Scientific Research Publishing

Advances in Molecular Imaging, 2025, 14(2), 27-39
https://www.scirp.org/journal/ami
ISSN Online: 2161-6752
ISSN Print: 2161-6728

In Situ Imaging Studies of Disease Markers and Small Molecules

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How to cite this paper: Feng, Q.C., Hu, Y.M. and Zheng, J. (2025) *In Situ* Imaging Studies of Disease Markers and Small Molecules. *Advances in Molecular Imaging*, **14**, 27-39.
https://doi.org/10.4236/ami.2025.142002

Received: January 5, 2025
Accepted: April 27, 2025
Published: April 30, 2025

Abstract

Rapid, precise, and sensitive detection of diseases can be realized by *in situ* imaging of disease markers or small molecules, which can provide prospective disease-related information to patients. This is important for early detection, prognosis, improvement of cure rate, and reduction of mortality. Methods: A DNA-coupled amphiphilic aggregation-induced emission probe (TPE-R-DNA) was synthesized based on nucleic acid exonuclease III-assisted target cycling technology for tumor tissue imaging and prognostic analysis. A dual-targeted DNA tetrahedral nanocarrier (MUC1-Td-AS1411) for breast cancer cell imaging and targeted drug delivery enables real-time imaging of breast cancer and drug therapy. Designed glutathione-gated DNA nanodevices with a highly specific strategy for visualizing miRNA fluorescence Flip-flop for proportional fluorescence imaging of small bioactive molecules in the nucleus. DNA nanoenzymes based catalyzed tyramine deposition reaction for *in situ* detection of HER2 homodimeric protein status on cell surface, among other methods.

Keywords

Disease Markers, *In Situ* Detection, *In Situ* Imaging, Fluorescence Imaging

1. Introduction

Strategy 1: Detection of ultra-low concentrations of mRNA is important for the prognosis of gene-related diseases. Wang [1] studied the synthesis of a DNA-conjugated amphiphilic aggregation-induced emission probe (TPE-R-DNA) based on nucleic acid exonuclease III-assisted target cycling technology for tumor tissue imaging and prognostic analysis. The fluorescent signal can be used to confirm the expression level of MnSOD mRNA in tumor tissues for *in situ* monitoring of mRNA expression in living cells and cancer tissues.

Strategy 2: In order to improve the efficacy of chemotherapy and enable real-time imaging of cancer cells, the development of nanocarriers with targeted drug delivery capability and fluorescence properties is crucial for cancer therapy. Here, Liu [2] constructed a dual-targeted DNA tetrahedral nanocarrier (MUC1-Td-AS1411) for breast cancer cell imaging and targeted drug delivery. The vector loaded DOX acted directly on the nucleolus through the cytosol, minimizing toxic effects and overcoming drug resistance while enabling real-time imaging of breast cancer cells.

Strategy 3: Glutathione (GSH) plays a key role in cell growth and function as the major antioxidant in mammalian cells [3]. Cancer cells contain approximately four times more glutathione than normal cells [4]. In addition, MicroRNAs (miRNAs), a class of non-coding RNAs, are much more accurate than messenger RNAs (mRNAs) in cancer diagnosis and are widely used as targets for cancer detection [5]. Based on this, Yan [6] proposed a glutathione-gated DNA nanodevice with high specificity for visualizing miRNA fluorescence imaging.

Strategy 4: The nucleus is the most important organelle in eukaryotic cells, and normal levels of endogenous bioactive molecules play an important role in transmitting information to the nucleus and maintaining normal cell morphology. However, unbalanced expression of endogenous bioactive molecule levels may lead to alterations in the genetic material within the nucleus and can affect the normal function of the nucleus, e.g., inhalation of SO_2 leads to abnormal nuclear function in several organs [7] and DNA damage [8] [9] leading to the development and progression of cerebral ischemic stroke. Based on this phenomenon, Wang [10] established a strategy called "charge-driven tripod flipping on DNA" for proportional fluorescence imaging of small biologically active molecules in the nucleus to directly or indirectly detect and track the flow of endogenous small molecules in the nucleus.

Strategy 5: Cell surface human epidermal growth factor receptor 2 (HER2) is an important class of cell surface receptors in the human epidermal growth receptor (HER) family also known as the type I receptor tyrosine kinase family. Abnormal dimerization of HER2 is a key factor in the development of breast cancer. Xu [11] based on the traditional tyrosine deposition reaction, designed a DNA nano-enzyme-catalyzed tyrosine deposition reaction based for a new method of *in situ* detection of HER2 protein status on the cell surface, which utilizes an aptamer specifically bound to the HER2 receptor to induce Hemin/G4 DNAzyme self-assembly on the cell surface. When Hemin/G4 DNAzyme with horseradish peroxidase activity is formed on the cell surface it can catalyze the generation of free radicals from tyramine residues, which in the presence of H_2O_2, in turn, triggers the deposition of tyramine at receptor proteins and emits bright fluorescence. This method can not only detect the expression of HER2, but also utilize the fluorescence to react the compositional status of HER2 dimer, which is valuable for the diagnosis and targeted therapy of breast cancer.

2. TPE-R-DNA for *In Situ* Imaging of Tumor Tissue

In past studies, researchers have demonstrated that intracellular cancer-associated mRNAs are specific markers of cancer, and their relative expression levels can provide valuable information about disease progression and prognosis. Therefore, a number of methods have been successfully used for the analysis of endogenous mRNAs, for example, Shi [12] *et al*. reported an mRNA gene expression signature for predicting the prognosis of patients using a quantitative real-time polymerase chain reaction (qRT-PCR) method. Chen [13] and colleagues devised a northern blotting (NB) assay specifically to identify the mRNA expression levels of PDCD4 mRNAs in human lung cancer of PDCD4 mRNA. However, these techniques still have different drawbacks, including high sample consumption, time-consuming or insufficient sensitivity.

Aggregation-induced emission fluorescent dyes (AIEgen) are a new class of organic dyes that barely fluoresce in solution but exhibit bright fluorescence in the aggregated state [14] [15]. Based on this, Wang [1] proposed a DNA-conjugated amphiphilic aggregation-induced emission probe (TPE-RDNA) with bursting-agent-free, long-wave red emission of AIEgen for *in situ* monitoring of mRNA expression in living cells and cancerous tissues. The TPE-R-DNA contains two components: a hydrophobic component, which is used as an "on " long-wave fluorescent developer (TPE-R-N3); and a hydrophilic single-stranded DNA (Alk DNA), which serves as the specific recognition portion of the target mRNA. In the absence of the target mRNA, TPE-R-DNA is almost non-fluorescent due to its high-water solubility. In contrast, in the presence of MnSOD mRNA, TPE-R-DNA was digested by exonuclease III (Exo III) to release hydrophobic fluoride (TPE-R-AT). Subsequently, TPE-R-AT formed aggregates and fluorescent signals could be clearly observed. In addition, the AIEgen in this probe emits red fluorescence, which does not emit light in the lower wavelength region of the visible spectrum, and due to their high self-absorption and autofluorescence, these fluorescent probes are susceptible to interference by biomolecules, especially in biological tissues. Therefore, TPE-R-DNA with the above characteristics can be used to predict the prognosis of cancer patients by detecting the expression level of mRNA in tissue microarrays, and provide a new aid for the follow-up treatment of patients (**Figure 1**).

3. Dual-Targeted DNA Tetrahedral Nanocarriers for Breast Cancer Cell Imaging and Drug Delivery

Breast cancer is a malignant tumor that develops in the epithelial tissue of the breast, threatening women's physical and mental health and even their lives [16]. Chemotherapy is a typical cancer treatment; however, the effectiveness of chemotherapy is often limited by low water solubility, nonspecific drug release and rapid clearance of chemotherapeutic agents [17] [18]. In order to improve the selectivity and efficiency of drug delivery to cancer cells, it is increasingly urgent to design and develop nanocarriers with targeted drug delivery and real-time imaging

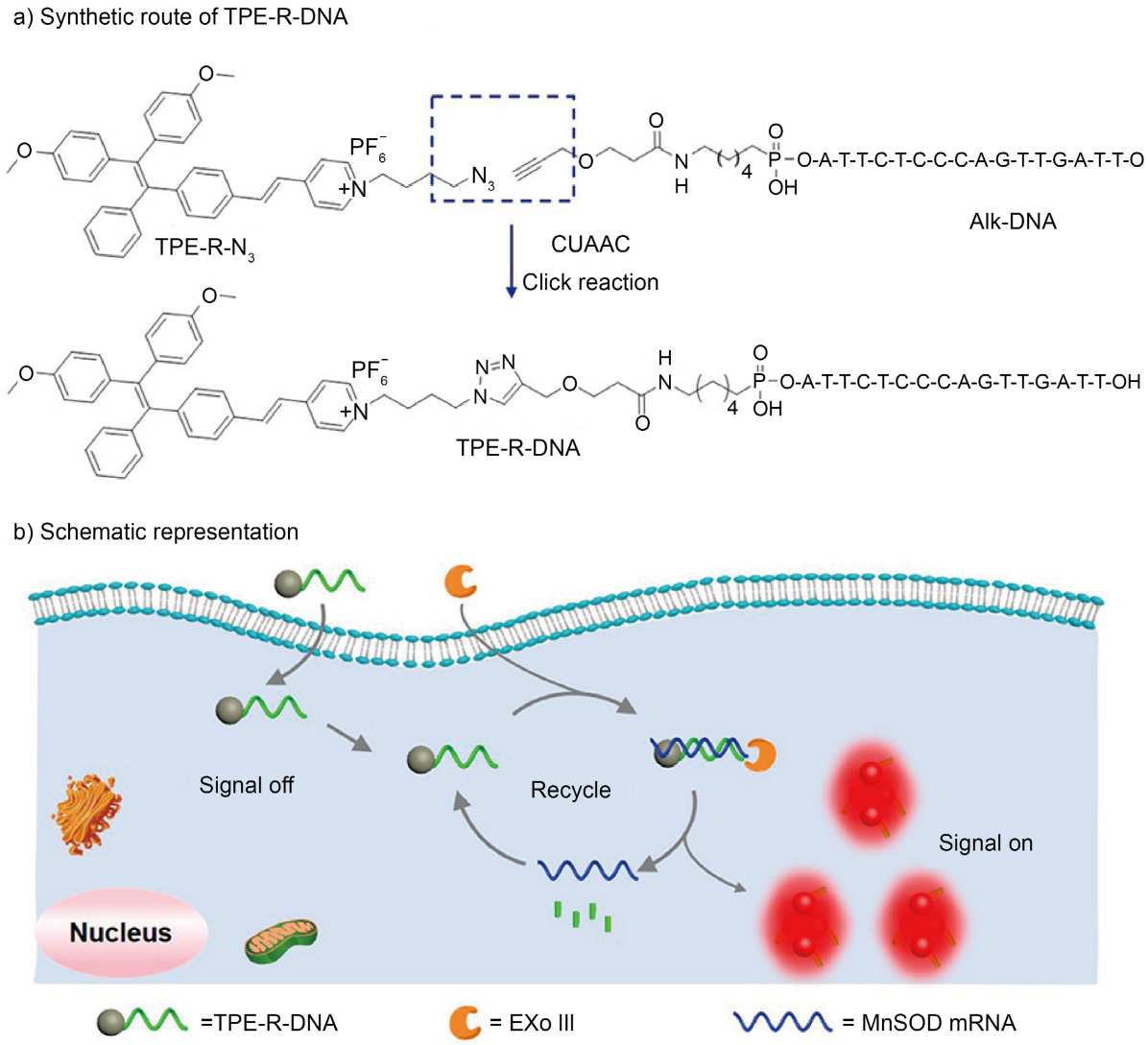

Figure 1. (a) Synthetic route of TPE-R-DNA and (b) schematic representation of TPE-R-DNA probe for detection of MnSOD mRNA.

capabilities. Zhu [19] and others developed aptamer pegylated nanoparticles as nanocarriers targeting cancer cell membrane receptors for drug delivery. This single-targeting application helps to improve the specificity of chemotherapeutic drugs and reduce toxic side effects. However, these nanocarriers could not eliminate drug resistance that occurs in chemotherapy. Zhang [20] *et al.* developed fluorophore-labeled DNA dendrimers for real-time imaging of drug delivery. However, these "always-on" fluorescent nanocarriers inevitably suffer from high background, low-contrast imaging and complex cleaning processes [21]. Here, Liu designed a dual-targeted DNA tetrahedral nanocarrier (MUC1-Td-AS1411) loaded with adriamycin (Dox@MUC1-Td-AS1411) for breast cancer cell imaging and drug delivery.MUC1-Td-AS1411 consists of three components: (1) a DNA tetrahedral nucleus for multivalent conjugation and loading of functional ligands; (2) an activatable DNA tetrahedral core for multivalent conjugation and loading

of adriamycin; and (3) an activatable DNA tetrahedral core for multivalent conjugation and loading of functional ligands. adriamycin; (2) an activatable MUC1 aptamer probe (MUC1 probe) consisting of a MUC1 aptamer sequence hybridized to a fluorophore extending from one vertex and to a complementary sequence to a bursting agent for targeting and imaging MUC1 proteins on cell membranes; and (3) an AS1411 aptamer, which is hybridized to prominences on three vertices by extended sequences and is used to bind riboprobes to effective target nuclei. Nucleoli is overexpressed in the nucleus and cytoplasm of cancer cells and can translocate between the nucleus and cytoplasm due to its bidirectional nuclear localization sequence.

First, in the presence of MUC1-positive cells (90% of breast cancer patients exhibit positive MUC1 protein), the MUC1 probe of the nanocarrier binds to the MUC1 protein, inducing a conformational change in the aptamer's MUC1 conformation, releasing a sequence that is complementary to that of the bursting agent, leading to fluorescence recovery. Subsequently, after internalization into the cell, the AS1411 aptamer portion of the nanocarrier selectively binds to the nucleoli protein, targeting the entire nanocarrier to the nucleus, which then releases Dox into the nucleus (**Figure 2**).

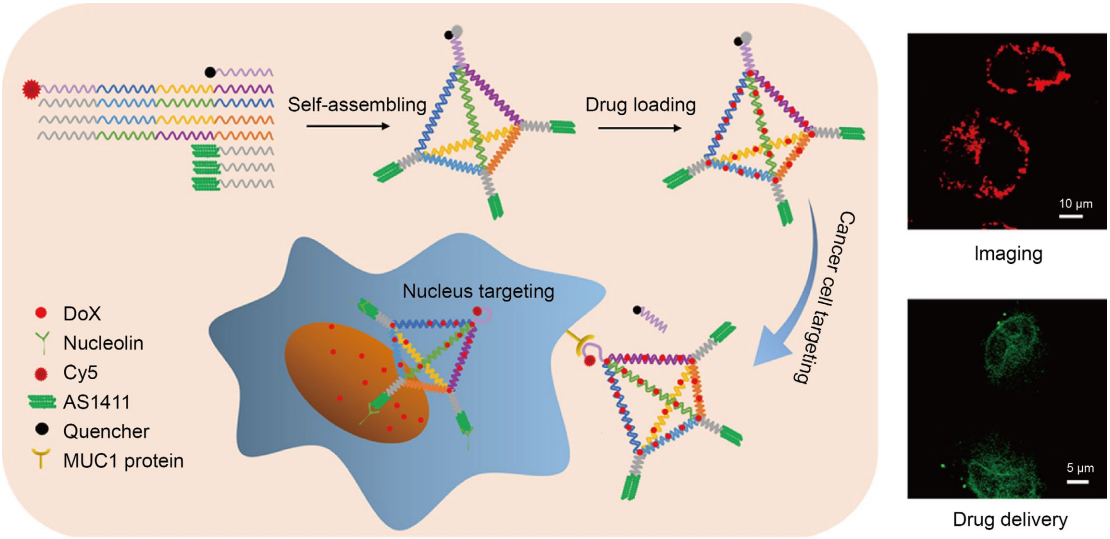

Figure 2. Schematic illustration of dual-targeting DNA tetrahedron nanocarrier loading doxorubicin for breast cancer cell imaging and drug delivery.

In contrast, for MUC1-negative cells, the MUC1 probe is not activated and remains in a burst state. Fluorescent imaging can distinguish MUC1-positive cells from MUC1-negative cells. Compared with normal use of Dox, Dox@MUC1-Td-AS1411 was less toxic to MUC1-negative HL-7702 cells, with a lethality roughly comparable to that of sensitized MCF-7 cells, and a higher kill rate for adriamycin-resistant MCF-7 cells. Furthermore, fluorescence co-localization analysis showed that MUC1-Td-AS1411 could evade the lysosomal pathway and avoid lysosomal acidification due to the integration of the AS1411 aptamer. Therefore, this dual-

targeted DNA tetrahedral nanocarrier, which integrates drug-delivery targeted therapy with fluorescence imaging, has great potential in cancer diagnosis and therapy.

4. GSH-Gated DNA Nanorods for *In Situ* Tumor Imaging

The presence of GSH overexpression in tumor patients suggests that GSH can also be used as an effective signaling molecule to enhance the specificity of tumor detection. In the reported systems regarding glutathione response, manganese dioxide nanoparticles showed superior reactivity when triggered by glutathione. Based on this, Yan proposed GSH-gated DNA nanodevices for miRNA-21 signal amplification. MnO_2 nanosheets loaded with single-stranded DNA, called fuels, were first synthesized as the signal-enhancing component in this nanodevice. In addition, each single-stranded DNA in a DNA duplex was labeled with a fluorophore (Cy5) and a bursting agent 2 (BHQ2). The fluorescence of this DNA duplex was burst at the beginning due to the fluorescence resonance energy transfer (FRET) effect between Cy5 and BHQ2. Modification of 4-imidazoleacetic acid hydrochloride (IAA) to polyethyleneimine (PEI-IAA) under electrostatic action. By ligand interaction between MnO_2 nanosheets and imidazole groups in PEI-IAA, GSH-gated DNA nanodevices, named MnO2@PEI-IAA nanodevices, were finally constructed (Figure 3).

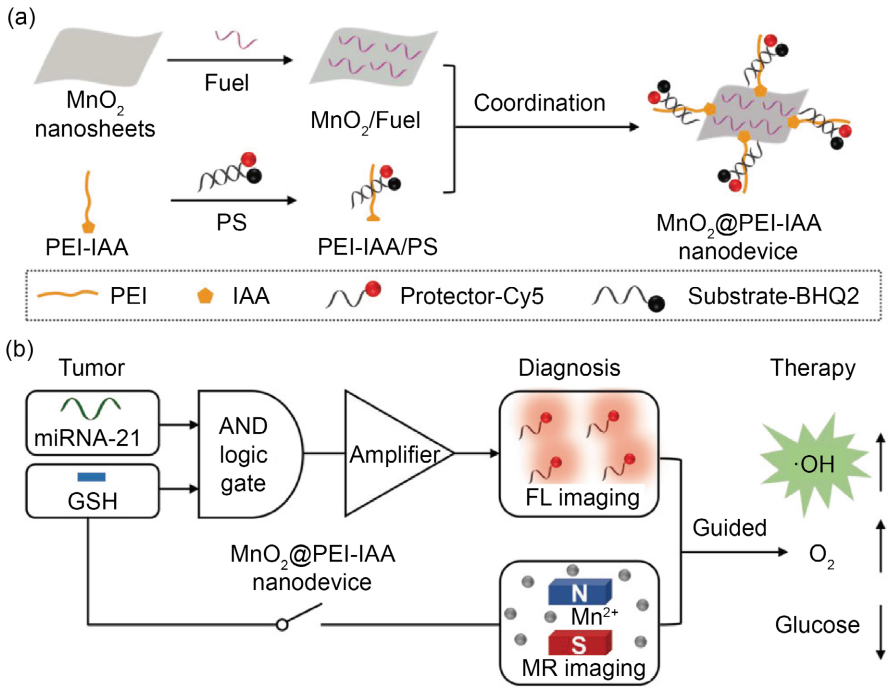

Figure 3. (a) Schematic illustration of the construction of the MnO_2@PEI-IAA nanodevice. (b) AND logic gated-MnO_2@PEI-IAA nanodevice for cascaded signal amplification of miRNA-21 (FL imaging) and GSH-activated MR imaging-guided theranostics.

GSH and miRNA were expressed at low levels in normal cells, while overex-

pression of GSH and miRNA existed in tumor cells. When GSH and miRNA were overexpressed, the nanodevices were activated *in situ*, and Cy5-labeled ssDNA in double-stranded DNA was released, which could be detected with significantly enhanced fluorescence signal and MR signal. On the contrary, the fluorescence signal was burst and the MR signal was kept at the background level with low miRNA and GSH (normal cells), which effectively reduced the false-positive signals by more than 50% with good feasibility. In addition, the nanodevice can be used for CDT, potential hypoxia relief and starvation therapy, providing new insights for designing smart therapeutic strategies.

5. Charge-Driven Tripod for Ratiometric Fluorescence Imaging of Endogenous Small Molecules in Nuclei

Currently, proportional fluorescence imaging is mainly performed by intracellular charge transfer in living cells, fluorescence resonance, and excited-state internal proton transfer. However, constructing probes using these strategies requires complex design and synthesis, which makes the difficulty and workload relatively large. On the other hand, due to the nucleic acid-induced fluorescence turn-on properties exhibited by many DNA and RNA binders, conjugation of two DNA binding motifs with different DNA affinities in a single probe may exhibit two different emission characteristics based on the DNA binding mode and DNA-modulated fluorescence. Then, the targeting problem of the cell nucleus can be solved by constructing a DNA-fluorescent probe with a DNA binding agent and a fluorescent probe [22]. Therefore, Wang established a ratiometric fluorescence imaging strategy called "charge-driven tripod flipping on DNA" by utilizing the nucleic acid-rich environment in the cell nucleus and the combination of two DNA binding motifs.

A tripod probe named $[PAST]^+$ was designed by conjugating dipyridyltriphenylamine fluorophores and benzothiazole salts through the vinyl group (reactive site with SO_2 derivatives), both of which are DNA-binding agents. Before encountering the HSO_3^-/SO_3^{2-} target analyte, the tripod produces a first emission feature (red fluorescence) from the positively charged benzothiazole portion by binding it to an uncharged neutral arm of dipyridyl triphenylamine in the DNA double-helix structure, while the other uncharged neutral arm of dipyridyl triphenylamine protrudes out. Upon meeting the negatively charged HSO_3^-/SO_3^{2-} target analyte for reaction, due to Coulomb repulsion between the negatively charged benzothiazole-SO_2 group and the DNA backbone, $[PAST]^+$ similarly rotates one unit to the right, grasping onto the DNA double helix structure through the two intrinsic neutral arms of bipyridyl triphenylamine, while the benzothiazole-SO_2 group protrudes outward, and the newly formed tripod $[PAT]^-$. This change in the DNA binding pattern leads to the disappearance of the first emission feature (red emission) and the formation of the second emission feature (yellow emission), resulting in proportional fluorescence spectra. Using this method, in vitro and *in vivo* ratiometric fluorescence imaging of changes in endogenous SO_2

derivatives in the nucleus was achieved for the first time (Figure 4).

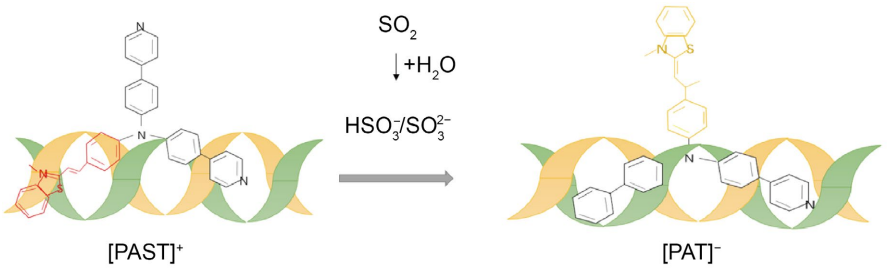

Figure 4. schematic illustration.

The schematic illustration of the strategy named "charge-driven tripod somersault on DNA" for ratiometric fluorescence imaging of small bioactive molecules in the nucleus: the tripodal probe $[PAST]^+$ is constructed by connecting two DNA binding moieties with an ethylene group; once the DNA-$[PAST]^+$ reacts with the negatively charged target analyte, the newly formed tripod $[PAT]^-$ gradually dissociates from the DNA system, freely rotates, and ultimately seizes back the DNA with different binding modes, thus resulting in the disappearance of the first emission feature from DNA-$[PAST]^+$ and the emergence of the second emission feature from DNA-$[PAT]^-$.

Furthermore, the change from positive to negative charge is not necessary and the formation of an electron-neutral response arm induced by the analyte is sufficient to change the DNA binding mode. Therefore, it is expected that this strategy can be further extended for scaled fluorescence imaging of a wide range of bioactive molecules in the nucleus.

6. DNA Nanoenzymes-Based Catalyzed Tyramine Deposition Reaction for *In Situ* Detection of HER2 Protein Status on the Cell Surface

The type I receptor tyrosine kinase family consists of four transmembrane protein receptors: EGFR, HER1, HER2, and HER3, and the ligands induce aberrant dimerization of the receptors, which in turn initiates downstream signaling pathways affecting, among other things, the growth and development of the organism. Among them, HER2 dimerization is the most important receptor in this family, and the abnormal dimerization of HER2 is divided into two scenarios, whether it is the HER2-HER2 homodimer or the heterodimer consisting of HER1-HER2/HER2-HER3, which is an important factor in the development of tumors such as breast cancer. Therefore, the novel markers of HER2 dimerization breast cancer are in urgent need of simple, efficient and rapid detection methods. Among the previous detection methods, the immunoprecipitation method requires lysis of cells and extraction of the proteins therein for detection, and this method is unable to detect the status of HER2 dimers. Currently, the immunohistochemistry (IHC) method is still used in pathology laboratories to detect

HER2 protein and other protein markers. However, this method can only analyze the total amount of HER2 dimers, and cannot accurately analyze the status of HER2 dimers. Therefore, the novel markers of HER2 dimerization breast cancer are in urgent need of simple, efficient and rapid detection methods.

An aptamer is a segment of nucleic acid sequence, which has the advantages of good stability, easy preparation and easy modification. The aptamers of HER family receptors have been screened [23]. G tetramer is a guanine (G)-rich DNA sequence, which can have horseradish peroxidase activity after binding to Hemin, which catalyzes the generation of free radicals from tyramine residues, which, in the presence of H_2O_2, in turn triggers the deposition of tyramine at receptor proteins (**Figure 5**).

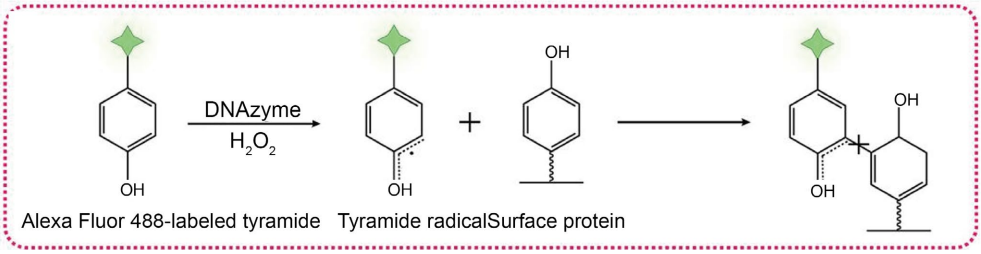

Figure 5. Alexa fluor 488-labeled tyramide, tyramide radical Surface protein.

In this method for detecting HER2, the specific aptamer (Apt2) [24] that binds HER2 was utilized with the G4 sequence to design an Apt2-G4 probe for analyzing the total amount of HER2 dimers. When Apt2 in the Apt2-G4 probe binds specifically to HER2 receptor proteins, the G4 sequence is exposed, and the G4 sequence in turn binds to Hemin, which catalyzes the *in situ* deposition of fluorescent tyramine substrates on the cell surface using the Hemin/ G4-bound formation complex DNAzyme [25] [26]. As the tyramine molecules are labeled with fluorescein, a large number of catalyzed fluorescent molecules deposited *in situ* on the cell surface emit fluorescence, which is used for signal amplification for imaging and analysis of membrane protein expression.

In addition, Xu followed the G4 consortium sequence, split into incomplete parts in a 4:8 ratio, and ligated with the HER2 aptamer Apt2, and designed Apt2-g4 and Apt2-g8 neighboring probes, which bind to the monomer of the HER2 homodimer, respectively, and are used to analyze the status of the HER2 homodimer. When Apt2-g4 and Apt2-g8 neighboring probes bind to HER2 homodimer monomers, respectively, their local concentrations increase, which leads to the proximity and binding of the g4 and g8 sequences to each other, which in turn binds to Hemin to form the Hemin/G4 DNAzyme with horseradish peroxidase activity. The self-assembly of the HER2 homodimers induced by the Apt2-g4 and Apt2-g8 neighboring effect was shown in the following table. The self-assembled DNAzyme induced by the neighboring effect subsequently catalyzes the deposition of fluorescent tyramine on HER2 homodimers on the cell surface. A new technology for precise molecular typing and accurate diagnosis and treatment of

breast cancer (Figure 6).

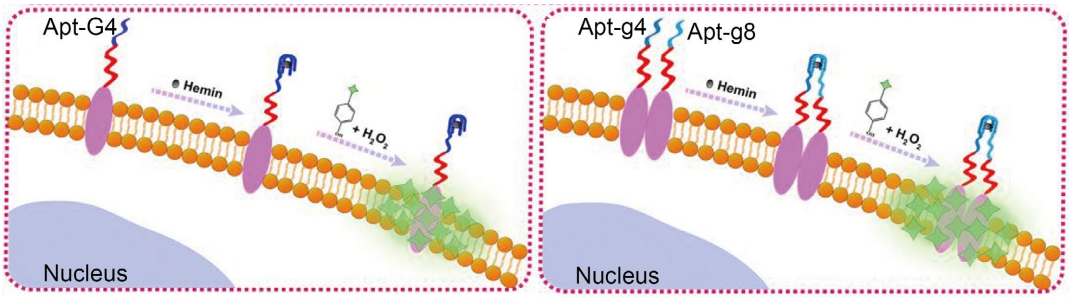

Figure 6. The imaging of total HER2 protein expression on the cell surface by Apt2-G4 probes. The imaging of HER2 homodimer on the cell surface by Apt2-g4 and Apt2-g8 probes.

In this review, we briefly introduce the basic principles and schematics of five strategies used for *in situ* imaging of disease markers. The TPE-R-AT probe approach provides an ultrasensitive biosensing platform for mRNA analysis and shows great potential for predicting overall survival in patients with genetically related diseases. However, to date, Exo III-assisted target cycling process-based mRNA detection in cancer tissues for prognostic assessment has not been explored.

Dual-targeted DNA tetrahedral nanocarriers for breast cancer cell imaging and drug delivery: We could observe the distribution of MUC1-Td-AS1411 in MUC1-positive cells by fluorescence imaging. Dox@MUC1-Td-AS1411 can deliver drugs into the nucleus by attaching AS1411 aptamers and has been shown to be effective in addressing the emergence of drug resistance. This study provides a novel strategy to design dual-targeted drug-carrying nanocarriers for imaging cancer cells and enhancing the efficacy of chemotherapy, which is important in cancer diagnosis and prognostic monitoring.

GSH's DNA-gated device establishes a new strategy for smart therapy, and this method can also guide photodynamic or photothermal therapies to achieve integrated imaging and sterilization. The nanodevice can be activated *in situ* by GSH overexpressed in the tumor cytoplasm and suppressed at background levels in normal tissues to enhance amplification specificity. The nanodevice represents a tumor-responsive therapeutic diagnostic strategy that provides a new perspective on the design of multifunctional nanoplatforms compared to conventional therapeutic diagnostic platforms.

Although the $[PAST]^+$ strategy can be further extended for scaled fluorescence imaging of a wide range of bioactive molecules in the nucleus, $[PAST]^+$ has some long-term cytotoxicity, causing certain defects and damage to cellular structure and function. The design of probes with low or no toxicity is of great help in this putative method, both to minimize the long-term damage of $[PAST]^+$ to cells and to reduce other diseases caused by side effects of toxicity on the organism.

In addition, due to the charge-driven DNA on the tripod for proportional fluorescence imaging is feasible, we have to further explore the modification group

or structure to improve the biocompatibility of the tripod probe. Good compatibility can improve the binding of the charge-driven DNA on the tripod pair to the organism cells, which is more favorable to the proportional fluorescence imaging strategy.

The aptamer co-localization triggered DNA mimicry enzyme self-assembly and catalyzed tyramine deposition reaction signal amplification system enables relative quantitative analysis of HER2 protein. The fluorescence imaging technique also enables in-situ imaging, including total HER2 protein expression and HER2 homodimer protein status, which provides a new way of thinking about the diagnosis and treatment of breast cancer. However, the Apt2-g4 and Apt2-g8 neighboring probes could only bind specifically to HER2 homodimers and could not bind and recognize HER1-HER2/HER2-HER3 heterodimers, which had a certain impact on the typing of breast cancer and the identification of HER2 homodimers.

Overall, although much progress has been made in the application of fluorescence imaging technology, further improvements are needed for specific uses. Optimizing existing methods of identification, diagnosis, and imaging to reduce toxic side effects, improve fluorescent probe utilization, increase detection efficiency, and provide new methods for patient screening, diagnosis, and prognosis.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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