Fluorescence Imaging of Metal ions Implicated in Diseases

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Introduction

Over the past decades, the abnormal homeostasis of metal ions in cells/tissues has been related to several diseases, such as neurodegenerative disease, cancer, and diabetes. Although their exact roles in disease pathologies remain unclear, a steadily growing number of diseases have been characterized with metal ion imbalance. In the diseased state, metal homeostasis is believed to be disrupted, resulting in poor control of potentially toxic metal ions. Abnormal accumulation of transition metal ions, such as copper, zinc and iron, has been observed in brain tissues with neurodegenerative diseases. Elevated copper ion levels have been shown to link to a variety of tumor and cancers. Irregular zinc ion concentrations have also been identified in cancers and diabetes. For example, the concentration of mobile zinc ions decreases considerably during the development of prostate cancer. Consequently, the identification and quantification of metal ions, ideally in a native physiological environment in tissues, cells, or even at the level of individual organelles and subcellular compartments, has become critical for understanding these diseases and developing diagnosis methods.

Since Tsien’s pioneering work using fluorescent Ca\(^{2+}\) sensors to study Ca\(^{2+}\)-involved biochemistry in cells and tissues, fluorescent imaging has evolved to become an essential tool for investigating the roles of metal ions in biology systems. Over the last thirty years, chemists have developed a variety of sensing mechanisms like photoinduced electron transfer (PET) and fluorescence resonance energy transfer (FRET) and established several molecular design criteria for making fluorescent metal sensors. Hence, various cell-permeable fluorescent sensors for different types of metal ions have been developed, by adopting different molecular recognition components and sensing mechanisms.

In this themed issue of Chemical Society Reviews, various fluorescent sensors have been reviewed, organized according to their target metal ions. In this highlight, the required functionalities of small-molecule sensors have been firstly examined, with an objective to understand the roles of metal ions in diseases development and diagnosis. Following that, we have surveyed recent progress in fluorescent imaging of metal ions by small-molecule sensors for understanding the role of metals in related diseases. We will also discuss challenges and opportunities for the design of small-molecule sensors for fluorescent detection of cellular metal ions as a potential method for disease diagnosis.

Functionality of fluorescent sensors

Metal ions play an important role in various cellular processes, such as proliferation, differentiation, and apoptosis. Yet, their intracellular distributions and dynamic changes are largely unknown. It remains challenging to quantify metal ion concentrations and detect the associated dynamics, which is critical for understanding different disease states.

To meet these objectives, fluorescent sensors should possess a few functionalities, including a high selectivity for target metal ions, a large dynamic range for in situ quantification of ion concentrations, and organelle-targetable ability to describe cellular distributions of metal ions.

To image intracellular metal homeostasis, genetically encoded fluorescent sensors feature highly, ascribing to a few advantages, such as extremely good selectivity, easy incorporation into cells, high region-specificity, controlled concentration and long imaging stability over days. However, there are also several disadvantage associated with this method, such as complicated manipulation, potential interference with the local system, and relatively weak fluorescence changes. In contrast, small-molecule fluorescent sensors with improved functionalities afford small sizes and versatile sensing strategies, thus representing promising tools in disease studies.

Selectivity

* Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See http://www.rsc.org/suppdata/xx/b0/b000000x/
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In nature, the coordination chemistry of metal ion-protein complexes forms an important foundation for metal ion discrimination. Accordingly, O, N or S-containing ligands have been mostly used for metal ion recognition in artificial fluorescent sensors. The specificity and stability of the resulted complexes depend on the properties of both the metal ion and the ligand. To design a selective complexation ligand, several factors should be considered, including donor atom preference, size and preorganization of polydentate ligand, and complex geometry. For example, sulfur-containing donors [i.e., thioethers (R₂S)] prefer soft metal ions, such as Cu²⁺, whereas oxygen donors (i.e., carboxylates and phenolates) are often used to detect hard metal ions, such as Fe³⁺.²⁰

In general, polydentate ligands show greater complex stability than monodentate; a polydentate ligand can thus be designed to bind metal ions selectively, according to the preferred binding geometry with a metal ion. More informations on selective ligand design can be found in several comprehensive reviews,²⁰ ²² while a few representative examples are shown in Figure 1.

In Figure 1a, a macrobicyclic cryptand incorporating an aniline group in 1 was designed as a selective K⁺ receptor over Na⁺.²³ Fluorescent sensor 1 has been used to image intracellular K⁺ distributions over a large concentration range. Imidodiacetic acid and derivatives such as bis(o-aminophenoxymethyl)-N,N',N''-tetraacetic acid (BAPTA) were used to construct fluorescent Ca²⁺ sensor. A BAPTA-based Near-infrared (NIR) sensor 2 using Si-rhodamine as the fluorophore has been adopted to image Ca²⁺ in brain slices with a high fluorescence off/on ratio of over 1000.²⁴ An azatetrahydrocrown ligand in 3 was reported firstly in 2005 to bind Cu²⁺ selectively over Cu²⁺ and other metal ions.²⁵ For zinc ions, di-2-picolylamine (DPA) in 4²⁶ has been used as the most popular receptor to develop fluorescent Zn²⁺ sensors.²⁷ For heavy metal ions, non-cyclic multi-N-coordination ligands in 5²⁸–³⁰ and 6³¹ displayed high selectivity for Hg²⁺ or Cd²⁺, respectively.

In another strategy, a reaction-based approach known as chemodosimeter has also been developed. This type of sensors usually exhibits extremely high selectivity, since they rely on the occurrence of specific chemical reactions with metal ions. The mostly used reaction to construct metal sensors is the sprioring-opening of xanthenes.³² In 7 (Figure 1b), the ring-opening reaction induced by Hg²⁺ triggered the FRET process from BODIPY to rhodamine.³³ Another recently developed reaction for designing metal sensors concerns the metal-catalyzed cleavage of C-O fluorescein-ether bond,³⁴ such as in the cases of 8, 9³⁵ and 10³⁶ (Figure 1b). Owing to the catalysis nature, this type of sensors can detect a trace amount of metal ions down to ppb level. Nevertheless, they are not suitable for imaging metal homeostasis, because these reactions are often irreversible.

It is also worth highlighting a nature-inspired molecular modification strategy to improve the metal ion selectivity of small-molecular sensors, via structure transformation induced by the tautomerization of a peptide bond. For example, an amide-containing DPA receptor in 11 displayed excellent selectivity to Zn²⁺ due to the specific tautomerization of the amide induced by zinc ions (Figure 1c). The excellent performance of 11 suggests that constitutional dynamic chemistry and adaptive chemistry³⁷ represents a promising route to design new fluorescent sensors with high selectivity.

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**Fig. 1. Representative types of receptors for selective binding of metal ions. (a) coordination-based; (b) reaction-based; (c) tautomerization-based.**

**Dynamic range**

The concentrations of cytosolic metal ions vary in various cells and at different disease states (even in the same cells). A precise measurement of metal ion concentrations is important for assessing the impact of metal ion deviations from their normal levels on cellular functions. As a result, fluorescent sensors should ideally possess a large dynamic range in order to quantify the highly dynamic changes of metal ion concentrations.

There are two aspects to be considered. The first one concerns the responding range of a sensor, which depends on its affinity with metal ions. A sensor can detect the concentration variations of metal ions only when its apparent dissociation constant is near the target concentration of metal ions. However, at higher or lower concentration regions, the fluorescence intensity of the sensor remains saturated or unaffected, rendering it ineffective in detecting metal ion concentrations. To cope with this problem, one possible strategy is to develop and collectively use a series of sensors,
each of which possessing varied metal ion affinities and hence working at different concentration ranges.\textsuperscript{38}

Another important consideration for quantifying metal ions regards the calibration of the fluorescent intensity on the concentration of the sensor itself. Different from genetically encoded sensors, the concentration of small-molecule sensors can not be controlled. Two methods have thus been developed to deal with this problem: ratiometric detection allows signal ratioing and has been widely applied in metal ion imaging and quantification;\textsuperscript{14-16} fluorescence lifetime imaging microscopy (FLIM), which is also sensor-concentraton independent, has recently been used to image Zn\textsuperscript{2+} (sensors \textsuperscript{12}\textsuperscript{30} and \textsuperscript{13}\textsuperscript{41}) and Cu\textsuperscript{2+} (sensors \textsuperscript{14}\textsuperscript{45} and \textsuperscript{15}\textsuperscript{42} Figure 2).

![Fig. 2. Fluorescence lifetime imaging sensors for Zn\textsuperscript{2+} (sensors 12 and 13) and Cu\textsuperscript{2+} (sensors 14 and 15).](image)

**Organelle-targetable**

The subcellular distribution of metal ions is highly heterogeneous. In view of the fact that many diseases are organelle phenotypic, it is essential to precisely define the identity of the cellular compartments being measured, when quantifying metal ions.\textsuperscript{43} Although it is difficult for a fluorescent sensor to obtain a full map of metal ion distributions across the entire cell,\textsuperscript{44} it is reasonable to unload and fix fluorescent sensors at a targeted cellular compartment by direct targeting or serendipitous localization. More informations about factors governing the localization of molecular sensors can be found in a recent review.\textsuperscript{15}

By attaching a sub-cellular targetable group, fluorescent sensors are able to detect metal ions in specific regions of a cell. For example, lipophilic delocalized cations, such as phosphonium ions or positively charged rhodamine derivatives, promote the accumulation of sensors in mitochondria. With the direction of triphenylphosphonium (TPP), sensors 16\textsuperscript{45} and 17\textsuperscript{46} are able to image Cu\textsuperscript{2+} and Zn\textsuperscript{2+} in mitochondria, respectively (Figure 3a). An exemplary sensor 18\textsuperscript{47} uses a cholesterol moiety for attaching to cell membrane and allows the detection of changing Zn\textsuperscript{2+} concentrations in a localized region. In another recent study, 2-morpholinoethylamine has been reported as a lysosome-targeted group, making 19\textsuperscript{48} a lysosomal Zn\textsuperscript{2+} sensor.

However, it is challenging for these small anchors alone to achieve sufficient accuracy in organelle targeting. To attain more accurate organelle localization, one attractive approach is to introduce a genetically encoded component to small-molecular sensors by transfection, viral transduction, or other transgenic technologies. This approach typically relies on the use of protein or peptide tags.\textsuperscript{49} Among various tags, SNAP is the only one used for contracting organelle targetable metal sensors at the present stage. The first example, sensor 20,\textsuperscript{50} is essentially a combination of zinc sensor ZP1 with a SNAP-tag to image zinc in mitochondria and Golgi. Later, SNAP-tag has also been incorporated in Ca\textsuperscript{2+} sensor 21\textsuperscript{51}.

![Fig. 3. Organelle-targetable sensors with (a) sub-cellular targetable groups; (b) SNAP tags.](image)

**Imaging in diseases**

Historically, the development of fluorescent sensors for metal ion imaging started with the fluorescent stain of Zn\textsuperscript{2+} in human plasma by 8-hydroxyquinoline in 1968.\textsuperscript{52} Living cell imaging of metal ions began with the Ca\textsuperscript{2+} sensor Quin2 by Roger Tsien in the early 1980s.\textsuperscript{13} In the field of metal homeostasis investigations at various disease states, Zn\textsuperscript{2+} and Cu\textsuperscript{2+} have attracted much more attentions than other metal ions, probably owing to the relatively mature design of fluorescent sensors for these two types of ions.\textsuperscript{14} To this end, recent work of zinc homeostasis imaging have demonstrated a strong potential in prostate cancer diagnosis.\textsuperscript{53,54}

**Neurodegenerative diseases**

The crucial role of metal ions in neurodegenerative diseases has been studied for many years. Metal ions such as copper, zinc and iron ions have been indentified as molecular aggregation modulators of some specific proteins that are directly linked to neurodegenerative diseases. In addition, altered metal homeostasis in the brain has been suggested as a possible cause for most of neurodegenerative diseases. However, the detailed biochemical mechanisms regarding the involvement of metal ions in neurodegenerative diseases are still largely unknown. Among fluorescent imaging of various metal ions, fluorescent Zn\textsuperscript{2+} imaging in the brain has been the hottest topic in the past decade.\textsuperscript{15} In a recent study, Khan et al. reported a two-photo fluorescent sensors 22 to image Zn\textsuperscript{2+} dynamics at a single mossy fiber termini of dentate gyrus neurons in an adult mouse hippocampal slices.\textsuperscript{55} This membrane-impermeant fluorescent sensor was loaded into presynaptic vesicles in hippocampal mossy fiber termini upon KCl-induced depolarization, which triggered subsequent endocytosis and vesicle restoration. Local tetanic stimulation decreased the Zn\textsuperscript{2+} signal observed at individual presynaptic sites, indicating the release of Zn\textsuperscript{2+} from the vesicles (Figure 4).
Cancers

Due to its close association with diseases, altered metal homeostasis is potentially a useful indicator for early diagnosis of diseases, disease progression monitoring, and drug activity tracking. Among various metal ions, previous research has identified Zn\(^{2+}\) as an excellent biomarker for prostate cells;\(^8\) fluorescent Zn\(^{2+}\) imaging have been performed for the early diagnosis of prostate cancer.\(^5,45\) Lippard\ et\ al.\ used\ the\ Zn\(^{2+}\)\ sensor\ ZPP1\ (23\(^{56}\))\ to\ image\ Zn\(^{2+}\) levels\ in\ the\ prostate\ cancer.\(^5\) A significant fluorescence decrease in prostate cancer cells were observed, because the concentrations of Zn\(^{2+}\) drop dramatically during the early stage of the prostate cancer. In contrast, the Zn\(^{2+}\)-binding induced green fluorescence intensity remained little changed in healthy prostate cells. Moreover, changes associated with mobile zinc ions in prostate were also monitored during the progression of prostate cancer in mice (Figure 5b). The substantially decreased fluorescence in the mouse model of the prostate cancer suggests that fluorescent Zn\(^{2+}\) imaging could potentially be used for early detection and progression tracking of the prostate cancer. The same group recently reported a new Zn\(^{2+}\) sensor 24 with improved properties used in differentiating prostate cancer cells from healthy prostate cells.\(^5\) The introduction of a TPP group allows the successful delivery of this sensor to mitochondria. Zn\(^{2+}\)-induced hydrolysis reaction of the acetyl groups ensure the excellent selectivity for Zn\(^{2+}\) over other metal ions. Sensor 24 represents an improved reaction-based version of metal ion sensor, since Zn\(^{2+}\) detection is a reversible coordination process (Figure 5a). The hybrid approach combining metal-dependent coordination and reaction-based assistance affords a promising option for the development of new metal ion sensors with enhanced properties.

Diabetes

Understanding insulin secretion at pancreatic islet \(\beta\) cells is of great interest for its importance in treating diabetes.\(^2\) Insulin is stored in secretory vesicles as a solid hexamer bound with two Zn\(^{2+}\) ions per hexamer. During exocytosis the Zn\(^{2+}\)-insulin complex dissolves and dissociates to release free Zn\(^{2+}\) and insulin. Therefore, detection of Zn\(^{2+}\) efflux could help to monitor insulin secretion. Kennedy\ et\ al.\ firstly\ used\ fluorescent\ zinc\ sensor\ Zinquin\ to\ image\ Zn\(^{2+}\) efflux\ from\ pancreatic\ \(\beta\)-cells.\(^3\) Following this work, several other zinc sensors, such as FluoZin-3,\(^3,59\) Rhod-Zin-3\(^60\) and ZnAF-2,\(^61\) were also applied to image the insulin release process. However, these sensors lack micro-localization abilities, thus displaying compromised sensitivity of detecting local Zn\(^{2+}\) release near the plasma membrane. Recently, a plasma membrane-targetable fluorescent sensor 25 has been developed to monitor Zn\(^{2+}\) release from cultured \(\beta\) cells and intact pancreatic islets after stimulation by high glucose.\(^62\) The two dodecyl side-chains quickly integrate into the outer cell membrane and allow 25 to anchor to the extracellular side of the cell membrane (Figure 6). When exposed to high glucose level and imaged by 25, the rat pancreatic islets \(\beta\)-cells do not exocytose Zn\(^{2+}\) homogenously. Rather, only a subpopulation of clustered \(\beta\)-cells exhibit robust secretion at any given time. These secretory clusters of \(\beta\)-cells were scattered throughout an islet along with other \(\beta\)-cells that show much weaker secretory activities. It has also been observed that Zn\(^{2+}\) release occurs in both homologous cell–cell contacts (\(\beta\)-\(\beta\)) and heterologous (\(\beta\)-\(\alpha\)) cell–cell contacts. In contrast, Zn\(^{2+}\) release is rare at other sites within the cell clusters. In future, more accurate location techniques, like protein tags, may help to further increase the spatial resolution of imaging Zn\(^{2+}\) release from pancreatic islet \(\beta\) cells.
Fluorescent sensors of metal ions are important tools to assist the further advance of biochemical and biomedical science. These sensors are able to detect various metal ions both in vitro and in vivo, demonstrating a strong potential in elucidating the roles of metal ions in disease development and treatment. Current efforts on fluorescent sensors have been focused on improving sensor sensitivity and selectivity, expanding the family of detectable metal ions, developing new sensing mechanisms, shifting the sensor excitation and emission spectra towards NIR region, and so forth. However, current research is still limited by the numbers and types of diseases under investigation.

It is expected that the future development of metal ion sensors will become more interdisciplinary and biomedical application driven, which requires a close collaboration between chemists and biologists. Furthermore, although challenging, it will be interesting and useful to image metal homeostasis in the whole organism in order to get a complete understanding about the functionalities of metal ions. In addition, more efforts should be directed to less explored areas. For example, fluorescent sensors for Ni²⁺ and Co²⁺ are still rare, in spite of their important biochemical and biomedical roles.最后，当前可实现的基于标准光谱的高灵敏度荧光成像方法为精确分界域内化。然而，最近发展起来的超高分辨率荧光成像技术已经创造了前所未有的新可能性为荧光传感器。快速开发在研究中的匹配金属离子传感器是相关应用的未来展望。

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Acknowledgement: X.Q. thanks the National Natural Science Foundation of China (21236002). Z.X. thanks financial supports from the National Natural Science Foundation of China (21276251, 21422606), Ministry of Human Resources and Social Security of PRC, the 100 talents program funded by Chinese Academy of Sciences.

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This highlight discusses recent advances in fluorescent imaging of metal ions for understanding the role of metals in related diseases.