Most recent advances on enzyme-activatable optical probes for bioimaging

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Abstract
Enzymes are essential biological elements that play vital roles in many key cellular events and physiological processes. The dysregulation of enzyme activity broadly occurs in a large number of diseases ranging from inflammation to neurodegenerative disorders to tumors. Molecular imaging allows accurate and noninvasive visualization of biological analytes/physiological processes of interest closely linked to human health at different levels. Among various imaging modalities, optical imaging stands out benefited from its high sensitivity, excellent spatiotemporal resolution, real-time mode, and facile accessibility. Diverse optical probes specifically activatable by disease-relevant enzymes have sprung up. In comparison to the “always-on” counterparts, the “off-on” imaging probes activated by enzymes hold great promise for precise diagnosis of diseases at early stage with high target-to-background ratio, dramatically improved specificity, and significantly enhanced sensitivity. Herein, the most recent advances in optical probes activatable by enzymes for biosensing and bioimaging are briefly reviewed emphasizing their molecular design, working mechanism, and biomedical applications. Besides, some important prospects and the current challenges to fully implement the potential of enzyme-activatable probes for precise and efficient theranostics in life science are also pointed out to hopefully arouse new insights into the development of new generation of theranostics.

KEYWORDS
aggregation-induced emission, bioimaging, chemiluminescence, enzyme-activatable, near-infrared fluorescence, photoacoustic, theranostics

INTRODUCTION
Developing accurate and sensitive analytical tools for selective imaging of diseases-associated biomarkers in life systems is of extreme significance for the clinical diagnosis, surgical interventions guide, and evaluation of therapeutic effect and prognosis. Nevertheless, such task remains very challenging because of the low concentrations of biomarkers in living systems. Optical imaging enjoying high sensitivity, ingenious spatiotemporal resolution, noninvasiveness, low-cost, ease operation, and real-time and in-situ imaging capacity stands out as a promising candidate.\[1,2\] Moreover, optical imaging based on nonionizing radiation, including UV, visible, and near-infrared (NIR) light, dramatically reduces the risk of harmful radiation related with such conventional imaging techniques as computed X-ray tomography (CT) and positron emission tomography (PET), and exhibits superior temporal resolution to CT, PET, and magnetic imaging (MRI), and higher spatial resolutions than PET, MRI, and ultrasound scanners.\[3-8\]

The optical imaging performance depends entirely on the target-to-background ratio (TBR).\[4,5,9,10\] In pursuit of superb optical imaging probes, researchers are always putting endeavors either on (1) maximizing the optical signal of the target, (2) minimizing the signal of background, or (3) doing both. Utilizing any of these means results in improved TBR, which in turn gives rise to improved sensitivity and specificity of disease diagnosis. An approach used for a long term to maximize the target signal is to develop “always-on” optical probes whose pharmacokinetics and biodistribution are altered by either active targeting via binding to a cell-surfaced receptor or passive targeting via the enhanced permeability and retention (EPR) effect to enhance the target accumulation in diseased tissues.

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Despite this, regardless of whether they approach the target tissue, “always-on” optical probes constantly exhibit unchanging optical signal. The “always-on” optical probes are thus still often suffering from low TBR as a result of the fact that although the target signal is increased, the background signal is still high (Figure 1A).[2–5,9] As a consequence, their sensitivity and specificity remain to be improved for disease diagnosis, especially early-stage diagnosis. In this context, the activatable optical probes whose signals change in response to the pathological parameters (i.e., pH, temperature, oxygen concentration, enzyme, redox, etc.) in target tissues emerged (Figure 1A). In comparison to the traditional “always-on” optical probes, the performance of activatable optical probes is completely dependent on activatable targeting, which simultaneously maximize the signal of target and minimize the signal of background.[3] In this way, higher TBR and lower limit of detection is provided, rendering sensitive real-time and in-vivo imaging of pathological parameters. Then, the underlying pathological mechanisms could be understood at the molecular level to optimize the therapeutic interventions. Besides that, distinct from the “always-on” probes which need the clearance of the probes nonspecifically distributing in normal tissues to minimize the background signal as to improve the TBR, what the activatable probes need is merely activation. Generally speaking, the activation time is very short that the images with high TBR could be fast formed, making the activatable probes perfect for clinical applications, such as real-time imaging-guided surgery/therapy. Moreover, the activatable probes are conducive to shed light on complex molecular pathways and biological mechanisms.[11–20]

As we all know, pathological microenvironment of diseased tissues, for example, redox, pH, temperature, or enzyme level, is distinctive from the physiological environment of the normal tissues. Variation of the physiological parameters existing in cells, organs, and tissues is closely intertwined with the pathological processes. More significantly, such pathological parameters are critical biomarkers for various kinds of diseases. For example, the overexpression of various enzymes is usually correlated with many diseases, including diverse cancers, inflammation, and tissue injuries.[21–26] Involved in almost all biological and metabolic processes, a large variety of enzymes serve as important biocatalysts and the prime protagonists in the chemistry of living organisms at a molecular level. Enzymes have thus recently emerged to be a promising triggering motif for the design of activatable probes.[11,26] As compared with the probes activatable by the other pathological parameters, enzyme-activatable probes exhibit several merits, such as superior selectivity and substrate specificity. It deserves to be mentioned that some enzymes can even effectively realize significant optical signal amplification. Currently, a spring of enzyme-activatable optical probes have been reported.

In the past couple of years, the optical probes activatable by enzymes are skyrocketing. In this review, the most recent advances since 2019 on enzyme-activatable optical probes equipped with fluorescence (FL), photoacoustic (PA), and/or chemiluminescence (CL) imaging capabilities and/or photodynamic therapy (PDT) or chemotherapy or photothermal therapy (PTT) ability are summarized (Figure 1B). The discussions are divided into three subsections in terms of the enzyme type (i.e., oxidoreductase, hydrolase, and transferase). The design principle, working mechanism, performance, and the applications of the enzyme-activatable optical probes in vitro/in vivo are discussed in details. Furthermore, the possible challenges and perspectives in developing optical probes activatable by enzyme are proposed as well.
ADVANCES OF ENZYME-ACTIVATABLE OPTICAL PROBES

Oxidoreductase activation

Oxidase

The oxidases so far used for the activation of optical probes are mainly myeloperoxidases (MPOs),[27] glucose oxidase,[28] and tyrosinase.[29] Herein, the MPO-activatable optical probe is introduced as a representative example.

Myeloperoxidase: As an important highly oxidative and proinflammatory enzyme, MPO plays a vital role in the mediation of damaging innate immune response.[30] The misdirected increased MPO activity or abnormal level of MPO is linked to a lot of diseases, including Alzheimer’s disease, atherosclerosis, multiple sclerosis, stroke, atrial fibrillation, and nonalcoholic steatohepatitis, among others. Although many species involved in the inflammatory cascade exert both damaging and beneficial effects relying on the microenvironment, MPO is merely secreted by proinflammatory cells, generating oxidative radicals damaging to biological tissues. Specifically evaluating MPO activity with high spatial-resolution in living organisms is thus very necessary but challenging. Current techniques for the detection of MPO activity in live organisms are scarce and often suffer from poor specificity, low spatial resolution, or insufficient tissue penetration. To combat these intractable obstacles, Chen’s group established a versatile imaging platform for the detection of MPO activity utilizing an activatable motif covalently bound to a biotin moiety.[27] The developed biotinylated sensor I activatable by MPO enables the monitoring of the innate immune response and its modulation at different settings and scales in live animals and in tissue sections (Figure 2).

MABS containing MPO-specific moiety and biotin group was designed and synthesized. MABS is activatable by MPO and preserved at the inflammation site via the binding to protein. Then, the subsequently administrated reporter-tagged streptavidin binds to the biotin in MABS to visualize MPO. The high utility of I for detecting MPO activity at microscopic and macroscopic scales to monitor the variation in the innate immune response was demonstrated by the FL and CT imaging. The specificity of I to MPO over other peroxidases in vitro and in vivo in multiple mouse models with
The structure and function of hepatic cytochrome P450 (CYP450) reductase, especially its role in drug metabolism and its impact on cytokines and thermoregulation. It has been shown that, when the liver is injured by alcohol, overexpression of CYP450 reductase takes place, along with enhanced degree of hypoxia. Alcoholic liver injury, including fatty liver, hepatic lesions, progressive fibrosis, steatohepatitis, cirrhosis, and even liver failure, is an early stage of alcoholic liver diseases. Detecting alcoholic liver injury is thus very important. Considering the increasing overexpression of hepatic CYP450 reductase together with hypoxia in the alcohol-induced liver injury cases, the hepatic CYP450 reductase could serve as an in-situ biomarker for liver injury induced by alcohol.

Establishing approaches for sensitively detecting CYP450-reductase in the hypoxic hepatic region would help diagnosing alcohol-induced liver injury at early stage and the effective treatment preventing the liver injury from worsening into severe alcoholic liver diseases. Integrating the NIR FL imaging with the optoacoustic (PA) imaging, Zeng and Wu et al. developed a water-soluble and hepatic CYP450-activatable probe for the detection of alcohol-induced liver injury by means of PA/FL dual-modal turn-on imaging.

In the developed probe (i.e., NIR-NO (2)), a benzothiazole-xanthene chromophore functions as the signaler, in the meantime, the N-oxide moiety serves as both the activation site and quencher for FL that is capable of undergoing bioreduction by CYP450 reductase with the deficiency in oxygen. Consequently, the N-oxide element is converted into an aromatic tertiary-amine group, making the probe turned into the chromophore NIR-NEt₂ that exhibits an intensified FL at 745 nm, red-shifted absorption from 550 to 718 nm, and an intense optoacoustic (PA) signal as well. With the conversion of the electron-withdrawing N-oxide moiety into an electron-donating diethylamino group, both the NIR FL and PA signals are triggered on. In-vivo FL and multispectral optoacoustic tomography (MSOT) imaging clearly suggests...
the quick response of the activatable probe to the CYP450 reductase overexpressed in the liver region. The FL signals in the liver region of the alcohol-treated mice groups are fairly prominent post the injection of probe and arrive to the maximum in around 10 min. Furthermore, the NIR FL signal becomes stronger along with the increasing dose (Figure 3B). The cross-sectional MOST images of the mice preadministered with alcohol (8 g kg\(^{-1}\)) at different time points after being treated with probe displayed in Figure 3C show that the PA signal intensity reaches the maximum at 10 min. As shown in Figure 3D, the mean signal intensities of the activatable probe signal in the region of interest over time offer quantified proof to the change in MSOT signals. It is clear from the simulated cryosection image of a male mouse that most signals from the activated probe lie in the liver region. As verified by the alanine transaminase level in the blood, the alcohol does induce the liver injury in the mice and the degree of injury correlates with the dose of alcohol. Moreover, when treated with a higher dose of alcohol, stronger signals are given out, suggesting that the CYP450 reductase level in the liver area is obviously higher than that of the contrast (Figure 3E).

Besides, the liver tissue sections with H&E staining provide more evidence to the in-situ and precise imaging ability of NIR-NO\(_2\) (2) to the alcohol-induced liver injury. It is noteworthy that the transformation of N-oxide to the aromatic tertiary-amine was also used as an activation mechanism of the PA probes constructed with a conformationally restricted aza-BODIPY for the detection of tumor hypoxia.[35]

**Nitroreductase:** Hypoxia, usually defined as the diminished level of molecular oxygen in tissues, is closely linked to a number of diseases, including the inflammatory diseases, cardiovascular diseases, acute ischemia, and tumors.[36] Diverse reductases are overexpressed in hypoxia, and nitroreductase (NTR) is noted as the most studied overexpressed flavin-containing reductases inside tumor tissue induced by hypoxia.[37] NTR usually acts as an effective biomarker for hypoxia. In other words, the evaluation of NTR level can be directly employed to assess the hypoxia extent, and as a result used for the determination of the status of disease and thus for diagnosis. Recently, quite a few NTR-activatable optical probes or theranostic systems have been reported.[38–49] The general working mechanisms are summarized in Figure 4.

One smart activation strategy is based on the caging-uncaging effect of the 4-nitrobenzyl moiety on the optical and/or therapeutic properties of the chromophore by the photo-induced electron transfer and the disruption of photo-induced electron transfer resulted from the NTR-triggered cleavage (Figure 4A). The NTR-activatable fluorescent probes developed via this approach are constructed with fluorophores varying from aggregation-induced emission (AIE) fluorogens (e.g., 3),[39–40] to thermally activated delayed fluorescence-active fluorophores (e.g., 4)[41] and to NTR emitter (e.g., 5).[42] Noteworthily, the heptamethine aminocyanine derivative 5 is an NTR-emissive photosensitizer with a long-lived triplet state, which can be activated by NTR under mild hypoxia and hence was able to be utilized in FL imaging and highly efficient PDT both in 2D and 3D HeLa cell culture models.

The mechanism illustrated in Figure 4B is very similar to the one depicted in Figure 4A. In this strategy, p-nitrobenzyl thioether attached to the fluorophore is reducible to p-aminobenzyl thioether by NTR, followed by a 1,6-elimination reaction (i.e., self-immolation) to release the required fluorophore, exhibiting dual-channel ratiometric FL and lighted-up NIR emission. 6 is a representative of the activatable optical probes established via this approach.[43]

The strategy shown in Figure 4C is the most direct as compared to the ones displayed in Figure 4A and B. In such a strategy, the FL of the probe is shielded by the aromatic nitro group. Upon the interaction with NTR, the electron-accepting aromatic nitro moiety is transformed into the electron-donating amino group. As a result, the probe is activated and the FL is unshielded. A number of activatable NIR FL probes (e.g., 7–9) have been successfully obtained by means of the nitro-to-amino transformation strategy, demonstrating the feasibility of this means.[44–49]

The work shown in Figure 5 is a good example to the strategy I. The nanoprobe developed by Zeng and Wu et al. through the self-assembly of Q-NO\(_2\) (i.e., compound 10) is NTR-activatable for the imaging of breast cancer metastases via NIR-I/NIR-II FL imaging and MSOT imaging.[39] 10 is composed of a hydrophobic strong electron donor (i.e., dihydroxanthene group), a hydrophilic electron acceptor (i.e., quinolinium), and a recognition element for NTR (i.e., nitrobenzoxylidiphenylamino). It is worth noting that the nitrobenzoxylidiphenylamino serves as the electron-withdrawing moiety which quenches the FL. The amphiphilic molecular structure of the synthesized chromophore 10 makes it readily form nanoprobe (NP-Q-NO\(_2\)) in aqueous media. In addition, due to the presence of the nitrobenzoxyl and triarylamino group, relatively loosely packed nanoaggregates of 10 were formed in water, which would aid in the enzymatic reaction mediated by NTR. As depicted in Figure 5A, the nanoprobe neither exhibits absorption nor displays FL in the NIR region in the absence of NTR. In sharp contrast, the strong electron-deficient nitro group is transformed to an electron-rich amino group by NTR, inducing a self-elimination to subsequently afford an electron-donating hydroxy group, which finally generates the activated probe with strong PA signal and the AIE-enhanced NIR FL extending beyond 900 nm (Figure 5B–F). Thus, the probe is capable of specifically responding to NTR.

The developed nanoprobe was employed to monitor and image the metastases from the orthotopic breast tumors to lymph nodes and then to lung in two model mice bearing breast cancers (Figure 5B). Furthermore, the treatment efficacy during chemotherapeutic course could be monitored by FL and MSOT imaging with the nanoprobe (Figure 5C). To be specific, NIR-I and NIR-II imaging for the mice administrated with 4T1 cells at the fifth pair of mammary fat pads were carried out first to evaluate the nanoprobe’s capacity to image distant metastasis. As can be seen from Figure 5B, the NIR-I and NIR-II FL images basically agree with the bioluminescent ones and more prominent as compared to the bioluminescent imaging. As depicted by the images in Figure 5C, overgrown tumors for the mice without treatment are distinguishable at the fifth pair of mammary fat pads at day 20 post cancer cell injection. In comparison, for the chemotherapy-treated mice, no metastases were observed even at day 20. The mice were sacrificed with partial skins removed and the pleura opened for better imaging (Figure 5D). The orthotopic tumors, and the metastasized tumors in lymph nodes and lungs are directly observable. The MSOT imaging capability of the nanoprobe for metastases of breast
cancer was evaluated on two mice models. As presented in Figure 5E and F, cross-sectional MSOT images at specific sites of the third and fifth pairs of mammary fat pads were obtained at different time points, demonstrating the superior performance of NP-Q-NO₂ for the MSOT imaging of metastases of breast cancer on different mice models. Therefore, all the results suggest that the nanoprobe is activatable by NTR related to breast tumor metastasis in the mouse models, and capable of imaging the sequential metastases from the orthotopic breast tumors to lymph nodes and then to lung, and monitoring the suppression on tumor metastasis during a chemotherapeutic course via the combination of NIR-I, NIR-II, and MSOT imaging.

Since the working mechanism of strategy II is very similar to that of strategy I, we will not discuss in details to avoid repetitive illustration. The practicality of strategy III displayed in Figure 4C is demonstrated by the works depicted in Figure 6. Compound 11 is a neomycin-based NTR-activatable fluorogenic probe, which is able to be used by Hu’s group to specifically identify bacterial infection from cancer. Compound 11 consists of a para-nitro aromatic group-decorated cyanine 7 (Cy 7) and a carboxylic ester-linked neomycin. The nitro moiety of the para-nitro phenyl sulfonic group in 11 was reduced to the amino group by NTR, giving rise to the light-up of the NIR FL of the latent Cy7. Probe 11 holds the capacity to quantitatively sense NTR in
FIGURE 5  (A) The working mechanism of the nitroreductase-activatable probe Q-NO₂ constructed by strategy I depicted in Figure 4. (B) Representative bioluminescence images, NIR-I (excitation filter: 675 nm, emission filter: 790 nm long pass) and NIR-II (excitation: 808 nm, emission filter: >900 nm, long pass) FL images, and the photoacoustic (PA) imaging photographs of the mice taken under halogen light (400–850 nm) at different days post injecting 4T1 cancer cell into the fifth pair of mammary fat pads. (C) Representative images at day 4 and day 20 post the injection of 4T1 cells for the mice with or without chemotherapy. (D) Representative bright-field, bioluminescence, NIR-I and NIR-II FL images and PA images for healthy mice (day 0) and the mice bearing tumor at day 17 (for the third pair model) or at day 20 (for the fifth pair model) with partial skin removed and pleura opened for better visualization. (E) Representative cross-sectional MSOT images of different locations for the control (day 0) and the mice with cancer cell injected into the third pair of mammary fat pads (17 days). (F) Representative cross-sectional MSOT images for the control (day 0) and the mice at 20 days post the injection of cancer cells into the fifth pair of mammary fat pads. Reproduced with permission. [39] Copyright 2020, Wiley-VCH.

The range of 0–150 ng mL⁻¹ in vitro with a limit of detection as low as 0.67 ng mL⁻¹ in a rapid and real-time fashion. In short words, the probe reported is (1) stable and water-soluble, (2) showing good performance, including ultrafast tests, outstanding selectivity, and ultrahigh sensitivity to NTR to precisely assess intracellular basal NTR activity in living bacteria both in vitro and in vivo, and (3) able to reliably and noninvasively differentiate bacterial infection sites from cancer within 30 min (Figure 6B). Since bacterial infection, inflammation, and cancer often have undesirable and significant overlap in the diagnostic features, the exclusively recognizing bacterial cells from white blood cells and cancerous
cells is very interesting, meaningful but far less investigated. Therefore, the work is of great significance to the understanding of bacterial infections relevant to molecular events as well as the accurate diagnosis of bacterial infections.

It is worth noting that the nitro group in strategy III is not necessarily attached to a phenyl ring. For example, the nitro group is linked to an imidazole moiety in probe IR1048-MZ (12) and still works very well in the NTR activation. The molecular probe 12 is obtained by conjugation of the nitro-attached imidazole moiety to the IR-1048 dye, where the 2-nitroimidazole group works as a specific hypoxia trigger and the IR-1048 dye acts as a NIR-II and PA signal reporter (Figure 6C–F). As stated above, NTR is closely associated with hypoxia, which is a key hallmark of solid tumors. Meanwhile, such an important feature is highly related to the resistance to radio-chemotherapy, which leads to poor prognosis and probability of tumor recurrence. The detection of hypoxia is thus very important and can be realized by an NTR-triggerable probe. 12 is such a probe that is hypoxia-switchable and NTR-activatable. As a consequence, based on 12, high-contrast dual-modal NIR-II and PA imaging for tumor and efficient hypoxia-activatable PTT has been realized. More specifically, the probe exhibited a fairly weak absorption in the range of 900–1060 nm, while an absorption peaked at around 980 nm emerged after NTR activation. In the meantime, IR1048-MZ hardly showed any FL at 1046 nm with a FL quantum yield ($\Phi_F$) as low as $1.88 \times 10^{-5}$, while when reacted with NTR, a 106.9-fold increment in the FL at 1046 nm was shown ($\Phi_F = 0.006$). Moreover, a good positive linear correlation between NTR (0–10 $\mu$g mL$^{-1}$) and the
FL intensity of 12 was displayed, with the detection limit of 43 ng mL\(^{-1}\). The increase in the NIR absorption of 12 after NTR activation results in an intense PA signal and high thermal efficacy. The PA signal is linearly boosted to 936 from 121, and the temperature of the detection system increased from 30 to 57.6 °C after the irradiation by a 980 nm NIR laser for 2 min, as the NTR concentration increased from 0 to 10 μg mL\(^{-1}\). The efficiency of photothermal conversion was determined to be 20.2%, which is about 5.5-folder higher than that reported for indocyanine green.

The feasibility of using 12 for NIR-II FL of tumors was studied by intravenously injecting 12 into subcutaneous A549 tumor-bearing BALB/c nude mice, as the A549 tumor is known to overexpress NTR under hypoxia. The NIR-II FL signal in the tumor site was clearly witnessed without any background at 10 h post administration and reached the maximum at approximately 14 h (Figure 6D). The hypoxic tumor detection was realized through the monitoring of the 3D PA signal of 12 with centimeter-level deep-tissue penetration and high-resolution \textit{in-vivo} imaging ability, for the first time. In good agreement with the FL imaging, the 3D PA signals in the cancerous areas were observable at 10 h after the injection, arrived to the maximum at around 14 h, and started to decrease afterward (Figure 6E). It has been demonstrated that the temperature of the treated tumor rose to 58 °C from 30 °C after being irradiated with 980 nm NIR laser for 2 min, which is sufficient to kill tumor cells, while the temperature of untreated tumor kept at 30 °C under parallel conditions (Figure 6F), suggesting a significant PTT effect for tumor ablation with no recurrence. Therefore, 12 might promisingly function as an excellent contrast agent and theranostic formulation for such hypoxia-associated diseases as tumor, inflammation, stroke, and cardiac ischemia.

\textit{Azoreductases}: Azoreductases are another class of reductive enzymes strongly associated with hypoxia.\[36,50\] A few of azoreductase-activatable optical probes varying from molecular probe to self-assembled nanoprobes to assembly system formed via host-guest interactions have recently been reported.\[51-56\] The representatives are exhibited in Figure 7. Their common working mechanism is based on the efficient reduction of the azo bond (N = N) in azobenzene compounds by azoreductase treatment in the hypoxia.

The molecular probe NR-azo (13) is not only an activatable NIR chromophore for FL and 3D MSOT imaging of tumor hypoxia but also a hypoxia-responsive prodrug for tumor inhibition (Figure 7A).\[51\] In this probe, the xanthene chromophore, azo linker, and nitrogen mustard function as the reporter, hypoxia-responsive moiety, and therapeutic drug, respectively. Notably, the incorporation of the anticancer drug nitrogen mustard into the probe greatly reduced its severe toxicity because the active drug would only be released under hypoxia. The NR-azo displays an absorption band peaked at ∼575 nm and exhibits negligible FL because of the covalent linkage to the electron-withdrawing and FL-quenching azo group. Under hypoxic conditions, the azo connector is cleaved, the chromophore NR-NH\(_2\) is consequently generated with the active drug nitrogen mustard simultaneously released. The dominated absorption at about 680 nm and the intense FL at ca. 710 nm of the resulted NR-NH\(_2\) makes it an ideal exogenous activatable contrast agent for MSOT and FL imaging of tumor hypoxia. Meanwhile, the released drug could be utilized to inhibit tumor growth. The results suggest that the dual-purpose system shows quick response to cancer hypoxia and realize substantial inhibition on tumor, which could be traced by both MSOT and FL imaging.

Azoreductase-activatable probe 14 is composed of an AIE-active luminogen (AIEgen), an azobenzene linker, and a poly(ethylene glycol) (PEG) tail (Figure 7B).\[52\] AIEgens have been recognized as appealing FL imaging agents. The AIE dots, which are nanoparticles constructed with an AIEgen as the core and biocompatible stuff as the shell, have been employed with great success in bioimaging thanks to their high brightness and biocompatibility. Novel environmentally sensitive AIE dots are needed to be developed for further expansion of the application scope of these exceptional FL nanooaggregates. As mentioned above, azobenzene derivatives always show strong absorption in the UV/Vis light region and thus often function as nonemissive energy acceptors to quench FL of the fluorophores in propinquity. Moreover, the azobenzene could be reduced efficiently to recover the FL of the probe system in hypoxic environment. The fluorophores containing reducible azobenzene group are hence could be employed as “off-on” FL probes for detecting tumor hypoxia. Although azobenzene-caged classical fluorophores have been developed as hypoxia-sensitive FL probes, the AIE FL probes caged by azobenzene are very rare. Azobenzene-caged PEGylated tetraphenylenethene (TPE) (14) is such an activatable AIE dot-based nanoprobe whose AIE effect could be switched on in response to the azoreductase. The amphiphilic macromolecule PEG-Azo-TPE (14), which has the azobenzene linker connecting a hydrophobic TPE head and a hydrophilic PEG tail, is able to self-assemble into micelles in aqueous solutions, affording azobenzene-caged AIE dots stabilized by biocompatible PEG. The obtained AIE dots are uniform with an average diameter of about 90 nm. In the hypoxic microenvironment, the azobenzene moieties are cleaved by the overexpressed azoreductase, with the FL of the AIEgen (i.e., TPE) recovered in the meantime. Moreover, the capacity of the explored activatable probe being utilized for “light-up” two-photon imaging with excitation by NIR light in the hypoxia tumor microenvironment was demonstrated. Given that AIE dots have dramatic merits over other fluorophores, this work offers new possibilities for tumor-specific imaging with AIEgens, such as deep-tissue imaging. Furthermore, owing to the EPR effect, the 14-based nanodots may have further advantages over conventional small molecular probes.

With the self-assembly of the hypoxia-activatable dimeric prodrug PTX\(_2\)-Azo and the peptide copolymer furnished with the photosensitizer chlorin e6 (Ce6), the light-boosted nanoprobe 15 was formed, realizing the combination of PDT, chemotherapy, and FL imaging (Figure 7C).\[53\] The nanoprobe 15 exhibited as ∼100 nm-sized well-defined spheres and shows effective tumor accumulation due to the EPR effect. The encapsulated paclitaxel prodrug PTX\(_2\)-Azo could be cleaved into free and potent PTX via the bioreduction by the overexpressed azoreductase and NTRs, the sequential 1,6-elimination, and decarboxylation reactions in hypoxia. The Ce6 coencapsulated in the nanoprobe could yield \(\text{O}_2\) to stimulate cell apoptosis and simultaneous oxygen consumption-promoted hypoxia, which further aggravated the release of PTX. As expected, the nanoprobe 15 possesses enhanced cytotoxicity against tumor cells and outstanding \textit{in-vivo} antitumor efficacy without evident systemic
FIGURE 7 The working mechanisms of the azoreductase-activatable probe systems 13–16. (A) Schematic illustration of the response of 13 to hypoxia in tumor-bearing mice model. Reproduced with permission.[51] Copyright 2019, Ivyspring International Publisher. (B) Fabrication of azoreductase-activatable probe based on polymeric AIE aggregates (14) self-assembled in aqueous solution. Reproduced with permission.[52] Copyright 2019, Wiley-VCH. (C) Schematic illustration of the mechanism and construction of the activatable nanoparticle (15) and its self-immolative drug release activated by light-boosted hypoxia for synergistic inhibition of tumor. Reproduced with permission.[53] Copyright 2020, Wiley-VCH. (D) The response mechanism of azo/β-CDP, the BHQ2 (16) response to hypoxia, and the imaging of hypoxia linked with IBD by a cascade amplifier based on cytoplasmic protein-powered FL cascade amplification. Reproduced with permission.[54] Copyright 2020, American Chemical Society

toxicity. Briefly speaking, the developed nanoprobe is a hypoxia-activatable theranostic system that makes use of the features of hypoxia and compensates the deficiency of PDT. Moreover, maximized therapeutic efficacy and minimized side effects are achieved by the synergetic PDT and chemotherapy, suggesting its great potential in clinical medicines.

It has been found that hypoxia is not only an important feature of tumor but also a typical hallmark of the inflammatory disorders. Inflammatory bowel disease (IBD) is one of the inflammatory disorders, and the exploration of effective tools to image hypoxia in vivo during the development of IBD is thus very significant to the precise diagnosis and treatment of this disease. However, the relatively less hypoxic state during IBD as compared to other pathological conditions makes the sensitive and precise imaging of IBD by traditional hypoxia-activatable FL probes remain a challenge. To address this issue, a hypoxia-activatable FL cascade amplifier powered by cytoplasmic protein (HCFA, 16) was designed by Yang’s group to image IBD-associated hypoxia in vivo (Figure 7D).[54] In their design, the 4-aminobenzoic acid (azo)–furnished mesoporous silica nanoparticle was employed as a container to load black hole quencher 2 (BHQ2) and the squarylium dye (SQ) binding to cytoplasmic protein; subsequently, 16 was formed through a host–guest interaction between the β-cyclodextrin polymer (β-CDP) and the azo group. Upon passively stagnating the inflamed tissue of IBD, the azo bond is cleaved under the hypoxic microenvironment, with SQ released to turn on the FL. Meanwhile, the free SQ can bind to cytoplasmic protein to show dramatic enhancement in the FL intensity, achieving the amplification of FL signal for the hypoxia imaging. By virtue of the high loading capacity of the mesoporous silica nanoparticles and the special properties of SQ, 16 is capable of sensing oxygen levels
ranging from 0% to 10%. As demonstrated by the FL imaging results, 16 is able to distinguish distinct levels of cellular hypoxia in a sensitive manner and monitor the variations of hypoxia, emphasizing that 16 is a promising detection tool for hypoxia relevant to IBD.

NAD(P)H Quinone Oxidoreductase 1 (NQO1): NQO1 is a kind of flavin protease that is ubiquitous in eukaryotic cells.\(^{[57]}\) It can specifically catalyze the intracellular two-electron reduction reaction, relieving the toxicity of quinones to cells so as to protect cells. It has been reported that NQO1 is overexpressed in many cancer cells, and thus selectively and sensitively detecting NQO1 with high signal-to-background ratio is of great significance to cancer diagnosis.\(^{[58]}\) Recently, a few NQO1-activatable optical probes have been explored for the purpose of disease diagnosis and/or therapeutic effect evaluation,\(^{[59–61]}\) among which, probe 17 is one of the most representative examples.

As the most powerful oxidant among the reactive oxygen species (ROS), the hydroxyl radical (‘OH) plays crucial role in initiating death of cancer cells and damages of tissues in the therapeutic modality mediated by ROS.\(^{[62]}\) In-vivo therapeutic feedback of ‘OH efficiency is usually unpredictable because of the high reactivity and intrinsic short lifetime of ‘OH, making the spatio-temporally reporting the therapeutic performance of ‘OH a major bottleneck of chemodynamic therapy (CDT), which catalyzes Fenton or Fenton-like chemical reaction in tumor for in-situ ‘OH generation. Guo et al. described a novel nanotheranostics system NQ-Cy@Fe&GOD (17) based on Fenton reaction for the reporting of intratumor OH-mediated treatment with high spatio-temporal resolution by creatively unifying dual-channel NIR FL and MRI signals (Figure 8).\(^{[59]}\)

To be more specific, Guo et al. engineered 17 through the encapsulation of dual-channel FL probe NQ-Cy (Figure 8A) and iron oxide nanoparticle (IONPs) with glucose oxidase (GOD), and the subsequent coencapsulation into the hydrophobic inner cavity of amphiphilic copolymer DSPE-PEG-FA (Figure 8B). Firstly, the dose distribution of NQ-Cy@Fe&GOD could be traced in situ and in real time by the MRI signal from IONPs. Once being delivered into
targeted cancer cells, the nanotheranostics system is promptly disassembled, turning on the NIR FL enhancement at 830 nm (NIR-1). Afterward, the oxidation reaction is initiated by the release of GOD to generate abundant H2O2 in situ. The subsequent intratumor production of ·OH from H2O2 is catalyzed by the Fenton reaction based on IONPs. The resulting oxidizing stress thereby triggers the significant overexpression of NQO1. Such a process is monitored in real time by the emission of CyK peaked at 650 nm (NIR-2) (Figure 8A and C). It is important that the Fenton reaction-based nanocomposite 17 is able to synchronously bridge dual-channel NIR FL and MRI imaging (Figure 8C), and derive several functions, including the mapping of its dose distribution by MRI signal, supervision of OH generation by virtue of dual channel FL signal without blind spot, and the achievement of real-time feedback from catalytic Fenton reaction in tumor. Given that, this work provides an attractive paradigm and paves a new avenue to integrate temporal and spatial resolution feedback on CDT for precise manipulation and reporting of therapeutic process dependent on the drug dose.

It is noteworthy that based on the similar mechanism shown in Figure 8A and the same recognizing moiety to NQO1 (i.e., quinone propionic acid) as that on NQ-Cy, ultrasensitive FL biosensor for activatable imaging of endogenous NQO1 was realized with conjugated oligomer framework,[60] and a highly specific NIR FL probe that is activatable by NQO1 and applicable to in-vivo imaging of tumor has also been developed by conjugating dicyanoisophorone fluoroaphore with the quinone propionic acid.[61]

Hydrolase activation

Esterase

Esterase is a class of enzymes extensively existing in tissue cells of diverse organisms, which regulate the hydrolysis of various esters as an enzymatic catalyst,[62,63] get involved in a large variety of metabolic functions, including ester metabolism,[64] detoxification,[65] protein metabolism,[66] and signal transmission, and are closely linked with several diseases, such as Wolman disease.[67] Moreover, esterase is one of the major factors determining cell metabolism and plays a critical role in cell viability and cytotoxicity evaluation. Considering this, it is extremely demanding to develop a reliable assay approach for quantifying the esterase activity as well as analyzing cellular status. In the past 2 years, several a reliable assay approach for quantifying the esterase activity.

To surmount the above-mentioned barrier, Su et al. have ingeniously created an enzyme-triggerable perylene monoamide-based nanocluster, which has deep tumor-penetration for activatable PDT when disassembled, by making full use of the high molar extinction coefficients, superb photostability, and remarkable self-assembling propensity (Figure 9B).[70] This nanocluster could act as an activatable and deep-tissue penetrable photosensitizer. Such a novel carboxylesterase-activatable amphiphilic NIR-emissive tetra-chloroperylene monoamide (19) was assembled with folate-functionalized albumins into nanoclusters, with a diameter of about 100 nm. Upon hydrolysis of 19 by the tumor-specific carboxylesterase, the nanoclusters readily disassemble into ultrasmall nanoparticles with a diameter of <10 nm, promoting the deep tumor penetration of this nanoprobing system. Moreover, the disassembly of nanoclusters triggered by the carboxylesterase gives rise to about eight-fold enhancement in the FL intensity and four-fold increase in the ability of singlet oxygen generation, rendering in-situ NIR FL imaging and promoted PDT with high accuracy and enhanced therapeutic outcome. Nanoclusters allow dramatic inhibition on tumor growth of 97.7% even with a single-dose intravenous injection and irradiation in vivo, with minimized side effects via imaging-guided activatable deep-tissue PDT. Such a work provides solid proof to the fact that the cascaded multifunctional control via enzyme-induced molecular disassembly is an effective approach for accurate theranostics of cancer.

Glycosidase

N-Acetyl-β-D-glucosaminidase (NAG): NAG is a lysosomal enzyme with a molecular weight of 13–14 kD, which is
widely distributed in various tissues and could not be filtered by the glomerulus. Kidney tissues especially renal tubular epithelial cells are rich in NAG, where the NAG concentration is much higher than that in ureter and lower urethra. When the kidney diseases occur, NAG overflows into the urine, leading to the elevated NAG activity in the urine. The activity of urinary NAG reflects renal parenchymal lesions, and is particularly sensitive to the acute injury and active periods of the kidneys, and thus can be used for early renal injury monitoring and disease course observation. At the same time, urine NAG test is more sensitive than measuring the urine protein, creatinine, and creatinine clearance rate in observing renal transplant rejection. Therefore, urine NAG test can also indicate renal transplant rejection at the early stage. Moreover, increased urinary NAG activity is seen in acute and chronic nephritis, chronic renal failure, lupus nephritis, nephrotic syndrome, toxic nephropathy, epidemic hemorrhagic fever, advanced liver cirrhosis, and so on.

In view of this, Pu’s group established an activatable dual-channel optical reporter ADR (20) with high renal-clearance to image contrast-induced acute kidney injury (CIAKI) in real time and noninvasively via CL and NIR FL, by leveraging the NAG activation (Figure 10). As it has been well recognized that the oxidative stress is an early characteristic of the CIAKI, the primary ROS superoxide anion ($O_2^{-}$) was chosen as one of the biomarkers in the work depicted in Figure 10. Apart from this, it is known that upregulation of ROS will switch on the pathways to lysosomal damage and trigger the release of NAG from kidney proximal tubular cells. Therefore, NAG was selected as the other biomarker. 20 is composed of a $O_2^{-}$-responsive trifluoromethanesulfonate-caged CL signal moiety and an NAG-triggerable
AGGREGATE

FIGURE 10  (A) The sensing mechanisms of N-Acetyl-β-D-glucosaminidase-activatable probe ADR (20) for the detection of contrast-induced acute kidney injury via NIR FL and CL. (B) Real-time duplex imaging (NIR FL and CL) of CIAKI in vivo. Representative CL (left panel) and NIR FL (right panel) images of living mice at 8 or 60 min after being injected with 20 at different timepoints after treatment. The kidneys and bladder in the dorsal and ventral side are indicated with white arrows, respectively. CL images acquired under bioluminescence mode at 180 s, and NIR FL images acquired at 720 nm when excited at 675 nm with IVIS spectrum imaging system. Reproduced with permission.[75] Copyright 2019, Wiley-VCH

N-acetyl-β-D-glucosamine-caged hemi-cyanine NIR FL unit, both of which are conjugated onto a renal-clearable scaffold, namely the (2-hydroxypropyl)-β-cyclodextrin (HPβCD) (Figure 10A). After being systemically administrated into living mice, 20 specifically enters the kidneys and gives out CL and NIR FL signals to report O₂•− and NAG levels, respectively (Figure 10B), exhibiting superb specificity and sensitivity to both biomarkers. Such an independent dual sensing ability of 20 prevents signal cross-talk, permitting the monitoring of two intercorrelated biomarkers in the kidneys of live mice before the glomerular filtration rate significantly decreasing and the tissue damage occurring during the progression of CIAKI. Moreover, 20 also displays minimal in-vivo metabolism and outstanding biocompatibility. 20 excels the typical clinical assay methods and detects the CIAKI at least 8 h and up to 16 h earlier via NIR FL and CL, respectively (Figure 10B). Therefore, 20 has great potential for diagnosing CIAKI at the early stage in the clinic and the screening of safe contrast media in preclinical settings.

β-Galactosidase (β-Gal): β-Gal is a hydrolytic enzyme in cell lysosomes, and the content is higher in renal proximal tubule epithelial cells as compared to those in other tissue cells. Urine β-Gal activity can reflect the early damage of renal parenchyma, especially renal tubules. It is measured together with urine NAG for urinary zymogram analysis,
which helps to observe the course of disease and prognosis evaluation. In the field of genetics, the diagnosis (including prenatal diagnosis) and basic research of human β-galactosidase deficiency are also important indications. Moreover, it has also been reported that β-Gal is associated with cancer, senescence, and the senescence-related diseases. As such, developing β-Gal-activatable probes is of great significance not only to the evaluation of β-Gal activity but also to the disease diagnosis. To this end, quite a few optical probes which could be activated specifically by β-Gal have been reported.[76–85] Probe 21–23 are exhibited in Figure 11 as the representatives to demonstrate the design rationale, working mechanism, and applications of β-Gal-activatable probes.

CL assays have drawn considerable attention by virtue of their remarkably high contrast and sensitivity, because the background emission is minimal as the light emission is initiated by a chemical reaction. CL-based imaging techniques have brought revolution in monitoring of biomolecules in vivo. However, the emitting species are short-lived, giving rise to uncontrollable photons release. It is even harder to meet the probe requirement in a biological milieu that is highly heterogeneous and dynamic. Consequently, achieving trigger-controlled, brilliant, and enriched CL signal has been limited. Recently, Guo et al. smartly established a dual-lock strategy using sequence-dependent triggers, providing a versatile tool for ultra-sensitive CL signal release and bright optical imaging with real-time FL signal (Figure 11A).[76] Each probe based on this strategy is composed of a luminophore, an adamantylide, and an analyte-activatable site. Taking DCM-gal-CF (21) as an example, when coexist with β-Gal, the masking β-galactosyl unit in 21 is readily removed by the β-Gal, unlocking the first lock and leading to the accumulation of prechemiluminophores with a typical twisting intramolecular charge transfer feature. Afterward, the electron-rich double bond is photoactivated to generate 1,2-dioxetane through an in-situ free-radical reaction, unlocking the second lock and giving out the enriched CL signal. The CL signals from 21 are undetectable after β-Gal-activation before photoactivation (Figure 11A). Under the subsequent irradiation by a portable LED light for 1 min, explosively growing and 460-fold enhanced CL signals are released from the activated prechemiluminophores derived from 21. Moreover, the β-Gal concentration and the CL intensity of 21 exhibit a nice linear correlation, indicating the quantifying capability of the designed sequentially activatable probe. In addition, the probe also works well in detecting the endogenous β-Gal activity in the human ovarian cancer cells, that is, SKOV-3 cells.

Developing FL probes for sensing specific biomarkers on site and tracing their activity for a long term is of particularly high need for the disease diagnosis at the early stage.
Regardless, the already developed small-molecular probes are either easy to penetrate the cell membrane or retain at the activation site, but always face up the aggregation-caused quenching effect. Given this, Guo and Zhu et al. reported an enzyme-activatable AIE probe 22 that consists of a hydrophobic AIEgen and a hydrophilic β-Gal-triggerable galactose group (Figure 11B). The probe is practically nonfluorescent in aqueous media, while the nanoaggregates formed in situ as a consequence of the AIE process of the liberated hydrophobic AIEgen when activated by β-Gal are highly fluorescent. Sensing endogenous β-Gal activity on site in living cells was thus realized. In particular, the feasibility of utilizing 22 for long-term visualization of ovarian cancer cells overexpressing β-Gal with high fidelity was further exemplified by virtue of the improved intracellular accumulation and retention of nanoaggregates. Such an AIE-active and enzyme-activatable probe not only acts as a powerful tool for studying the functions of β-Gal in biological systems, but also provides an enzyme-modulated liberation strategy for exploring preclinically applicable multifunctional probes.

Since the dysregulation of the enzymatic activity is closely relevant with many diseases, including inflammation, neurodegenerative disorders, and cancer, the identification of the precise distribution and regulatory mechanisms of an enzyme under a specific cellular environment lays a foundation for unveiling the correlation between its expression and a pathological process. Exactly determining the intracellular localization of an enzyme is still a challenge owing to the diffraction limit of light and undesirable spatial-temporal resolution of the existing FL probes. The recently developed super-resolution imaging technologies have brought forth revolution to FL-based imaging with their capability to visualize basic subcellular structures with nanoscale precision surpassing the diffraction limit of light. For stochastic optical reconstruction microscopy (STORM) technique, reversible photo-switchable systems are highly demanded because they permit the same activated fluorophore to experience multiple photo-switching cycles to afford images with super resolution and potentially fewer toxicity issues. Zhang et al. have developed such a photochromic FL probing platform for imaging biologically crucial biomarkers, including β-Gal with super resolution (Figure 11C). The probe NpG (23) is constituted of a substrate for β-Gal (i.e., β-galactosyl), a photochromic unit (i.e., merocyanine), and a fluorescent naphthalimide unit which is bindable to human serum albumin (HSA). NpG was prebound to HSA to form the hybrid of probe and protein (i.e., NpG@HSA). An evident increase in FL intensity at 520 nm resulted from the formation of hybrid was observed, attributed to the attachment of the fluorescent naphthalimide moiety to HSA. It enabled the FL emission of spiropyran to be visualized in aqueous media. This probe/protein hybrid approach provided a unique imaging platform with improved cell permeability. Moreover, the cellular uptake of NpG@HSA was able to be tracked prior to its activation by β-Gal. β-Gal-regulated cleavage of the galactose part within the NpG@HSA hybrid leads to the NpM@HSA formation as well as an enhancement in the red FL emission centered at 620 nm (Figure 11C). The resulted merocyanine unit was then capable of experiencing photoisomerization between merocyanine and spiropyran to facilitate the STORM imaging with negligible phototoxicity, outstanding photostability, and reversibility. With the aid of STORM, NpM@HSA could determine the subcellular β-Gal distribution between different cell lines with nanoscale precision with the help of the β-Gal-activatable feature and the photochromism of merocyanine. It can be envisioned that the β-galactosyl unit could be replaced with other reactive motifs to detect biomarkers, such as H2O2, esterase, and so on.

Apart from the examples shown in Figure 11, β-Gal-activatable theranostics have also been reported. For example, Pu’s group reported a macrootheranostic probe that is triggerable by β-Gal, showing disease-activated NIR FL, PA, and PTT signal in the ovarian cancer cells (SKOV3) for imaging-guided therapy in 2018. The probe CyGal-P comprises of a D-galactose-caged NIR-emissive hemicyanine dye attached with a long PEG chain, where the D-galactose unit could be exclusively cleaved by β-Gal at the glycosidic bond, while the PEG chain enables the enhanced hydrophilicity for improved biodistribution. CyGal-P initially neither showed FL nor PA due to the diminished electron-donating capacity of the oxygen atom. When systemically administrated, CyGal-P passively targets the tumor and can be specifically switched on by β-Gal with efficient NIR FL and PA signals. It is notable that this β-Gal-activatable multimodal phototheranostics is the first example of β-Gal-triggerable theranostics.

In addition to NAG and β-Gal, other glycosidases, such as β-glucosidase, have also been applied as triggers to the enzyme-activatable probes.

Alkaline phosphatase

As one of the subfamily of phosphatases catalyzing the hydrolysis of the phosphate ester group in a diversity of nucleic acids, proteins, and small molecules, alkaline phosphatase (ALP) is known to be extensively distributed in cancer cells and tissues and has been utilized as a biomarker in the cancer diagnosis. In view of its overexpression in tumors and its importance in early diagnosis and therapy of cancers, such as metastatic prostate cancer, several ALP-activatable probes have been explored for the specific imaging or theranostics of cancer. In addition to NAG and β-Gal, other glycosidases, such as β-glucosidase, have also been applied as triggers to the enzyme-activatable probes.
efficient fluorogenic reaction uncapping 24 by ALPs bound on membrane is triggered, liberating hydrophobic dephosphorylated residue CyFF-Gd which fluoresces in the NIR region at 710 nm. The FF dipeptide-containing CyFF-Gd is prone to undergo molecular self-assembly aided by the efficient intermolecular interactions offered by the FF dipeptide, and thus fluorescent and magnetic nanoparticles are generated. The nanoparticles have a much larger molecular size as compared to 24, potentially hampering molecular rotation and prolonging tumbling time of Gd-chelates and enhancing $r_1$ relaxivity. The NIR FL (over 70 folds) and $r_1$ relaxivity (ca. 2.3 folds) are simultaneously enhanced, allowing
imaging and localizing the ALP activity in live tumor cells and mice in real time and with superb sensitivity, high spatial-resolution using NIR FL imaging, and MRI (Figure 12B). Furthermore, foci of orthotopic liver tumor could also be delineated (Figure 12C), which eases the efficient real-time resection of tumor tissues guided by imaging in intraoperative mice (Figure 12D). Integrating activatable NIR FL via a fluoro-enzyme activity and locations in real time.

Designing other activatable bimodal probes for imaging strategy established by Ye et al. provides a versatile platform for delineating (Figure 12C), which eases the efficient real-time resection.

Furthermore, foci of orthotopic liver tumor could also be imaged with high resolution on a two-photon microscopy platform (Figure 12G). DQM-ALP also rendered discriminating tumor from normal tissue in situ, implying that 25 might act as a potent tool to aid surgeons during tumor resection.

In addition to the above-introduced ALP-activatable probes employed for imaging, theranostic systems triggerable by ALP have also been reported.[90] The enzyme-activatable theranostic probe ETP is composed of a NIR emitter, namely the hemicyanine dye, a mitochondria-targeting hydrophobic triphenylphosphonium group, and a phosphate ester group. In this probe, the hemicyanine contributes to the NIR FL/PA signal of the hemicyanine in its original form. Once being dephosphorylated by ALP, the probe ETP is rapidly activated, providing NIR FL and PA signals for the imaging of ALP activity. Simultaneously, the cleaved residue self-assembled in situ into a supramolecular network structure due to the transformation of ETP from hydrophilic to hydrophobic. The aggregates generated could arouse cell apoptosis and death. In the meantime, the energy dissipation from the incident NIR light could be hindered by the conformational restriction of the hemicyanine moiety, enabling the enhancement in the photothermal conversion efficiency for PTT. The tumor-derived enzyme-activation, bimodal NIR FL/PA imaging, and aggregation-enhanced PTT endows ETP with great potential as an outstanding theranostic probe.

Carboxypeptidase

Carboxypeptidases (CPs), cleaving one or more amino acids from the C-terminal of peptides or proteins, are a family of proteases which play vital roles in physiological processes and a variety of diseases.[94,95] For instance, carboxypeptidase A (CPA) and carboxypeptidase B (CPB) generated in the pancreas account for the degradation of proteins, and angiotensin converting enzyme regulates peptide hormone activity and thus controls blood pressure. CPs are expressed in various diseases at varied levels. For example, CPA and CPB are mainly expressed in pancreatitis and pancreatic cancer, while carboxypeptidase N is preferably expressed in breast cancer. Nevertheless, the methodology to track the CP activity in real time is of great significance but insufficiently developed. In spite of their potential utility for the enzymatic functions studying and disease diagnosis, there are merely a few activatable FL probes for CPs reported, mainly due to the difficulty in the design of suitable substrate analogues of CPs (Figure 13).

CPA/CPB: To address the aforementioned challenge, Urano’s group proposed a straightforward and versatile molecular design strategy for activatable FL probes for CPs through directly translating chemical structural conversion from aliphatic carboxamide to aliphatic carboxylate catalyzed by CPs into dynamic FL activation (Figure 13A).[96] Such a translation was implemented by making use of the intramolecular spirocyclization of rhodamines, as it had been found that such a molecular mechanism is highly suited to convert the aliphatic carboxamide-to-carboxylate transformation to FL activation. FL probes for clinically important CPs were thus successfully developed. Notably, CPA which recognizes C-terminal bulky amino acid such as phenylalanine and CPB that recognizes C-terminal basic amino acids like arginine are individually detected with the developed FL probes merely by altering the conjugated amino acid moieties attached on the rhodamine scaffold (Figure 13A). Moreover, it was shown that 26 and 27 are highly available for detecting in-vitro and ex-vivo pancreatic fluid leakage (Figure 13B and C), one of the most severe complications post digestive surgery that can lead to death. This work was the first report of a design approach which is of high practicality for constructing activatable FL probes for CPs. The developed probes have various potential applications, such as serving as tools for medical diagnosis and elucidating the biological roles of CPs, as well as screening and evaluating CP inhibitors. It is particularly worth mentioning that the rhodamine scaffold should also be widely usable for other enzymes, which could induce chemical transformations resulting in decreased electron-withdrawing capability. It is, therefore, believed that such a platform could also be applicable to exploring activatable FL probes for diverse other enzymes, which remain untargetable thus far.

Prostate-specific membrane antigen (PSMA): PSMA, also known as a glutamate carboxypeptidase II or N-acetyl-L-aspartyl-1-glutamate peptidase I, is a type II transmembrane glycoprotein overexpressed in prostate cancer.[97,98]
Moreover, the expression level of PSMA is upregulated with the disease progression and Gleason score. PSMA has thus drawn broad interest recently from researchers as a target for imaging and therapy of prostate cancer, which is among the most leading male cancers all over the world. As a consequence, facile methods for detecting cancerous lesions and precisely determining their localization in the tissue during operation are of high utility. In view of this, following their work in developing CPA or CPB-activatable FL probes, Urano et al. reported a newly proposed strategy for designing activatable FL probes to visualize the CP activity (Figure 13D and E). 28 is such a first-in-class activatable FL probe for CP activity of PSMA designed and synthesized using this strategy and has been successfully applied for FL visualization of prostate cancer in cultured cells and clinical specimens. 28 consists of a fluorescein framework where the glutamate unit was connected to a benzene group via an azoformyl linker at the 5-position and the methyl group was substituted at 2'-position. When interacted with PSMA, 28 is hydrolyzed through the catalysis by PSMA, releasing the cell-permeable product, that is, activated-28, and enabling the visualization of PSMA-positive cells. When probe 28 was employed to the resected specimens from patients with prostate cancer, significant FL activation was observed and the degree of activation...
corelated well with the pathological localization of prostate cancer. It is believed that probe 28 might render prostate resection with more minimal invasiveness. In addition, it was reported recently that PSMA also exists in the neovascularization of other cancers, including cancers of breast, gastric, colorectal, lung, and bladder with high levels, and it is also expressed in various neurological diseases. Therefore, it can be speculated that 28 could also be a useful tool for studying the functions of PSMA in the pathophysiology of these diseases and in developing therapeutics.

Aminopeptidase

Aminopeptidases (APs for short, EC 3.4.11) is a type of exopeptidase that can selectively cleave amino acid residues from the N-terminus of proteins and polypeptides to produce free amino acids. AP is widely found in different species of organisms, including mammals, plants, microorganisms, and so on. Moreover, there are a large variety of APs. Recently, activatable probes for the evaluation of the activity of APs, including leucine AP and aminopeptidase N (APN), have been reported.[101–104]

Leucine aminopeptidase (LAP): LAP is among the important proteolytic enzymes, which is associated with the development of a large number of pathological disorders as a well-identified biomarker.[105] LAP has the capability to catalyze the hydrolysis of leucine residues from the amino termini of protein or peptide substrates. To quantitatively detect the distribution and dynamic changes of LAP, Guo and Zhu et al., designed an NIR FL, that is, DCM-Leu, for tracking LAP activity in a ratiometric and quantitative manner in different living cell lines.[101] DCM-Leu consists of an NIR-emissive dicyanomethylene-benzopyran (DCM) as a signaler, a L-leucine as an activatable unit, which are connected together via an amide bond. Responding to LAP activity, high-contrast ratiometric NIR FL signal was achieved, giving rise to the quantification of endogenous LAP with negligible background interference as well as “build-in” calibration. Particularly, the LAP-triggerable probe DCM-Leu holds striking merits, such as a Stokes shift of up to ~205 nm, high photo-stability, and superb selectivity. In addition to the exemplification of DCM-Leu in monitoring of endogenous LAP activity in cells, the intracellular distribution of LAP is ambiguously witnessed from different perspectives for the first time, thanks to the high signal-to-noise ratio of such a ratiometric NIR FL response. Collectively, it has been demonstrated that DCM-Leu possesses potential preclinical value of acting as an NIR FL probe favorable for early diagnosis of LAP-relevant diseases and the screening of inhibitors for LAP.

Most recently, by taking advantage of an LAP-activatable probe, drug-induced liver injury (DILI) was diagnosed.[102] DILI caused by therapeutic drugs or other chemicals poses a severe threat to human health, as LAP is a cytosolic enzyme existing in liver tissue. In general, liver diseases, for example, liver cirrhosis, liver necrosis/injury, and hepatitis, usually cause an elevation in the activity of LAP in serum. It has also been recognized that DILI also leads to the increase of LAP level in serum. Undoubtedly, LAP could function as a new biomarker for the diagnosis of DILI. Wu’s group smartly designed a molecular LAP-activatable probe DLP for DILI diagnosis by assessing the raised LAP level in liver tissue of mice with both PA and FL imaging.[102] In DLP, the chromene-benzindolium chromophore acts as the signal reporter, the N-terminal leucyl unit functions as the recognizing element, and the 4-aminobenzylalcohol serves as the connector, respectively. The N-terminal leucyl moiety is cleaved by the hepatic LAP and a cascade of reactions are induced, the probe is thus activated and the sensitive probing of LAP is realized. The high extinction coefficient at 705 nm and strong FL at 733 nm exhibited by the activated probe make DLP an effective molecular probe for imaging of LAP in vivo. Benefited from the FL nature of the probe activated by LAP, liver injury was fluorescently imaged. By virtue of the MSOT, not only was the liver injury-associated elevation in LAP level evaluated noninvasively, but also the injury foci was identified and located via 3D images.

Apart from the above-discussed LAP-activatable probes, Zhang’s group recently reported an intriguing molecular probe which is activatable by two enzymes via a “double-locked” strategy.[105] Imaging and identifying diseases in a very accurate fashion with single-enzyme activatable molecular probes is not easy to be realized. By means of attaching a leucine to a propylamine unit via a pseudo-peptide bond, which in turn is connected to the silenced NIR hemicyanine moiety through an ether bond via an eliminative spacer, namely a para-hydroxybenzyl group, Zhang et al. engineered a “double-locked” probe, which is successively triggerable by LAP and monoamine oxidase. In this probe, the leucine unit acts as the first “lock” and the propylamine group functions as the second “lock.” The pseudo-peptide bond is cleavable by the first “key,” that is, LAP, while exposing the resulted amino group to the second “key,” that is, monoamine oxidase would lead to the oxidation and β-elimination to liberate the NIR emitter and recover its FL emission peaked at 720 nm. Such a probe can only be activated by the coexistence of LAP and monoamine oxidase, while keeps silent when either enzyme is inhibited. In this manner, accurate bioimaging and hepatopathy differentiation could be realized. To be specific, when tested in serum, the probe exhibited dramatic differences in mouse models of both liver cirrhosis induced by CCL4 and DILI. Significantly, the probe can be used to precisely identifying serum samples from clinical patients with distinct hepatopathies. More importantly, the probe designed via the “dual-lock” approach is more accurate for the imaging of DILI as compared to the “single-locked” probe. Such a smart strategy shows great potential for hepatopathy diagnosis and clinical transformation.

**CD13/APN:** APN is a member of the zinc metallopeptidase family and known as a dimeric membrane protein participating in many physiological and pathological processes, such as neuromodulation, signal transduction, antigen processing, and immunological responses.[106,107] APN has been taken as a biomarker for cancer because of its important role in tumor growth, migration, angiogenesis, and metastasis. Imaging endogenous APN levels in situ is of considerable significance for studying APN and its diverse functions.

Fan’s group developed a novel two-photon excited FL probe with NIR emission (i.e., DCM-APN) for APN detection through simple combination of L-alanine as the recognizing moiety with the DCM fluorogen.[103] Enzymatically hydrolyzing the amide bond by APN converts DCM-APN (29) into DCM-NH2 which is fluorescence-inactive.
strong electron donor, and releases the NIR FL signal at 664 nm due to the enhanced ICT effect (Figure 14A). Ultra-high sensitivity with a detection limit of as low as 0.25 ng mL\(^{-1}\) was achieved with DCM-APN. Noteworthily, a Stokes shift of up to 194 nm greatly boosted the ability to collect valid signals. Moreover, DCM-APN was utilized to discriminate cancer cells from normal cells as well as hepatocellular carcinoma tissue from normal liver tissue through 3D imaging by means of two-photon excited FL (Figure 14B). The \textit{in-vivo} imaging of transplanted tumor in nude BALB/c mice suggested that the APN-activatable probe DCM-APN could serve as a promising tool for assessing the activity of APN and thereby aid in the diagnosis at early stage. Most recently, novel FL probe activatable by APN for metastatic cancer tracking and image-guided surgery through \textit{in-situ} spraying was reported by Yoon et al.
Matrix metalloproteinases-2

Matrix metalloproteinases (MMPs), known as important biomarkers, are a kind of proteases involved in the onset of tumor, invasion, progression, metastasis, and angiogenesis of cancers. Among them, matrix metalloproteinases-2 (MMP-2) is one of the most important, because it is often abnormally expressed in most of the solid tumors in such as bladder, breast, prostate, colon, and stomach cancers, rendering assessing in vivo MMP-2 activity closely linked to the clinical diagnosis and therapeutic evaluation of cancers. Most recently, a few probes that are activatable by MMP-2 have been designed and used for bioimaging applications. Taking the probes shown in Figure 15 for examples, the feasibility and applicability of MMP-2-activatable probes for the imaging of tumor will be demonstrated.

Probe QSY21-GPLGVRG(125I)Y-Cy5.5 (31) is an MMP-2-activatable NIR FL/PA probe which is employable to quantitative in-vivo visualization and analysis of the MMP-2 activity. As can be seen from Figure 15A, 31 contains a peptide sequence GPLGVRG, which is the substrate to MMP-2, an NIR- emissive dye Cy5.5, and the QSY21 which is an effective quencher to Cy5.5. In 31, GPLGVRG also acts as a covalent linker to connect QSY21 and Cy5.5. Owing to the presence of more hydrophobic QSY21 and the more hydrophilic Cy5.5, the resultant activatable 31 molecules tend to undergo self-assembly to form uniform nanoparticles in aqueous solution (Figure 15B). It was revealed that the nanoparticles of 31 in the intact state practically emitted no detectable FL. In sharp contrast, intense FL was readily switched on when incubated with MMP-2, and showed obvious dependence on the concentration of MMP-2. In addition to the high-performance in the FL imaging of MMP-2-overexpressed tumor (Figure 15B), the nanoparticles of 31 also unexpectedly displayed a PA signal at 680 nm. The PA signal @ 680 nm reduced linearly against the MMP-2 concentration, whereas the PA signal peaked at around 730 nm kept almost unaltered and thus served as an internal reference, due to the change in the aggregated state of both Cy5.5 and its quencher as a result of cleavage. Such a unique feature enables the nanoparticles of 31 to possess high practicability for functioning as ratiometric PA imaging probes for quantitative monitoring of the MMP-2 activities in vivo (Figure 15C). This proof-of-concept work is anticipated to provide a versatile platform for noninvasively studying the in-vivo malignant behaviors of tumor-relevant proteases.

31 is a representative of MMP-2-activatable molecular imaging probes, while T-MAN (32) represents the MMP-2-activatable nanoprobe-based theranostics. The tumor-targetable and MMP-2 activatable T-MAN is elaborately designed (Figure 15D). T-MAN is composed of Gd-doping CuS nanodisks (i.e., Gd/CuS), a PEG2000-furnished amphiphilic phospholipid polymer (i.e., DSPE-PEG2000) layer, a tumor-targeting cRGD moiety, and an NIR emitter Cy5.5 along with a QSY21-decorated peptide substrate to MMP-2 (i.e., QSY21-GGPLGVRGK-Cy5.5-SH), which is quite similar to 31 (i.e., QSY21-GPLLVRG(125I)Y-Cy5.5). Owing to their small size, efficient NIR absorption, and high photothermal conversion efficiency, the Gd/CuS nanodisks are applied as photothermal element in this nanoprobe. Besides, the introduction of paramagnetic Gd3+ endowed the nanoprobe system with T1-shortening effect and bright MR contrast for high-resolution in-vivo imaging of deep-lying tumors, such as orthotopic gastric cancer. The DSPE-PEG2000 encapsulating Gd/CuS nanodisks into micelles aids in improving water miscibility and biocompatibility, prolonging blood circulation, and hampering immunogenic and antigenic responses. What is more important is that the micellar encapsulation is also able to promote close packing of the flat Gd/CuS nanodisks inside the micelles, probably inducing the geometrical confining effect, boosting the rotational correlation time, and enhancing r1 relaxivity, as a result of the restriction of molecular rotations of the Gd/CuS nanodisks. Analogue to the case of 31, Cy5.5 was selected as the FL reporter in view of its strong NIR FL, QSY21 was chosen as the quencher owing to its high quenching efficiency on the NIR FL of Cy5.5, and in the meantime, GPLGVRG was applied as the substrate for MMP-2 considering its superb capability to be split by MMP-2, then initiating the liberation of QSY21 from T-MAN to restore the NIR FL of Cy5.5.
Different from 31, the covalent conjugation of cRGD to the micelles in T-MAN facilitates the active delivery into tumor cells for one thing and for another, and helps to orient the peptide substrate for efficient cleavage by MMP-2.

At the initial state, T-MAN (32) displays high MRI signal but silent NIR FL owing to the quenching effect of QSY21. When intravenously administrated, 32 is efficiently transported to gastric cancer tissues via αvβ3 integrin-regulated active delivery. Once entering the gastric cancer tissues, the αvβ3-bound overexpressed MMP-2 recognizes and cleaves the peptide sequence, yielding T-MAN-cle together with the recovery of NIR FL of Cy5.5 (Figure 15D). The brilliant MR contrast with an r1 relaxivity as high as ~60.0 mM⁻¹ S⁻¹ per Gd³⁺ at T1 provided by 32 offered superb resolution and deep-tissue penetrating ability to noninvasively track the accumulation of 32 in the gastric cancer tissues (Figure 15E). Meanwhile, the activated NIR FL with a turn-on ratio up to ~185 folds could give an ultrasensitive signal for imaging MMP-2 activity relevant to gastric cancer cells in real time. With the guide by bimodal FL/MR imaging, irradiation with an 808 nm-NIR laser at gastric cancer sites can afford hyperthermia to ablate gastric cancer cells both in vitro and in vivo, with a photothermal conversion efficiency of about 70.1%. Moreover, 32 can preferentially accumulate in gastric tumors with about 23.4% ID% g⁻¹ at 12 h. Consequently, combining dual biomarker recognizing ability and bimodal imaging capability enables T-MAN to draw a clear and accurate picture of primary gastric cancer tissues and lymph node metastasis, further be capable of guiding the efficient in-vivo PTT of gastric cancer. This is the first time
that orthotopic gastric tumors in intraoperative mice are accurately detected and ablated through laser irradiation-initiated photothermal effect, shedding insight on further design of theranostics integrating dual/multiple biomarker recognition and dual/multiple imaging modalities.

Fibroblast activation proteins

Fibroblast activation protein (FAP) is a surface antigen expressed by activated fibroblasts in matrix. It is a type II membrane binding protein and belongs to serine proteolytic enzyme family. It has dipeptidyl peptidase (DPP) activity and collagenase activity. It can hydrolyze extracellular matrix (ECM) components, such as DPP, gelatin, and type I collagen, playing a vital role in the growth, invasion, and metastasis of tumors. It has been reported that FAP can also assist in clinical diagnosis.

For example, fibroblast activation protein-alpha (FAPα) shows overexpression in activated keloid fibroblasts compared to normal ones. FAPα acts a vital role in the overgrowth of the wound boundaries of keloids, which is relevant to the “invasiveness” of keloids. Detecting skin diseases, including keloids at an early stage, has great implication for the effective treatment. Regardless, the FL molecular probes that competent for this task are scarce. Pu’s group reported the first activatable NIR FL molecular probe for specific and sensitive imaging of keloid cells derived from abnormal scar fibrous lesions. Although both are FAP-triggerable, different from FAPα, the cleavage of the amide bond between the self-immolative linker and the peptide substrate readily takes place to produce the intermediate aniline (i.e., activated-33).

Following this, 1,6-elimination and the subsequent spontaneous cyclization of N,N′-dimethylatedenediamine occur and eventually give rise to free CyOH, the uncaged form of 33 with boosted electron-donating ability from the oxygen atom (Figure 16A). As such, NIR FL at 710 nm is immediately turned on in response to FAPα by at 45-fold, enabling the effective and selective discrimination of keloid cells from normal skin cells with rapid activation kinetics. Integrating 33 with a facile microneedle-aided topical application allows the probe to sensitively detect keloid cells in metabolically active human skin tissue with a theoretical detection limit of as few as 20,000 cells (Figure 16B). Thus, it is believed that 33 should possess the potential to catch early symbols of abnormal scarring prior to developing into mature keloid scars. It is worth mentioning that few probes specific to FAPα had been reported before probe 33, and none of them had been applied to detect keloids.

Although both are FAP-triggerable, different from 33 which is a molecular probe used for NIR FL imaging, probe 34 shown in Figure 16C is a nanotheranostics, which has been employed for functional and anatomical image-guided PTT for tumors. Methodologies for effective tumor detection and oncotherapy are urgently needed due to the serious threat exerted by malignant tumors on public health.

Complementary information, including molecular, pathological, and anatomic information on tumor features, could be offered by multimodal imaging, and hence, multimodal imaging has great potential for precise diagnosis, monitoring drug distribution, and trafficking therapeutic effect. Nowadays, establishing nanoplatforms is proved to be an effective means to achieve multimodal imaging systems. The above discussed probe 32 and the to-be-discussed probe 34 are perfect examples.

The extendable star-like nanoplatform GNS@PDA (34) was built on the basis of a polydopamine-coated gold nanoparticle. Such a nanoplatform can introduce different imaging modalities as demanded via a simple constructing procedure for multimodal imaging-guided PTT (Figure 16C). As an ideal photothermal conversion agent, the inner GNS with tunable plasma peak in the NIR region of 600–1100 nm was chosen as not only an outstanding contrast agent for two-photon excited FL, CT, and PA imaging but also an efficient quencher to NIR FL. The versatility of the PDA shell renders facile diversification of this simple but powerful nanoplatform to achieve further function extensions. More specifically, thiol-bearing peptide could be introduced to the quinone groups in PDA via Michael addition, facilitating the design of tumor-related protease-activatable FL probe. Cyanine-7-decorated peptide (Cy7-KTSGPNPC) was attached to GNS@PDA to afford an FAP-specific NIR FL probe as a paradigm. Moreover, PDA holds natural ability to bind with a variety of metal ions to act as contrast agents. GNS@PDA chelated with Fe3+ was demonstrated as a contrast agent for T1-weighted MRI in this work. Such a nanoplatform showed high photothermal conversion capability and superior photothermal stability, achieving efficient internalization into cells and accumulation in tumors. It is clear that with a single nanosystem GNS@PDA, intrinsic CT, PA, two-photon-excited luminescence (TPL), infrared thermal imaging (IRT), extended FAP-activatable NIR FL, as well as MR imaging, for precise theranostics to cancers have been realized (Figure 16D).

Detailed positional information of tumors is provided by the CT, PA, and Fe3+-MR imaging, efficient cellular uptake and distribution of GNS@PDA is confirmed by the TPL imaging, precious pathological information on tumor is unveiled by the switch-on NIR FL imaging, and real-time therapeutic response during PTT is monitored by the IRT imaging. The results clearly elucidated that this versatile and extendable nanotheranostics not only enabled multimodal imaging for the visualization of tumor structures and behaviors, but also achieved homogeneous photothermal ablation of bulky solid tumors (~200 mm3) in a single treatment with satisfactory tolerance, with the aid of accurate image-guidance. Given that Fe3+ could be replaced by many other imaging-related metal ions and the FAP-cleavable peptide could be displaced by other tumor-relevant protease-specific substrates, it can be envisaged that further development of GNS@PDA-based nanotheranostics for anatomical image-guided PTT could be easily achieved.

Cathepsin B

Secreted extracellularly, cathepsin B is a crucial hydrolase mainly locating in the lysosome and able to cause degradation of the ECM, and thus facilitate invasion and angiogenesis.
FIGURE 16 The design and working mechanisms of (A) FNP1 (33) and (C) 34 for fibroblast activation protein. (B) Schematically illustrating microneedle-assisted penetration of FNP1 for FAPα imaging in keloid disease models. Upper panel: (i) Skin tissue which is pretreated with microneedles to afford microchannels, (ii) microchannels facilitating the penetration of 33, and (iii) FL imaging. Middle panel: FL imaging and FL intensities of unmodified skin, HaCaT-implanted skin, 33-treated normal dermal fibroblasts or 33-treated keloid-derived fibroblasts cells. Lower panel: FL imaging and FL intensities of skin implanted with different concentrations of keloid-derived fibroblasts cells. Reproduced with permission.[116] Copyright 2018, Wiley-VCH. (D) In-vivo anatomical (CT/PA/MR) and optical (NIR FL) imaging. Reproduced with permission.[117] Copyright 2019, American Chemical Society

of tumors.[119] Moreover, cathepsin B is also a protease of papain family, which is overexpressed in various cancers with a concentration ranging from 4.8 to 10.7 ng mL\(^{-1}\). In recent years, cathepsin B has been found to be associated with tumor invasion and metastasis. Cathepsin B can directly dissolve or indirectly activate and dissolve ECM, such as collagen, laminin, basement membrane, and other components, promoting tumor cells to infiltrate deep tissues, thus opening a channel for cancer cells to move, in the process of cancer metastasis. Studies have found that cathepsin B is highly expressed in a variety of tumor cells, such as cells of gastric, lung, colorectal, breast, prostate, kidney, and other cancers. The expression of cathepsin B is increased in peritumoral stromal cells and vascular endothelial cells. Under normal conditions, cathepsin B can also be secreted by interstitial fibroblasts and macrophages. However, the expression of cathepsin B is significantly increased in cancer stage, indicating that the excessive synthesis and leakage of cathepsin B in macrophages during the invasion and metastasis of carcinoma may also be involved in the diffusion mechanism of cancer cells. Considering the critical roles of cathepsin B in tumor-associated processes, a series of cathepsin B-activatable probes have been recently reported for bioimaging or theranostics.[120–124] The most recently reported probes 35 and 36 are depicted in Figure 17 to elucidate the design principle, working mechanism, and performance of cathepsin B-activatable probes.

It has been found cathepsin B shows aberrant expression or abnormal activity in triple negative breast cancer, the most aggressive subtype of breast cancer.[119] With a high relapse
FIGURE 17 The design and working mechanisms of (A) 35 and (C) 36 for cathepsin B detection. (B) MR and NIR FL dual-modal imaging of mice bearing MDA-MB-231 and MCF-7 tumors, and in-vivo PDT of triple negative breast cancer using probe 35. Reproduced with permission. Copyright 2020, American Chemical Society. (D) In-vitro sensing ability of cathepsin B and the in-vivo therapeutic applicability of 36. Schematic illustration of FL recovery and ROS generation activated by CaB. Representative photographs of xenograft tumors in mice at day 14 after being treated with saline, or saline with NIR irradiation, or unconversion nanoprobe, or upconversion nanoprobe with NIR irradiation. Reproduced with permission. Copyright 2020, American Chemical Society.

risk, poor overall survival, high proliferative index, and the highest rate of metastasis, patients with triple negative breast cancer are presently the subgroup with the worst outcome, yielding a key need for developing novel efficient therapeutic treatments for triple negative breast cancer. Moreover, diagnosing triple negative breast cancer in a precise manner is crucial for early therapeutic intervention. Due to the short of specific targets, endocrine therapy or conventional targeting therapy is unable to exert positive effect on the triple negative breast cancer. Even worse, the already existing diagnostic and therapeutic methods are of limited value for the triple negative breast cancer. Exploring theranostic probes specific to the triple negative breast cancer is thus of vital importance. Wang et al. utilized a sequential strategy to improve the specificity of theranostics, that is, Pep-SQ@USPIO (35), for MR/NIR FL bimodal imaging-guided PDT for triple negative breast cancer (Figure 17A and B). In the cathepsin B-activatable theranostic system 35, the squaraine (SQ) derivative is both a photosensitizer and an NIR fluorophore, and the cleavable substrate of cathepsin B, namely Gly-Phe-Leu-Gly (GFLG) peptide, and the fibronectin-targetable Cys-Arg-Glu-Lys-Ala (CREKA) were conjugated to SQ. The resulting Pep-SQ was linked to maleimide-DSPE-PEG-decorated ultrasmall superparamagnetic iron oxide (USPIO) through covalent attachment between cysteine unit of Pep-SQ and maleimide to produce Pep-SQ@USPIO (35), where the MRI contrast agent USPIO also functions as the quencher or energy acceptor of SQ. Once being intravenously administrated, 35 could accumulate at triple negative breast cancer cells by virtue of the CREKA ligand, with the in-vivo accumulation behaviors monitorable by MRI. Once entering the tissues of the triple negative breast cancer, the cleavage of GFLG occurs upon interaction with the overexpressed endogenous cathepsin B, and the SQ
moiety detaches from USPIO, recovering the prequenched NIR FL and photodynamic activities of SQ, and switching on the NIR FL imaging and PDT (Figure 17B). Remarkably, it is demonstrated that this tumor-ECM-targeting and endogenous enzyme-activatable nanoprobe paves a new way for the diagnosis and therapy of the triple negative breast cancer.

Probe 36 is another nanotheranostics based on the cathepsin B activation (Figure 17C and D). 36 was designed to realize activatable PDT with capability of self-corrected predicting in-situ therapeutic effect.\textsuperscript{123} PDT, as an emerged promising approach for cancer therapy that is noninvasive, highly spatiotemporal selective, almost nondrug-resistant, its real clinical application is hampered by the “always-on” feature of most photosensitizers and the corresponding “off-target” phototoxicity during light irradiation. Thus, PDT systems specifically activatable by tumor intracellular triggers, such as enzyme with low “off-target” damage and simultaneous prediction of therapeutic effect, are extremely attractive. The upconversion nanoprobe 36 was smartly developed for this purpose.

36 is made of five main components: antenna molecule 800CW-modified multishelled upconversion nanoparticles (MUCNPs), namely \( \text{NaYF}_4: \text{Gd}@\text{NaYF}_4: \text{Er,Yb}@\text{NaYF}_4; \) Nd,Yb; which show enhancement in luminescence under NIR irradiation (808 nm), Rose Bengal (RB) that serves as photosensitizer for PDT, Cy3 for the prediction of therapeutic effect, a QSY7 quencher, and a substrate for cathepsin B cleavage (Figure 17C). The activator \( \text{Er}^{3+} \) is confined closely to the surface of MUCNPs, which promotes the luminescence resonance energy transfer (LRET) from MUCNPs’ emission at 540 nm to the Cy3 and RB, which are immobilized on the surface. The subsequent LRET from Cy3 and RB to the QSY7 at the terminus of peptide quenches the emission of Cys and deactivates RB to achieve the cathepsin B-triggerable PDT nanoprobe. When transfected into cells, the intracellular cathepsin B specifically cleaves QSY7 from the MUCNPs’ surface, correspondingly activating RB for ROS generation and restoring the FL of Cy3 to image cathepsin B to predict therapeutic effect in situ (Figure 17D). In other words, these two continuous LRET processes from MUCNPs to QSY7 through Cy3 and RB enhance the efficiency of energy transfer, and give rise to sensitive imaging of cathepsin B and efficient PDT. Remarkably, the latter LRET process from Cy3/RB to QSY7 does not alter the MUCNPs emission at 540 nm, hence the constant emission during the cleavage process functions as an internal reference for correcting Cy3 luminescence and the FL intensity ratio of Cy3 over MUCNPs \(( F_{530}/F_{540} )\) is measured for self-corrected prediction of treatment effect. Undoubtedly, such a work provides clues for future precise and efficient therapy of tumors.

Urokinase-type plasminogen activator

Urokinase-type plasminogen activator (uPA) is a glycoprotein belonging to the family of serine proteases. The uPA system is expressed much more in various malignant tumors in comparison to normal tissues, and serves an extremely critical role in the invasion and metastasis of malignant tumors, including breast cancer, digestive system tumors, bladder cancer, endometrial carcinoma, liver cancer, prostate cancer, lung cancer, pancreatic cancer, glioma, and nasopharyngeal carcinoma.\textsuperscript{126,127} In the treatment of malignant tumors, it can inhibit the invasion and metastasis of tumor by interfering with different action pathways of uPA system. Therefore, the detection of uPA expression in tumor tissue can be used to determine the degree of disease progression and prognosis, because of its critical role in the prevention, diagnosis, treatment, and prognosis of malignant tumors. However, very few uPA-responsive probes have been reported and not to mention to be utilized for the in-vivo detection of malignant tumors. Most recently, a uPA-activatable probe was developed for NIR FL and PA imaging of invasive cancer (Figure 18).\textsuperscript{128}

The activatable polymeric reporter P-Dex (37) is equipped with four elements to guarantee high sensitivity and specificity to uPA as well as the ideal renal clearance: (1) the dextran backbone which enhances the hydrophilicity, enables renal clearance, and promotes the enzymatic cleavage as compared to the small-molecular counterpart, (2) the NIR-fluorescent hemicyanine dye (CyN3OH) which is the signaling moiety for FL and PA imaging, (3) the p-aminobenzyl alcohol acting as a self-immolative linker, and (4) the peptide carbobenzyloxy-Gly-Gly-Arg-OH (Cbz-Gly-Gly-Arg-OH), which is a biomarker-responsive substrate recognizable and cleavable by uPA (Figure 18A).\textsuperscript{128} The small-molecule-based probe SP without the dextran backbone is utilized as the contrast. Neither FL nor PA is shown by P-Dex and SP as the hydroxyl groups of both probes are capped with reduced electron-donating ability. The ingenious molecular design enables P-Dex to passively target tumors. When interacts with uPA, the amide linker between the peptide and the self-immolative linkage is readily cleaved, following which is the spontaneous 1,6-elimination to liberate CyN3OH-Dex or CyN3OH. Thereby, attributed to the strengthened electron-donating capability of the oxygen atom on the uncaged free dye, the NIR FL and PA signals are triggered on. In other words, the probes hold great capability to specifically activate the NIR FL and PA signal responding to uPA (Figure 18B). By taking advantage of rapid activation kinetics and outstanding specificity to uPA, malignant breast cancer cells, that is, MDA-MB-231 are discriminated from other breast cancer cells, that is, MCF-7 (Figure 18C). After being systemically administrated, P-Dex shows passive accumulation in cancer tissue, and in the meantime, the FL and PA signals are specifically activated to report the expression level of the invasive cancer biomarker uPA (Figure 18D). The subsequent efficient renal clearance minimizes the toxicity potential, while the small-molecular probe SP is harder to be cleared by renal system due to its relatively poor hydrophilicity. This is the first polymeric reporter for distinguishing invasive breast tumor from noninvasive one. Considering the overexpression of uPA in metastatic breast tumor lesions, P-Dex is envisioned to hold great promise to be explored for imaging breast tumor metastasis at early stage.

Transferase activation

\( \gamma \)-Glutamyl transpeptidase

\( \gamma \)-Glutamyl transpeptidase (GGT), as a crucial cell-surface peptidase that participates in the cellular homeostasis of glutathione and cysteine, is closely associated with a large
FIGURE 18  (A) The design and (B) working mechanism of 37 for the detection of urokinase-type plasminogen activator (uPA). (C) The FL intensities and PA spectra of MDA-MB-231 or MCF-7 cells after being treated with SP, 37 or 37 together with uPA inhibitor (4-CPG) for 8 h. (D) In-vivo NIR FL and PA imaging of an uPA-overexpressed tumor. Representative NIR FL and PA images of mice bearing MDA-MB-231-tumor at different time points after being intravenously administrated with 37, SP, and 37 together with 4-CPG, respectively, and mice bearing MCF-7 tumor after being intravenously injected with 37. Reproduced with permission. Copyright 2020, Wiley-VCH

number of essential physiological processes, playing a critical role in the maintenance of the cellular redox equilibrium. It has been reported that the level and activity of GGT is dramatically elevated in diverse diseases, such as asthma, hepatotoxicity, diabetes, bacterial infection, and cancer, and thus the abnormal expression of GGT could be taken as a potential biomarker for cancer. Accurately monitoring of GGT activity is of urgent need for early diagnosis and prediction of therapy efficiency. Consequently, a lot of GGT-activatable probes have been developed for the tracking of GGT activity, with the majority of which designed by simply attaching \( \gamma \)-glutamate (\( \gamma \)-Glu), a GGT-cleavable substrate, to a quenched fluorogen. The FL could be turned on from the off state by the GGT-triggered removal of \( \gamma \)-Glu from the probes, rendering the real-time reporting of GGT activity. In the light of the recent advances in the development of optical imaging probes for GGT and their applications in the detection of tumors in animals have been summarized by Ye et al., to avoid repetition, herein, only probes 38 and 39 would be illustrated as examples (Figure 19).

GGT-activatable and tumor-specific NIR-emissive probe 38 is capable of specifically tracking activity of GGT in the tumors of live mice. As the molecular structure shown in Figure 19A, 38 is made of a GGT-specific substrate \( \gamma \)-Glu, the NIR fluorogen merocyanine (mCy-Cl), a self-immolating spacer linking \( \gamma \)-Glu and mCy-Cl (i.e., \( p \)-aminobenzyl alcohol), and the cRGD, the targeting ligand for \( \alpha_\text{v} \beta_3 \)-integrin receptor. Naturally, \( \gamma \)-Glu in 38 is employed to enable the specificity to GGT. cRGD is used to facilitate the active uptake of the probe by \( \alpha_\text{v} \beta_3 \)-positive tumor cells via the endocytosis process. Furthermore, the negative charge and hydrophilicity of cRGD could also enhance the solubility of
FIGURE 19 The design and working mechanism of (A) 38 and (C) 39 for the detection of γ-glutamyl transpeptidase (GGT). (B) FL imaging of GGT in vivo with the dual-target NIR FL probe 38. Real-time FL imaging of mice bearing U87MG-tumor after being intravenously injected with 38 (25 μM, 150 μL), 38-ctrl (25 μM, 150 μL), 38 together with intratumorally injected GGsTop (5 mM, 100 μL), or 38 together with intratumorally injected free c-RGD (2 mM, 100 μL). Reproduced with permission. Copyright 2018, American Chemical Society. (D) Real-time NIR FL and PA imaging of cisplatin-induced acute kidney injury with 39. Left panel: Representative NIR FL images of living mice at 1 h after intravenous administration of 39 (6.5 μM kg⁻¹ body weight, 200 μL in saline) in different treated groups (excitation wavelength = 675 nm, emission wavelength = 720 nm). N-Acetyl-L-cysteine-protected mice, real-time imaging was carried out at 48 h after being treated with cisplatin. Right panel: Representative PA images of mice transverse section at 120 min after being intravenously injected with 39 in different treated groups (700 nm). Reproduced with permission. Copyright 2020, Wiley-VCH

38 under physiological conditions and hinder free diffusion into the αvβ3-deficient normal tissues after being activated by GGT. 38 is initially at an FL “off” state due to the quenching effect of p-aminobenzyl alcohol capping on the mCy-Cl. The presence of GGT will immediately cleave the γ-Glu moiety from probe 38, subsequently induce the spontaneous elimination of the self-immolative linker, and ultimately release activated-38, which emits strongly in the NIR region peaked at 712 nm (Figure 19A).

When intravenously injected, the extravasation and penetration of probe 38 into tumor tissues will be promoted by the small size and hydrophilicity of 38. The subsequent FL turn-on in the tumors is realized via two plausible processes: (1) In process I, the αvβ3 integrin overexpressed on the cell surface of tumor tissue first recognizes the cRGD ligand in 38 and facilitates its attachment to the tumor cells. The GGT bound to cell membrane can then cleave the γ-Glu in 38, producing activated-38 attached at αvβ3. (2) In
process II, probe 38 is hydrolyzed first by the GGT bound on cell membrane and transformed into a strongly fluorescent activated-38. The cRGD ligand in activated-38 will be recognized by the αvβ3 receptor on the surface of tumor cells, promoting its binding to the tumor cells. Then, activated-38 can be delivered into the tumor cells via endocytosis mediated by αvβ3-receptor and accumulate in lysosomes, eventually giving rise to intense NIR FL in tumor cells (Figure 19B). Such strong activation of FL in tumor could be restricted by either GGSTop or free cRGD, verifying the mechanism illustrated above. In sharp contrast, thanks to the deficiency in either αvβ3-integrin or GGT, intense intracellular FL could not be induced in normal cells. By virtue of combining the dual biomarker-recognizable peptide substrates in 38, tumor-targeted delivery and activation is enhanced, the selectivity of 38 to tumor imaging after a systemic administration is substantially improved, finally helping to detect tumor tissue in vivo. A negative-control probe (38-ctrl) was also prepared via esterification of the γ-Glu in 38 by an allylic unit to prevent the cleavage of γ-Glu by GGT after its binding to cancer cells, rendering the substantiation of the proposed mechanism. Notably, it should be feasible to explore other dual-target probes for noninvasively detecting purposes by similar principles.

Probe FPRR (39) is a GGT-activatable NIR FL/PA polymeric reporter for bimodal imaging of acute kidney injury in real time. Receiving up to 25% cardiac output and working as the essential site for metabolizing and excreting body wastes, kidney is vulnerable to toxic exposure and easy to be damaged because of the local high concentration of medicines and metabolites. Therefore, it is very important to develop renal-clearable optical imaging agents for imaging and diagnosing kidney diseases. It is recognized that GGT, which is highly expressed in proximal tubular epithelial cells, is released by exocytosis or leakage after proximal tubular epithelial cell damage and can be immediately detected in urine. PA imaging is attractive but currently encounters the issues of slow body excretion and low signal specificity. To overcome this problem, Pu’s group developed probe FPRR,[133] which has the same polymeric framework and fluorogenic scaffold as those in probe 37. The only difference between probes FPRR and 37 lies in the enzyme-responsive moiety. As displayed in Figure 19C, similar to 37, in FPRR, the polymerizable moiety dextran is a renal clearance enabler, the hemicyanine functions both as an NIR FL and PA signal reporter, and the p-aminobenzyl alcohol serves as a self-immolative linker. However, the uPA-responsive moiety in probe 37 is replaced by the GGT-specific substrate in probe FPRR. As a result, when interacted with GGT, the amide bond adjacent to γ-Glu of FPRR is readily cleaved to subsequently initiate 1,6-immolation and spontaneous release of the p-aminobenzyl alcohol. Consequently, dextran-substituted hemicyanine Dex-CD is afforded with the NIR FL and PA signals simultaneously triggered to report the onset of acute kidney injury. Combing the merits as superior specificity and sensitivity to the biomarker of acute kidney injury, and the renal clearance efficiency as high as 78% at 24 h post-injection, FPRR is competent for detecting cisplatin-induced acute kidney injury at 24 h after drug treatment through both real-time NIR FL/PA dual imaging and optical urinalysis (Figure 19D), which is 48 h earlier than the elevation of serum biomarkers, such as creatinine, the decline of glomerular filtration rate, and the histological changes. FPRR is the first reported activatable PA probe that is able to imaging acute kidney injury in real time. Moreover, such a research work offer possible guide for designing polymeric optical agents with efficient renal clearance, which has great implication for clinical transformation.

A large number of optical probes that are activated by other enzymes besides the ones discussed in this review article have appeared in various journals,[142–163] indicating the rapid development of enzyme-activatable optical probes and implying that developing biomarker-activatable probes has emerged as a new direction of research in the fields of chemistry and materials.

**SUMMARY AND PERSPECTIVE**

In this review, we briefly overviewed the most recent progresses made on the optical probes activated by enzymes in the past two years. The enzyme-activatable optical probes, including small molecule-based ones and those in nanoscale, are introduced. The probes specifically eluted are classified into three categories in accordance with the type of enzymes, namely the oxidoreductases-activatable probes, hydrolases-triggered probes, and transferases-activatable ones. More specifically, the optical probes are individually employed to track the activity of enzymes, including MPO, cytochrome P450 reductase, NTR, azoreductase, NAD(P)H quinone oxidoreductase 1, esterase, N-acetyl-β-D-glucosaminidase, β-galactosidase, ALP, CPs, APN, MMP-2, fibroblast activation protein-alpha, cathepsin B, uPA, and GGT. It has been clearly demonstrated that the enzyme-activatable optical probes enjoy ultra-high sensitivity, superb specificity, and high TBR. Moreover, such probes could be tailored to serve for various purposes by integrating different substrates according to the disease-associated enzyme, introducing dual or multiple imaging modalities, including FL imaging, MRI, PA imaging, CT, and CL imaging, and/or combing distinct therapeutic techniques, such as PDT, PTT, and chemotherapy. Undoubtedly, enzyme-activatable probes have perfectly shown their charm and power in biosensing, bioimaging, and theranostics.

In spite of the inviting advances achieved in the pursuit of enzyme-activatable probes, the research relevant to this aspect is still at the initial stage. To reach the full potential of enzyme-activatable optical probes, more efforts should probably be devoted to overcome the following challenges: (1) Developing enzyme-activatable optical probes with AIEgens. The unique merits, such as high luminosity, superior photostability, large Stokes shift, and good biocompatibility, enable AIEgens to emerge as a class of powerful luminescent materials for biological applications.[164–177] Very importantly, it can be envisaged that the turn-on attribute of AIEgens could be fully used to develop enzyme-activatable optical probes with higher performance. So far, only a handful of AIEgens have been utilized in the construction of activatable probes not to mention the enzyme-activatable ones, leaving large room for further investigation. (2) Diversifying the NIR-emissive scaffolds used to construct activatable optical probes. By far, the most frequently used NIR fluorophores for the development of enzyme-activatable are basically limited to the hemicyanine derivatives, cyanine dyes, and
squanryium dyes. Although these NIR emitters are already highly performed, the diversification of the NIR moieties in the activatable optical probes could possibly bring forth new choices and chances for activatable optical probes and subsequently promote their further development. (3) Achieving activatable NIR-II FL probes whose excitation and emission lying in NIR especially NIR-II region. The already-developed enzyme-activatable NIR fluorescent probes primarily display FL in the NIR-I region (650–900 nm). Attributed to the reduced photon scattering, tissue autofluorescence, and optical absorption at longer wavelength, detecting NIR-II photons (900–1700 nm) is more suitable for in-vivo FL imaging application. Moreover, compared with the energy of visible light and NIR-I photons, the energy of NIR-II photons is evidently lower and results in lower photodamage to bio-substrates and deeper tissue-penetration, making developing enzyme-activatable FL probes with activation and emission in NIR-II region a long-lasting goal. (4) Exploring enzyme-activatable FL probe with in-vivo quantitation ability of pathological parameters. The majority of the currently available enzyme-activatable probes can only realize activation by biomarkers or diseased cells in target sites in a qualitative fashion, but are not able to realize the measurement of biomarker or diseased cells in a quantitative manner. Though a few of work strove to quantification of biomarker, only limited success has been achieved. Quantitative evaluation of biomarkers in vivo, for example, enzymes, can assess the disease process, therapeutic effect, and prognosis. Developing such enzyme-activatable FL probes capable of quantifying biomarkers in vivo would thus be a long-term pursuit. (5) Developing “dual-locked”/“multilocked” activatable probes. As we mentioned above, single-parameter activatable probes have some limitations in really accurate bioimaging and disease diagnosis, making the exploration of probes that are activatable by dual or even multiple parameters, including enzymes and other pathological parameters, in a successive manner fairly attractive.

In summary, the enzyme-activatable probes are anticipated to function as powerful and useful tools for advancing the in-vivo biomedical imaging, making the research of enzyme-activatable probes a new emerging direction in the field of bio-probes and advanced materials. It is hoped that this review will simulate more attention and insights into the enzyme-activatable probes and the biomedical area.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. J.A. Thomas, Chem. Soc. Rev. (2015), 44, 4494.
2. A. Fernández, M. Vendrell, Chem. Soc. Rev. (2016), 45, 1182.
3. S.H. Yun, S.J.J. Kwok, Nat. Biomed. Eng. (2017), 1, 0008.
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