Serial Review

Copper Biology in Health and Disease
Guest Editor: Hirokazu Hara

Advances in reaction-based synthetic fluorescent probes for studying the role of zinc and copper ions in living systems

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(Received 29 August, 2022; Accepted 1 October, 2022; Released online in J-STAGE as advance publication 15 December, 2022)

Recently, the behavior of essential trace metal elements in living organisms has attracted more and more attention as their dynamics have been found to be tightly regulated by metallothionines, transporters, etc. As the physiological and/or pathological roles of such metal elements are critical, there have been many non-invasive methods developed to determine their cellular functions, mainly by small molecule fluorescent probes. In this review, we focus on probes that detect intracellular zinc and monovalent copper. Both zinc and copper act not only as tightly bound cofactors of enzymes and proteins but also as signaling factors as labile or loosely bound species. Many fluorescent probes that detect mobile zinc or monovalent copper are recognition-based probes, whose detection is hindered by the abundance of intracellular chelators such as glutathione which interfere with the interaction between probe and metal. In contrast, reaction-based probes release fluorophores triggered by zinc or copper and avoid interference from such intracellular chelators, allowing the detection of even low concentrations of such metals. Here, we summarize the current status of the cumulative effort to develop such reaction-based probes and discuss the strategies adopted to overcome their shortcomings.

***Key Words*: reaction-based fluorescent probe, signal amplification, zinc, copper, glutathione**

Essential trace metal elements have long attracted much attention due to their nutritional importance. Recently, their various biological roles have been studied at the molecular level. Among them, zinc is the second most abundant metal element after iron, and copper is the third. We focus on these two elements in this review not only for their abundance among trace elements but also for their critical physiological and/or pathological roles. In addition, crosstalk between and imbalance of zinc and copper have received increasing attention mainly in the area of the central nervous system,[1,2] providing further motivation for us to discuss them.

First, we focus on zinc. Zinc is involved in biological processes as a cofactor of various enzymes such as alkaline phosphatase, carboxy peptidase, and carbonic anhydrase.[3] Zinc works as a hydrolysis or hydration catalyst in these enzymes. Zinc is also used as a component of the zinc finger domain, often observed in transcription factors to maintain protein structure.[4] Given the importance of zinc, it is not surprising that intracellular zinc is tightly controlled. Zinc transporter proteins ([Zrt-, Irt-like proteins (ZIPs)] and ZnTs) and zinc-transport proteins (metallothionines, etc.) closely regulate its dynamics. Recently, it has been proposed that loosely bound or free zinc functions as a mediator in signaling pathways, and there are many reports which suggest the involvement of mobile zinc in the regulation of mast cell-, basophil-, and T cell-mediated allergic responses,[5] the secretion of insulin in pancreatic β cells,[6] intercellular signaling in the central nervous system,[7] and the prognosis of prostate cancer.[8]

Next, we discuss copper. Contrary to the oxidation state of zinc which is constantly divalent, that of copper interconverts between monovalent and divalent. This redox feature of copper makes it a useful redox cofactor in many enzymes such as superoxide dismutase, cytochrome *c* oxidase, and tyrosinase.[9] Reactions of copper entrapped in enzymes are controlled, but the unregulated interaction of copper with oxygen molecules (O_2) generates reactive oxygen species (ROS, via the Fenton and Haber–Weiss reaction) due to their close redox potential. These ROS have toxic effects which lead to many disease states. To harness such a dangerous metal and make it useful in biological systems, many copper-related proteins such as copper transporters (CTR1, CTR2, ATP7A, and ATP7B), copper chaperones (ATOX1, CCS, and COX17), and copper-transport proteins (ceruloplasmin, metallothionein) work cooperatively.[10] Collapse of copper homeostasis leads to many diseases such as the genetic Menkes and Wilson's diseases.[11,12] Additionally, copper dysregulation is suspected as an accelerator or marker of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. It is thought that the formation of Amyloid beta (Aβ) peptide and α-synuclein aggregates (Aβ plaque and Lewy bodies respectively) are accelerated by copper which acts as an oxidative stress inducer to form neurotoxic masses.[13] The role of copper signaling is being investigated in neurobiology, immunology, cancer biology, and the regulation of fat metabolism.[14] Also, as the chemical properties of Cu(I) and Cu(II) are quite different, it is critical to distinguish them from each other. In an intracellular environment, copper exists almost exclusively as reduced Cu(I) due to abundant glutathione (GSH), while copper in extracellular environments exists mostly as oxidized Cu(II).[15]

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doi: 10.3164/jcbn.22-92

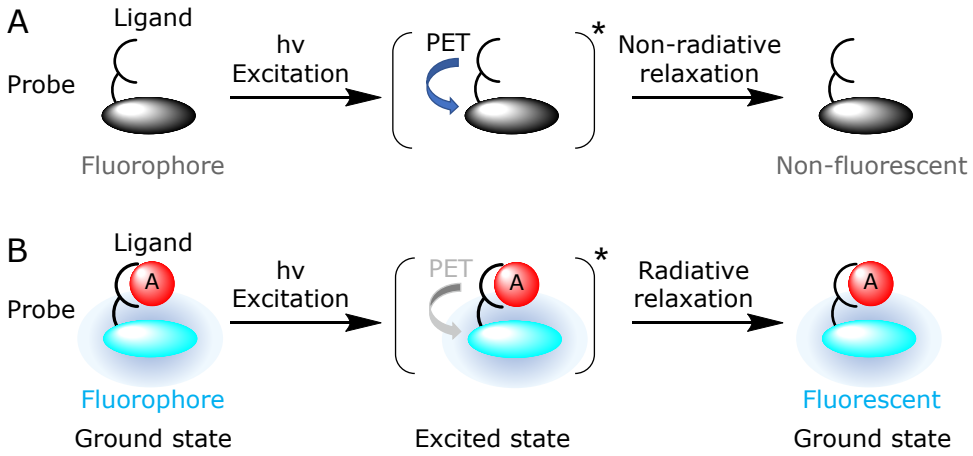

Fig. 1. (A) An electron transfer from the empty ligand to the photo-excited fluorophore creates the quenched, OFF state of the probe. (B) Electron transfer from the occupied ligand is blocked resulting in the fluorescent, ON state of the probe. A: the target analyte.

To quantitatively analyze zinc and copper in samples, inductively coupled plasma mass spectrometry (ICP-MS)[16] and atomic absorption spectroscopy (AAS) have been used.[17,18] These methods are well-known to be applicable to metals including zinc and copper with high selectivity and sensitivity, but they cannot be used with live samples. Moreover, they are unable to distinguish between valence differences, making it unsuitable for biological copper analysis. Alternatively, bioimaging techniques have been developed to visualize zinc and copper in living systems to sufficiently understand their intracellular functions.

Chemical Design and Working Principles of Synthetic Fluorescent Probes for Metal Imaging

Much effort has been paid to developing fluorescence (FL) imaging platforms to detect target analytes *in vitro* and *in vivo* with high sensitivity and selectivity. With such a technique, we can observe subjects of interest in its living state. FL imaging has several advantages over other imaging modalities: low cost, moderate sensitivity (10^{-9}–10^{-12} M) and expedient resolution.[19] Concerning sensitivity and resolution, FL imaging has no major drawbacks compared to other analytical methods. There are various methods for bioimaging other than fluorescent probes, such as luminescence imaging,[20] positron emission tomography,[21] single-photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI).[22,23] Each of these has its own advantages and disadvantages, but neither they nor fluorescent protein-based genetically-encoded sensors[24] will be discussed in this review.

To monitor a target analyte selectively and sensitively with fluorescent imaging probes, the molecules usually have at least two substructures; first, a recognition site specific for the target and second, a fluorophore moiety which changes emission intensity and/or wavelength of excitation/emission upon detection of the analyte. When the target is a metal ion, chemists use their knowledge of coordination chemistry (hard-soft acid-base theory, coordination geometry, etc.) to design a recognition site using suitable ligands. Notably, metal ions with unpaired electrons in their d-orbitals such as divalent Cu(II) naturally quench the emission of neighboring fluorophores.[25] Therefore, such targets are essentially FL quenchers that fundamentally "turn-off" fluorescent probes—a trait not amenable to bioimaging. To develop "turn-on" fluorescent probes for such paramagnetic metal ions, analyte-bound probe complexes are usually cleaved from the quenched fluorophores, i.e., reaction-based probes. Nevertheless, copper exists in its monovalent Cu(I) form in an intracellular environment, and Cu(II) imaging in the field of molecular cell biology is of little use. We will not present them in detail in this review. Readers who wish to understand the current status of the development of Cu(II) recognition-based fluorescent probes are encouraged to refer to a recent review.[26] The majority are turn-off probes with few examples of turn-on probes. In contrast, much effort has been paid to developing reaction-based fluorescent probes to visualize Cu(II). Typically, hydrolysis or an oxidation reaction is used to turn on FL.[27]

In the case of Zn(II) and Cu(I), which do not have quenching properties, we can elaborate turn-on fluorescent probe mechanisms. FL is a phenomenon in which molecules excited by light release energy as emitted light upon returning to their ground state. A proximal free ligand can quench FL from the excited fluorophore via photoinduced electron transfer (PET) (Fig. 1A) if the frontier energy orbitals are in close proximity. In this state, the excited fluorophore's energy is converted to molecular kinetic energy resulting in non-radiative relaxation. Once a complex forms between the ligand and the target metal analyte, PET quenching no longer occurs, and FL is restored (Fig. 1B).[28] This is the most commonly used principle behind recognition-based "turn-on" fluorescent probes, which are the most prevalent fluorescent metal ion probes. Internal charge transfer (ICT) and Förster resonance energy transfer (FRET)[29] are also widely used for the design principle of ratiometric probes[30] as they can modulate the excitation/emission wavelengths. Ratiometric fluorescent probes are more quantitative than simple turn-on probes as the FL intensity at multiple wavelengths provides an internal standard.[31]

Many "recognition-based" probes for zinc and copper have been developed and used to elucidate the metals' biological functions using bioimaging techniques.[32,33] However, recognition-based probes have major drawbacks in terms of sensitivity, as described below.

As metal-ligand complex formation is reversible, dissociation of the analyte metal ions from the ligand moiety of a probe and capture by competing intracellular chelators hinders the FL readout of the target analyte (Fig. 2A). In the case of zinc and copper, which we highlight in this review, examples of such intracellular chelators are GSH and metallothionein. Particularly, intracellular GSH is present at concentrations up to 10 mM,[34] interfering with recognition-based zinc or copper probes greatly. As a result, the sensitivity of recognition-based fluorescent probes in the intracellular environment decreases dramatically compared to those in simple aqueous buffers, severely impairing

doi: 10.3164/jcbn.22-92

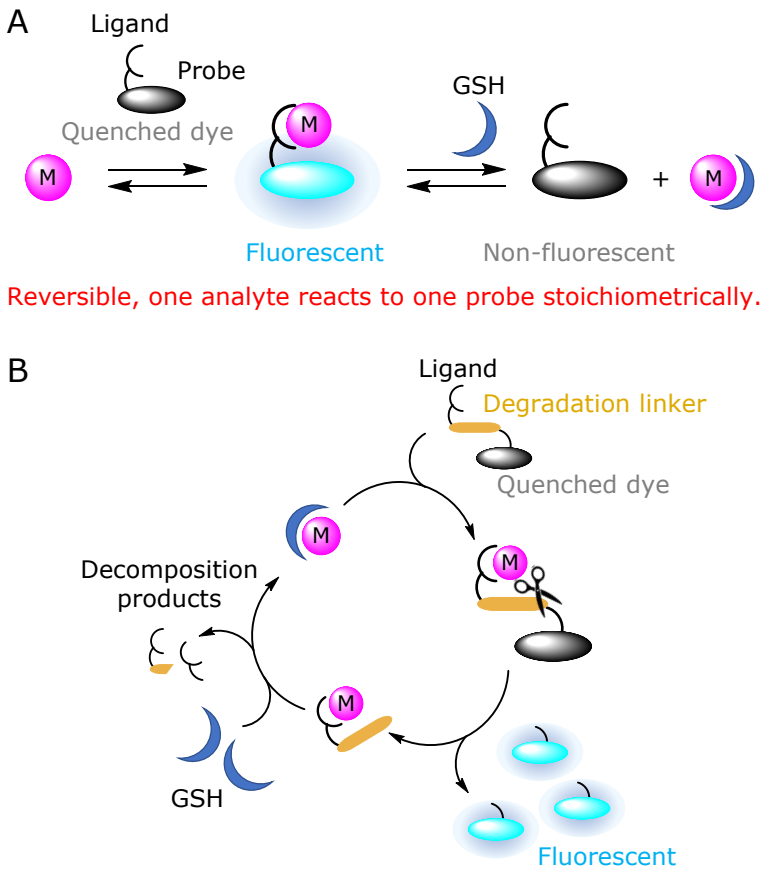

Fig. 2. (A) Concept of recognition-based fluorescent probes. (B) Concept of reaction-based fluorescent probes. M: target metal ion analyte.

the probes' practicality for in-cell bioimaging. Also, one target analyte can react to only one probe at best. Only dramatic intracellular changes (e.g., addition of large quantities of metal ions in medium) of zinc or copper can be visualized. Although live-cell imaging using recognition-based probes have revealed dramatic states and roles of target analyte metal ions during physiological and pathological events,[35–37] it is natural to imagine that even low concentrations of zinc or copper, which recognition-based probes cannot detect, play a role in the cellular environment.

Reaction-based fluorescent probes provide a promising alternative that address this issue. Most of the reaction-based probes are comprised of a recognition site for the target analyte, a fluorophore moiety, and a cleavable moiety whose dissociation is catalyzed by the bound target metal ion. Contrary to the recognition-based fluorescent probes which provide an immediate FL readout upon metal complex formation, the fluorophore and ligand components of reaction-based probes first must dissociate via a chemical reaction (hydrolysis, oxidative elimination, etc.) after metal binding. This is a disadvantage in terms of response speed compared to the recognition-based probes. Also, as reaction-based probes rely on an irreversible process for their FL readout, they cannot track reversible changes of the target analyte level over extended periods of time. Nevertheless, this irreversibility minimizes the interference from intracellular chelators and enables the highly sensitive FL readout of target metal ions. To take advantage of this merit, efforts have been made to develop reaction-based zinc and copper fluorescent probes.

To design such reaction-based probes, chemists must be more strategic than when designing recognition-based probes. In addition to the conventional steps of designing recognition-based fluorescent probes such as selecting a fluorophore with suitable excitation/emission wavelengths, a recognition site for the target analyte, good cell permeability and low cytotoxicity, the metal binding moiety must be highly selective to ignite the chemical reaction upon complex formation to release the fluorophore and quickly elicit a FL response. An additional advantage is that catalytic signal amplification by the target analyte metal ions is possible as one metal ion can react with multiple fluorescent probes, affording high sensitivity and superior detection limit (Fig. 2B). In the case of subcellular investigation, organelle specificity must also be considered.[38]

Overview of the Recognition-Based Synthetic Fluorescent Probes for Zn(II)

N-(6-methoxyquinolin-8-yl)-4-methylbenzenesulfonamide (TSQ) and its related analogues (e.g., Zinquin and its ethyl ester) are spearhead molecules in the field of recognition-based probes for Zn(II) (Fig. 3).[39–41] They bind to Zn(II) to form 2:1 complexes and their FL turn-on mechanism is based on ICT and chelation-enhanced FL. They are commercially available and useful for histochemical applications. Although they can be used for live-cell imaging,[42] they require ultraviolet light for excitation and therefore are not ideal for such purposes. Such short wavelength light is not only harmful to cells, it also induces autofluorescence from intracellular components {e.g., reduced nicotinamide adenine dinucleotide (phosphate) [NAD(P)H], flavins}. At the beginning of 21st century, the Nagano and Lippard groups developed ZnAFs and Zinpyrs probes, respectively, based on

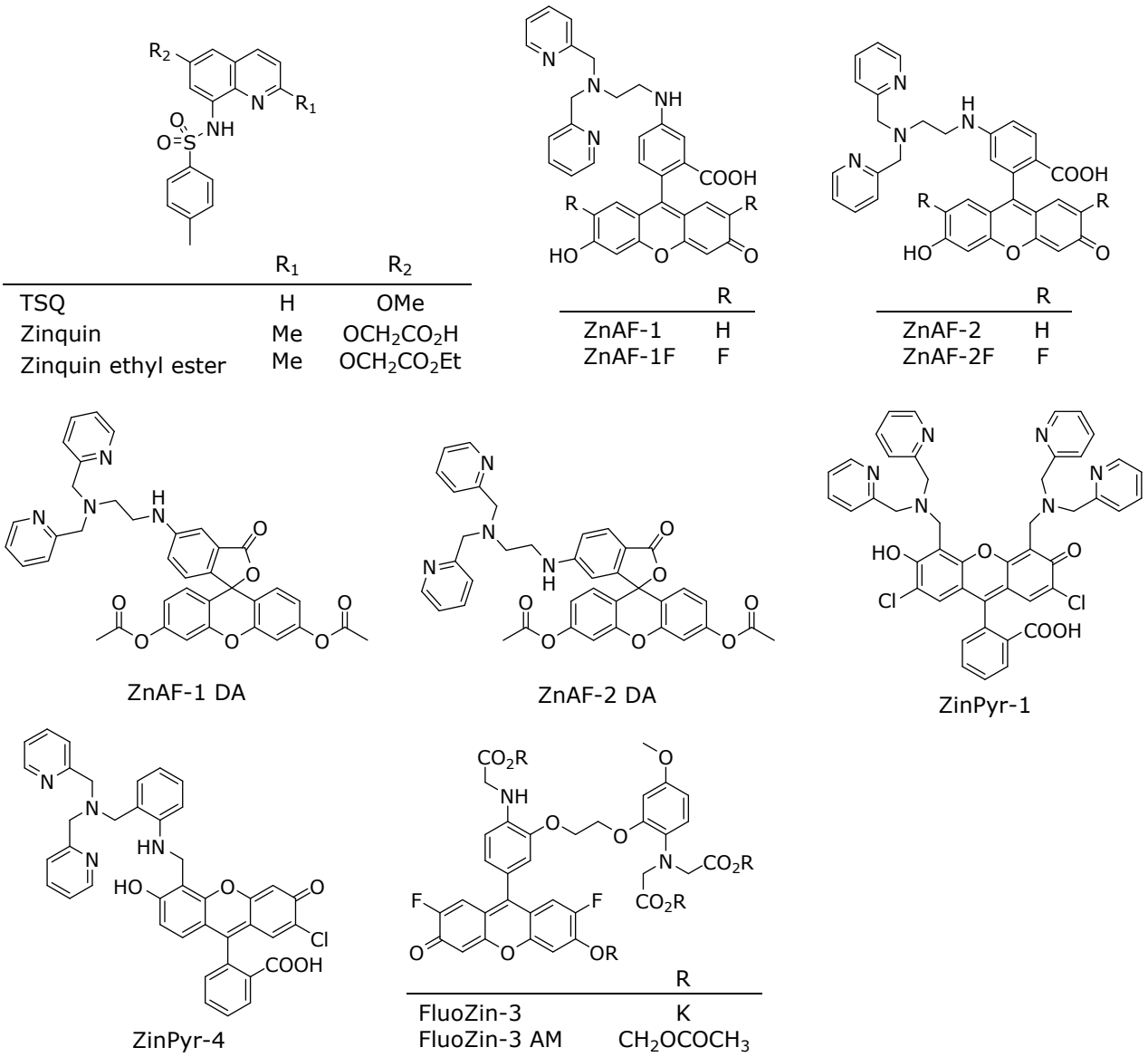

Fig. 3. Representative recognition-based Zn(II) fluorescent probes which are commercially available.

fluorescein, which are excitable with visible light and suitable for application in live cells.[43–46] Once the Zn(II)-selective di(2-picolyl)amine (DPA) moiety binds the target ion, the PET-quenching is disengaged and FL is restored. These probes are now commercially available. Subsequently, many successor Zn(II) probes have been developed to yield various types of turn-on probes such as ratiometric fluorescent probes, two-photon fluorescent probes,[47] and panels of probes which emit at various wavelengths. Many organelle-specific (mitochondria, lysosome, etc.) probes have also been developed.[38] We also show commercially available FluoZin-3 and its cell permeable analogue, FluoZin-3 acetoxymethyl (AM), which were developed by the Kennedy group and Molecular Probes, Inc. to detect Zn(II) with a distinct ligand moiety.[48] Readers who wish to gain detailed insight into this field are encouraged to read some related reviews.[33,49]

Overview of the Reaction-Based Fluorescent Probes for Zn(II)

Zn(II) works as a Lewis acid to decrease the pK_a of zinc-bound water and increase its nucleophilicity.[50,51] Actually, a number of enzymes use zinc as a cofactor to catalyze hydration (e.g., carbonic anhydrase) or hydrolysis (e.g., alkaline phosphatase) reactions as has already been mentioned in the introduction.[3] Exploiting this property of Zn(II), chemists can design a turn-on fluorescent probe composed of a fluorophore and quencher, which is cleaved by a Zn(II)-catalyzed reaction. Actually, aside from those reported by the Lippard and our groups, very few reaction-based probes for Zn(II) have been reported[52–55] in contrast to the large number of recognition-based probes (Fig. 4).

The strategy used by the Lippard group is shown in Fig. 5. DPA was employed as the PET-quenching Zn(II)-selective ligand. Moreover, acetylation of the electron-donating aromatic hydroxy group was performed for even more complete quenching of the probe (Fig. 5A). In other words, diacetyl (DA)-ZP1 is the diacetylated derivative of its predecessor ZinPyr-1.

doi: 10.3164/jcbn.22-92

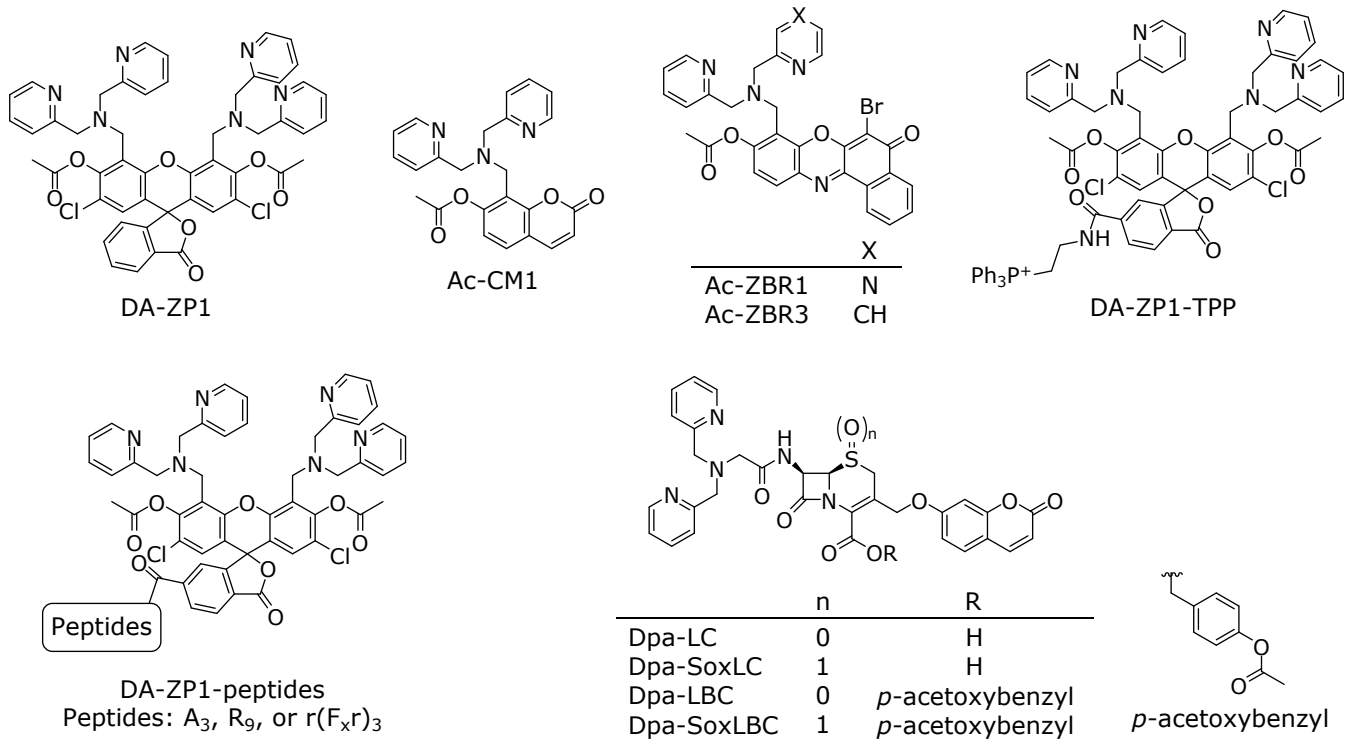

Fig. 4. Reaction-based fluorescent probes developed for Zn(II) so far.

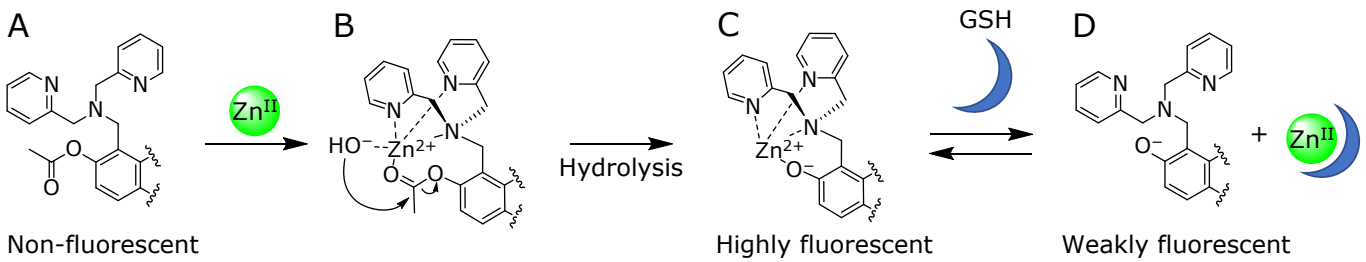

Fig. 5. Reaction-based FL turn-on mechanism of DA-ZP1 and its derivatives.

This ester is fairly robust and impervious to intracellular esterases for over 90 min. Once a complex of the ligand moiety and Zn(II) is formed, nucleophilic zinc-bound water attacks the acetyl moiety to cause ester hydrolysis (Fig. 5B), leading to the same fluorescent turn-on state as that of Zinpyr-1 (Fig. 5C). They showed that DA-ZP1 accumulates in the Golgi apparatus of HeLa cells and displays high FL in tissue slices from endogenous zinc. Other probes based on the same mechanism, Ac-CM1 and Ac-ZBR-1/3, have also been developed to emit blue and red light to achieve multicolor imaging. Obviously, dissociation of zinc from the hydrolyzed probe by competing intracellular chelators such as GSH hinders FL readout (Fig. 5D). It is hard to say that these probes are taking full advantage of the reaction-based probe premise as their FL response is reversible and no signal amplification mechanism exists. In other words, these probes are recognition-based probes, which depend on zinc-catalyzed chemical reactions. The Lippard group also developed organelle-localizable DA-ZP1 derivatives, DA-ZP1-TPP with triphenyl phosphonium (TPP) as the mitochondrial targeting moiety,[52] and DA-ZP1-peptides with modular targeting vector peptides which were directed to the cytoplasm/nucleus, vesicles, or mitochondria in live HeLa cells.[53] DA-ZP1-TPP proved to be a useful tool for investigating mobile Zn(II) in the mitochondria of healthy versus cancerous prostate cells.

As a Lewis acid, zinc can enhance the electrophilicity of carbonyls as well as decrease water's pK_a, which we have already seen in DA-ZP1. Taking advantage of these properties, we have recently developed signal amplification zinc fluorescent probes (Dpa-SoxLC and its cell permeable derivative, Dpa-LBC) which employ a zinc-catalyzed hydrolysis reaction inspired by β-lactamase (Fig. 6A). The probe is composed of an antibiotic cephem core, a zinc-ligand moiety (DPA), and a FL-quenched umbelliferone. Once Zn(II) forms a complex with DPA, it also coordinates to the carbonyl of β-lactam to activate its reactivity. Nucleophilic zinc-bound water attacks β-lactam to hydrolyze the amide bond, leading to sequential electron transfer to release the dye which fluoresces. Simultaneously, the remaining molecular skeleton is broken down into small pieces to release Zn(II). Therefore, a single atom of zinc is recycled and reacts with numerous probe molecules in a catalytic cycle to enhance the FL signal (see also Fig. 2B). The ability of this molecule to detect zinc was maintained even in the presence of GSH at intracellular concentrations, and it was possible to detect zinc sensitively with a change in FL intensity of more than 40-fold. It was determined

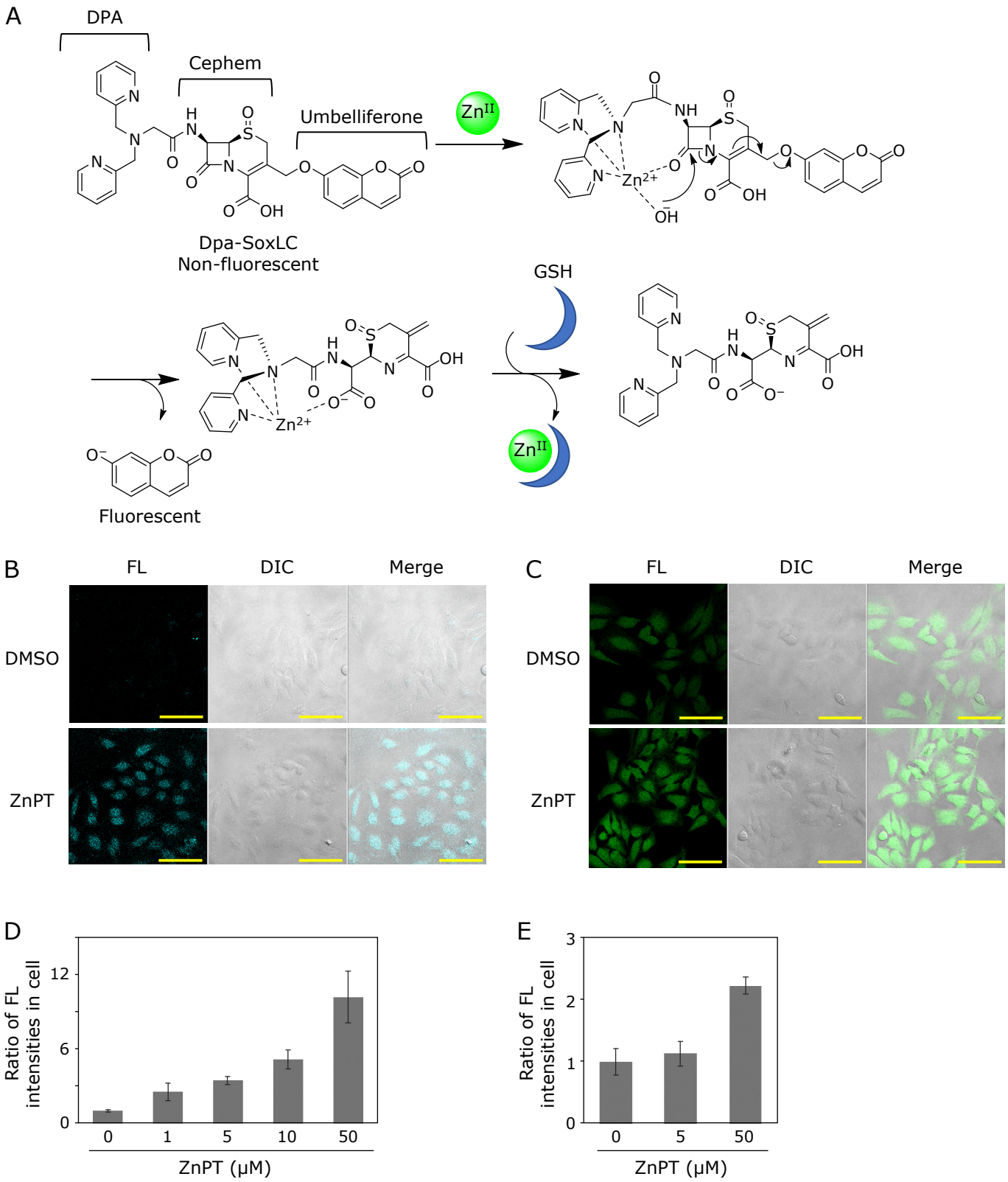

Fig. 6. (A) Mechanistic illustration of the Zn(II) catalyzed reaction of Dpa-SoxLC. (B)–(E) Fluorescent confocal imaging of HeLa cells for the detection of zinc, (B) cells treated with 3 μM Dpa-LBC and 50 μM ZnPT, (C) cells treated with 3 μM ZnAF-2 DA and 50 μM ZnPT, scale bar: 100 μm, (D) FL intensities in the intracellular region 30 min after treatment with 3 μM Dpa-LBC and ZnPT ($n = 5$), (E) FL intensities in the intracellular region 5 min after treatment with 3 μM ZnAF-2 DA and ZnPT ($n = 5$). FL channel: Dpa-LBC, $\lambda_{ex} = 405$ nm, $\lambda_{em} = 420$–530 nm, ZnAF-2 DA, $\lambda_{ex} = 488$ nm, $\lambda_{em} = 510$–630 nm. DIC, differential interference contrast.

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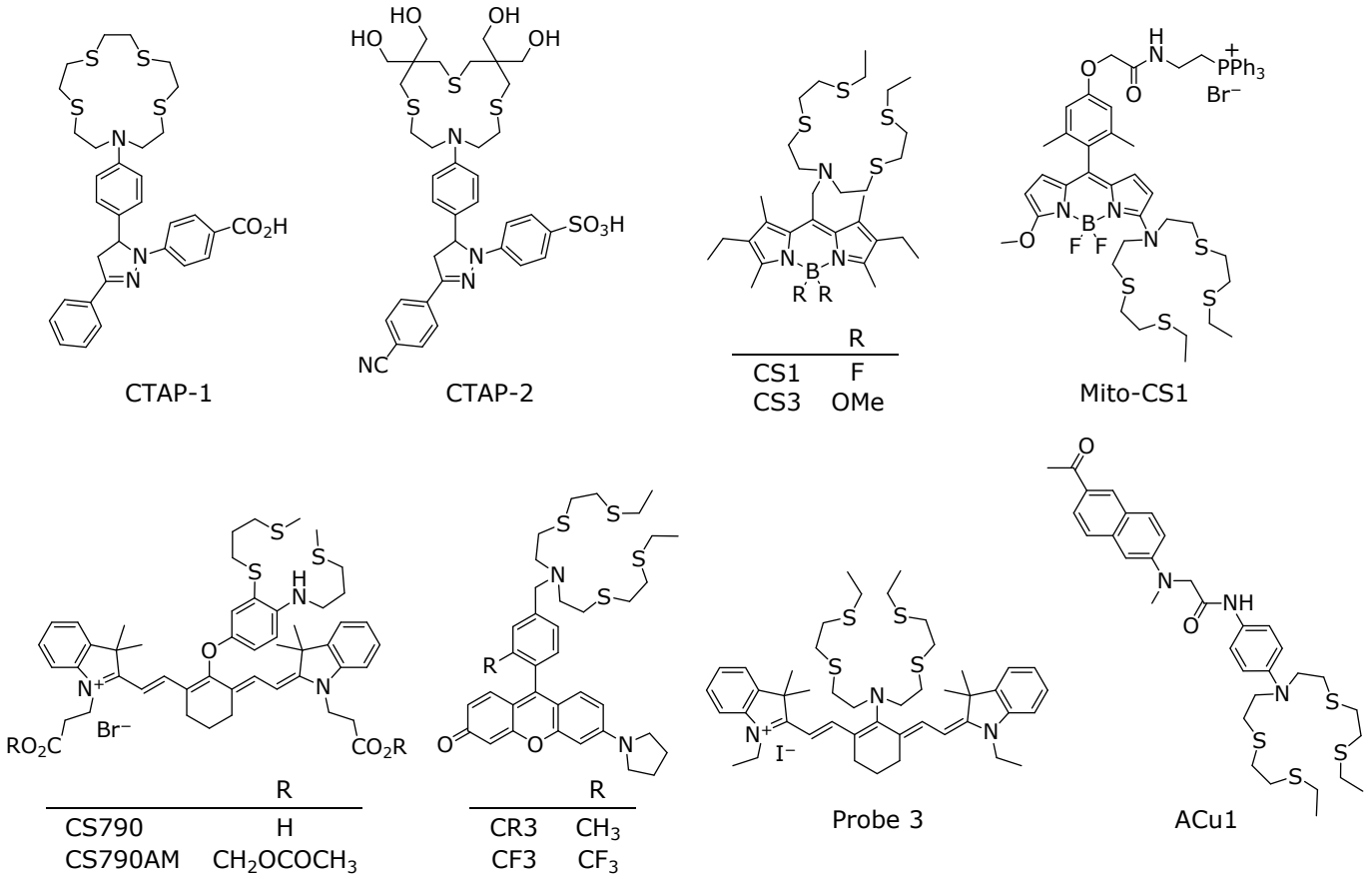

Fig. 7. Representative recognition-based Cu(I) fluorescent probes.

from the FL intensity that zinc reacts with 2.8 Dpa-SoxLC molecules per hour, proving that the system is a catalytic reaction. The detection limit of Dpa-SoxLC was lower than that of commercially available ZnAF-2 and Zinpyr-1 both with 2 mM GSH and without. In the presence of GSH in living HeLa cells, this molecule could detect intracellular zinc [administered as zinc pyrithione (ZnPT)] at low concentrations (even 1 μM) with better sensitivity than conventional fluorescent probes (e.g., ZnAF-2 DA, cell permeable derivative of ZnAF-2), indicating that this molecular design is useful for creating highly sensitive fluorescent probes (Fig. 6B–E). Also, we have found in our laboratory that umbelliferone can easily be replaced in the synthetic route with other fluorophores possessing various wavelengths for further biological applications.

Overview of the Recognition-Based Synthetic Fluorescent Probes for Cu(I)

Before we review reaction-based probes for Cu(I), we will take a look at recognition-based probes for Cu(I). To achieve selective recognition of Cu(I), many researchers have viewed the extreme softness of Cu(I) as soft acidity,[56] pairing it with soft base ligand moieties such as multidentate thioethers to tune the coordination size and geometry over other soft acid analytes [e.g., Ag(I), Pb(II), and Hg(II)]. One key feature in the design of such Cu(I) turn-on probes is PET (Fig. 1A). These dyes are composed of a PET-quenched fluorophore,[28] a thioether ligand moiety, and a linker moiety that contains a nitrogen atom. The highest occupied molecular orbital (HOMO) of the ligand moiety plays a role as a PET modulator to cancel the FL emission from the excited fluorophore. Once a complex forms between the ligand moiety and Cu(I), PET quenching is disengaged, and FL is restored (Fig. 1B).

Here we show representative recognition-based Cu(I) fluorescent probes (Fig. 7). The Fahrni and Chang groups have mainly explored these types of probes. Fahrni's CTAP-1 and Chang's CS1 both visualized intracellular copper which was supplemented in medium.[57,58] The Fahrni group later developed more hydrophilic CTAP-2 for imaging in-gel protein-bound copper.[59] The Chang group improved the efficiency of CS1 to give CS3, which showed that the endogenous Cu(I) distribution depends on calcium levels.[35] They also devised the machinery for developing Mito-CS1 to visualize Cu(I) in mitochondria specifically with the aid of a TPP moiety,[60] CS790AM for *in vivo* imaging with the aid of a near infrared (NIR) cyanine fluorescent dye,[61] and CR3/CF3, aided by the hydrophilic rhodol scaffold, to establish the importance of copper as a fundamental and dynamic component of brain circuitry.[37] Another NIR fluorescent probe, Probe 3, and a two-photon fluorescent probe, ACu1, were developed by the Lin and Cho groups respectively.[62,63] Readers who wish to gain detailed insight into this field are encouraged to read some related reviews.[32,64]

Overview of the Reaction-Based Fluorescent Probes for Cu(I)

Taking advantage of the unique chemical reactivity of Cu(I) to catalyze the azide-alkyne click reaction even in the biological environment, Viguier and Hulme devised a combination of an alkynyl Eu(III) complex and *N*-(2-azidoethyl)-5-(dimethylamino)naphthalene-1-sulfonamide ("dansyl azide") as a Cu(I) fluorescent probe (Fig. 8).[65] A Huisgen 1,3-dipolar

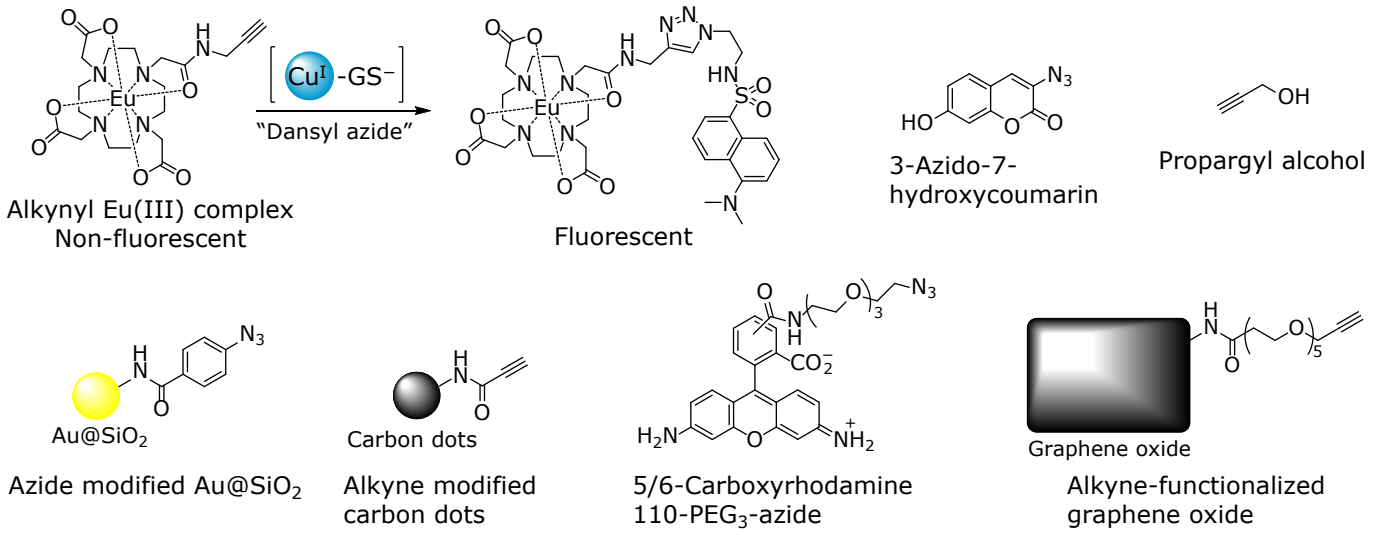

Fig. 8. Reaction-based split-type Cu(I) fluorescent probes based on the Cu(I)-catalyzed click reaction.

cycloaddition reaction between them was catalyzed by micromolar [GS⁻—Cu(I)], a common biological copper complex, to emit a 10-fold enhancement of europium FL by energy transfer from the dansyl moiety. However, they did not demonstrate that it is applicable in live-cell imaging. Later, others reported a similar fluorescent Cu(I) detection system comprised of 3-azido-7-hydroxycoumarin and propargyl alcohol, or azide-modified Au@SiO_2 and an alkyne group modified on carbon dots using ascorbic acid as a reductant, without evidence of utility for live-cell imaging.[66,67] Also, the Jiang group developed a FL report system consisting of alkyne-functionalized graphene oxide and 5/6-carboxyrhodamine 110-polyethylene $glycol_3$ (PEG_3)-azide in which Cu(I) triggered a click reaction to quench the FL of rhodamine.[68] This system was applied successfully to live-cell imaging, but a turn-off probe has limited value.

Recently, the Han and Bu group reported a turn-on fluorescent sensor system triggered by a Cu(I)-catalyzed click reaction.[69] They adopt gold nanoparticles (AuNP) as quenchers for the conjugated fluorophores (Cy3), and Cu(II) reduced to Cu(I) by exogenous ascorbic acid triggers the click reaction which assembles the 3D DNA walker, which is subsequently nicked by an endonuclease (N.BstNBI), releasing Cy3 to fluoresce. Although the addition of ascorbic acid is required for this sensor system, they successfully showed live-cell imaging of exogenously administered Cu(II) with high sensitivity (as low as 6 μM).

Another reaction-based strategy to detect Cu(I) fluorescently is Cu(I)-mediated biomimetic oxidative bond cleavage, which was first applied successfully by the Taki group (Fig. 9).[70] Their FluTPA1 and FluTPA2 consist of *O*-Me dihydrofluorescein derivatives and tris(2-picolyl)amine (TPA) connected by a benzylic ether tether at the fluorescein phenol group. Once the complex between Cu(I) and the TPA moiety is formed, molecular oxygen coordinated to Cu(I) is reductively activated via electron donation by the metal, leading to oxidative C-O bond cleavage to release the quenched fluorophore precursors which undergo spontaneous oxidation to restore the fluorescent fluorescein skeleton. Although the TPA ligand has high affinity to various heavy metal ions including Zn(II),[71] the probe's high Cu(I) selectivity is ensured because only Cu(I) activates coordinated O_2 to ignite the reaction, yielding over a 100-fold increase in FL. It should be noted, however, that high concentrations of GSH (2 mM) is required for this reaction to report FL. Although there is generally plenty of GSH (1–2 mM) in the intracellular environment,[34] GSH fluctuations caused by various physiological and pathological events will convolute Cu(I) analysis. Also, the need for high levels of GSH implies that much of the activated O_2 by the redox-active Cu(I) (1 or 5 μM)-TPA complex generates ROS with only a small portion performing the oxidative cleavage of the linker. Therefore, repetitive regeneration of Cu(I) from Cu(II) by GSH is necessary for the reaction to emit FL. In this process, GSH is oxidized to glutathione disulfide (GSSG), and consequently the probe itself interferes with GSH/GSSG balance in the presence of Cu(I). As the GSH redox status has widespread influence on live cells,[72] such interference may complicate the interpretation of FL imaging. Another concern is that hypoxic conditions are not compatible with this probe as O_2 is indispensable for the reaction. Considering that copper interferes with the adaptive response to hypoxia,[73,74] careful attention should be paid to the FL response, especially in the field of hypoxia biology.[75] Nevertheless, FluTPA2 was successfully applicable to imaging Cu(I) fluorescently in living cells. FluTPA2 is now commercially available as "BioTracker Green Copper Live Cell Dye" (Merck KGaA, Darmstadt, Germany). Similarly, using this Cu(I)-TPA complex-catalyzed oxidative cleavage reaction, the Taki group subsequently reported a Cu(I) fluorescent probe RdlTPA-Et_2 and its mitochondria-targeted derivative RdlTPA-TPP to visualize mitochondrial copper in live HeLa cells.[76] In this probe design, they employed *O*-alkylated hydroxymethyl rhodol to suppress background FL of the probe, which we also employed in the design of a Fe(II) reaction-based fluorescent probe.[77] The TPP moiety was employed as the mitochondrial targeting component in the design of RdlTPA-TPP. There are many successor probes employing this unique Cu(I)-specific reaction, such as one equipped with a different fluorophore, ResCu,[78] ratiometric fluorescent probes, HBTCu and Probe 1,[79,80] a two photon fluorescent probe, Xan-Cu,[81] and a NIR fluorescent probe, TPACy.[82] Cu(I)-catalyzed oxidative cleavage followed by Pinner cyclisation was employed as the turn-on mechanism of CP1, another Cu(I) fluorescent probe.[83] The Chang group developed a ratiometric FRET probe FCP-1 consisting of rhodamine, quenched fluorescein, and a TPA moiety connecting the two fluorophores.[84] It revealed oncogene-driven changes in labile copper pools induced by altered GSH metabolism by live-cell imaging. Interestingly, substitution of TPA with 2-hydroxyethyl[bis(2-picolyl)]amine converted the Cu(I) probes to Co(II) probes.[78,79]

As we have seen so far, Taki's Cu(I)-catalyzed oxidative

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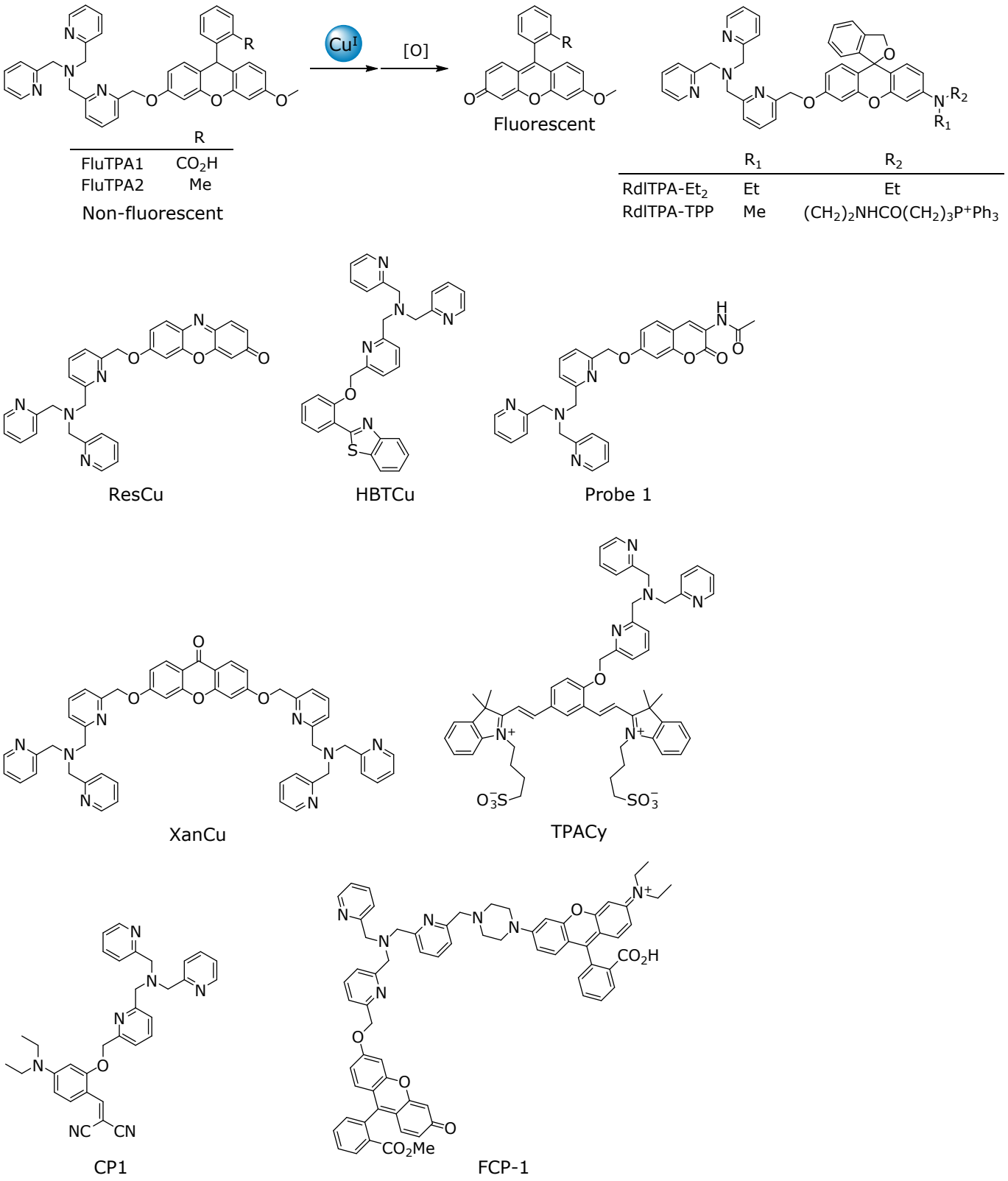

Fig. 9. Reaction-based Cu(I) fluorescent probes based on Cu(I)-catalyzed oxidative benzylic C–O bond cleavage.

cleavage strategy with TPA to develop a Cu(I) fluorescent probe is quite useful for biological studies. Nevertheless, careful attention should be paid to interpreting the FL response due to its O_2 dependence and interference of GSH metabolism, which we have already discussed. To overcome this drawback, hydrolytic cleavage-based turn-on fluorescent probes similar to the reaction-based Zn(II) probes may be useful, in which Cu(I)-complex-catalyzed hydrolysis[85] may be implemented.

Conclusion and Perspectives

In this review article, we have summarized fluorescent probes that detect Zn(II) and Cu(I) mainly based on a chemical reactions to turn on FL. Recognition-based fluorescent zinc and copper probes have proven to be useful imaging tools to analyze biological events such as the zinc spark in fertilization[86] in addition to those discussed in the preceding section.[35–37,52] We suspect that there may be regions of zinc and copper signal propagation that act at even lower concentrations than previously thought, in which existing fluorescent probes cannot detect because of insufficient sensitivity. Reaction-based fluorescent probes, which have an advantage in terms of sensitivity, can be a powerful tool for analyzing the biological functions of zinc and copper at such low concentrations. As demonstrated by our Dpa-SoxLC and Dpa-LBC probes,[55] reactive fluorescent probes exhibit a much greater FL enhancement than recognition-based probes and have a superior detection limit. Additionally, the signal amplification system in which zinc or copper enhances the signal as it reacts catalytically increases sensitivity further. The major disadvantage of reaction-based probes (relatively slow response times) can be addressed by adjusting the structure of the ligand moiety to accelerate the necessary reaction. We are eager for the opportunity to unravel unexplored biological phenomena using such improved fluorescent probes.

Author Contributions

KO: study concept, drafting of the manuscript, obtained funding; IT: study concept and design, obtained funding, revision of the manuscript for important intellectual content; AT: revision of the manuscript.

Acknowledgments

Reproduced from Ref. 55 with permission from the Royal Society of Chemistry. This work was supported in part by JSPS KAKENHI (Grant Numbers JP19K23816 to IT and JP19K07035 to KO), the Zero Emission Research Program, Institute of Advanced Energy, Kyoto Univ. (Grant Number ZE2020C-06 and ZE2021C-03 to IT), and the Vehicle Racing Commemorative Foundation (Grant Number 6110, 6231, and 6297 to KO).

Abbreviations

A	L-alanine
AAS	atomic absorption spectroscopy
Aβ	amyloid beta
AM	acetoxymethyl
ATOX1	antioxidant protein 1
ATP7A	copper-transporting ATPase 1
ATP7B	copper-transporting ATPase 2
AuNP	gold nanoparticles
CCS	copper chaperone for superoxide dismutase
COX17	cytochrome *c* oxidase copper chaperone
CTR1	copper transporter 1
CTR2	copper transporter 2
Cy3	cyanine 3
DA	diacetyl
DIC	differential interference contrast
DPA	di(2-picolyl)amine
em	emission
ex	excitation
FL	fluorescence
FRET	Förster resonance energy transfer
F_x	L-cyclohexylalanine
GSH	glutathione
GSSG	glutathione disulfide
HOMO	highest occupied molecular orbital
ICP-MS	inductively coupled plasma mass spectrometry
ICT	internal charge transfer
MRI	magnetic resonance imaging
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
NIR	near infrared
PEG	polyethylene glycol
PET	photoinduced electron transfer
R	L-arginine
r	D-arginine
ROS	reactive oxygen species
SPECT	single photon emission computed tomography
TPA	tris(2-picolyl)amine
TPP	triphenyl phosphonium
TSQ	*N*-(6-methoxyquinolin-8-yl)-4-methylbenzenesulfonamide
ZIP	Zrt-, Irt-like protein
ZnPT	zinc pyrithione
ZnT	Zn transporter

Conflict of Interest

No potential conflicts of interest were disclosed.

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doi: 10.3164/jcbn.22-92