Fluorescent probes and functional materials for biomedical applications

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Abstract Due to their simplicity in preparation, sensitivity and selectivity, fluorescent probes have become the analytical tool of choice in a wide range of research and industrial fields, facilitating the rapid detection of chemical substances of interest as well as the study of important physiological and pathological processes at the cellular level. In addition, many long-wavelength fluorescent probes developed have also proven applicable for in vivo biomedical applications including fluorescence-guided disease diagnosis and theranostics (e.g., fluorogenic prodrugs). Impressive progresses have been made in the development of sensing agents and materials for the detection of ions, organic small molecules, and biomacromolecules including enzymes, DNAs/RNAs, lipids, and carbohydrates that play crucial roles in biological and disease-relevant events. Here, we highlight examples of fluorescent probes and functional materials for biological applications selected from the special issues “Fluorescent Probes” and “Molecular Sensors and Logic Gates” recently published in this journal, offering insights into the future development of powerful fluorescence-based chemical tools for basic biological studies and clinical translation.

Keywords fluorescent probes, imaging, cell, biomedicine, biomolecules

1 Introduction

Fluorescence-based techniques have been extensively exploited in a number of research and industrial areas. The key agent to this technique is small-molecule fluorescent probes, which have proven advantageous in sensing and imaging applications due to their simplicity in design and synthesis, good sensitivity, selectivity, and minimal invasiveness when used for cell imaging [1,2]. Fluorescent probes have also been shown to be amenable to disease diagnosis and therapy; for example, the fluorescence-guided localization and stimulus-activated ablation of malignant tumors has been achieved in vivo [3–6]. With the rapid advancement of biophysical and analytical techniques, fluorescence-based agents have already become the most popular chemical tool for the detection, monitoring and tracking of biologically important species including ions [3], small molecules [7–9], reactive species and biomacromolecules including DNAs/RNAs, proteins, carbohydrates, and lipids, facilitating interdisciplinary studies at the interface of physics, chemistry, biology, and pharmaceutical and medical sciences [10]. The use of fluorescent probes in these basic research areas has led to the elaboration of a variety of previously unknown biological and diseases-relevant cellular processes at the molecular level [11,12].

Fluorescence is a photophysical phenomenon originating from the fast release of photonic energy of excited electrons as light, as they relax from the excited to ground state, and is typically observed for organic compounds with large conjugated structures [13]. Fluorescent
molecules, when modified with a receptor or reactive unit, can respond to a specific analyte of interest with a change in fluorescence intensity (fluorescence quenching or enhancement) or shift in fluorescence wavelength (ratiometric), thus achieving the fluorescence-based “sensing” of the analyte. In addition, by incorporating small-molecule fluorescent probes into functional material backbones, performance-enhanced (such as sensitivity, stability, and the ability to enter target cells more effectively) functional materials can be constructed to achieve the sensing as well as interruption of cellular processes with molecular precision, thereby aiding disease diagnosis and theranostics [14–16]. The following text will summarize recent progress in the development of fluorescent probes and functional materials for the detection and imaging of ions, reactive species, and biomacromolecules including enzymes DNAs/RNAs, lipids, and carbohydrates, and some other important small-molecular organic molecules at the cellular level and in vivo [17–19]. To date, several advanced methods for the analysis of binary images have been developed beyond the traditional image-processing and computer-vision approaches. Segmentation theory is widely exploited, and through clustering and probabilistic segmentation, convolutional networks for object recognition and semantic segmentation have been made possible. In addition, machine learning plays a vital role in image analysis, facilitating feature selection, classification, and the discovery of latent structures [20]. Examples are mainly selected from those published in recent special issues “Fluorescent Probes” (year 2020) and “Molecular Sensors and Logic Gates” (year 2021) published in this journal.

2 Fluorescence probes for metal ions

Fluorescent probes for metal ions including toxic metal ions and those that are essential to life have been extensively developed over the past 20 years. While real-time and accurate detection of toxic metal ions such as mercury and lead have proven useful for monitoring environmental pollution, the spatiotemporal tracking of the dynamic changes in the concentration and distribution of biologically important metal ions such as copper and zinc help advance our understanding of disease biology. Traditional methods for the analysis of ions in aqueous solution include liquid chromatography, atomic absorption, and mass spectrometry, which provide quantitative information of the analytes, and are costly and time-consuming. Fluorescent probes have been one of the most effective tools developed for the fast and sensitive detection of ions [21].

Detection of copper ions (mainly in the form of Cu$^{2+}$) is particularly important since unmonitored release can lead to environmental pollution and can be toxic to the human body. Significantly, the concentration of Cu$^{2+}$ ions in drinking water must be below 2 mg·L$^{-1}$ (30 μmol·L$^{-1}$) as regulated by the World Health Organization. Although there are many reports on the selective detection of copper ions, most of them are based on coordination chemistry, which are susceptible to interference from competitive metal ions. To address this issue, Deng et al. [22] reported a “ligandless” N-cyclohexyl carbamate-based fluorene-alt-phenylene copolymer (PFPNCC) with good water solubility for the highly selective detection of Cu(II) (Fig. 1). The fluorescence of PFPNCC decreased gradually with increasing Cu(II) due to a photo-induced electron transfer process from the excited PFPNCC to Cu(II) in nucleophilic solvents such as N,N-dimethylformamide and dimethylsulfoxide. These findings provided a new strategy for developing polymer-based probes for various analytes.

Sasaki et al. [23] designed a focused fluorescent polymer sensor array based on two different carboxylic acid salts of polythiophenes, which was used to simultaneously detect 8 metal ions. Upon metal ion binding, the polythiophenes exhibited changes in fluorescence intensity or shifts in fluorescence emission wavelengths to different extents. The sensor array was also coated onto glass chips for the quantitative differentiation of metal ions in aqueous solution (Fig. 2). Similarly, a small library of C-bridged benzodiazoles

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**Fig. 1** (a) Structure of PFPNCC; (b) Plausible mechanism of the fluorescence quenching of PFPNCC in the presence of Cu$^{2+}$ in nucleophilic solvents. Reprinted with permission from Ref. [22], copyright 2020, Springer Nature.
were synthesized by de Moliner et al. [24] as a new class of near-infrared (NIR) fluorescent dyes (Fig. 3), which were used in the construction of fluorescent probes able to discriminate $\text{Fe}^{2+}$ and $\text{Fe}^{3+}$ ions based on the differential aggregation profiles in aqueous solutions that contained $\text{Fe}^{2+}$ or $\text{Fe}^{3+}$ ions.

3 Fluorescence probes for reactive species and small molecules

Reactive oxygen species (ROS) are transient substances that are constantly being produced and consumed in all living creatures. In mammalian cells, ROS are a product of mitochondrial respiration and electron transfer, whose levels maintain the redox balance. However, abnormalities in ROS levels can cause cell dysfunction, leading to serious diseases including cancer, inflammation, diabetes, arthritis, and neurodegenerative diseases [25,26]. Superoxide anion ($\text{O}_2^{-}$) is a prototypical type of ROS with short half-life, high reactivity found at low concentrations in most cells and organs. Previously reported ROS probes are generally for a single analyte, and the development of multi-functional probes for the detection of more than one ROS that co-exist in the complicated cellular environment remains a challenging task. In addition, the ability to capture ROS selectively is demanding due to transient nature and short half-life and remains a significant challenge.

Chen et al [27] developed a method based on microchip electrophoresis laser-induced fluorescence (MCE-LIF) for the rapid and simultaneous measurement of $\text{O}_2^{-}$ and NO in the mitochondria of cells (Fig. 4). The presence of $\text{O}_2^{-}$ and NO converted non-fluorescent compounds 2-chloro-1,3-dibenzothiazolinecyclohexene (DBZTC) and 3-amino,4-aminomethyl-2',7'-difluorescein (DAF-FM) to the fluorescent products DBZTC oxide (DBO) and DAF-FM triazole (DAF-FMT), respectively. Based on the ratiometric changes of DBO and DAF-FMT in their respective electropherograms, the electropherographic peaks assigned to DBO and DAF-FMT were identified, which corresponded to the presence of $\text{O}_2^{-}$ and NO, respectively. In addition, the authors achieved the simultaneous tracking of the dynamic changes of $\text{O}_2^{-}$ and NO concentrations during resveratrol-induced cell apoptosis.

Hypochlorite ($\text{ClO}^{-}$), which is endogenously produced by the peroxidation of chloride ions in the presence of myeloperoxidase, plays a vital role in a variety of physiological and pathological events. Endogenous $\text{ClO}^{-}$ in living cells, such as neutrophils, macrophages, mononuclear cells, may damage some invasive microorganisms when existing in excess. However, excessive $\text{ClO}^{-}$ can also lead to a variety of human diseases such as cancer, cardiovascular disease, and neurodegeneration. Therefore, it’s very important to monitoring intracellular levels of
Fig. 3  Synthetic route to a focused library of C-bridged benzodiazoles. Reprinted with permission from Ref. [24], copyright 2021, Springer Nature.

Fig. 4  Simultaneous determination of superoxide anion and NO in mitochondria of apoptotic cells based on MCE-LIF. Reprinted with permission from Ref. [27], copyright 2012, American Chemical Society.
The detection of ClO⁻ in living cells was achieved by Li et al. [30] using a redox-switchable UiO-type fluorescent sensor based on metal-organic frameworks (MOFs) synthesized by the direct ligand modification approach (Fig. 5). MOFs are porous materials formed by the self-assembly of inorganic metals with appropriate organic ligands. Due to their large specific surface area, high porosity, and adjustable pore structure, MOFs have been widely used for biosensing applications [31,32]. A p-methoxyphenol-containing redox-switchable ligand termed H₂L-ol was mixed with ZrCl₄ and benzoic acids under solvothermal conditions to afford the MOF material UiO-68-ol. Upon the addition of ClO⁻, the phenol group in UiO-68-ol was oxidized, leading to the transformation of H₂L-ol to H₂L-one resulting in fluorescence quenching. This MOF-based material was used to detect intracellular ClO⁻ in RAW264.7 (Abelson leukemia expressed in mouse macrophage) cells.

Glucose is a diagnostic marker for diabetes, and the traditional approach to monitor glucose levels relies on glucose oxidase-based electrochemical sensors. However, this approach has some disadvantages in terms of the relatively low thermal stability of enzymes and stability issues when used under non-physiological conditions. Hashimoto et al. [33] developed a supramolecular system consisting of a phenylboronic acid-based fluorescent probe and γ-cyclodextrin (γ-CyD) for the recognition of glucose over other monosaccharides. The selective detection of glucose was achieved through the formation of pyrene- and anthanthrene-excimers in the hydrophobic cavity of γ-CyD upon binding with glucose in a stoichiometry of probe:glucose = 2:1 (Fig. 6). In a similar study, Xu et al. [34] developed a hydrogel system in which anthracene-modified boronic acid probes were embedded for the detection of monosaccharides. Based on the binding between boronic acid derivatives and carbohydrates, the boronic acid-containing hydrogel exhibited a ~16-fold fluorescence enhancement in the presence of fructose.

H₂S is an endogenous signaling molecule that modulates a myriad of physiological and pathological processes in the human body. A recent review by Zhou et al. [35] comprehensively summarized progress made in the construction of small-molecular fluorescent probes for the detection and imaging of H₂S in cells and in vivo. Shi et al. [36] synthesized a mitochondria-targeting ratiometric fluorescent probe. In the presence of H₂S, a Michael-type addition of HS⁻ to the vinyl bridge of the probe led to a ratiometric fluorescence change of the system (Fig. 7). The probe was used for imaging HeLa cells and nude...
mice on addition of exogenous H$_2$S a fast response time (within 30 s) was observed. Moreover, the probe was suitable for mitochondrial imaging in HeLa cells. Brito da Silva et al. [37] synthesized a fluorogenic H$_2$S probe based on azido-benzoxazole derivatives. When the azide group was reduced to an amine, the fluorescence of the probe was recovered due to the inhibition of the ESIPT (excited-state intramolecular proton transfer) process (Fig. 8). The probe was successfully used for the fluorescence imaging of T98G (human glioblastoma) cells.

A biothiol/thiophenol detection assay was developed by Xie et al. [38] using fluorescent probes based on resorufin and fluorescein modified with biothiol- and thiophenol-reactive groups, respectively (Fig. 9). The probes enabled the fluorescent imaging of both exogenously-added biothiols and thiophenol in HepG2 (human liver cancer) cells, and the imaging of endogenously generated biothiols in nude mice (Fig. 9). Similarly, a two-photon red-emitting fluorescent probe based on a naphthalene-BODIPY conjugate to which 2,4-dinitrobenzenesulfonate was introduced as a thiophenol-reactive site was reported by Liu et al. [39]. The probe was suitable for the fluorescence imaging of thiophenol in live HeLa cells.

### 4 Fluorescent probes for enzymes

Enzymes are essential catalytic species involved in the biochemical processes in all living organisms. Traditional analytical methods for enzymes are mainly immunosorbent and colorimetric assays, which are not suitable for sensing enzymes in live cells and in vivo. The exploitation of small-molecule fluorescent probes enables the effective tracking of the expression level as well as activity of enzymes involved in many biologically important processes. Therefore, the development of small-molecule based fluorescent probes for functionally diverse enzymes has become a topical research area [40]. For instance, Gurram et al. [41] used the commercially

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**Fig. 7** (a) Synthesis of probe 1, and (b) its proposed sensing mechanism for HS$^-$. Reprinted with permission from Ref. [36], copyright 2021, Springer Nature.

**Fig. 8** Structure and biological application of azido-benzoxazole derivatives for the fluorogenic detection of H$_2$S based on ESIPT. Reprinted with permission from Ref. [37], copyright 2018, American Chemical Society.

**Fig. 9** (a) Structure and imaging application of Res-Biot and Flu-Pht for biothiols and thiophenol, respectively. (b–e) Fluorescence imaging of endogenous biothiols in nude mice. The mice were injected with (b) vehicle, (c) N-ethylmaleimide (1 mmol·L$^{-1}$, 200 μL), (d) lipopolysaccharide (1 mg·mL$^{-1}$, 200 μL), and (e) L-buthionine sulfoximine (BSO, 1 mmol·L$^{-1}$, 200 μL) followed by injection of Res-Biot (100 μmol·L$^{-1}$, 200 μL). Reprinted with permission from Ref. [38], copyright 2017, American Chemical Society.
available Nile Blue (NB) and Celecoxib (CCB) to produce a fluorescent probe, NB-C6-CCB, connected using an alkyl linkage. CCB is a selective inhibitor for cyclooxygenase-2 (COX-2). The probe was used for the selective detection of COX-2 in the Golgi apparatus of cancer cells. Based on their differences in expression level of COX-2, cancer and normal cells were effectively differentiated using the fluorescence from the probe localized in the Golgi apparatus (Fig. 10).

Odyniec et al. [42] designed a simple dual-responsive luciferase probe (PF3-GLC) for the simultaneous detection of β-glucoside enzyme (β-GLC) and H$_2$O$_2$ (Fig. 11). The “AND”-logic probe consisting of a fluorescein core as the fluorescence reporter and boronate ester and glucosyl group installed onto both sides of the fluorophore was used for the simultaneous sensing of β-GLC and H$_2$O$_2$ via a fluorescence “turn-on” signal. In vitro fluorescence dual-response of both CelTec2 (β-glucoside) and H$_2$O$_2$ was observed, indicating the potential of the probe for monitoring glucose metabolism.

Real-time imaging of UDP-glucuronosyltransferase 1A8 (UGT1A8, a UGT isoform involved in the biotransformation of environmental toxins and steroids) activity in living cells and tissues was achieved by Zhu et al. [43]. UGT1A8 adds a glucuronic acid to the fluorescent BDMP probe, based on the boron-dipyromethene (BODIPY) scaffold, resulting in a strong fluorescence emission at 580 nm. The probe was used for the real-time imaging of endogenous UGT1A8 in HCT-15 (human colon cancer) cells and in LoVo (human colon cancer) tumor tissues (Fig. 12).

Xie et al. [44] developed a dual-function fluorescent “turn-on” probe termed KTLip for the evaluation of lysine delipoylation. The probe acted as a substrate for recombinant NAD$^+$-dependent histone deacetylases Sirt1/Sirt2 to undergo a tandem delipoylation/intramolecular nucleophilic exchange reaction enhancing fluorescence (Fig. 13). KTLip was able to measure the activity of these enzymes in living cells.

![Fig. 10](image1.png) (a) Synthesis of NB-C6-CCB. (b) Confocal microscopic images of (i) normal African green monkey kidney COS-7 cells and (iii) human breast cancer MCF-7 cells co-cultured following staining using NB-C6-CCB. Fluorescence images of (ii) co-cultured cancer (MCF-7) and (iv) normal cells (COS-7). Reprinted with permission from Ref. [41], copyright 2020, Springer Nature.

![Fig. 11](image2.png) Fig. 11 Structure of a fluorescein-based dual-activated “AND”-logic probe and its sensing mechanism towards β-GLC and H$_2$O$_2$. Reprinted with permission from Ref. [42], copyright 2020, Springer Nature.
of recombinant Sirt1/Sirt2 and could potentially be used for the screening of inhibitors of these enzymes, which may lead to the discovery of potential therapeutically active drugs. In addition, the probe was able to fluorescently label Sirt2 in a complex lysate environment.

5 Fluorescence probes for other biomolecules

The in-situ and real-time monitoring of mitochondrial DNA (mtDNA) damage was achieved by Feng et al. [45] (Fig. 14) using an environmentally sensitive fluorescent probe, MBI-CN consisting of 2-methyl-1H-benzo[d]imidazole and terephthalaldehyde. MBI was selected as the fluorophore because it can be easily implanted into the mtDNA double-stranded structure without causing DNA damage. However, when mtDNA is damaged, the malononitrile group in the MBI-CN could undergo degradation, and the resulting aldehyde-based product MBI-CHO with dual fluorescence emission exhibited a decreased fluorescence at both 437 and 553 nm.

A two-photon fluorescent probe that can be excited by light within the first biological optical window (650–950 nm) was developed by Kim et al. [46] (Fig. 15) based on the 6-acetyl-2-(dimethylamino) naphthalene skeleton for the imaging of amyloid-β (Aβ) plaques, a well-known biomarker for Alzheimer’s disease (AD). The good tissue penetration depth of the probe facilitated the in vivo imaging of the Aβ plaques in an AD mouse model. Chen et al. [47] developed lysosome-targeting anticancer fluorescent agents for imaging zebra fish. Three probes based on an identical 4-(2-(bis(2-chloroethyl) amino) ethoxy) benzaldehyde skeleton were developed, and zebra fish embryos and larvae were used as models to validate the imaging capacity of the probes. In addition, these probes were shown to be selectively localized in the lysosomes of living cells and suitable for the in vivo tumor imaging of BxPC-3 (human pancreatic adenocarcinoma) tumor-bearing mice.

6 Conclusions

As effective chemical tools to selectively monitor biospecies associated physiological and pathological processes, fluorescent probes have been extensively developed facilitating the study of biochemistry, chemical
biology, and biomedicine. Fluorescent probes with the ability to simultaneously react with two independent bio-species have been developed in recent years to overcome problems associated with single-response fluorescent probes [48–55]. Probes have also been developed that are able to monitor ions, reactive species, biomacromolecules, and small-molecule organic molecules which are important signaling molecules and are implicated in a range of physiological and pathological processes in living organisms [56–60]. In this short review, recent progresses towards the fluorescence-based sensing of several molecular targets have been discussed and summarized. We anticipate that this short report will encourage others to develop fluorescence probes and materials with improved characteristics suitable for monitoring biological systems.

To conclude, we would like to provide some guidance regarding appropriate research areas where development of fluorescent probes with enhanced capabilities for biomedical applications is required. These improved probe systems should: (1) Be capable of imaging in vivo biomarkers and as such need to overcome the limited tissue penetration exhibited by visible fluorescence. One effective approach is to develop probes exhibiting long fluorescence emission wavelengths. Recent successful examples include the extension of emission wavelengths to the NIR-II region (1000–1700 nm), resulting in substantially enhanced imaging properties of organic dyes in terms of tissue penetration depth, spatial and temporal resolution, imaging contrast and biocompatibility [61]. Alternatively, multimodal imaging can be used to multiplex the tissue penetration advantages of imaging modalities such as computed tomography and magnetic resonance imaging, with the imaging precision of fluorescence [62]. (2) Be suitable for the quantitative analysis of trace amounts of endogenous metal ions, free radicals, anions, and enzymes in biological systems. As such, the judicious design of nanomaterial-based probes could be one solution to achieve this goal. For example, a dual-emission water-soluble g-C$_3$N$_4$@AuNCs-based nanomaterial probe was designed by Guo et al. [63] for the label-free, quantitative analysis of trace Fe$^{2+}$ and Cu$^{2+}$ in the presence of competing cations. (3) Be capable of multi-organelle-targeting and able to interrogate the spatial-temporal localization of biomarkers with sub-organelle precision [64]. We have reported a fluorescent glycoprobe-human serum albumin (HSA) hybrid system with reversibly tunable fluorescence properties as a super-resolution imaging agent for intracellular β-galactosidase (β-Gal) activity. Using this probe, we found that while β-
Gal is evenly distributed in the cytoplasm of ovarian cancer cells, that the enzyme is specifically expressed in the lysosomes of senescent cells, thereby offering insight into the delineation of disease biology [65]. (4) Should have undergone extensive assessment of their biocompatibility to ensure non-toxicity and mildness towards the tissues undergoing clinical evaluation [66–68]. As such, the host–guest inclusion of probe molecules with HSA, an endogenous protein abundantly existing in the human blood, could represent a facile approach to minimize the toxicity of highly conjugated dyes [69].

Fluorescent probes with good water solubility are often required to enable high sensitivity, selectivity, and accurate imaging of endogenous biomarkers in living cells. The introduction of cationic units, anionic units or both, to organic molecules using simple chemical modifications is a particularly effective strategy that has been used to achieve this goal. For example, modification of quinoline-malononitrile using a sulfonate enabled Shao et al. [70] to substantial enhance the water solubility of their dye, thus improving the binding efficacy with Aβ aggregates. While, the addition of hydrophilic biomolecules such as carbohydrates to fluorescent dyes improves not only the water solubility, but in addition enhances the targeting towards specific cells with carbohydrate receptors [71−74].

**Acknowledgements** The authors thank the National Natural Science Foundation of China (Grant Nos. 21907030 and 22108077) and the International Cooperation Program of Shanghai Science and Technology Committee (Grant No. 19410712600). De-Tai Shi would like to thank the Natural Science Foundation of Jiangxi (Grant No. 20161BAB213067) and the Scientific Research Fund of Jiangxi Provincial Education Department (Grant No. GJJ170807). Tony D. James wishes to thank the Royal Society for a Wolfson Research Merit Award and the Open Research Fund of the School of Chemistry and Chemical Engineering, Henan Normal University for support (Grant No. 2020ZD01).

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