}$ values of 90–110 GM at 780 nm (two-photon absorption maximum) in the presence of Ca^{2+} in buffered solutions. The probes detected dynamic levels of intracellular free Ca^{2+} in living cells and tissues (down to ~150 μm depth) without interference from other metal ions. Using probe **22b**, they visualized the intra- and intercellular calcium ion waves in cultured astrocytes using TPM. Furthermore, they confirmed that calcium waves propagate by the astrocytic process to the soma, from TPM imaging experiments for astrocytes in hypothalamic slices of a rat labelled with probe **23b**. Probe **23e** was used to TPM imaging of near-membrane Ca^{2+}.

Magnesium ion (Mg^{2+}). As the fourth-most-abundant metal ion in cells, magnesium ion is involved in diverse cellular functions. For example, ATP, the main source of energy in cells, is indeed used in the Mg^{2+}-bound form. Also, the triphosphate moiety of DNA and RNA involved in all enzymatic processes is stabilized by association with Mg^{2+}. Magnesium deficiency in human has been associated with cardiovascular disease, diabetes, high blood pressure, anxiety disorders, migraines, osteoporosis, and cerebral infarction.[103] Accordingly, a number of one-photon fluorescent probes for magnesium ion have been developed including Fura-

mag-2 and Magnesium Green;[104] however, most of them show a higher affinity for Ca^{2+}. Owing to this limit, those probes can used to sense Mg^{2+} in cells where the resting level of Ca^{2+} is below 1 μM.[105]

Cho and co-workers developed a two-photon probe for Mg^{2+} by introducing a known Mg^{2+} ligand, *o*-aminophenol-*N,N,O*-triacetic acid (APTRA), to the acedan fluorophore (**AMg1**, **25**).[106] **AMg1** showed turn-on response to Mg^{2+} in Tris buffer solution (10 mM, pH 7.05). Although **AMg1** showed a higher K_d value to Mg^{2+} (1.4±0.1 mM) over Ca^{2+} (9.0±0.3 μM), the probe detected the intracellular free Mg^{2+} in the mM range with little interference from Ca^{2+} because the intracellular Mg^{2+} concentration (0.1–6.0 mM) is much higher than that of Ca^{2+} (10 nm–1 μM). **AMg1**–Mg^{2+} complex showed a 7-fold larger TPACS value of 125 GM at 780 nm than that of Fura-mag-2–Mg^{2+} complex. **AMg1** was used to detect the labile Mg^{2+} in endogenous stores in live tissues at 100–300 μm depth by TPM.

Another turn-on type two-photon fluorescent probe developed by the same group is benzocoumarin **26**, which provides a *β*-diketone binding site to Mg^{2+}.[107] This probe showed a similar level of dissociation constants to Mg^{2+} (1.3 mM) and Ca^{2+} (3.6 mM), allowing two-photon imaging of free Mg^{2+} in endogenous stores at 100–300 mm depth, without interference from Ca^{2+}. The TPACS value of **26**–Mg^{2+} complex was determined to be 107 GM at 880 nm.

Sodium ion (Na^+). Sodium ion is necessary for regulation of the electrolyte level, cation transport, blood volume and pressure, and cell volume, osmotic pressure and pH, nerve conduction, and muscle and heart contraction.[108] The intracellular free $[Na^+]$ is in the range of 5–30 mM, and that of extracellular $[Na^+]$ is >100 mM. In a resting cell, Na^+ influx is regulated by Na^+/Ca^{2+} exchanger, Na^+/H^+ exchanger, and Na^+ channels, whereas the efflux is mediated by Na^+/K^+-ATPase (sodium pump).[109]

Cho and co-workers developed a two-photon fluorescent probe for Na^+ by incorporating known Na^+-binding 1,7-diaza-15-crown-5 moiety into acedan fluorophore (probe **27**).[110] Probe **27**–Na^+ complex showed a TPACS value of 95 GM at 780 nm in pH 7.0 MOPS buffer, a higher value than those of the commercially available probes, Sodium Green and SBFI. Probe **27** was used to image the Na^+ change in astrocytes upon addition of Ouabain, a steroid hormone that increases the cytosolic free Na^+ concentration by inhibiting Na^+/K^+-ATPase, and glutamate, respectively. Probe **27** allowed fluorescent imaging of sodium ions in live tissues at 100–200 μm depth by TPM.

Dual imaging of Na^+/Ca^{2+} and Ca^{2+}/Mg^{2+}. The Na^+/Ca^{2+} exchanger, found in the plasma membrane, mitochondria, and endoplasmic reticulum, plays an important role in Ca^{2+} homeostasis, along with the plasma membrane Ca^{2+}-ATPase. Na^+/Ca^{2+} exchanger removes a single calcium ion in exchange for the import of three sodium ions.[111]

Cho and co-workers studied the Na^+/Ca^{2+} exchange (NCX) process by using two different two-photon probes (probe **27** for Na^+) and (probe **24** for Ca^{2+}) and observing at different emission wavelength channels:[112] Na^+ probe **24** and Ca^{2+} probe **27** emitted in the range of 390–450 nm and 500–560 nm, respectively. The new Ca^{2+} probe **24** showed turn-on response with K_d value of

78±5 μM, and thus are suitable for detecting near-membrane concentration of Ca^{2+} that can reach greater than 100 μM upon activation. TPM fluorescent images of HeLa cells co-labelled with both the probes **24** and **27** showed distinguished distribution of Ca^{2+} and Na^+ in the plasma membrane and cytosol, respectively. Furthermore, they investigated Na^+/Ca^{2+} exchange in live cells in the presence of histamine that stimulates the cells to release Ca^{2+} from intracellular stores; rapid changes in the two-photon fluorescence intensity were observed in the plasma membrane followed by in the cytoplasm regions, which indicated Ca^{2+} efflux followed by Na^+ influx. The combined use of Na^+ and Ca^{2+} probes was also applied to two-photon imaging of both cations in rat hippocampal tissue; the results showed not merged fluorescent images of the two cations.

Ca^{2+}/Mg^{2+} exchange is vital to the muscle contraction and relaxation. Also, Mg^{2+} promotes Ca^{2+} influx in various tissues.[113] Ca^{2+} and Mg^{2+} compete for the divalent cation binding sites in DNA and RNA.[114] Cho and co-workers developed a pair of two-photon probes for dual-colour cell imaging of Ca^{2+} and Mg^{2+}. For the dual imaging of Mg^{2+}/Ca^{2+} in cells and tissue, Mg^{2+} probes **28** and **26** were used, along with Ca^{2+} probe **24**.[115] Probes **28** and **29** showed TPACS values of 87 and 76 GM at 740 nm, respectively, in the presence of excess Mg^{2+}, with the maximum emission wavelength at 525 nm. HepG2 cells co-labelled with probes **24** and **29b** were imaged by TPM through two channels at 400–450 nm (Ch1 for **24**–Ca^{2+}) and 525–600 nm (Ch2 for **29b**–Mg^{2+}), which showed distinct colours in the plasma membrane (green) and in the cytoplasm (red). When the probe-labelled cells were treated with calicimycin, an ionophore that allows Ca^{2+} to cross cell membrane, epidermal growth factor (EFG) that induces a PLCγ1-dependent Ca^{2+} influx, and Mg^{2+} (exogenous), the two-photon fluorescence intensity increased sharply in the plasma membrane and then decreased to the baseline level; a similar trend was observed in the cytoplasm, albeit at a slower rate. When the same experiment was carried out in the absence of exogenous Mg^{2+}, a similar fluorescence change with reduced intensity was observed in the plasma membrane but no change in the cytoplasm. The depletion of Mg^{2+} thus reduced the EGF-induced influx rate of Ca^{2+} whereas nullifying that of Mg^{2+}. The approach of dual probes allowed simultaneous imaging of Ca^{2+} (membrane) and Mg^{2+} (cytoplasm) at 100–200 μm depth in rat hippocampal tissues by TPM.

Zinc ion (Zn^{2+}). Zinc ion is found in hundreds of enzymes (carbonic anhydrase, carboxypeptidase, etc) and proteins (zinc fingers),[116] serving as structural ions and plays ubiquitous biological roles such as in the metabolism of RNA and DNA, signal transduction, gene expression, and apoptosis. Most zinc ions are in the brain, muscle, bones, kidney, and liver. In the brain, a few millimoles of intracellular free Zn^{2+} ions stored in the presynaptic vesicles, which are released upon synaptic activation to modulate brain excitability.[117] A typical ligand for Zn^{2+} ion is DPEN (*N, N*-di-(2-picolylethylene-diamine) and its analogues, which were independently developed by Nagano and Lippard groups, respectively.[118] Numerous fluorescent probes for Zn^{2+} have been developed by incorporating DPEN ligands to fluorophores such as rhodamine [119] and fluorescein dyes.[120]
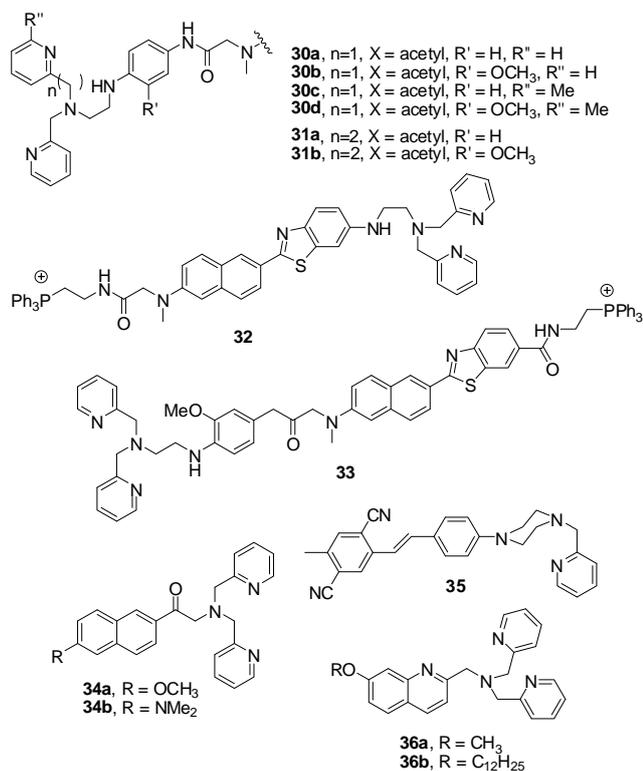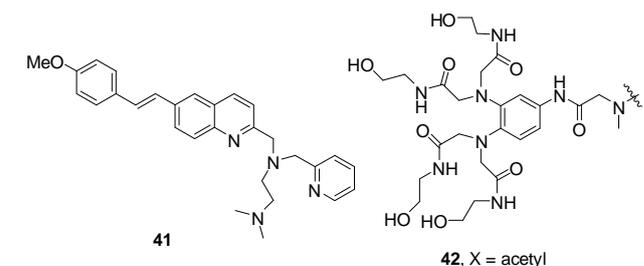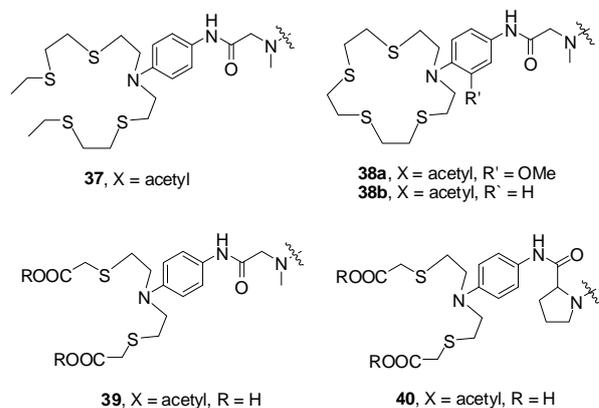
Scheme 16. Two-photon probes for Zn^{2+} (**30–36**).

Cho and co-workers developed acedan-derived two-photon probe **30a** for Zn^{2+},[121] which showed turn-on fluorescence change. **30a**–Zn^{2+} complex showed a TPACS value of 90 GM at 780 nm and the maximum emission wavelength at 500 nm (Scheme 16). Probe **30a** was predominantly located in the cytosolic compartments, and thereby could detect Zn^{2+} ion in live cells without interference from the membrane-bound probes. In further studies, *p*-methoxy-modified DPEN moieties were developed, which reduced residual fluorescence of probes (**30c** and **31**).[122] Also, reported are two-photon fluorescent probes for Zn^{2+} ion with a triphenylphosphonium group that targets mitochondria (**32** and **33**).[123] In addition, simple acedan derivatives that contain the DPEN moiety on the electron-acceptor part (**34**),[124] styrene-based probe (**35**)[125] and quinolone-based probe (**36**)[126] are reported as two-photon fluorescent probes for Zn^{2+}. These probes showed appreciable water solubility, cell permeability, high selectivity, high photo-stability, pH insensitivity at pH>7, and short response times, along with significant TPACS values in the presence of Zn^{2+}.

Copper ion (Cu$^+$). Copper is an essential trace metal found in all living organisms in the oxidized Cu^{2+} and reduced Cu$^+$ states.[127] It is used as a cofactor by many cytosolic, mitochondrial, and vesicular oxygen processing enzymes, including cytochrome-C oxidase, copper/zinc superoxide dismutase, and metallothionein.[128] Free copper ion levels are tightly controlled in the cells, as disruption in copper homeostasis can cause various symptoms including neutropenia, increased incidences of infections, osteoporosis, hyperthyroidism, Menkes and Wilson's disease, and Alzheimer's disease.[129] Fluorescence detection of copper ions in most cases results in quenching owing to a ready interconversion between Cu$^+$ and Cu^{2+} (paramagnetic): metal-mediated quenching

can occur through enhanced spin–orbit coupling or competing energy and electron transfer processes.[130]

Chang and co-workers developed a Cu$^+$-selective fluorescent probe based on an azatetrathia receptor.[131] Cho and co-workers introduced this moiety into an acedan fluorophore to develop the corresponding two-photon probe **37** (Scheme 17).[132] **37**–Cu$^+$ complex showed a TPACS value of 67 GM at 750 nm, with the maximum emission wavelength at 500 nm and 4-fold fluorescence enhancement. The probe was insensitive to pH in the biologically relevant pH range, and was used to detect Cu$^+$ in live cells and living tissues at 90–220 μm depth by TPM

Scheme 17. Two-photon probes for Cu$^+$ (**37**), Hg^{2+} (**38**), Ni^{2+} (**39**, **40**), and Cd^{2+} (**41**, **42**).

Mercury ion (Hg^{2+}). As mentioned in the reaction-based probes for Hg^{2+}, mercury species are highly toxic; hence, efforts to develop efficient detection and analysis tools for them have witnessed a great number of fluorescent probes. Only a few fluorescence probes, however, showed two-photon imaging capability such as the ratiometric probe **20** developed by Ahn and co-workers. Cho and co-workers developed acedan-based two-photon probes **38** for Hg^{2+} by incorporating the sulphur-bridged 5-crown ether ligand that was developed by C. J. Chang (Scheme 17).[133] Probe **38** showed turn-on response to Hg^{2+}; and **38**–Hg^{2+} complex showed a TPACS value of 78 GM at 750 nm with the maximum emission wavelength at 500 nm. Probe **38** was used to imaging of Hg^{2+} in live cells and fish organs at 80–170 μm depth by TPM, with little interference from other metal ions.

Nickel ion (Ni^{2+}). Nickel is used in various industrial and consumer products; stainless steel, magnets, coinage, rechargeable batteries, and special alloys.[134] The toxicity of nickel is low but excess nickel accumulation can cause lethal effects in aquatic ecosystems. The polluted water by Ni ions can effects on human as well as living species likes fish.[135]

A number of fluorescent probes for Ni^{2+} ions have been developed based on BODIPY,[136] coumarin,[137] and other dyes.[138] Cho and co-workers developed an acedan based two-photon fluorescent probes by incorporating the Ni^{2+} binding CTEA ligand (*N, N*-bis[2-(carboxylmethyl)thioethyl]amine (**39**, **40**, Scheme 17).[139] Ni^{2+}-complexes of **39** and **40** showed TPACS values of 32 and 90 GM at 750 nm, respectively. Probe **40** showed 26-fold-enhanced fluorescence in the presence of Ni^{2+}; it was used to detect Ni^{2+} ions in live cells and fish organs at depths of 80–150 μm by TPM, without interference from other metal ions or membrane-bound probes.

Cadmium ion (Cd^{2+}). Cadmium ion is a highly toxic and its half-life in the human body is estimated to be 15–30 years.[140] Excessive exposure to Cd^{2+} will lead to pulmonary and also probably some non-pulmonary cancers such as prostatic and renal cancers.[141]

So far, a few fluorescent Cd^{2+} probes have been reported;[142] however, most of these probes have limited solubility in water or pH dependent.[143] Liu and co-workers reported a selective two-photon probe for Cd^{2+} (probe **41**, Scheme 17).[144] **41**–Cd^{2+} complex showed a TPACS value of 109 GM at 740 nm, with the maximum emission wavelength at 500 nm. Guo and co-workers developed an acedan-based two-photon probe for Cd^{2+} by introducing a new Cd^{2+}-binding ligand, *N,N*-dimethyl-*N*-(pyridine-2-ylmethylethan-1,2-diamine (**42**, Scheme 17).[145] **42**–Cd^{2+} complex showed a TPACS value of 200 GM at 720 nm, with the maximum emission wavelength at 510 nm. Probe **42** was successfully applied to intracellular imaging of Cd^{2+} in live HepG2 cells by TPM.

Biophosphates

ATP. Adenosine triphosphate (ATP) is perceived as the "molecular unit of currency" for intracellular energy transfer in the living cells; it transports chemical energy within the cells for metabolism.[146] The average concentration of ATP in normal and tumour cells was determined to be 2.5 and 3.1 mM, respectively.[147] To name a few, the glycolysis, the Krebs cycle, transporting activities of proteins, modulating ion channels and activating signalling cascades are the fundamental activities of life that rely on ATP energy.[148] For the analysis of ATP, a commercialized "luciferin-luciferase" bioluminescence assay is available, together with a variety of colorimetric and fluorescent probes.[149] The bioluminescence assay poses limitation in the case of *in vivo* imaging of ATP. Also, the known fluorescent probes are only useful for one-photon microscopy.[150]

Ahn and co-workers developed a two-photon fluorescence probe for ATP/ADP based on mononuclear zinc complex and acedan fluorophore (**43**, Scheme 18).[151] Zn^{2+}-complex **43** was found to be chemically stable and non-fluorescent in solution ($\Phi_F = 0.02$) but became highly fluorescent in the presence of ATP (2 equivalents; $\Phi_F = 0.546$, 1.0 N H_2SO_4) and ADP. The competitive binding of Zn^{2+}-complex **43** to ATP/ADP seemed to be responsible for the emission change. A comparison of the binding constants and fluorescence enhancement factors of Zn^{2+} complex **43** toward ATP (6.2×10^6 M^{-1}; 8.9-fold), ADP (3.2×10^5 M^{-1}; 7.0-fold), AMP (5.0×10^3 M^{-1}; 1.9-fold), and pyrophosphate (PPi, 4.0×10^6 M^{-1}; 2.8-fold) suggested that the fluorescence change was also influenced by other factors such as π–π stacking

interactions between the nucleobase and the naphthalene ring, in addition to the binding constant.
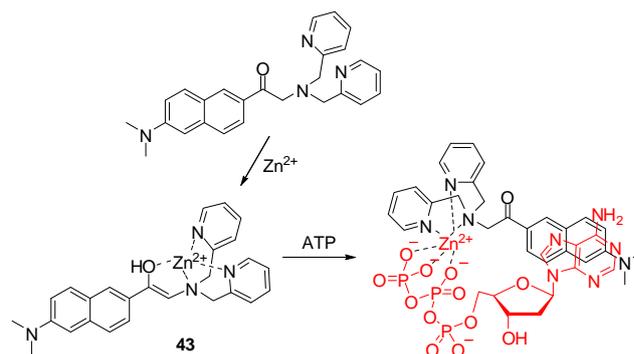
Scheme 18. Schematic illustration for the binding mode between Zn(DPA) complex **43** and ATP.

Upon addition of ATP to Zn^{2+} complex **43** in pH 7.4 HEPES buffer (10 mM; containing 1% CH_3CN), green fluorescence increased with a shift in the maximum emission wavelength from 553 to 537 nm under excitation at 370 nm (at λ_{abs} of acedan). Probe **43** was biocompatible, permeable to cell membrane, able to detect ATP lower than 1.0 μM level, and allowed two-photon imaging of ATP/ADP in live cells for the first time. Ahn and Jung further used ATP probe **43** to obtain higher quality of fluorescence images of ATP in lung, spleen, liver, and spinal cord tissues, compared to commercial quinacrine dihydrochloride.[152]

DNA. The detection of nucleic acid by fluorescent probes has become increasingly important for a variety of analytic and diagnostic applications.[153] For such applications, a probe with a higher affinity for DNA than for other cellular components is required.[154] Also, a large increase in quantum yield should be induced by the binding event. So far, a few fluorescent probes evaluated with one-photon fluorescence microscopy are known.[155] Feng and co-workers reported a carbazole-based pyridinium salt **44** as two-photon probe for double-stranded DNA (dsDNA), which showed high sensitivity and fluorescence turn-on response (Scheme 19).[156] Intercalation of pyridinium salt **44** between dsDNA base pairs served clear TPM images in HeLa cells with specific subcellular localization without interference from other materials.

RNA. RNA is a ubiquitous family of large biological molecules that perform multiple vital roles in the coding, decoding, regulation, and expression of genes.[157] Thus, fluorescent probes for RNA in nuclei and cytoplasm are essential tools in biochemistry and biomedicine.[158] For instance, fluorescence imaging of RNA in parasites increases parasite detection, improves the spatial and temporal resolution of the parasite under drug treatments, and resolves morphological changes in an individual cell.[159] But, DNA staining dyes cannot give additional information regarding the parasite stage affected by the drug and a better indication of the drug's action mode.[160]

Li and co-workers screened a large combinatorial library of fluorescent styryl molecules (1336 compounds) to find several RNA-selective probes including **44** and **45**.[161] Song and co-workers reported indole-based mono- and di-cationic salts such as a V-shaped **45** (Scheme 19), which possessed higher affinity to

RNA over DNA.[162] These compounds exhibited marginal fluorescence enhancement upon binding to RNA. RNA-bound **45** showed a small TPACS value (20 GM at 800 nm) with the maximum emission wavelength at 550 nm.
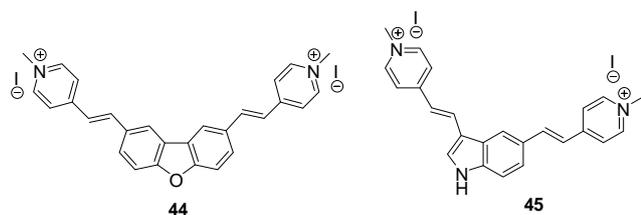
Scheme 19. Two-photon probes for DNA (**44**) and RNA (**45**).

Conclusion

Owing to the several advantageous features of two-photon microscopy in fluorescence imaging of tissue, a variety of two-photon fluorescent probes have been developed in recent years. Various two-photon fluorescent probes with bio-imaging capability are overviewed in this review categorized by the sensing modes: the reaction-based and coordination- or binding-based approaches. As demonstrated by Cho and co-workers with various examples, the coordination-based sensing approach is particularly useful in the development of two-photon fluorescent probes for metal cations (Ca^{2+}, Mg^{2+}, Na^+, Na^+/Ca^{2+}, Ca^{2+}/Mg^{2+}, Zn^{2+}, Cu^+, Hg^{2+}, and Ni^{2+}) where the photo-induced electron-transfer process from the heteroatom (mostly nitrogen) lone-pair electrons of the coordinating ligand was utilized to induce turn-on fluorescence change. For neutral and anionic species (RSH, MAOs, tyrosinase, phosphatase, F^-, ATP/ADP, DNA, and RNA), the reaction-based approach has proven to be promising in the development of two-photon fluorescent probes for realizing high selectivity and turn-on or ratiometric fluorescence change. The use of chemical conversions applies to enzyme catalysed reactions, leading to the so-called activity-based probes. The fluorescently quenched activity-based probes, notably the modular system of quencher–enzyme-substrate–fluorophore offers a general platform of two-photon enzyme probes, as demonstrated by Yao and co-workers.

As shown in the examples, biologically essential metal ions such as Ca^{2+}, Mg^{2+}, Na^+, Zn^{2+}, and Cu^+ have received much attention in contrast to other metal cations including heavy metal species such as Ni^{2+}, Hg^{2+}, and Cd^{2+}. As one-photon fluorescent probes for other metal cations are abundant, obviously the corresponding two-photon probes are expected to appear in near future. On the other hand, it is a more challenging but demanding task to develop two-photon probes for biologically important enzymes, nucleosides (ATP, ADP, AMP, c-GMP, etc) and nucleic acids (DNA and RNA) by securing high selectivity among the competing biomolecules. Efforts toward this direction will be greatly recognized by researchers in biological and biomedical science areas. Also, it should be noted that most of the two-photon probes essentially belong to the intensity-based probes, which may limit their use in quantification of analytes in biological systems because the intensity of fluorescence is susceptible to the environmental parameters such as pH, polarity, temperature, and several instrumental parameters. In this regards, more efforts to develop ratiometric two-photon probes (the ICT

or FRET based) are necessary even though it requires creative modulation of ICT geared with the binding or the conversion by analytes or synthetic elaboration (for the FRET based).

In addition, most of the two-photon probes are based on acedan and its derivatives that show the maximum absorption wavelength around 370 nm or so, rather in the short wavelength region, which cause limited light penetration as well as substantial autofluorescence and photo-bleaching in tissue imaging under the two-photon excitation conditions. Thus, there is a strong need for new two-photon excitable dyes that can be excited at longer wavelength (~900 nm or greater). In this regard, the linear benzocoumarin derivatives developed by Ahn and co-workers are promising for the development of two-photon probes. Surely, other types of novel two-photon fluorescent probes are also expected to enter the stage in coming years as more chemists become interested in this area.

Finally, a number of other issues should be considered in designing two-photon probes, which include solubility in aqueous media, cell permeability, toxicity, organelle specificity, in addition to chemical stability and photo-stability. Most of the two-photon probes described in this review have an adequate solubility (1–10 μM in aqueous buffer) for biological studies solubility in aqueous media.

Notes and references

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Abbreviations: OPM; one-photon microscopy, TPM; two-photon microscopy, TPACS; two-photon absorption cross-section, GM; Göppert-Mayer, HEPES; 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-sulfonic acid buffer, PBS; Phosphate buffered saline, DMSO; dimethyl sulfoxide, ROS; reactive oxygen species, MOPS; 3-morpholinopropane-1-sulfonic acid buffer.

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Table of Content
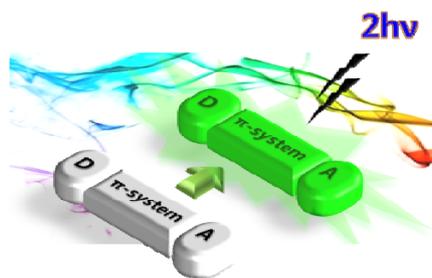
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