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Advances in Nitroreductase-Activated Fluorescent Probes Based on Photophysical Response Mechanisms

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Abstract: Nitroreductase (NTR) is a flavin mononucleotide-dependent oxidoreductase that selectively reduces aromatic nitro compounds to hydroxylamine or amino derivatives in the presence of NAD(P)H. Tumor hypoxia is often accompanied by upregulation of NTR levels, making it an ideal biomarker for hypoxia assessment. This article briefly reviews recent advances in NTR-activated fluorescent probes based on photophysical response mechanisms. According to their design principles, these probes are mainly classified into three categories: probes based on photoinduced electron transfer (PET), which utilize the nitro group to quench fluorescence and achieve a turn-on signal upon reduction; probes based on intramolecular charge transfer (ICT), which incorporate the nitro group into a donor-acceptor electronic system and produce spectral responses upon changes in electronic properties following reduction; and ratiometric probes based on Förster resonance energy transfer (FRET), which employ an internal reference channel to eliminate environmental interference. In addition, reversibly binding probes that block electron transfer by forming hydrogen bonds with the reduced cofactor represent a new mode of non-reactive detection. Finally, this review summarizes the core challenges faced by current probes and outlines future directions.

Keywords: Nitroreductase; Fluorescent Probe; Photoinduced Electron Transfer; Intramolecular Charge Transfer; Förster Resonance Energy Transfer; Hypoxia

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1. Introduction

Hypoxia is a prominent hallmark of solid tumors. In most solid tumors, the median oxygen partial pressure (pO_2) is approximately 4%, with local regions even dropping to 0% [1,2]. This hypoxic state arises from the relative lag in angiogenesis due to rapid tumor cell proliferation, leading to an imbalance between oxygen supply and demand [3]. Hypoxia not only reduces the efficacy of radiotherapy by attenuating oxygen-dependent free radical generation but also significantly diminishes the effectiveness of chemotherapy by upregulating drug efflux pumps and inhibiting apoptotic pathways [4,5]. Nevertheless, this unique hypoxic microenvironment also provides an opportunity for precision theranostics of tumors, as the selectively upregulated reductase activity in hypoxic regions can be exploited to achieve selective imaging and targeted therapy [6].

NTR is a class of flavin mononucleotide-dependent oxidoreductases that catalyze the reduction of nitroaromatic compounds using NAD(P)H as an electron donor [7]. Based on their oxygen dependence, NTRs can be classified into oxygen-insensitive (Type I) and oxygen-sensitive (Type II) enzymes. Among these, Type II NTR exhibits efficient reductive activity only under hypoxic conditions, as its one-electron-reduced intermediate is rapidly reoxidized under normoxia [8,9]. Given that NTR is

significantly overexpressed in hypoxic tumor cells and its expression level directly correlates with the degree of hypoxia, it has emerged as an ideal biomarker for assessing tumor hypoxia [1,10].

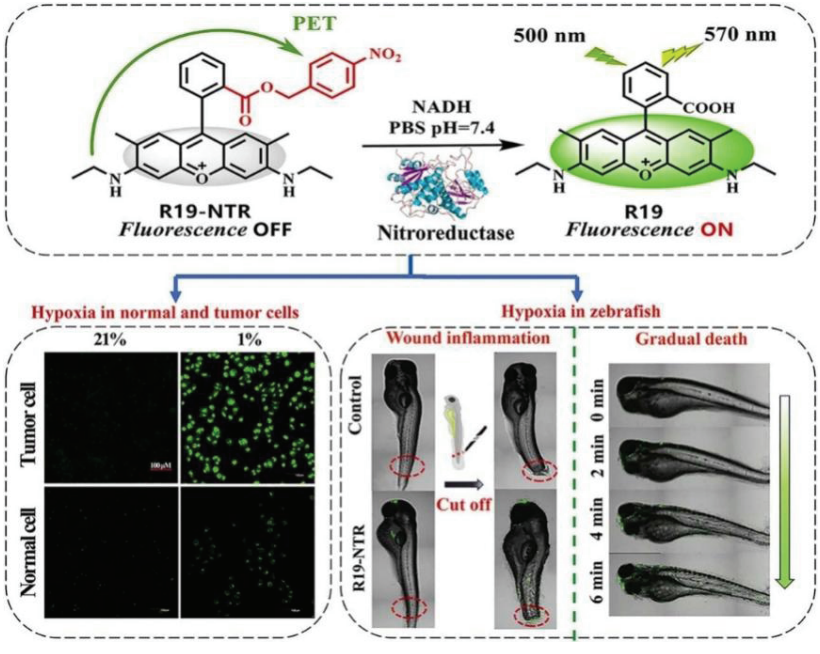
To enable accurate monitoring of NTR activity and its associated hypoxic status, fluorescence imaging techniques have been widely employed due to their advantages such as high sensitivity, high spatiotemporal resolution, and non-invasiveness [11,12]. In parallel, researchers have developed a large number of NTR-activatable fluorescent probes based on distinct photophysical mechanisms. PET and ICT represent the two most prevalent design strategies among them [13,14]. PET-based probes utilize the strong electron-withdrawing ability of the nitro group to quench fluorescence via electron transfer; upon NTR-catalyzed reduction to an amino group, this process is blocked, resulting in a turn-on signal [15]. ICT-based probes, on the other hand, embed the nitro group into the donor-acceptor electronic system of the fluorophore; the alteration in electronic properties before and after reduction leads to a significant spectral shift or enhancement [16]. Furthermore, the FRET mechanism has been employed to construct ratiometric probes. By introducing an internal reference fluorophore that is not perturbed by NTR, these probes effectively eliminate quantitative errors arising from uneven probe distribution and environmental interference [17]. Following the above mechanisms as the primary classification framework, this article systematically reviews recent advances in the design principles, performance optimization, and biological applications of NTR fluorescent probes, and provides perspectives on the challenges and future directions in this field.

2. PET-Based NTR Fluorescent Probes

The PET mechanism is the most widely utilized mechanism in the design of NTR fluorescent probes. Its fundamental principle is as follows: in the probe molecule, the nitro group serves as a strong electron-withdrawing group. Upon excitation of the fluorophore, the excited-state electron is transferred to the lowest unoccupied molecular orbital (LUMO) of the nitro group, resulting in fluorescence quenching. After NTR-catalyzed reduction converts the nitro group to an amino group, the electron-donating nature of the amino group blocks the PET process, leading to fluorescence recovery.

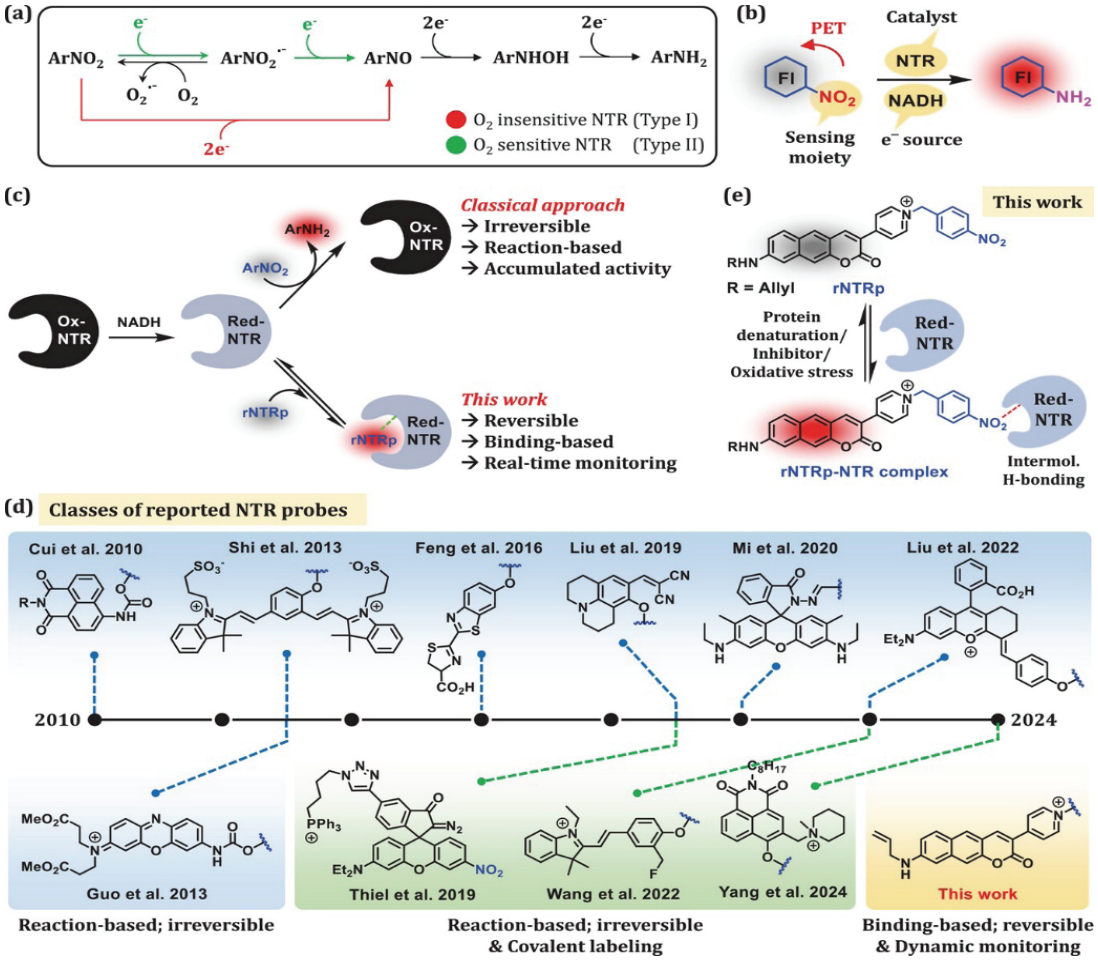
An et al. [18] developed the probe R19-NTR based on a rhodamine platform, in which a p-nitrobenzyl group was linked via an ester bond as the NTR-responsive moiety. The probe exhibited almost no fluorescence due to the PET process (absolute quantum yield 1.6%), and upon reaction with NTR, the fluorescence was enhanced approximately 114-fold. Notably, this probe displayed an extremely fast response, with a distinct fluorescence increase observed within 1 min and a plateau reached within 5 min. The d-PET quenching mechanism was verified by synthesizing a nitro-free control compound, R19-Ben. As the fluorescence of R19-Ben was nearly identical to that of free R19, it was demonstrated that the nitro group is the key moiety responsible for PET quenching.

Figure 1. Response process of the rhodamine platform-based probe R19-NTR to NTR.



Distinct from conventional reactive probes, Sarkar et al. [19] reported a reversibly binding NTR probe, rNTRp, which represents an entirely new detection mode. rNTRp employs benzocoumarin as the fluorophore and recognizes the reduced state of NTR (red-NTR) through a (nitrobenzyl) pyridinium moiety. Its mechanism does not involve enzymatic reduction; instead, the nitro group of the probe forms hydrogen bonds with the cofactor $FMNH_2$. This hydrogen bonding suppresses the photoinduced electron transfer (PET) from the nitro group to the fluorophore while simultaneously lowering the reduction potential of the probe to -761 mV, thereby substantially resisting the possibility of enzymatic reduction. Consequently, rNTRp generates fluorescence upon reversible binding exclusively to red-NTR without undergoing irreversible chemical transformation. Experimental results demonstrated that this probe can distinguish red-NTR from ox-NTR, monitor dynamic changes in intracellular red-NTR levels in real time, and exhibit reversible binding behavior as evidenced by fluorescence decrease upon addition of 1,4-benzoquinone and fluorescence recovery upon subsequent addition of NADH.

Figure 2. Reversible recognition process of the benzocoumarin platform-based probe rNTRp toward NTR.



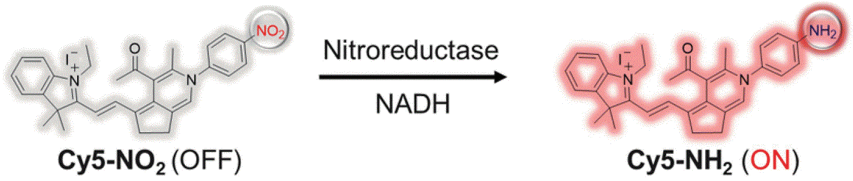
3. ICT Mechanism-Based NTR Fluorescent Probes

The ICT mechanism is the second most prevalent probe design mechanism after PET. Probe molecules typically constitute a D-π-A (donor-π-acceptor) push–pull electronic system. The nitro group, serving as a strong electron-withdrawing moiety, acts as the acceptor (A) terminus and participates in the ICT process across the conjugated system. When NTR reduces the nitro group to an amino group, the strongly electron-withdrawing terminal is converted into an electron-donating one, resulting in a dramatic alteration in intramolecular charge transfer characteristics, which leads to a spectral shift and fluorescence enhancement.

Liu et al. [16] constructed a probe, Cy5-NO_2, using a pentamethine cyanine skeleton by directly incorporating a p-nitrophenyl group into the conjugated system through meso-heterocyclization. The core design of this probe lies in embedding the nitro group at the terminus of the donor-acceptor electronic system of the cyanine dye, thereby forming a D-π-A structure. Theoretical calculations indicate that, in the excited state of Cy5-NO_2, the highest occupied molecular orbital (HOMO) is

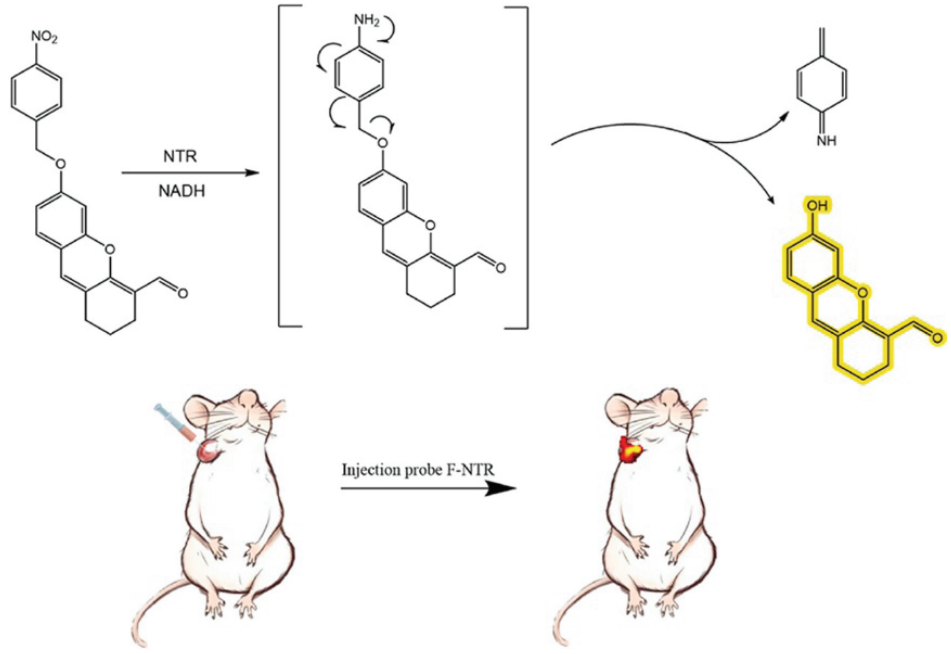
primarily localized on the benzoindole and the central heterocycle, whereas the LUMO is almost entirely concentrated on the nitrobenzene unit. This pronounced spatial separation between HOMO and LUMO is characteristic of an ICT process, with an oscillator strength (f) of only 0.0014, resulting in fluorescence quenching. Upon reduction by NTR, the generated Cy5-NH_2 exhibits both HOMO and LUMO localized on the benzoindole-heterocycle region, and the transition becomes a locally excited state (f = 0.8750), with a fluorescence enhancement of approximately 30-fold at 620 nm. This probe has a detection limit of 10 ng/mL and has been successfully applied for fluorescence imaging of live bacteria, including E. coli and S. aureus.

Figure 3. Fluorescence turn-on response process of the pentamethine cyanine platform-based probe Cy5-NO_2 toward NTR.



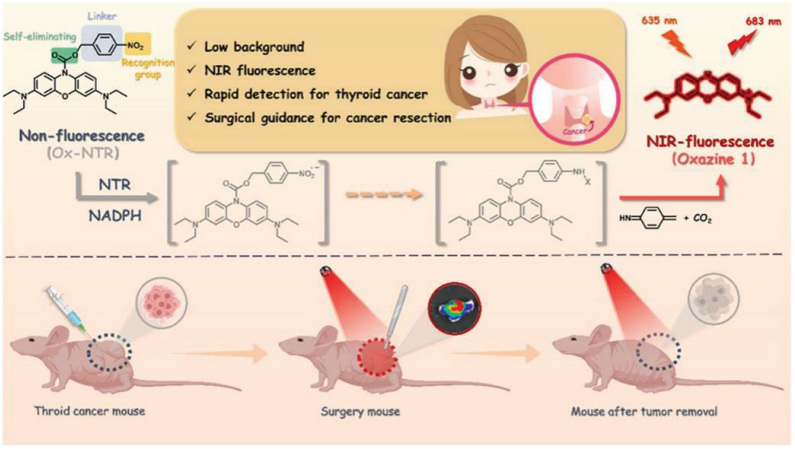
Gao et al. [20] developed F-NTR, which employs a xanthene fluorophore linked to a p-nitrobenzyl group via an ether bond as the NTR recognition site. The electron-withdrawing effect of the nitro group weakens the push-pull electronic characteristics of the fluorophore, resulting in fluorescence quenching. Upon reaction with NTR in the presence of NADH, reduction of the nitro group triggers a 1,6-elimination, releasing the free xanthene fluorophore and generating strong fluorescence at 540 nm. This probe exhibits a detection limit of 5.651 ng/mL and has been successfully applied for fluorescence imaging of tumor hypoxia both in vitro and in vivo.

Figure 4. Response process of the xanthene platform-based probe F-NTR toward NTR.



Zhang et al. [21] proposed a unique ‘conjugation disruption’ strategy. They designed Ox-NTR using oxazine 1 as the fluorophore core, with the NTR recognition group introduced via a p-nitrobenzyloxycarbonyl linker. The key design lies in the fact that the linker interrupts the original rigid conjugated planar structure of oxazine 1, rendering the probe nearly colorless and completely non-fluorescent prior to reaction—representing a thorough disruption of ICT. Upon NTR-catalyzed reduction of the nitro group triggering a 1,6-elimination, the intact conjugated system of oxazine 1 is restored (ICT re-established), generating a strong near-infrared fluorescence signal (approximately 13-fold enhancement at 683 nm). Control experiments demonstrated that oxazine 1 itself is highly fluorescent, whereas Ox-NTR is almost entirely in a dark state. This strategy of first ‘disrupting’ and then ‘reconstructing’ the ICT core endows the probe with quasi-zero background signal in its inactive state, making it highly suitable for bioimaging scenarios that demand an exceptionally high signal-to-background ratio.

Figure 5. Zero-background fluorescence turn-on response process of the oxazine 1 platform-based probe Ox-NTR toward NTR.

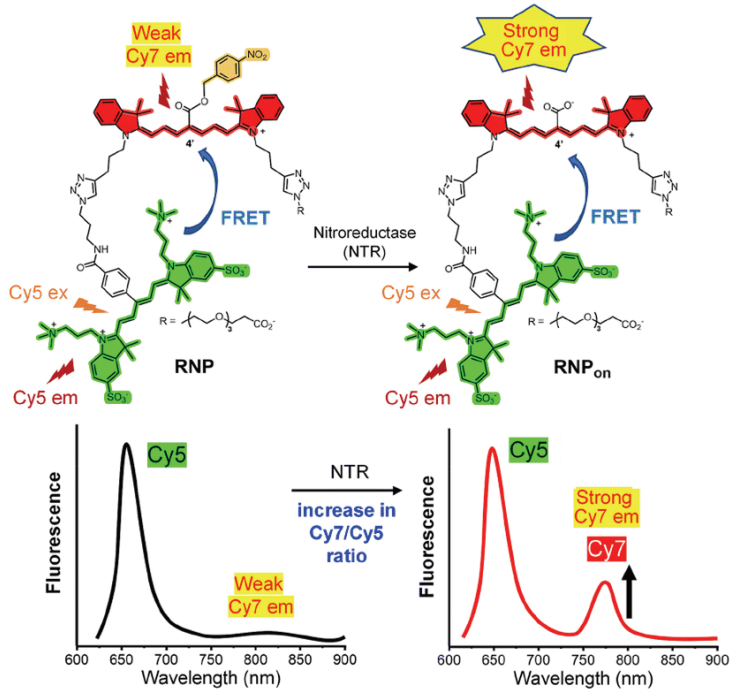


4. FRET Mechanism-Based Ratiometric NTR Probe

The FRET mechanism enables ratiometric response through intramolecular energy transfer between two fluorophores—the donor fluorescence, unaffected by NTR, serves as an internal reference, while the acceptor fluorescence varies with NTR activity. The ratio between the two effectively calibrates signal fluctuations caused by factors such as probe concentration, excitation intensity, and environmental polarity.

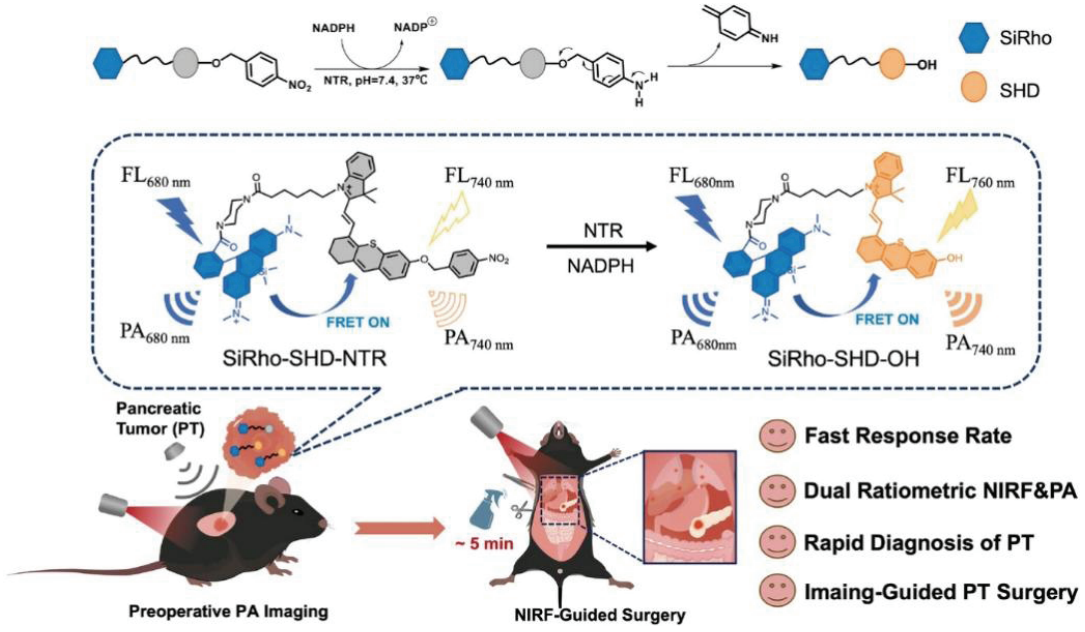
RNP, developed by Morsby et al. [17], represents a representative FRET-based ratiometric probe. RNP consists of Cy5 (donor) and Cy7 (acceptor) linked via a covalent chain. The Cy7 moiety is modified with a p-nitrobenzyl ester: prior to response, Cy7 is in a dark state (with extremely low quantum yield), serving only as a FRET acceptor without emitting fluorescence; upon NTR-catalyzed reduction of the nitro group, a self-immolative reaction is triggered, converting the 4'-ester group on Cy7 to a 4'-carboxylate, which substantially enhances the fluorescence quantum yield of Cy7. Spectroscopic measurements showed that upon excitation of the Cy5 donor at 620 nm, NTR induced an approximately 8-fold enhancement in Cy7 emission, while the Cy5 fluorescence remained essentially unchanged; the Cy7/Cy5 ratio increased nearly linearly with NTR concentration. When Cy7 was directly excited at 720 nm, the emission enhancement reached approximately 40-fold. RNP exhibits excellent water solubility and low protein binding, and has been successfully applied for the visualization of hypoxic cells in A549 cell 2D monolayer cultures and HT-29 tumor spheroid 3D models.

Figure 6. Response process of the Cy5-Cy7 FRET platform-based ratiometric probe RNP toward NTR.



Hu et al. [22] further integrated FRET and ICT into a dual-ratiometric near-infrared fluorescence/photoacoustic (NIRF/PA) probe, SiRho-SHD-NTR. This probe employs silicon rhodamine (SiRho) as the FRET donor and semicyanine (SHD) as the FRET acceptor. A p-nitrobenzyl group modified on the hydroxyl group of SHD serves as the NTR recognition site to regulate the acceptor: prior to response, both the fluorescence and PA signals of SHD are weak; upon NTR-catalyzed reduction and release of the hydroxyl group, the ICT of SHD is greatly enhanced, while the FRET efficiency remains approximately 60%, resulting in an approximately 18-fold enhancement of acceptor fluorescence at 760 nm and an approximately 3-fold enhancement of PA signal at 740 nm. Meanwhile, the fluorescence (680 nm) and PA signal (680 nm) of the SiRho donor remain stable as internal references. The probe exhibits outstanding kinetic performance, reaching a plateau within only 5 min of reaction with NTR. Using an orthotopic pancreatic cancer mouse model, topical spraying of the probe onto the abdominal cavity allowed discrimination between tumor and normal pancreatic tissue within approximately 5 min, and successfully enabled fluorescence-guided surgical resection of both orthotopic tumors and metastatic lesions.

Figure 7. Response process of the SiRho-SHD platform-based ratiometric probe SiRho-SHD-NTR toward NTR.



5. Conclusion

Leveraging the ability of NTR to selectively reduce nitro groups to hydroxylamine or amino derivatives, researchers have developed a variety of optical probes for NTR based on mechanisms such as PET, ICT, and FRET. PET-type probes offer high turn-on ratios and low background signals, but their products are prone to diffusion. ICT-type probes enable ratiometric detection through spectral shifts, and certain 'disruption–reconstruction' strategies can achieve zero background. FRET-type probes utilize an internal calibration channel to overcome tissue environment interference, albeit with relatively complex designs. The integration of these probes with fluorescence imaging techniques has preliminarily revealed biological phenomena such as hypoxia-induced NTR upregulation and the spatial distribution of NTR.

Nevertheless, current NTR probes still face two core challenges: First, the vast majority of probes are developed using bacterial NTR as the screening target, resulting in a species-specific gap with respect to endogenous mammalian reductases such as POR; the molecular target responsible for the endogenous cellular signal remains ambiguous. Second, there is still a lack of multichannel probes capable of sequentially monitoring NTR activity and the subsequent ·OH burst, making it difficult to elucidate the cascade pathological mechanism of 'hypoxia → reductive activation → oxidative damage'. In the future, the development of specific probes for human POR, the construction of two-color sequential response platforms for NTR/·OH, and the integration of NTR-activated imaging with Type I photodynamic therapy represent important directions toward clinical translation in this field.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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