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Application of enzyme-responsive fluorescent probes in the precise diagnosis of Hepatocellular Carcinoma

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Abstract:

Hepatocellular carcinoma (HCC) is a highly lethal malignacy with rising global incidence, where early diagnosis is essential for improving patient outcomes. However, conventional imaging techniques (ultrasound, CT, MRI) and serum biomarkers (e.g., AFP) remain limited in early-stage lesion localization, detection of micro-metastases, and real-time intraoperative navigation. In recent years, enzyme-activatable fluorescent probes, designed around the aberrant expression or activity of tumor-associated enzymes, have shown rapid advances and offer promising strategies for highly sensitive, real-time, and targeted molecular imaging. These probes exploit specific enzymatic activities within the tumor microenvironment, converting non-fluorescent or weakly fluorescent precursors into activated fluorophores or amplifying signals to achieve high-contrast imaging. Their molecular design integrates a recognition unit, fluorophore, and signal-modulation module, enabling switch-on or ratiometric outputs optimized for different enzymatic targets. Compared with traditional molecular imaging, these probes provide faster response, reduced background interference, and enhanced stability in vivo. Emerging developments, including multimodal imaging (e.g., photoacoustic–fluorescence) and dual-enzyme logic-gated systems, further improve diagnostic precision. This review summarizes the design principles, key enzymatic targets, and representative applications of enzyme-activatable fluorescent probes in HCC, and discusses future directions for multimodal integration and clinical translation.

Keywords: Enzyme-activatable fluorescent probes, Hepatocellular carcinoma, Enzyme activity imaging, Near-infrared probes, Intraoperative navigation

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1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of cancer-related mortality worldwide. Owing to the low rate of early diagnosis, patient prognosis remains poor (Asanuma et al., 2015). Traditional imaging modalities provide valuable anatomical information but are limited in detecting small or early-stage lesions, delineating tumor margins, and supporting real-time intraoperative navigation. Serum AFP, although widely used clinically, suffers from inadequate sensitivity and specificity and is frequently negative in patients with early or small HCC (Yuan et al., 2022; Lu et al., 2022).

Molecular imaging offers an opportunity to visualize tumor microenvironmental features and molecular signatures through tumor-specific biomarkers. Among these approaches, enzyme-responsive fluorescent probes have emerged rapidly as a promising strategy. Their key advantage lies in the generation of high-contrast, zero-background signals achieved by exploiting specific enzymatic activities to convert non-fluorescent or weakly fluorescent precursors into strongly emissive products (Habibollahi et al., 2012; Ryu et al., 2014; Chen et al., 2021). Beyond providing sensitive fluorescence activation, enzyme-responsive systems can also reflect the metabolic status, invasive potential, and therapeutic responsiveness of tumors, underscoring their potential for functional molecular diagnosis.

Recent advances in HCC imaging have expanded the application of enzyme-responsive fluorescent probes from basic visualization to intraoperative navigation, targeted drug delivery, and treatment-response assessment. In particular, integration with near-infrared (NIR-I/II) imaging technologies has significantly enhanced tissue penetration, spatial resolution, and imaging speed (Gao et al., 2024; Lian et al., 2022; Liao et al., 2025). /Concurrent progress in biomaterials and organic synthesis has further improved probe photostability, biocompatibility, and pharmacokinetic profiles, providing a strong foundation for preclinical evaluation and future clinical translation.

2. Molecular design principles and response mechanisms of enzyme-responsive fluorescent probes

2.1 Basic Design Elements

The design of enzyme-responsive fluorescent probes depends on the careful integration of multiple functional modules that act together to generate measurable signal changes in response to enzymatic activity. Fluorophores are typically selected from dye families that combine high photostability, strong quantum yields, and good biocompatibility. Hemicyanine dyes, BODIPY, and rhodamine derivatives are frequently employed, while near-infrared (NIR) dyes are especially valuable because they minimize tissue autofluorescence and allow deeper penetration into biological tissues (Khatun et al., 2020; Zhou et al., 2023). In addition, structural motifs that enable intramolecular charge transfer (ICT) or excited-state intramolecular proton transfer (ESIPT) have been incorporated to enhance imaging contrast and improve the signal-to-noise ratio.

The enzyme-responsive unit is the core determinant of selectivity, typically composed of substrates or protective groups that can be specifically recognized and cleaved by target enzymes. For example, alkaline phosphatase (ALP) substrates often feature phosphate ester groups, aminopeptidase N (APN) favors N-terminal cleavage sites of short peptide chains, and γ-glutamyltransferase (GGT) selectively recognizes γ-glutamyl moieties (Xiao et al., 2024). Because enzyme expression varies across tumor microenvironments, these differences provide crucial diagnostic information for disease classification and lesion localization.

Signal modulation modules are designed to minimize background fluorescence and amplify the signal once enzymatic activation occurs. One common strategy is the incorporation of quenching groups to construct “off–on” probes, which remain silent until triggered by enzymatic cleavage. Another approach involves introducing hydrophobic segments that drive self-assembly into nano-aggregates after activation, thereby enhancing localized fluorescence intensity (Zhang et al., 2020). More recently, ratiometric probes have been developed to measure enzyme activity through the relative intensity change of two emission wavelengths. This ratiometric approach reduces the influence of environmental fluctuations and produces more stable and reliable imaging outcomes.

2.2 Common Response Mechanisms

2.2.1 ALP Response Mechanism

Alkaline phosphatase (ALP) is frequently elevated in liver diseases and hepatocellular carcinoma. Its catalytic activity enables the cleavage of phosphate ester bonds, thereby releasing a “locked” fluorophore and shifting the fluorescence signal from weak to strong (Yan et al., 2019). Beyond simple activation, some studies have exploited the hydrophobicity of ALP cleavage products to induce na-

noscale self-aggregation at the cell membrane or intracellular protein interfaces. This aggregation effect amplifies the local fluorescence signal, providing enhanced sensitivity for imaging applications.

2.2.2 APN Response Mechanism

Aminopeptidase N (APN) plays a critical role in tumor invasion and angiogenesis. APN-responsive probes are typically designed with short peptide sequences that serve as cleavable sites. Upon enzymatic digestion, the fluorophore is either exposed or undergoes conformational rearrangement, resulting in increased fluorescence intensity or a shift in emission wavelength (Habibollahi et al., 2012; Ryu et al., 2014). Because APN is highly expressed at tumor margins, these probes hold particular promise for delineating surgical boundaries and guiding tumor resection.

2.2.3 GGT Response Mechanism

Gamma-glutamyltransferase (GGT) is markedly upregulated in liver cancer cells but downregulated in normal hepatocytes and other tissues, making it a valuable biomarker for distinguishing malignant lesions from healthy tissue (Nakamura et al., 2017). Probes designed with γ-glutamyl protecting groups remain quenched in the absence of enzymatic activity. Once GGT cleaves the protecting group, fluorescence is rapidly restored, enabling real-time signal enhancement. This mechanism has been applied to intraoperative navigation, allowing surgeons to identify tumor margins with high precision.

2.2.4 CTSB Response Mechanism

Cathepsin B (CTSB), a lysosomal protease, is often overexpressed during tumor invasion and metastasis. Probes targeting CTSB typically incorporate peptide sequences such as Gly-Phe-Leu-Gly, which are selectively cleaved by the enzyme. Enzymatic digestion activates fluorescence or photoacoustic signals, providing versatile imaging modalities (Ni et al., 2019). More recently, CTSB has been exploited in the development of dual-mode photoacoustic/fluorescence probes, which enable accurate localization of deep-seated lesions and improve diagnostic precision.

2.2.5 β-gal Response Mechanism

β-galactosidase (β-gal) is highly expressed in certain tumors and senescent cells, making it a useful biomarker for both cancer and aging-related studies. Probes incorporating β-galactosidase protecting groups remain non-fluorescent until hydrolyzed by the enzyme, at which point they undergo a sharp transition to a highly fluorescent state (Asanuma et al., 2015). This mechanism offers excellent sensitivity for detecting small lesions and heterogeneous tumor regions, thereby facilitating high-resolution imaging of complex biological environments.

3. Enzymatic Characteristics and Target Value of Liver Cancer Microenvironment

Enzyme expression within the liver cancer microenvironment exhibits pronounced spatiotemporal heterogeneity. Metabolic abnormalities in tumor cells often lead to acidification of the extracellular milieu, increased lysosomal activity, and accelerated degradation of the extracellular matrix. These changes result in the aberrant activation of numerous hydrolases, transferases, and proteases, which collectively shape the tumor microenvironment and influence disease progression.

Among these enzymes, alkaline phosphatase (ALP) is closely associated with hepatocellular injury and cholestasis. Its elevated activity provides a useful biomarker for liver function assessment, and ALP-responsive probes have been employed to visualize lesion localization and disease status (Yan et al., 2019). Aminopeptidase N (APN), a key mediator of tumor angiogenesis, reflects both the invasiveness and malignancy of hepatocellular carcinoma (HCC). Probes targeting APN can therefore provide valuable insight into tumor aggressiveness (Habibollahi et al., 2012; Ryu et al., 2014). Gamma-glutamyltransferase (GGT) plays a central role in oxidative stress regulation and glutathione metabolism. Its high expression levels are linked to tumor drug resistance and enhanced cellular antioxidant capacity, making GGT a critical molecular marker for therapeutic evaluation (Nakamura et al., 2017). Cathepsin B (CTSB) overactivation is strongly correlated with tumor migration and metastatic potential. CTSB-responsive probes are particularly useful for monitoring high-risk or metastatic HCC lesions, enabling early detection of aggressive disease phenotypes (Ni et al., 2019). In addition, β-galactosidase (β-gal) demonstrates tumor-specific expression patterns that are advantageous for identifying intratumoral heterogeneity and minimal residual disease. This specificity enhances the sensitivity of imaging approaches and supports precision oncology strategies (Asanuma et al., 2015).

Taken together, these enzymatic features highlight the diagnostic and therapeutic value of enzyme-responsive probes in liver cancer. By constructing multimodal systems that integrate imaging and therapeutic functions, it is possible to establish a comprehensive treatment chain encompassing early diagnosis, intraoperative navigation,

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and post-treatment efficacy monitoring. Such approaches hold significant promise for improving clinical outcomes in hepatocellular carcinoma.

4 Representative Enzyme Response Probes and Research Progress

4.1 β-gal Response Probe

The HMRef-βGal probe developed by Asanuma et al. represents a milestone in enzyme-responsive probe design, achieving high-sensitivity detection of micrometastatic tumors smaller than 1 mm (Asanuma et al., 2015). Its core mechanism relies on β-galactosidase-mediated cleavage, which triggers a "dark-to-bright" signal amplification effect at the cellular level. Structural optimization of the fluorophore has yielded a high quantum yield and minimal background interference in the near-infrared (NIR) region, thereby enhancing imaging performance. Subsequent studies confirmed that β-gal expression is significantly elevated in hepatocellular carcinoma (HCC) cell lines compared with normal hepatocytes, establishing a strong foundation for early diagnostic applications.

A key innovation of HMRef-βGal lies in its anchoring mechanism: once activated, the probe covalently binds to intracellular proteins, prolonging signal retention and markedly improving the signal-to-noise ratio. This strategy enables luminescence to persist for more than 60 minutes during peritoneal metastasis imaging. Building on this design, Nakamura et al. introduced a pyrroloindole conjugated group to further enhance NIR penetration depth. Moreover, combining β-gal-responsive probes with metabolic tracers has proven effective in differentiating tumor subtypes, paving the way for multidimensional lesion characterization (Fig. 1).

Beyond diagnostic imaging, β-gal probes show considerable promise in intraoperative navigation. Integrated with surgical fluorescence imaging systems, they allow real-time visualization of micrometastases in living models, thereby facilitating the identification of minimal residual disease (Fig. 2). Improved designs such as SPiDERβGal exhibit enhanced anchoring stability after activation, preventing probe loss and increasing both safety and accuracy during surgical procedures. The design principles underlying β-gal probes have also inspired the development of other glycosidase-responsive systems, including β-glucosidase and β-glucanase probes, which hold potential for mapping metabolic pathways in liver cancer.

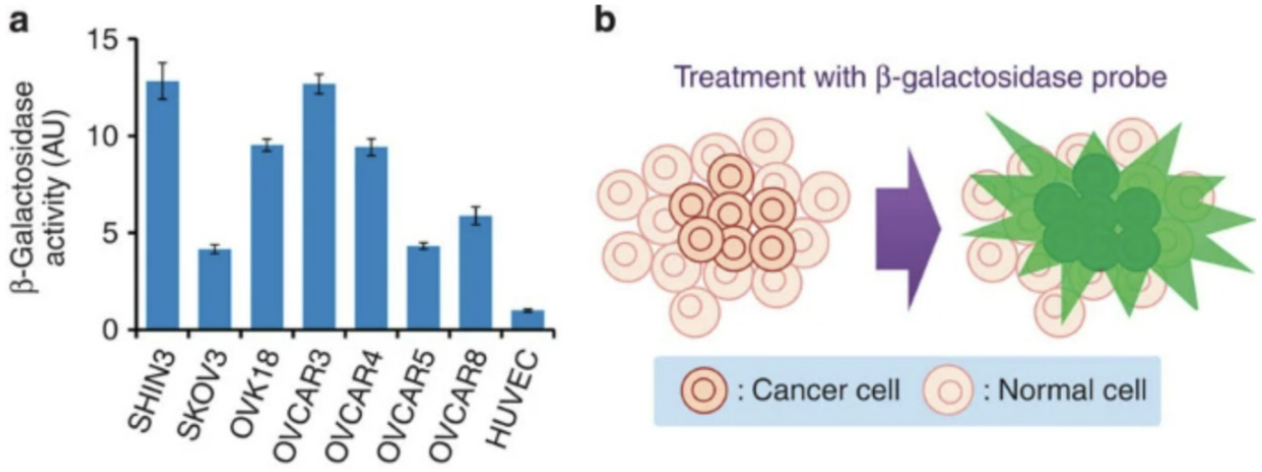

Fig. 1 β-gal overexpression in tumor cells

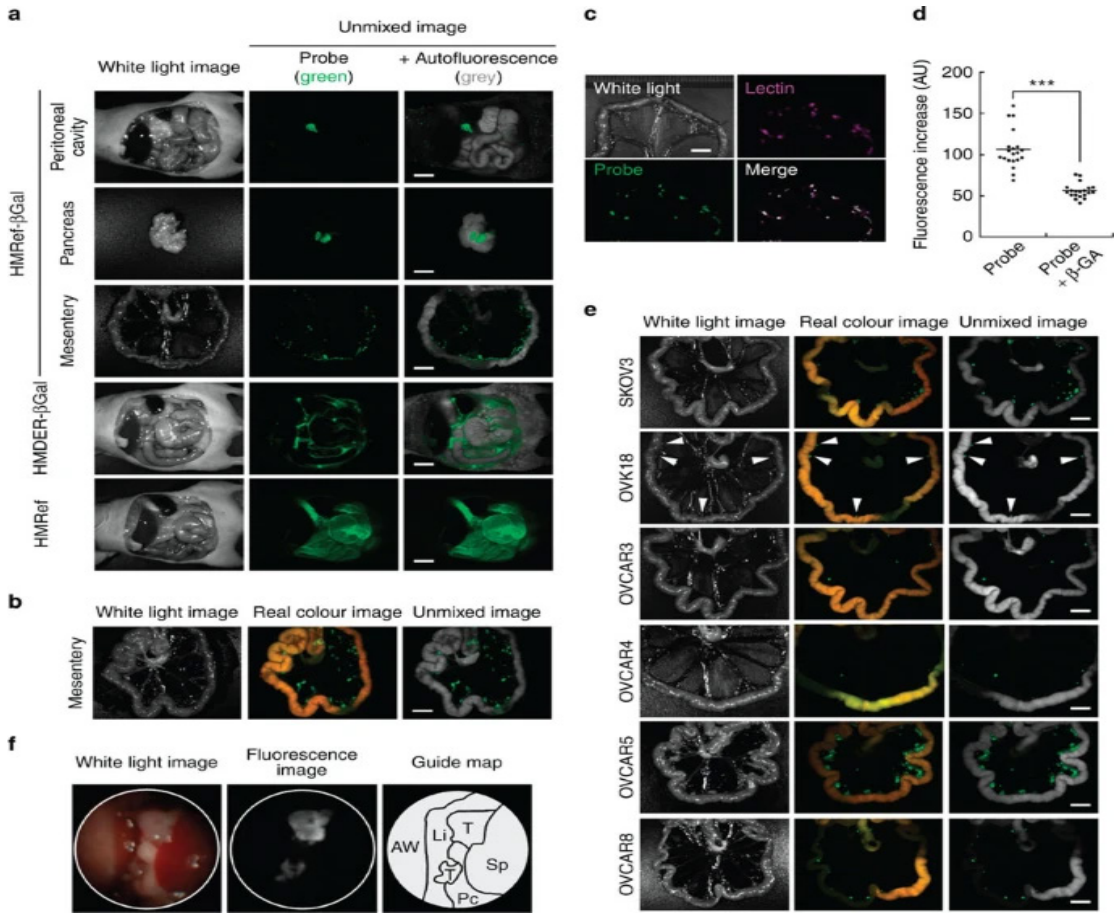

Fig. 2 Imaging illustration of HMRef-βGal in an animal model

4.2 ALP-Responsive NIR Probe

Alkaline phosphatase (ALP) is highly expressed in liver cancer tissues, particularly on tumor cell membranes and in bile duct-associated regions, making it an attractive target for probe development. Zhao et al. reviewed a series of ALP-responsive near-infrared probes designed to generate ratiometric signals and enable rapid in vivo imaging (Zhao et al., 2023). These probes typically employ phosphate ester protecting groups to maintain a "light-off" state until enzymatic cleavage occurs. A representative example is DCM-2F-HP, which releases a hydroxyl fluorophore upon ALP hydrolysis, shifting its emission peak from 620 nm to 710 nm. This ratiometric signal enhancement improves detection sensitivity and supports quantitative analysis.

Zhang et al. (2020) advanced this concept by incorporating a self-assembly module into probe design. Following enzymatic digestion, the probe forms nano-aggregates that prolong signal duration and improve tissue retention (Zhang et al., 2020). In vivo studies demonstrated strong tumor targeting, with fluorescence signals persisting for more than 12 hours. Khatun et al. further expanded the application of ALP-responsive probes by developing Mito-Phos, which integrates the mitochondrial-targeting moiety TPP+. This probe revealed abnormal ALP activation within mitochondria of liver cancer cells, uncovering metabolic dysfunctions and altered enzyme activity at the subcellular level (Fig. 3).

In addition to tissue imaging, Zhou et al. validated the feasibility of ALP-responsive probes for non-invasive diagnostics by applying them in serum detection assays (Zhou et al., 2023). Collectively, these studies demonstrate that ALP-responsive probes have evolved from tools for tissue-level imaging into versatile platforms applicable to body fluid analysis, intraoperative navigation, and subcellular metabolic investigations.

4.3 CTSB-Responsive Fluorescence/Photoacoustic Dual-mode Probe

The CTSB-activatable probe developed by Chen et al. enables both fluorescence and photoacoustic imaging, thereby enhancing tumor recognition and diagnostic accuracy (Ni et al., 2019). Cathepsin B (CTSB), a lysosomal cysteine protease, plays a pivotal role in the invasion and migration of liver cancer cells, making it an ideal target

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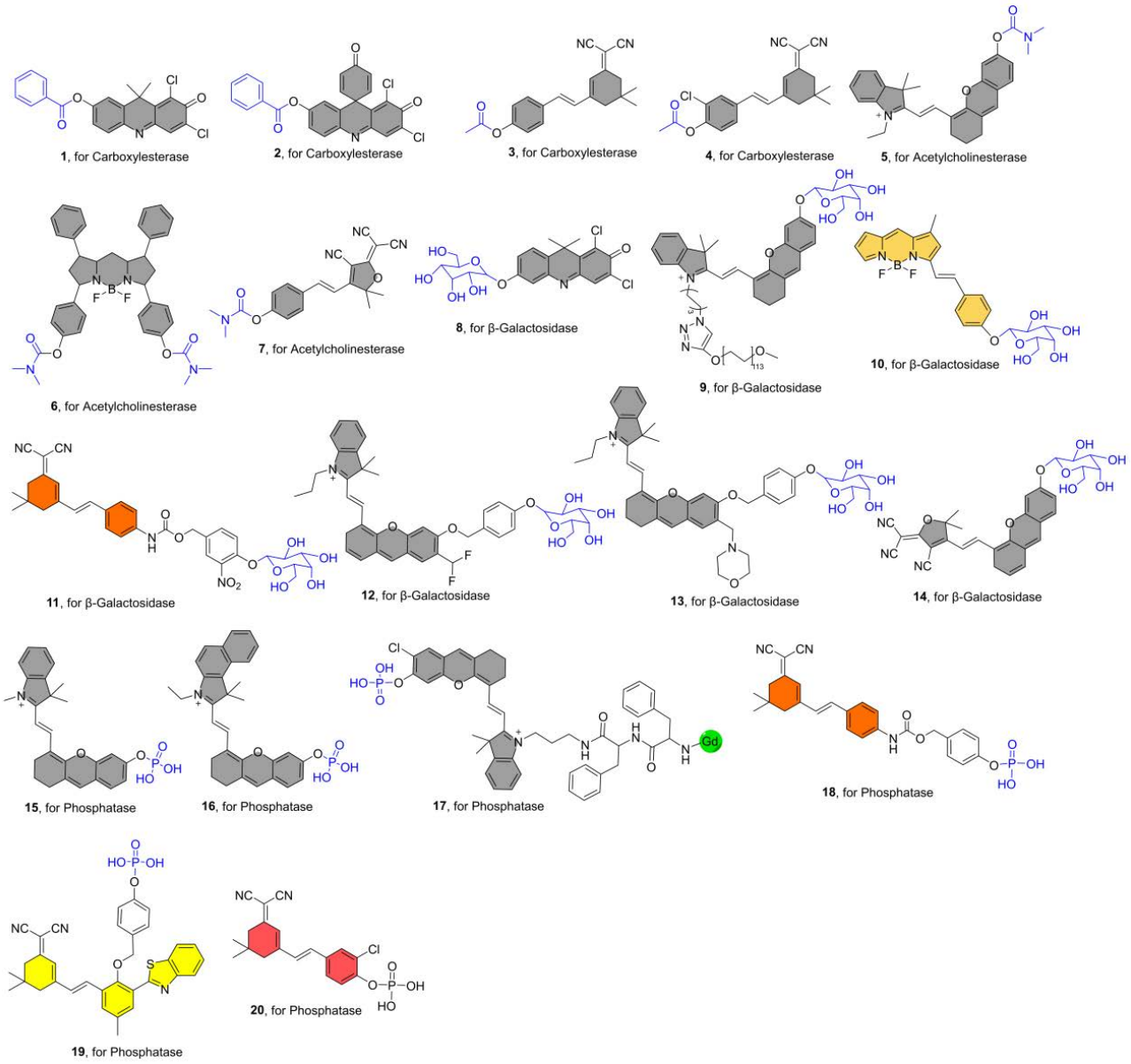
within the tumor matrix and lysosomal compartments. Chen et al. designed their probe using the CTSB-specific peptide sequence Val-Cit, which, upon enzymatic cleavage, releases near-infrared (NIR) fluorophores together with photoacoustic-sensitive units to achieve dual-mode imaging. Compared with conventional fluorescence imaging alone, the photoacoustic modality offers superior tissue penetration and higher spatial resolution, thereby enabling the detection of deep-seated lesions. Complementary work by Ni et al. introduced a bioluminescent CTSB probe that employs an energy transfer mechanism to generate a "dark-to-bright" signal with minimal background interference, further improving sensitivity for tumor visualization (Ni et al., 2019). Collectively, these multi-mode designs provide new strategies for the accurate detection of liver cancer lesions at varying depths.



Probe Number	Original Name	Trigger	Solvent [a]	Signal Transduction Mode	Excitation, Maximal Emission (nm)	LOD [b]	K_m [c]	Localized Organelles	Application	Ref.
1	DDAB	CE	PBS	"turn-on"	F646Ex, 662Em	0.07 ug/mL	1.927 ± 0.31 uM	n.a.	Live cells and mice	[38]
2	DSAB	CE	PBS	"turn-on"	F630Ex, 678Em	0.03 ug/mL	6.55 ± 0.49 uM	n.a.	Live cells and high-throughput screening	[39]
3	CE-1	CE	PBS/DMSO = 4:1, v/v	"turn-on"	F535Ex, 665Em	2.76 × 10^{-3} U/mL	n.a.	n.a.	Live cells and mice	[40]
4	ZM-1	CE	PBS	"turn-on"	F495Ex, 665Em	0.287 × 10^{-3} U/mL	5.4 uM	n.a.	Live cells and mice	[41]
5	CyN	AChE	PBS	"turn-on"	F670Ex, 700Em	0.1173 U/mL	25.36 uM	n.a.	Live cells and Zebrafish	[42]
6	BD-AChE	AChE	HEPES	"turn-on"	F710Ex, 740Em	0.21 U/mL	85 uM	n.a.	Live cells and mice aging models	[43]
7	EW3	AChE	PBS	"turn-on"	F560Ex, 690Em	0.17 U/mL	n.a.	n.a.	Live cells and Zebrafish	[44]
8	DDAOG	β-gal	PBS	"turn-on"	F636Ex, 659Em	n.a.	n.a.	n.a.	Live cells and mice	[45]
9	CyGal-P	β-gal	HEPES	"turn-on"	F675Ex, 720Em	n.a.	48.3 uM	n.a.	Live cells and photothermal therapy in mice	[46]
10	BODIPY-βgal	β-gal	PBS/DMSO = 1:1, v/v	ratiometric	F560Ex, 575Em/ F660Ex, 730Em	4.6 U/L	n.a.	n.a.	Live cells and mice	[47]
11	TMG	β-gal	PBS	ratiometric	F410Ex, 580Em/ F445Ex, 660Em	0.86 U/L	24.04 uM	n.a.	Live cells	[48]
12	NIR-BG2	β-gal	PBS	"turn-on"	F596Ex, 709Em	n.a.	9.3 uM	n.a.	Live cells and mice	[49]
13	Lyso-Gal	β-gal	PBS/DMSO = 4:1, v/v	"turn-on"	F690Ex, ~720Em	22 U/L	n.a.	lysosome	Live cells	[50]
14	DMC-βgal	β-gal	PBS/DMSO = 7:3, v/v	"turn-on"	F725Ex, 770Em	0.298 U/L	n.a.	n.a.	Live cells and mice	[51]
15	NALP	ALP	Tris-HCl/DMSO = 19:1, v/v,	"turn-on"	F680Ex, 706Em	0.28 U/L	52.45 uM	n.a.	Live cells and mice	[52]
16	CyP	ALP	Tris-HCl	"turn-on"	F690Ex, 738Em	3 U/L	9.32 uM	n.a.	Live cells and mice	[53]
17	PCyFF-Gd	ALP	Tris	"turn-on"	F680Ex, 710Em	0.017 U/L	13.14 uM	n.a.	Fluorescence/MRI bimodal imaging in mice	[54]
18	APT	ALP	Tris-HCl	ratiometric	F410Ex, 580Em/ F445Ex, 650Em	0.89 U/L	1.64 uM	n.a.	Live cells and Zebrafish	[55]
19	HP	ALP	Tris-HCl	ratiometric	F398Ex, 556Em/ F423Ex, 689Em	3.98 U/L	2.93 uM	n.a.	Live cells and Zebrafish	[56]
20	SWJT-3	ALP	PBS/DMSO = 4:1, v/v	ratiometric	F405Ex, 590Em/ F405Ex, 670Em	0.87 U/L	8.89 uM	n.a.	Live cells and mice	[57]

[a] Detailed composition in the original literature. [b] Limit of detection (LOD). [c] Michaelis–Menten kinetics (K_m) values. n.a = not available.

Fig. 3 A comparison of the performance of ALP-responsive near-infrared probes under various models and their in vivo imaging effects.

Beyond imaging, CTSB-responsive probes can be integrated with nanocarrier systems to establish "theranostic" platforms that combine diagnosis and treatment. In such designs, chemotherapy drugs are covalently linked to CTSB-responsive units, allowing selective release at the tumor site following enzymatic activation. This approach enables precise drug delivery while simultaneously providing real-time imaging feedback. Research in this area lays the groundwork for the future realization of "visualized treatment" strategies in liver cancer, where therapeutic interventions are guided and monitored by enzyme-responsive probes (Fig. 4).

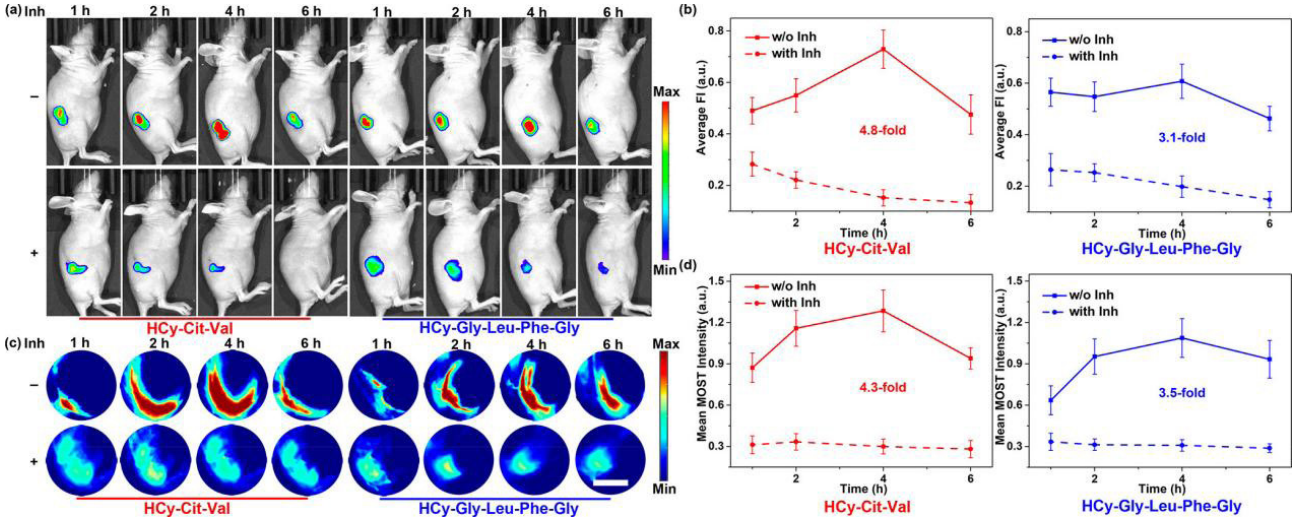


Fig. 4 In vivo fluorescence and photoacoustic dual-mode imaging performance of CTSB-responsive probes HCy-Cit-Val and HCy-Gly-Leu-Phe-Gly.

4.4 Dual-Enzyme Logic-gated Probes

Dual-enzyme logic-gated probes represent an advanced strategy to improve imaging specificity by requiring simultaneous activation from two enzymes or from an enzyme in combination with microenvironmental cues. This design significantly reduces false-positive signals (Luo et

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al., 2025). Lu et al. proposed an AND-logic probe that responds only under double-positive conditions of GGT and CTSB, thereby producing fluorescence exclusively in tumor regions where both enzymes are active. In liver cancer xenograft models, this probe demonstrated high tissue selectivity, with background signals reduced to approximately 20% of those observed in single-enzyme probes (Lu et al., 2022). Gao et al. further expanded the concept by developing a dual-color probe that emits red and green fluorescence at distinct wavelengths, enabling differentiation of tumor metabolic states (Gao et al., 2024). These innovations integrate molecular logic computation into probe design, allowing precise discrimination within complex physiological environments and greatly enhancing the specificity of imaging-based diagnosis.

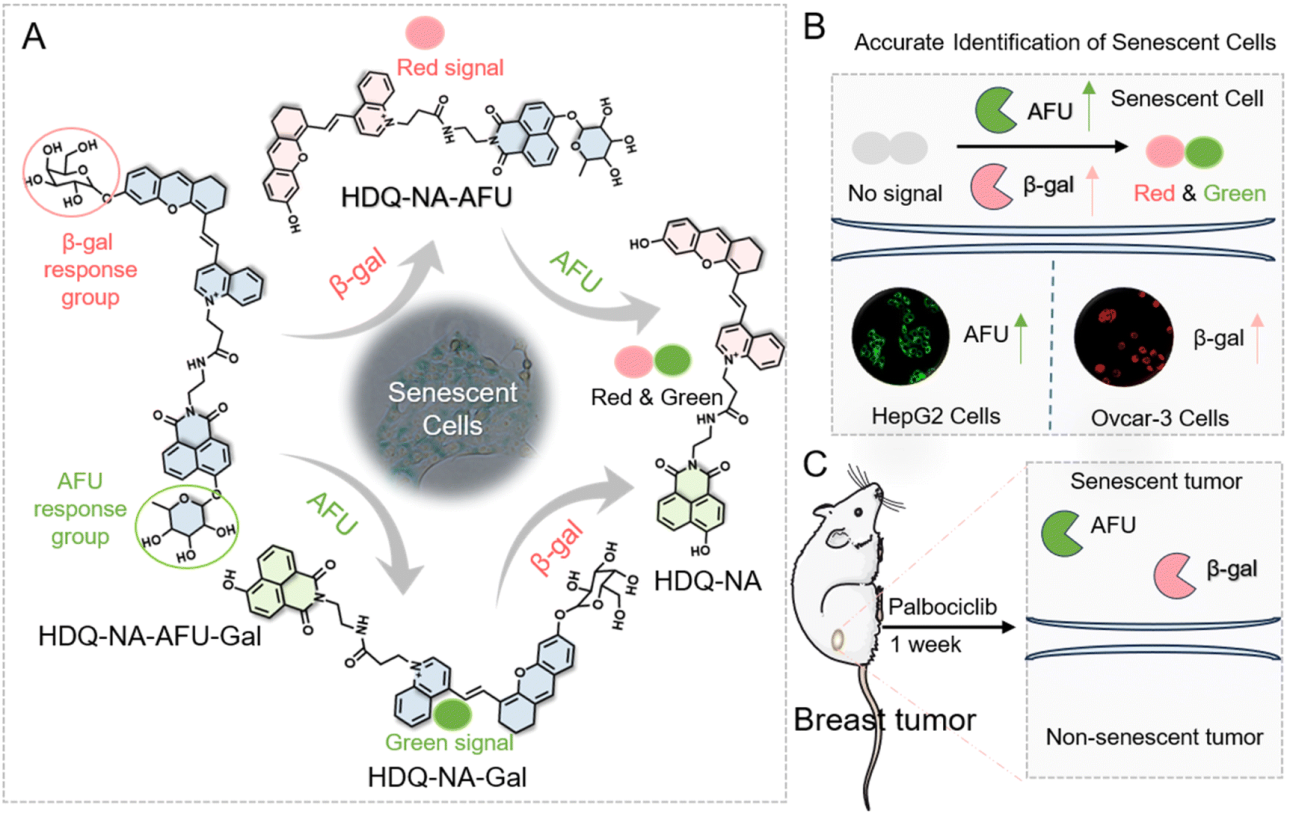

Fig. 5 Schematic diagram of the working principle of a dual-enzyme activation logic-gated fluorescent probe

4.5 Intraoperative navigation and deep imaging

Near-infrared (NIR) probes have become indispensable tools in fluorescence-guided surgery, particularly when combined with dual-mode imaging modalities such as fluorescence and photoacoustic (FL/PA) techniques. This integration allows accurate localization of tumor boundaries and micrometastases during surgical procedures (Zhang et al., 2020). Probes operating in the NIR-II window (1000–1700 nm) have attracted considerable attention due to their reduced tissue autofluorescence and superior penetration depth, making them highly suitable for intraoperative applications. Ye and colleagues demonstrated the use of ALP-triggered NIR/MR dual-mode probes to achieve centimeter-scale visual imaging in animal models, highlighting the potential of enzyme-responsive systems for deep-tissue navigation (Yan et al., 2019).

Moreover, multi-mode navigation platforms that incorporate photoacoustic signal analysis enable real-time monitoring and quantitative assessment of tumor margins, thereby improving the completeness of surgical resection. Looking ahead, enzyme-responsive probes compatible with clinically established platforms such as indocyanine green (ICG) fluorescence are expected to accelerate clinical translation. Such developments may represent the first generation of enzyme-activated probes to be routinely applied in surgical oncology, bridging the gap between experimental innovation and clinical practice.

5. Limitations and Technical Challenges of Enzyme-Response Probes

5.1 Selectivity and False Positives

A major challenge in enzyme-responsive probe design lies in achieving sufficient selectivity. Single enzymes are often expressed across multiple tissues, which can lead to false-positive signals. To address this, multi-enzyme logic gating strategies or targeted delivery systems are required (Luo et al., 2025). The complexity of the hepatic metabolic enzyme network further complicates detection, as inflammatory responses and drug metabolism can interfere with probe specificity. Structural modifications using tumor-specific ligands or antibody fragments offer a promising solution. For example, conjugation with RGD peptides or GalNAc groups can enhance probe uptake by tumor cells while reducing non-specific background signals, thereby improving diagnostic accuracy.

5.2 Biocompatibility and Pharmacokinetics

Balancing clearance and retention remains another technical hurdle. Small-molecule probes are rapidly cleared from circulation, but their signals often diffuse, reducing spatial precision. In contrast, nanoparticle-based probes generate stronger signals yet risk deposition and long-term accumulation (Habibollahi et al., 2012; Ryu et al., 2014). Future strategies emphasize the use of degradable polymers or ester-linked structures to enable self-limited metabolism. Surface modifications such as PEGylation or liposome encapsulation can extend plasma circulation time and mitigate toxicity. Additionally, natural polysaccharide carriers, such as hyaluronic acid, have been explored to construct “soft” probes with improved biocompatibility and reduced immunogenicity.

5.3 Penetration Depth and Imaging Depth

Optical penetration depth is a critical limitation for in vivo imaging. While NIR-I light provides limited tissue penetration, NIR-II probes and photoacoustic imaging modalities enable visualization of deeper structures (Ni et al., 2019). Future probe designs must balance enhanced optical properties with molecular stability to ensure reliable performance. Techniques such as Förster resonance energy transfer (FRET) and upconversion can broaden excitation bands and improve signal quality. Moreover, composite imaging systems that integrate photoacoustic and fluorescence modalities can simultaneously capture structural and molecular information, thereby improving tumor localization and diagnostic precision.

5.4 Quantitative Ability

Accurate quantification of enzyme activity remains an unmet need. Ratiometric probes offer a partial solution by reducing background interference and enabling relative measurement of enzymatic activity (Yan et al., 2019). However, further improvements are required to ensure reproducibility across different imaging platforms. Fluorescence lifetime imaging (FLIM) provides a robust method for quantifying dynamic changes, while machine learning algorithms can be employed to establish standardized models for signal calibration. Together, these approaches may enable cross-platform quantitative comparisons and enhance the reliability of enzyme-responsive imaging in clinical applications.

6. Clinical Translation and Future Prospects

6.1 Preclinical to Clinical Pathway

The successful clinical translation of enzyme-responsive probes requires a rigorous and systematic approach encompassing toxicology studies, pharmacokinetic evaluation, GMP-compliant manufacturing, and the design of regulatory-compliant clinical trials. At present, most probes have demonstrated efficacy only in mouse models, with limited validation in large animal studies or human subjects. Future research must therefore prioritize long-term toxicity and immunogenicity assessments, as well as detailed investigations into metabolic pathways within liver and kidney tissues. Establishing a GLP-compliant quality control framework will be an essential prerequisite for clinical trial registration and eventual approval.

6.2 Technological development trends

Emerging technological directions highlight several promising strategies for advancing enzyme-responsive probes. Multimodal imaging platforms that integrate NIR-II, photoacoustic, and magnetic resonance modalities are expected to provide complementary diagnostic information. Dual-enzyme or multi-marker logic gating systems can significantly reduce false positives by requiring simultaneous activation, while enzyme-triggered self-assembly mechanisms offer powerful signal amplification. The use of degradable nanocarriers is another important trend, designed to minimize long-term accumulation risks and improve biosafety. In clinical practice, intraoperative navigation represents the most immediate application, with gradual expansion toward routine diagnostic use.

Artificial intelligence is poised to play a transformative role in this field. Image recognition algorithms can auto-

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matically delineate fluorescent boundaries, thereby assisting surgeons in real-time decision-making. As the stability of NIR-II probes improves and optical instrumentation advances, real-time three-dimensional navigation and prediction of residual lesions will become feasible. Looking ahead, the integration of enzyme-responsive probes with immunotherapy markers may enable a comprehensive "diagnosis–treatment–evaluation" paradigm, offering a unified approach to precision oncology.

7 Conclusion

Enzyme-responsive fluorescent probes represent a powerful emerging technology for the precise diagnosis and intraoperative navigation of liver cancer. Probes targeting enzymes such as ALP, GGT, CTSB, and APN have already demonstrated high sensitivity, strong imaging contrast, and excellent tumor selectivity in preclinical studies. The incorporation of ratiometric designs and multimodal imaging systems has further enabled quantitative signal analysis and deep-tissue visualization, expanding their potential clinical utility.

Looking ahead, future development should prioritize the construction of multimodal probes, the application of dual-enzyme logic gating strategies, and comprehensive preclinical safety evaluations, followed by early-phase clinical trials. These efforts will be essential to translate laboratory advances into reliable clinical tools capable of guiding real-time surgical decision-making.

With continued progress in synthetic chemistry, imaging engineering, and surgical medicine, enzyme-responsive fluorescent probes are poised to bridge the gap between molecular diagnostics and therapeutic intervention. Ultimately, their integration into clinical practice could significantly improve early detection rates, enhance surgical precision, and deliver meaningful survival benefits for patients with liver cancer.

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