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Recent advances in activatable fluorescence imaging probes for tumor imaging

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Fluorescence imaging is superior in sensitivity and resolution compared with other imaging modalities; however, its application is hindered by high background noise. Tissue-selective strategies, such as passive, active, and activatable targeting, hold great promise in accelerating clinical translation by significantly improving the tumor:background ratio (TBR) and, in turn, the sensitivity and contrast of fluorescence imaging. Compared with the 'always on' contrast agents, activatable probes, which remain nonfluorescent until being activated by tumor-specific molecular targets, further enhance TBR and at the same time provide additional molecular information that can be related to tumor staging and therapy response. In this review, we summarize recent advances in the development of activatable fluorescence probes and provide insights into their advantages and limitations when used for tumor imaging.

Introduction

Cancer is the leading cause of death worldwide and is expected to increase as a result of the growth and aging of the world's population, as well as an increasing prevalence of established risk factors, such as smoking, obesity, physical inactivity, and changing reproductive patterns [1]. Early detection of cancer is crucial for preventing or delaying cancer-related death; for example, the 5-year survival rate of patients with lung cancer improved from 4% when detected at a late stage to 55% with early detection [2]. Imaging modalities, such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT), can provide anatomical and functional information of tumor in a noninvasive way, and are becoming increasingly important for the early detection of cancer and surveillance of reoccurrence. Nevertheless, all of these techniques have limited spacial resolution and contrast for visualizing infiltrating cancer boarders or detecting metastasis, leading to a high rate of repeated surgery because of incomplete dissection of a tumor during the initial surgery, although this varies significantly among doctors and institutions [3]. Fluorescence imaging, which has superior resolution and sensitivity for the imaging of small tumor nodules compared with the techniques listed above, bridges this technical gap. The development of imaging systems and contrast agents over the past few decades has improved the clinical relevance of this technique [4]. Clinical studies have demonstrated the feasibility of using fluorescence contrast agents, such as indocyanine green (ICG), methylene blue (MB), and 5-ALA-induced PpIX, for sentinel lymph node mapping, tumor imaging, and imaging of vital structures during surgery [5]. Intraoperative fluorescence imaging facilitates the detection of small metastases that can not be detected by preoperative CT, MRI, or intraoperative ultrasonography. In addition to open surgery, the fluorescence imaging systems can be integrated with minimally invasive surgical techniques, such as laparoscopic, thoracoscopic, and robot-assisted surgery, in which there is diminished tactile feedback. Nevertheless, the clinically available fluorescence dyes were not originally designed for cancer imaging; their tumor selectivity largely depends on the

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administration route and intrinsic pharmacokinetics, resulting in low TBR. For instance, the near-infrared (NIR) fluorescent dye ICG is mainly investigated for hepatobiliary cancer because of its exclusive excretion into the bile and subsequent passive accumulation in tumors; however, when applied for the intraoperative imaging of other types of tumor, unexpected false-positive results were observed [6–8]. Although the observations need to be verified with larger patient samples, these pilot studies raised concern about the specificity of ICG. Most promising new probes are designed to have features that enable tumor targeting; and TBR is considered an important criterion for the evaluation and selection of fluorescence contrast agents.

The design of tumor-selective probes exploits the characteristic traits of cancer. The neoplastic cells interact with stroma cells, such as endothelial cells, pericytes, and tumor-associated immune cells, to remodel the extracellular matrix (ECM), building a microenvironment that promotes tumor growth, proliferation, angiogenesis, and metastasis [9]. The gaining of hallmarks leads to changes in the level of cell receptors and signaling molecules, structure of blood vessels, composition of ECM, and tumor-associated immunity. All these alterations distinguish tumors from surrounding normal tissues, and could be used for tumor targeting. The current strategies that target either the cancer cells or the tumor microenvironment have been categorized as passive targeting, active targeting, and activatable targeting [10].

Passive targeting

Passive targeting was first observed 30 years ago by Matsumura and Maeda, who reported that macromolecules larger than 30 kDa could preferentially accumulated in the tumor interstitium because of the presence of fenestrations in the neovasculature and poor lymphatic drainage [11]. Small molecules that bind to serum proteins and nanoparticle-based fluorescence probes usually have sizes exceeding this value and are capable of passive targeting. For example, albumin-bound ICG was shown to have more rapid accumulation and slower clearance in tumors than in normal tissue, resulting in a gradual increase in tumor contrast [12]. Recent evidence suggests that passive targeting is more complex than originally assumed; its effectiveness varies among individuals and tumor types as well as with the biological and physiochemical properties of the nanoparticles [13]. For example, nanoparticles that are too big to leak into pancreatic tumors could enter colorectal and ovarian tumors because of the naturally more leaky blood vessels [14]. Fluorescent nanoprobes that rely solely on passive targeting could have a short retention time; for example, the NIR fluorescence signal of ICG that was used for endoscopic gastric cancer imaging was visible for only 3 min in tumor tissue, limiting its application in surgical settings [6].

Active targeting

Active targeting is the selective binding of probes to surface molecules or receptors overexpressed on tumor cells and the subcellular organelles, improving the tumor retention time and TBR. Active targeting probes could be fluorophores that are linked to tumor-selective ligands, such as proteins, peptides, nucleic acids, and small molecules; or they could be fluorophores with structure-inherent targeting capabilities [15]. Intraoperative imaging with intravenously injected folate-conjugated fluorescein (folate-FITC) was shown to assist the detection of tumor deposits <1 mm (TBR = 3.1 ± 0.8), leading to improved tumor staging and cytoreductive surgery of ovarian cancer in a pilot clinical study [16]. In another clinical investigation involving 25 patients, topical administration of a fluorescently labeled synthetic peptide (ASY*-FITC) that selectively binds esophageal adenocarcinoma (EAC) cells was shown to reach 75% sensitivity and 97% specificity in detection of early-stage EAC, which is normally difficult to detect because of the flat appearance of the new lesions [17]. Achieving an adequate TBR is challenging for most targeted contrast agents because of their nonspecific binding and uptake by normal tissue. Choi *et al.* improved TBR by deliberate neutralization of the net charge of targeted zwitterionic NIR fluorophore ZW800-1; the reduction in background could be attributed to reduced nonspecific interaction and uptake [18].

Activatable targeting

Activatable targeting probes further reduce background signal and improve TBR by remaining in a nonfluorescent state until they are activated by tumor-specific molecular targets. High TBR is crucial for the detection of small tumor nodules, because even with adequate inherent sensitivity and resolution for cellular imaging, the tumor would be invisible if the background was too high [19]. The quenching of fluorescence of activatable probes usually involves mechanisms such as photon-induced electron transfer (PeT), Förster resonance energy transfer (FRET), aggregation caused quenching (ACQ) and aggregation induced emission (AIE) (Fig. 1) [20,21]. PeT is an intramolecular mechanism whereby the electron transfer from donor to excited fluorophore quenches the fluorescence. The fluorescence can be recovered when the electron donor is cleaved from the fluorophore or inactivated by pH, oxidation, or specific metals that change the HOMO or LUMO energy status of the donor, inhibiting the electron transfer. FRET is the transfer of energy from excited donor fluorophores to acceptors by nonradiative dipole–dipole coupling, leading to quenching of the donor fluorescence. The efficiency of FRET is sensitive to the distance between the donor and acceptor; and it can generally be induced when the distance is within 10 nm. Separation of the FRET pair by enzymatic cleavage is a common strategy for fluorescence recovery. ACQ occurs because of nonradiative energy transfer between aggregated fluorophores that have a small Stoke's shift and a large overlap between the absorption and emission spectra. Fluorophores conjugated to a polymer platform form aggregates by self-assembly; upon activation, disassembly of the platform results in separation of fluorophores and recovery of fluorescence. In contrast to ACQ, in the aggregation state, AIE fluorophores undergo significant increase in fluorescence emission because of the restriction of intramolecular rotations, which block the nonradiative pathway and activate the radiative channels for energy dissipation [22,23]. We discuss these mechanisms below, summarizing recent advances in extracellular and intracellular stimuli-activated fluorescence imaging probes and providing insights into their advantages and limitations when used for tumor imaging.

Extracellular stimuli-activated probes

The tumor microenvironment comprises stroma cells and the ECM. To provide a niche for the progression of malignancy, the

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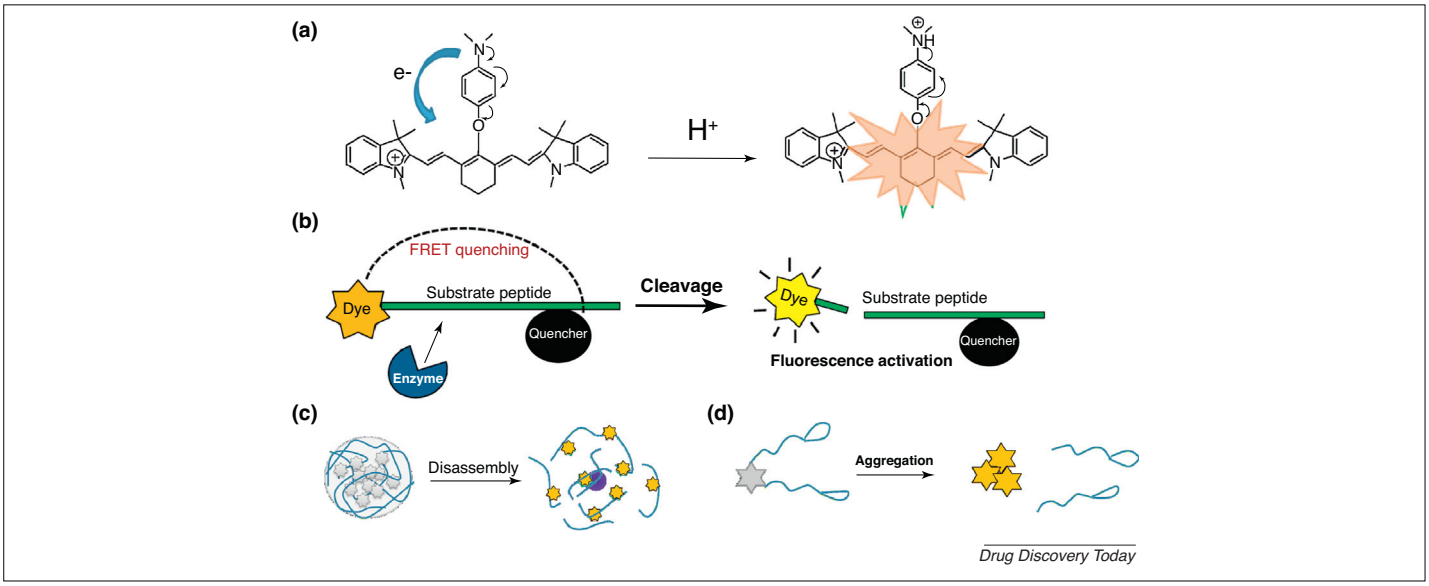

FIGURE 1

Schematic illustration of activation mechanisms of activatable fluorescence probes. (a) Photon-induced electron transfer (PeT), (b) Förster resonance energy transfer (FRET), (c) aggregation-caused quenching (ACQ), and (d) aggregation-induced emission (AIE). Adapted, with permission, from Refs [23,30].

tumor microenvironment differs from the surrounding tissue in terms of its vasculature, pH, oxygenation, and metabolic state. The dysregulation creates tumor-specific molecular activators that are in the ECM or bound to the membrane of cancer and stroma cells that can 'turn on' the silent fluorescence probes.

Extracellular pH

Compared with normal differentiated adult cells, which have an intracellular pH (~7.2) slightly lower than the extracellular pH (~7.4), cancer cells maintain a higher cytoplasm pH (≥7.4) by excreting lactic acid, overexpressing H^+ efflux pumps, the high activity of carbonic anhydrases, and poor perfusion, creating an acidic tumor microenvironment with a pH ranging from 6.7 to 7.1 [24]. The low pH in the tumor microenvironment is an adaptive feature of most cancers, enabling them to gain survival benefits and promoting metastasis. pH-activatable fluorescence probes are designed by introducing 'ionizable' chemical groups, such as amines, carboxylic acid, and phosphoric acid, which are protonated at a low pH, inhibiting PeT; or by using acid labile chemical linkers, such as hydrazone, acetal, and ester bonds, which are cleaved in low pH to separate FRET pairs or aggregated dyes, leading to the recovery of fluorescence. Most of the pH-responsive probes reported in the literature are designed for sensing lysosomal pH (5.0–5.5), and only a few are claimed to be activated in the tumor microenvironment. For example, introducing an ionizable amino group to the IR-775 dye quenched its fluorescence via PeT, creating a small-molecule fluorescence dye that can be activated by both the tumor extracellular and intracellular acidic environment [25]. Although the pH in the tumor environment only induces an approximately 10% recovery of fluorescence, it enables the quick development of signal for real-time imaging after topical administration. The imaging of extracellular pH requires a responsive pH range to match the pH in the tumor microenvironment and the inhibition of cell penetration. This has been achieved by conjugating a pH low-insertion peptide (pHLIP) to a pH-activatable fluorescence dye [26]. The pHLIP facilitates transmembrane insertion but not internalization, exposing the fluorophore on the cell surface. The authors argued that the evaluation of cell surface pH is more informative because pH is at its lowest near the cell surface and increases with distance from the cellular membrane. pHLIP and pH-activatable fluorophore-modified dendrimers were shown to stain orthotropic breast and brain tumors in mice with TBR >1 (Fig. 2) [27].

Extracellular enzymes

Cancer cells and stroma cells express and secrete elevated levels of enzymes, such as matrix metalloproteinases (MMPs) and cathepsin B (CB), to promote proliferation, angiogenesis, and metastasis. MMPs are upregulated in most human cancers, and have a wide spectrum of substrates, including ECM components, growth factor precursors, and cell adhesion molecules [28]. CB, a lysosomal cysteine protease in normal cells, is upregulated and secreted to the pericellular region of tumors [29]. Enzymatic activation is usually achieved by enzymatic cleavage of the peptide that links a FRET pair. For example, Park *et al.* synthesized a self-quenched fluorescence probe by connecting a FRET pair Cy5.5 and black hole quencher-3 (BHQ-3) via a MT1-MMP (a membrane-type MMP) cleavable peptide; the probe was further conjugated to a magnetic nanocrystal for dual-model MRI and fluorescence imaging [30]. NIR fluorescence was detected in MT1-MMP-expressing xenografts; however, the fluorescence was significantly reduced after the first hour,probably because of the fast clearance of Cy5.5. Although this approach shows a well-demonstrated proof-of-concept study, TBR and toxicological studies are still needed to fully reveal the translational potential of this approach. Similarly, Ryu *et al.* developed a CB-sensitive nanoprobe (CB-CNP) by connecting Cy5.5 and BHQ-3 via a CB-cleavable peptide, with subsequent conjugation to the surface of chitosan nanoparticles [31]. The intravenously administered CB-CNP was shown to light up metastases in liver, lung, and peritoneal metastatic mice models;

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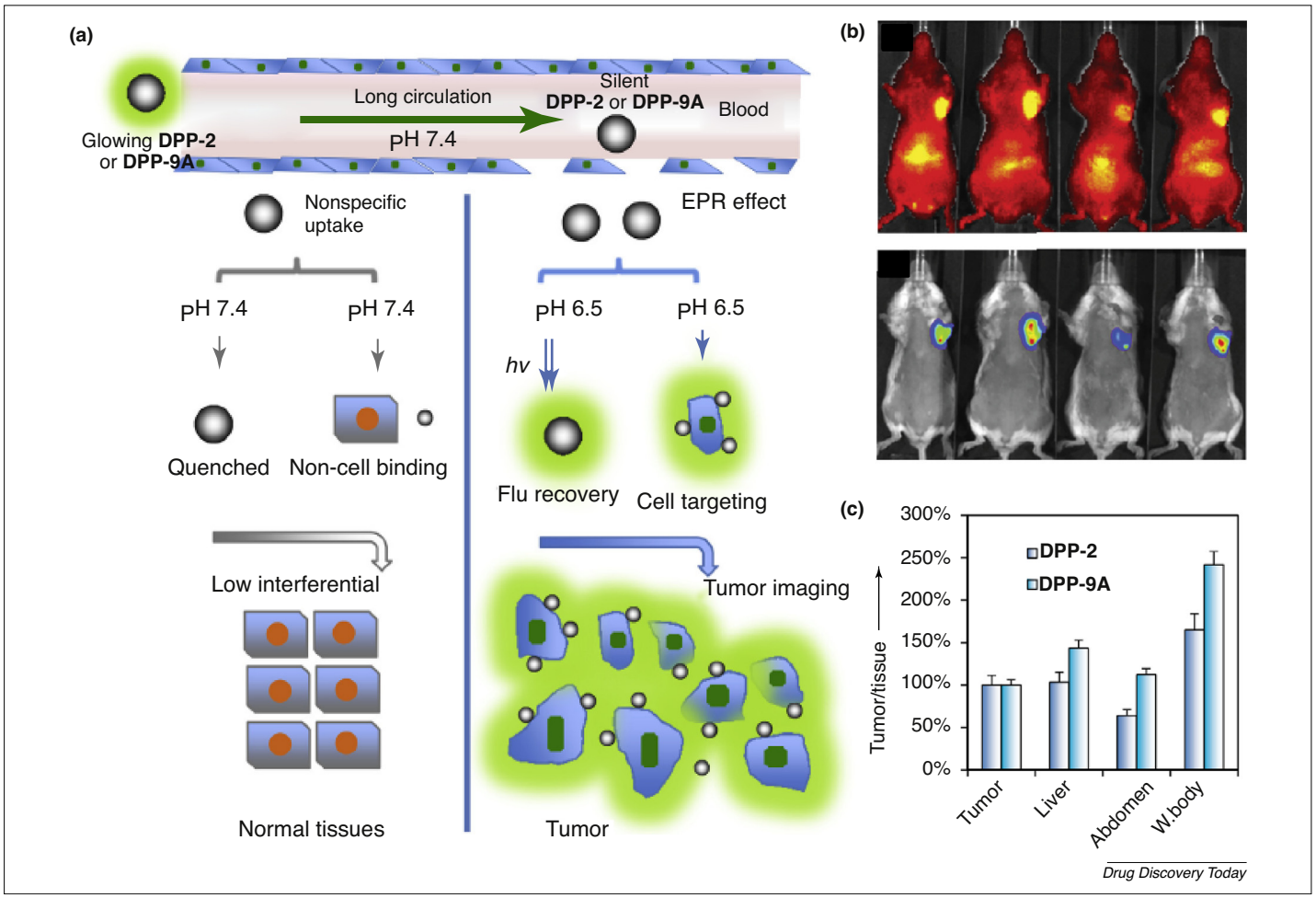

FIGURE 2

pH low-insertion peptide (pHLIP)-mediated pH-activatable fluorescence imaging in the tumor microenvironment. (a) The passive tumor accumulation of two dendrimer-based nanoprobes with surface conjugation of pHLIP and pH-activatable fluorophores: DPP-2 and DPP-9A. pHLIP-induced membrane insertion of the probes and fluorescence activation by the acidic pH in the tumor microenvironment. (b) *In vivo* imaging of mice administered with DPP-9A. (c) Calculated tumor:background ratio (TBR). Adapted, with permission, from Ref. [25]. Abbreviation: EPR, enhanced permeability and retention.

however, the TBR was not shown, making it difficult to compare with currently available contrast agents. A comparison of the CB-activated and MMP-activated Cy5.5-peptide-BHQ-3 probes suggested that the MMP probes are superior in specificity, possibly because they were activated in ECM, whereas the CB-probe was activated predominantly in the lysosome [32]. In addition to MMP and CB, fibroblast activation protein-α (FAP-α), a membrane-bound serine protease that is highly and selectively expressed by cancer-associated fibroblasts (CAFs) and pericytes, has also been exploited for the activation of fluorescence probes. Ferritin-based nanocages were formed by the assembly of FAM-tagged and quencher-tagged single ferritin molecular units, leading to the quenching of fluorescence by FRET [33]. Upon activation, FAM that was connected via a FAP-α cleavable peptide to the nanocage was detached, resulting in fluorescence recovery. The accumulation and activation of the activatable nanocage were shown in mice xenografts that contained a large number of CAFs.

Intracellular stimuli-activated probes

As mentioned above, tumor microenvironment-activated probes usually suffer from limited fluorescence recovery and short tumor imaging time because of inadequate activation by the extracellular stimuli and their potential diffusion away from the tumor site. Therefore, fluorescence probes that can selectively image cancer cells with high and stable TBR are desirable. Intracellular stimuli-activated probes that have their fluorescence recovered after internalization by cancer cells with improved intracellular retention can realize small tumor imaging with stable TBR to provide accurate delineation of tumor margins for surgeons during cancer resections. Usually, the intracellular stimuli-activatable probes should have features, such as good cancer cell permeability, to ensure selective, efficient, and prolonged exposure to stimuli, and a sensitive and specific response to intracellular stimuli, such as pH and enzymes. Here, we briefly introduce representative intracellular stimuli-activated fluorescence probes used for tumor imaging.

Activatable cell penetration

To obtain tumor imaging with optimized TBR, the fluorescence probes should be maximally accumulated in cancer cells and minimally internalized by normal cells. Different strategies have been developed to optimize the endocytosis of fluorescence probes by cancer cells [34]. Among them, polyethylene glycol

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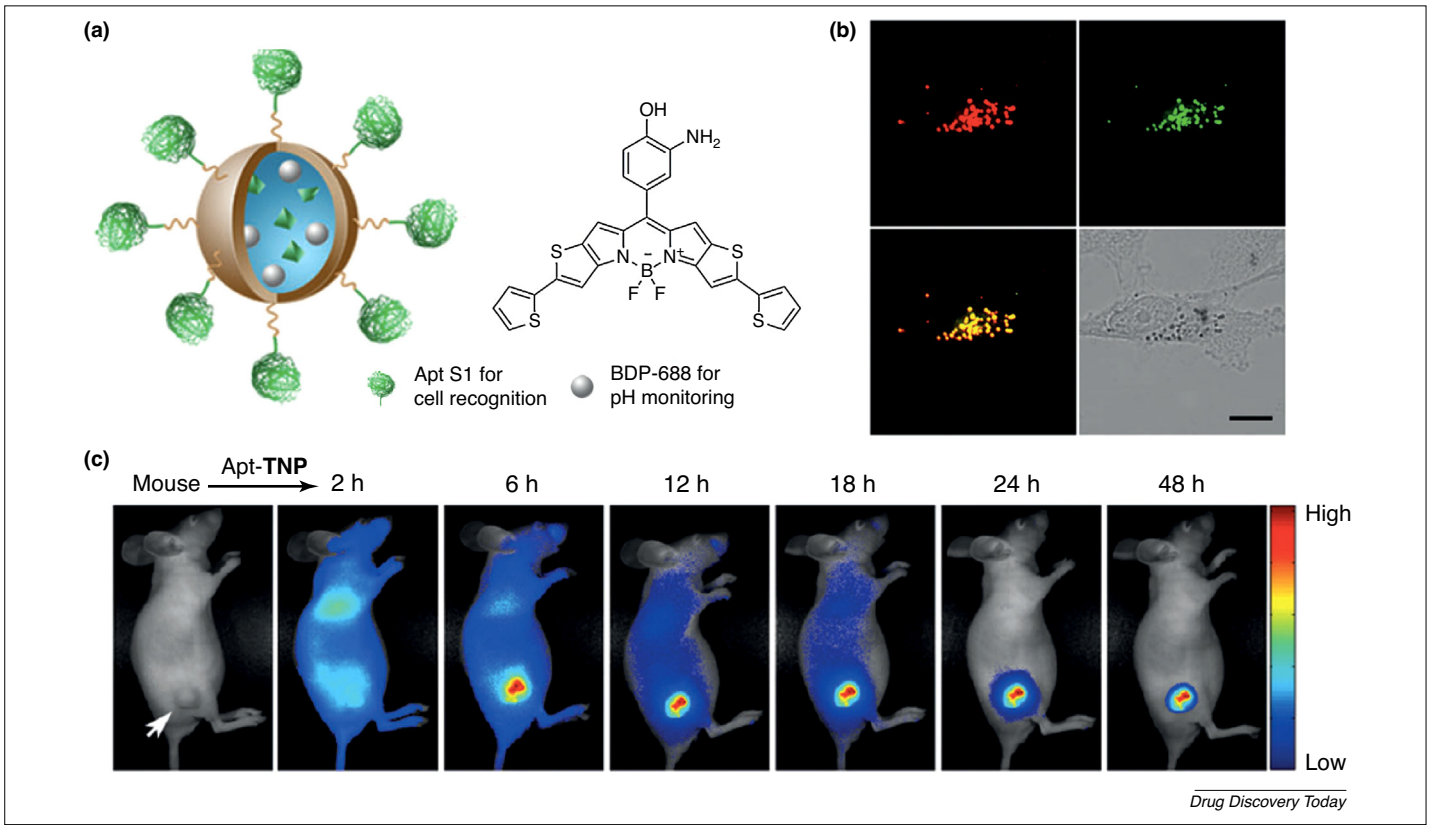

FIGURE 3

Intracellular pH-activatable fluorescent probes for tumor imaging. (a) Aptamer-modified nanomicelle containing the fluorescent probe BDP-688 (Apt-TNP). (b) Co-localization of the fluorescence signal from Apt-TNP (red) and LysoTracker Green. Scale bar = 10 mm. (c) Time-dependent *in vivo* fluorescence images of tumor-bearing mice after intravenous injection of Apt-TNP. Adapted, with permission, from Ref. [38].

(PEG)-sheddable PEGylated activatable cell-penetrating fluorescence probes hold great promise because they have a prolonged circulation time for improved accumulation at the tumor site via the enhanced permeability and retention (EPR) effect and then immediately respond to the tumor microenvironment stimuli to detach PEG for more efficient cancer cell endocytosis [35,36]. Therefore, these fluorescence probe-labeled cancer cells can realize ideal tumor imaging with high TBR.

Intracellular pH

Activatable fluorescence probes designed for tumor microenvironment imaging usually encounter an extracellular pH of 6.5–7.1. By contrast, the activatable fluorescence probes designed for intracellular pH imaging will be subjected to pH gradients (pH 5.9–6.2 in early endosomes and pH 5.0–5.5 in late endosomes and lysosomes) that are much lower [37]. Great efforts have been made to realize activatable intracellular pH imaging based on different pH-responsive fluorescent molecules, whose fluorescence intensities increase dramatically in an acidic environment. The fluorescence initially quenched by PeT [38] can be recovered by protonation of the amino group in a low pH environment. Apart from amine groups, phenol/phenolate interconversion has also been used as a pH-responsive switch to 'turn on' fluorescence in lysosomes via PeT. NIR fluorophore BF2-azadipyrromethenes designed based on this mechanism facilitated imaging of cellular trafficking and tumor xenografts in mice [39]. However, without conjugation of targeting ligand, normal cells can also uptake and activate these pH-responsive fluorescent molecular probes in endosomes and lysosomes, resulting in false-positive fluorescence and background signals. Therefore, fluorescence probes that can selectively recognize cancer cells and realize lysosome-based cancer-specific imaging are required. Recently, Tian *et al.* developed a cancer-specific aptamer-decorated nanomicelle encapsulated with a pH-activatable fluorescent molecule BDP-688 (Apt-TNP) as the intracellular pH-activated probe for selective recognition of cancer cells and lysosome-based cancer-specific imaging [40]. After 24-h administration of Apt-TNP in tumor-bearing mice, the fluorescence signal from the tumor region was around ten times higher than that from other regions (Fig. 3) [40].

Intracellular enzymes

The proteases significantly overexpressed in cancer cells (e.g., NQO1, COX2, and cathepsins) provide a base for the design of intracellular activatable fluorescence probes to specifically image tumors [41–43]. One common strategy is the intracellular protease-triggered linker cleavage, which would release the fluorescence of the probes that is quenched by PeT, FRET, or ACQ from a covalently attached substrate for activatable tumor imaging [41,44,45]. For example, Asanuma *et al.* developed the β-galactosidase-triggered fluorescence 'turn-on' probe (HMRef-βGal) (Fig. 4a) to image peritoneal metastases from ovarian cancers [46]. With the help of HMRef-βGal, peritoneal metastases <1 mm in

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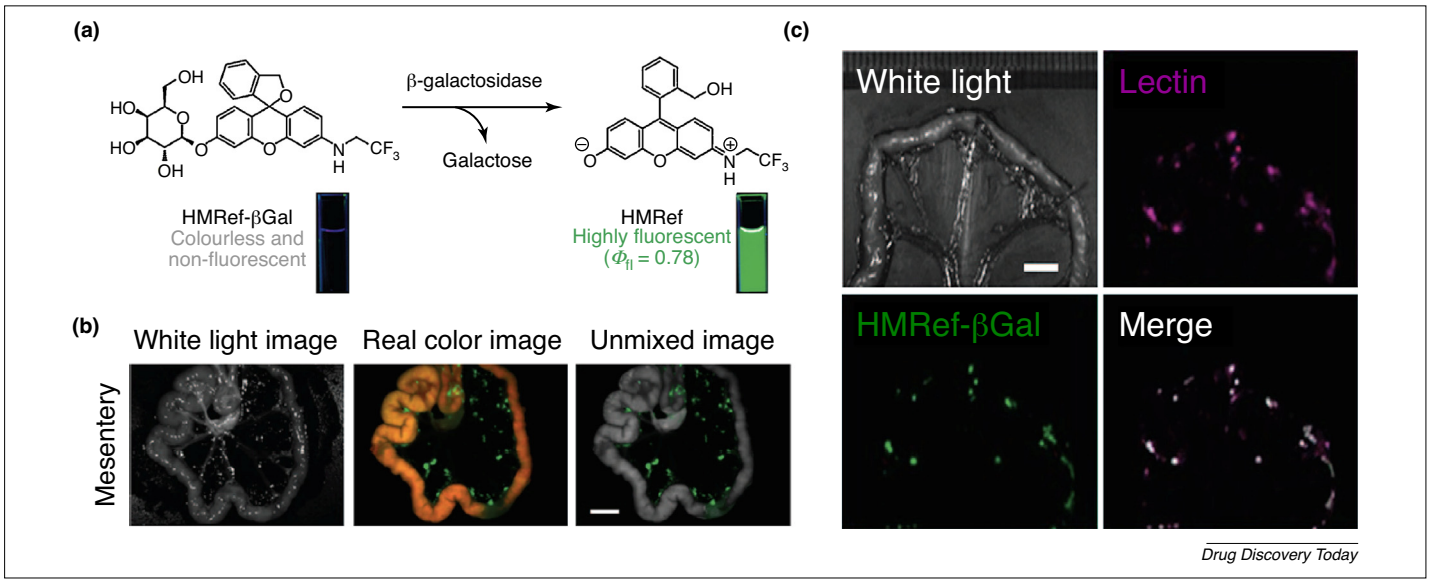

FIGURE 4

Intracellular enzyme-activatable fluorescent probes for tumor imaging. (a) β-galactosidase-triggered activation of HMRef-βGal. (b) Naked-eye observation of peritoneal metastases irradiated with 470 nm light after 1 h administration of HMRef-βGal (green). (c) Co-localization of fluorescence signal from HMRef-βGal (green) and metastasis-specific lectin staining (purple), confirming the accuracy of the probe in the detection of ovarian metastatic cancers. Adapted, with permission, from Ref. [44].

diameter could be clearly visualized by the naked eye when irradiated with 470-nm light (Fig. 4b). The co-localization between the fluorescence from HMRef-βGal and metastasis-specific lectin staining confirmed the selectivity of the synthesized activatable fluorescence probe in tumor imaging (Fig. 4c). However, most activatable molecular probes are hydrophobic and, thus, would naturally aggregate in aqueous media because of their π-π interactions [42,47], leading to fluorescence quenching and reduced TBR when used for tumor imaging. In contrast to ACQ, the fluorochromes with aggregation-induced emission (AIE) have emerged as powerful tools in realizing activatable tumor imaging with a stable and high TBR [48]. Yuan *et al.* developed a cancer-targeted fluorescence probe based on an AIE fluorogen that can be activated by CB overexpressed in the lysosomes of cancer cells. The intracellular activatable probe enables real-time and triggered light-up cancer cell imaging, which has great potential in clearly identifying tumor margins *in vivo* [49].

Hypoxia

Hypoxia, which is caused by an inadequate oxygen supply, is a feature of cancer; it affects many aspects of tumor biology, such as selection of genotypes favoring survival under stress, enhancement of tumor angiogenesis, the epithelial-to-mesenchymal transition, invasiveness, and metastasis. Biological responses to hypoxia have two distinctive intracellular processes: stabilization of hypoxia-inducible factor-1 (HIF-1) and accelerated bioreductive reactions. Most of the activatable fluorescence probes that target hypoxia contain nitro, quinone, and azo groups that could be 'turned on' by oxygen-sensitive reductases [50–52]. For example, an electron-withdrawing aromatic nitro group that was conjugated to the NIR fluorescence dye Cy7 quenched its fluorescence by PeT [50]. Nitroreductase, which is overexpressed in hypoxic tumors, could reduce the nitro group to an amino group, inhibiting intramolecular electron transfer and inducing the recovery of fluorescence. The fluorescence enhancement in the tumor region was shown to correlate well with the degree of hypoxia.

Concluding remarks and future perspectives

Recent advances in tumor biology, chemistry, nanotechnology, and molecular imaging provide the impetus for the development of activatable fluorescence probes that keep silent until they encounter their molecular targets, leading to a superior TBR that enables high sensitivity and high-contrast tumor imaging. Multiple parameters need to be taken into consideration for the design of activatable fluorescence probes. First, the *in vivo* safety of the probes, in terms of pharmacokinetics (i.e., absorption, distribution, metabolism, and secretion) and adverse effects on vital organs, is important if their translation into clinic is the ultimate goal. In the literature, the safety information of activatable probes is seldom provided. Given that the fluorescence intensity of organs does not reflect the real distribution of the probes, additional quantification methods are necessary. In addition, the probes need to be designed to have sufficient fluorescence recovery in the target site to provide a detectable signal. Furthermore, it is beneficial to design activatable contrast agents with passive and active targeting properties to facilitate maximum accumulation (i.e., maximized signal intensity in the target site). Design strategies, such as increasing hydrophilicity, reducing net charge, and incorporation of targeting moieties, are also applicable in the design of activatable probes.

The tumor microenvironment has intra- and intertumor heterogeneity and changes over time as a result of disease progression and treatment intervention. The ever-changing concentration of molecular activators in the heterogeneous microenvironment poses challenges for the design and implementation of

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fluorescence probes. Selection of patients who could benefit from activatable fluorescence imaging is not easy despite the fast development of sequencing techniques. The dynamic tumor microenvironment also provides opportunities for monitoring cancer progression and therapy responses. For example, tumor microenvironment acidity was shown to correlate with metastasis; and metastatic tumors were shown to have more acidic microenvironments than their non-metastatic counterparts [53,54]. In addition, upregulation of extracellular proteins (e.g., a 76-fold increase in MMP-1) was observed in the tumor microenvironment after genotoxic treatment, suggesting the gaining of therapy resistance [55]. Thus, MMP-responsive probes have the potential to reflect the MMP level *in vivo*, and assist treatment selection.

Although fluorescence imaging is powerful in imaging cancer-specific molecular targets with high resolution and sensitivity, it suffers from two major drawbacks: limited penetration depth and an inability to provide anatomical information. Many efforts have been made to extend the excitation and emission wavelength of fluorescence probes to and beyond the NIR-I region (650–950 nm) to further reduce absorption, scattering, and autofluorescence [56,57]. The autofluorescence could also be eliminated by time-gated imaging using probes that have emission lifetimes that are longer than the tissue autofluorescence [58]. Alternatively, the issue could be solved by multimodal imaging, which yields complementary information and offers synergistic advantages. The combination of NIRF imaging with nuclear imaging techniques, such as PET and SPECT, allows increased penetration depth and more accurate quantification. The combination of NIRF imaging with anatomical imaging modalities, such as MRI and CT, facilitates the integration of anatomical and molecular information, enabling both cancer detection and intraoperative guidance.

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