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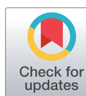
Review Article

Recent advances in living cell nucleic acid probes based on nanomaterials for early cancer diagnosis



Xuyao Liu [a], *Qi Shi* [a], *Peng Qi* [a], *Ziming Wang* [a], *Tongyue Zhang* [a], *Sijia Zhang* [b], *Jiayan Wu* [b], *Zhaopei Guo* [b], *Jie Chen* [b], *Qiang Zhang* [a,*]

[a] *Department of Thyroid Surgery, General Surgery Center, The First Hospital of Jilin University, Changchun 130021, China*

[b] *Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China*

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A B S T R A C T

The early diagnosis of cancer is vital for effective treatment and improved prognosis. Tumor biomarkers, which can be used for the early diagnosis, treatment, and prognostic evaluation of cancer, have emerged as a topic of intense research interest in recent years. Nucleic acid, as a type of tumor biomarker, contains vital genetic information, which is of great significance for the occurrence and development of cancer. Currently, living cell nucleic acid probes, which enable the *in situ* imaging and dynamic monitoring of nucleic acids, have become a rapidly developing field. This review focuses on living cell nucleic acid probes that can be used for the early diagnosis of tumors. We describe the fundamental design of the probe in terms of three units and focus on the roles of different nanomaterials in probe delivery.

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1. Introduction

Malignant tumors are a life-threatening disease with high morbidity and mortality rates. The current treatment strategies for malignant tumors include chemotherapy, surgical resection, radiotherapy, immunotherapy, and targeted drug therapy [1–4]. The early diagnosis, precise localization and characterization of tumors all contribute to the selection of tumor treatment methods and to patient survival [5]. Conventional cancer localization and diagnosis strategies mainly include tissue biopsy and imaging methods such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). These strategies have limitations for early and accurate cancer diagnosis. Such techniques rely primarily on the physical characteristics of tumors, rendering it challenging to accurately locate small and concealed tumors and determine their properties. Moreover, while biopsy remains a viable option for determining the properties of tumors, its disadvantages include high costs, high risk, operator-dependence, and a propensity for false negative results [6,7]. Recently, tumor biomarkers, including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and peptides, exosomes, metabolites of cellular biological

* Corresponding author.
E-mail address: zqiang@jlu.edu.cn (Q. Zhang).
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processes, circulating tumor cells, and circulating immune cells, have been used for the early diagnosis of diseases. The detection of tumor markers holds significant reference value in guiding treatment strategies, assessing therapeutic effectiveness, and monitoring prognosis [8–12]. Nucleic acid molecules, including DNA and RNA, participate in the expression and regulation of genes and play vital roles in life processes [13–15]. RNA can be divided into messenger RNA (mRNA), microRNA (miRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), long noncoding RNA (lncRNA), and other small RNA molecules. Currently, the commonly used methods of detecting nucleic acid molecules include Southern blotting, Northern blotting [16], DNA microarray technology [17] and polymerase chain reaction (PCR) analysis [18]. These techniques, which all rely on cell lysates and high nucleic acid concentrations, can only average the data from all cells, making it impossible to measure and precisely identify individual cells in real-time. The process is also complicated.

In recent years, nucleic acid probes have developed rapidly as a class of DNA/RNA components that can enable *in situ* nucleic acid molecular imaging. The probes often include two parts: a targeting unit and an imaging unit. Rationally designed probes can enter cells and recognize specific targets [19,20]. Currently, there are two types of nucleic acid imaging probes. One type is the probes designed for fixed cells. Due to the enhanced cell membrane permeability of fixed cells, the probe can directly enter the cells for recognition and imaging. The most commonly used technique is fluorescence *in situ* hybridization (FISH). Femino et al. [21] proposed a single-molecule fluorescence *in situ* hybridization (smFISH) technique that allows for *in situ* imaging. The other type is living cell probes, which are delivered into the cell interior via endocytosis or manual techniques such as microinjection or electroporation [13]. Compared with the probes in fixed cells, living cell probes do not damage cells and have more promising applications. However, the physiological environment of living cells is more complex; for example, intracellular DNase I cleaves single- and double-stranded DNA, leading to the generation of false-positive signals. Additionally, the efficiency of cellular endocytosis significantly affects the ability of the probe to function [15]. To improve this situation, many studies have been conducted on combining nanomaterials with nucleic acid probes to enhance their delivery efficiency, stability, and sensitivity [22,23].

Living cell nucleic acid probes include a biometric identification element (targeting unit) for targeting a specific nucleic acid sequence, a signal-transducing element (imaging unit) for imaging and a carrier element (delivery unit) for delivering a probe to the cell [24]. The targeting unit usually consists of nucleotide sequences that can hybridize with the target nucleic acid chain, and common imaging units include fluorescence reporting molecules and Raman reporting molecules. Because some nucleic acids, such as miRNA, are present in low amounts under physiological conditions, the design of the imaging unit must address the signal amplification technology. Delivery units commonly include electroporation, microneedle injection [25], and nanomaterials such as liposomes, polymers, DNA nanostructures, gold nanoparticles, carbon-based nanomaterials, etc. [26–32]. In this review, we discuss the construction of nucleic acid probes based on their three basic units. Regarding the targeting unit, we introduce the basic design of current probe recognition components and common target nucleic acids. Regarding the imaging unit, we introduce fluorescence and Raman imaging of live cells, as well as common signal amplification technologies. Afterwards, we focus on the application of different nanomaterials in cell delivery and imaging in the delivery unit (Fig. 1).

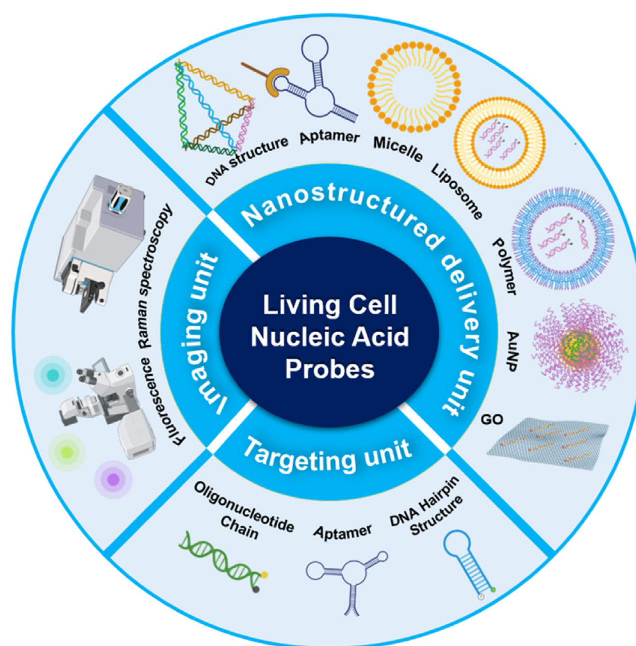

Fig. 1 – Nanomaterials-based living cell nucleic acid probes for early cancer diagnosis.Part icons from Biorender.com.

2. Targeting unit

Targeting unit is responsible for recognizing and binding to specific nucleic acids. Currently, the design of this unit commonly used in living cells can be classified into three types: oligonucleotide chains, aptamers and DNA hairpin structures [33]. In order to enhance chain stability, overcome complex intracellular physiological environments, and optimize probe performance, researchers often add chemical modifications to this foundation. Herein, we focus on providing a general overview of targeting unit design and list different nucleic acids commonly used for tumor targeting (Table 1).

2.1. *Design of the targeting unit*

2.1.1. *Oligonucleotide chain*

The oligonucleotide chain is the earliest and most foundational design for nucleic acid probes, based on the Watson-Crick pairing principle, for the recognition of target DNA, mRNA, miRNA, and other biomolecules. While the design and utilization of oligonucleotide chains as probes are widespread in non-living cell environments, their

Table 1 – Some targeted nucleic acid chain selection of probes.

Targeted nucleic acid	Tumor type	Cell	Introduction	Ref
C-myc mRNA	breast cancer hepatocellular cancer	MCF-7 HepG2	C-myc, proto-oncogene transcription factor. Overexpression of the C-myc gene often causes malignant transformation of multiple cells, which can drive and maintain the occurrence and development of tumors.	[34–36]
C-raf-1 mRNA	lung cancer	A549	Proto-oncogene, expressing serine–threonine kinase Raf-1 protein, is used as a tumor marker and antisense therapy target.	[37,38]
hTERT mRNA	cervical cancer hepatocellular cancer osteosarcoma	HeLa HepG2 U2OS	hTERT gene expresses the TERT protein, which is used to maintain telomere length. In most cancers, it is overactivated to induce continuous cell proliferation.	[39–41]
TK1 mRNA	hepatocellular cancer	HepG2	Overexpression of TK1 can be found in various cancers, including lung, colorectal and breast cancer. It is an early event in cancer progression.	[42,43]
MDR1 mRNA	ovarian cancer	OVCAR8	Encoding the drug efflux transporter protein Pgp, which is often overexpressed in many cancer cells and is associated with the development of MDR.	[44]
MDM2 mRNA	breast cancer	MCF-7	MDM2 encodes MDM2 protein, which is a multifunctional oncoprotein. The gene is amplified in many malignant tumors.	[45,46]
MMP-2 mRNA	breast cancer	MCF-7 MDA-MB-231	MMP-2 is often overexpressed in tumor tissues and is closely related to the progression and metastasis of cancers.	[47,48]
MnSOD mRNA	breast cancer	MCF-7	Encodes MnSOD, an antioxidant enzyme that can mediate the death signaling pathway of various cancer cells.	[49,50]
RAB-22a mRNA	breast cancer	MCF-7 MDA-MB-231	Rab22a is a member of the RAS oncogene family, overexpressed in human breast cancer, and related to cell proliferation, migration and invasion.	[47,51]
Survivin mRNA	breast cancer colorectal cancer lung cancer	SKBR3 HCT116 SW480 A549	Impede apoptosis and modulates cellular division, when its expression is upregulated.	[30,52–55]
miRNA-20a	hepatocellular cancer	Huh-7	miR-20a is overexpressed in a variety of cancers. It can be utilized as a tumor diagnosis and prognosis marker.	[56,57]
miRNA-21	breast cancer bladder cancer cervical cancer hepatocellular cancer lung cancer malignant cancer	MCF-7 MDA-MB-231 Biu-87 HeLa HepG2 A549 A375	miRNA-21 is highly expressed in a variety of cancers and is related to promoting tumor cell proliferation and inhibiting apoptosis.	[58–66]
miRNA-155	DLBCL breast cancer	DLBCL MCF-7	Highly expressed in lung, renal, hepatocellular, colorectal and breast cancers et al. Promote the stemness of cancer cells, increase drug resistance to chemotherapy and radiotherapy, and can be used for tumor immunotherapy.	[20,27,67,68]
miRNA-1246	lung cancer	A549	miRNA-1246 can exert oncogenic roles in various cancers.	[58,69]
miRNA let-7a	hepatocellular cancer breast cancer	HepG2 MCF-7	Mature let-7a is typically downregulated in lung cancer, breast cancer and other cancers.	[63,70–72]

human telomerase reverse transcriptase (hTERT); thymidine kinase 1 (TK1); P-glycoprotein (Pgp); multidrug resistance (MDR); mouse double minute 2 (MDM2); Matrix metalloproteinases (MMPs); Manganese superoxide dismutase (MnSOD); diffuse large b-cell lymphoma (DLBCL).

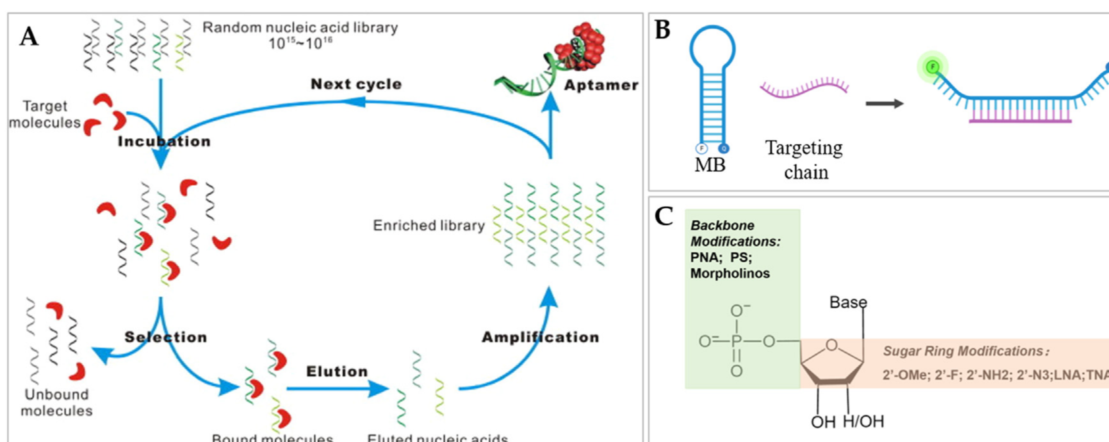

Fig. 2 – (A) The SELEX enrichment process. Reproduced with permission [76], Copyright 2007, Elsevier. (B) Basic design of molecular beacon. (C) The common modifications applied to targeting unit.

stability within cellular environments is notably lacking. To address this limitation, researchers currently rely on linear nucleotide chains as the foundational design, employing methodologies such as HCR and CHA to engineer more intricate binding chains or construct spatial DNA structures to attain added functionality [64,73–75]. Further details on these advancements will be discussed in the following chapters.

2.1.2. *Aptamers*

Aptamers, also known as chemical antibodies, are single-stranded nucleic acid oligomers produced through systematic evolution of ligands by exponential enrichment (SELEX) process [76,77] (Fig. 2A). In SELEX, the target molecule is incubated with a nucleic acid library. The bound nucleic acid is extracted and amplified by PCR. Nucleic acids with high affinity for the target molecules are ultimately isolated and enriched via repeated cycles. When combined with the target receptor, the aptamer forms unique secondary and tertiary structures. An aptamer usually has a high affinity for its target, and aptamers can be designed to effectively and selectively bind various targets, including metal ions, small organic molecules, peptides, proteins, and even whole cells and tissues [78–81]. Aptamers find wide application in diverse fields owing to their small physical size, adaptable structure, versatile chemical modifications, exceptional stability, and non-immunogenic nature. Sunbul et al. [82] have developed an SRB-2 aptamer capable of enabling real-time localization imaging of 5S rRNA in Hela cells. Yan et al. [83] have engineered a fluorogenic allosteric aptamer (FaApt) for real-time imaging of β-actin mRNA localization in living cells. The designs of nucleic acid-targeted aptamers often involve molecular beacon (MB) conjugation to achieve enhanced performance [84]. Ying et al. [85] engineered a MB-based luminescent RNA aptamer capable of enabling real-time imaging of miR-21 in Hela cells. Meanwhile, many aptamer probes used for cancer diagnosis target specific proteins or cancer cells. Several comprehensive reviews have detailed these advancements extensively [86–88], which will not be detailed here.

2.1.3. *DNA hairpin structure*

DNA hairpin structures typically consist of a stem structure with reversible complementary base pairing, a ring sequence, and a group of fluorescent and quenching markers. The most common DNA hairpin structure is the MB, which was first reported in 1996 [89]. The stem is usually composed of 5–7 base pairs and the ring sequence comprises 15–25 nucleotides [24,90] (Fig. 2B). This special hairpin structure design has high target specificity, single-base mismatch recognition ability, and good stability, and it is extensively used in detection, biosensors, imaging, gene therapy, and other fields. Numerous nucleic acid probes utilized for live cell detection are designed based on MB. For instance, Zhang et al. [44] developed an MB probe to specifically target MDR1 mRNA in ovarian cancer cells. In pursuit of enhanced performance, MB frequently incorporates chemical modifications or more intricate structural designs. For example, Ratajcza et al. [55] engineered sophisticated MB probes that integrate MB with hairpin-structured target oligonucleotides to detect survivin mRNA in colon cancer cells.

2.2. *Chemical modification of the targeting unit*

Nucleotides consist of three subunits, namely a nitrogen-containing base, a five-carbon sugar moiety, and a phosphate “backbone”. Currently, there are relatively well-developed technologies for the chemical modification of the various parts of nucleotides to obtain better performance and wide-ranging applications. In this section, we introduce the common modifications applied to nucleic acid probes (Fig. 2C).

2.2.1. *Backbone modifications*

(1) Peptide nucleic acid (PNA)

PNA is a synthetic DNA analog in which the sugar-phosphate backbone is replaced by a peptide backbone. PNA strands can be hybridized with complementary single-stranded DNA, RNA, or other PNA strands to form stable double-stranded molecules. PNA is currently widely employed in the design of nucleic acid probes. Liao and colleagues [91] developed a PNA probe that utilizes π-π stacking

interactions to bind with a delivery unit, enabling the detection of miR-18a within liver cancer cells. Compared to DNA, PNA's special structural framework provides it with enhanced hybridization characteristics, such as faster hybridization, greater stability, higher sequence selectivity, and strong resistance to nuclease and protein hydrolytic degradation. This allows PNA to maintain its high stability in complex biological environments [73,92–94].

(2) Phosphorothioate (PS)

When an oxygen atom in the phosphate group of a nucleotide is replaced with a sulfur atom, a thiophosphate ester is produced. Phosphorothioate can improve the resistance of nucleotide chains to nucleases, and thereby increase their stability in the intracellular environment [95]. They are commonly utilized in the modification of MB probes [96,97].

(3) Morpholinos

Morpholino oligonucleotides (MO) are nonionic DNA analogs based on a morpholine ring through base pairing. Chen et al. [98] developed an MO-based MB probe for RNA imaging within live cells. Compared to DNA or RNA, the backbone connections of MO exhibit alterations. The absence of a negative charge in the nucleotide backbone enhances the specificity of target and resistance of nuclease, and reduces nonselective interactions with intracellular proteins [99,100].

2.2.2. *Sugar ring modifications*

(1) Common chemical modifications at the 2′-O position

In pursuit of bolstering sequence stability within the intracellular milieu, chemical modifications targeting the 2′O position of the sugar ring are commonly employed, involving the introduction of functional groups such as fluorine (-F), amino (-NH2), azide (-N3), or methoxy (-OMe) to supplant the 2′O position [101]. This modification method can improve the probe's affinity for the target and is resistant to specific nuclease degradation in the cellular environment [102,103]. Li et al. [104] constructed a DNA probe completely modified by 2′-OMe, which realized the targeting and inhibition of miRNA-21 in breast cancer cells. Compared with normal DNA, this 2′ -OMe DNA has higher stability and resistance to exonuclease.

(2) Locked nucleic acid (LNA)

LNA is a new type of synthetic oligonucleotide that contains at least one LNA monomer. The 2′ oxygen in the modified nucleic acid base is bridged to a 4′ carbon, that is, a 2′-O, 4′-C-methylene-β-D-furan ribonucleotide. LNA has an excellent binding affinity for complementary DNA and RNA, high mismatch recognition ability, nuclease resistance, and satisfactory stability in complex biological environments. Zhu et al. [105] constructed an LNA-modified MB probe to identify miRNA-155 in lung cancer cells.

(3) Threose nucleic acid (TNA)

Compared to DNA, the sugar ring of TNA has changed from a five-carbon ribose to a four-carbon threose. TNA can complement DNA, RNA, and itself, thus being considered a precursor of RNA [106]. Wang and colleagues [107] prepared a TNA probe for detecting and imaging miR-21 in ovarian cancer cells. The TNA probe exhibits superior target recognition ability, enhanced resistance to nucleases, and improved thermal stability.

3. Imaging unit

The probe recognizes specific targets in the cell, allowing the signal-transducing element to visualize the signal and achieve *in situ* imaging of the cell. The most common probe imaging technology is fluorescence and electrochemical sensing. Although electrochemical sensing has the characteristics of high selectivity, high sensitivity, simplicity, reliability and low cost, it is rarely used in living cells or *in vivo* [33,108–111]. Nucleic acid probe imaging techniques commonly used in living cells include fluorescence and Raman imaging [33]. To improve signal detection sensitivity, many probes use signal amplification strategies to reduce the background signal, and detect the low-abundance targets, such as certain miRNAs. In this section, we list cancer cell imaging strategies and common signal amplification techniques for probes.

3.1. *Cancer cell imaging strategies*

3.1.1. *Fluorescence imaging*

Fluorescence imaging is the most commonly used imaging technique for cancer cells and other living organisms. Numerous fluorescent materials have been discovered, each with its own advantages and disadvantages. The use of different fluorescent materials in the probe yields different imaging results.

Fluorescence recovery after quenching is a commonly used technique. The probe is in the "off" state when inactive and switches to the detectable "on" state when it binds to the target. This is usually achieved via fluorescence resonance energy transfer (FRET) or collision-quenching interactions. FRET refers to the process of energy transfer from an excited chromophore (donor) to another chromophore (acceptor). FRET reduces the fluorescence of the donor, and enhances or quenches the fluorescence of the receptor depending on its optical properties. Most MBs use different fluorescent (F) and quenching (Q) groups to obtain on/off signals by changing their structural state. Additionally, some chain probes with special structural designs exhibit fluorescence recovery after quenching [24,90,112–114]. Common basic F–Q designs include organic dyes and quenched molecules, organic dyes and quenched nanomaterials, and fluorescent nanomaterials and quenched nanomaterials (Table 2).

Organic dyes are fluorophores that emit in the ultraviolet–visible (UV–vis) and near-infrared (NIR) regions, and they are small, water-soluble, and easy to use for biological coupling [118]. Common types include the BODIPY compounds [119], fluorescein [120], cyanine family [121], and rhodamine family [122]. Among them, 4-(4′-dimethylaminophenylazo) benzoic acid (Dabcyl) and the black hole quenching agent (BHQ) are often used as quenching agents. They often have wide absorption spectra and are suitable for quenching a variety of dyes [64,115].

In addition, nanomaterials are used by researchers in fluorophore quenching. In this approach, the probe identifies the specific target and detaches from the nanomaterials to initiate luminescence. Currently, the most common nanomaterials used for this purpose are gold nanoparticles

Table 2 – Common probe fluorescence and quenching signal design.

Fluorescence signal	Quenching signal	Ref
Alexa-488	Dabcyl	[44]
ATTO 647N	BBQ650	[52]
Cy3	BHQ2	[49]
Cy5.5	BHQ2	[115]
FAM	BHQ1; BHQ2; Dabcyl; TAMRA	[58,64,70]
JOE	Dabcyl	[55]
ROX	BHQ1; BHQ2	[58,70]
TAMRA	BHQ2	[54]
FITC	AuNP	[62]
Alexa-488	AuNP	[47]
CY3	AuNP; GO	[29,36]
CY5	AuNP; GO	[47,63]
DA	AuNP	[116]
FAM	AuNP; GO; Fe3O4	[29,56,63]
FAM	MnO2	[117]
QD	AuNP	[66]
CQD	BHQ1	[65]

(AuNPs) [62], graphene oxide (GO) [29], carbon nanotubes [91], quantum dots (QDs) [123], molybdenum disulfide (MoS2) [124], etc. Nanomaterials that can function as quenching agents have better probe-binding abilities, light-bleaching resistance, and enzymatic degradation abilities than other quenching agents and result in higher detection sensitivity.

QDs, a representative fluorescent nanomaterial, are semiconductor nanocrystals with a diameter range of 1–10 nm. They have great advantages in the NIR light region and have been used in different biological imaging applications owing to their strong and persistent photoluminescence (PL). Additionally, they exhibit excellent photostability and effectively resist photobleaching [125]. However, the inherent toxicity of QDs inhibits their application *in vivo*. Currently, QDs are often modified for imaging applications in living cells or tissues. Zhang et al. [126] engineered non-toxic InP/ZnSe/ZnS QDs based on aminophosphate precursors. These high-quality lnP QDs enable the recognition and imaging of AFP in liver cancer cells. This non-toxic quantum dot probe demonstrates great potential for cancer diagnosis. He et al. [66] designed a AuNP-QD assembly probe. In the inactive state, the PL of the QDs was quenched by nearby AuNPs based on the FRET principle. Upon the recognition of miR-21 in cancer cells, the imaging signal was acquired. Carbon QDs are nanostructured carbon that can be obtained using low-cost manufacturing technology with good biocompatibility, low toxicity, and a strong quantum size effect [127]. Mahani et al. [65] designed an MB labeled with carbon QDs to detect miR-21.

Forced intercalation (FIT) probes are designed as a quenching-free probe imaging method [74]. In 2005, a thiazole orange (TO) family dye developed by the Seitz group was used as an alternative probe to the nucleic acid base group [128]. When the probe was in the inactive state, the TO dye rotated internally and did not generate a fluoresce signal. While the "intercalation" of the dye blocked the rotation of the secondary methyl bridge, fluorescence was emitted in the presence of the target. Compared to MBs, FIT probes have good properties, such as strong single-base mismatch recognition ability and fluorescence enhancement, and avoid the production of false positive signals [129,130].

3.1.2. *Raman imaging*

Raman spectroscopy is a powerful nondestructive spectroscopy technique. It has the characteristics of a narrow spectrum, light stability, and NIR excitation; however, its low signal intensity significantly limits its application in biological imaging [131,132]. Recently, surface-enhanced Raman spectroscopy has become commonly used as a new strategy to enhance imaging signals [133]. In cancer cell imaging, Raman probes are mainly used to target the aptamers of proteins and antibodies in cells [132,134]. Additionally, nucleic acid-based probes were designed and developed. Ma et al. fabricated a GO-AuNP system using splint DNA, which simultaneously monitored the epithelial cellular adhesion molecule (EpCAM) and miR-21 in living cells via circular dichroism (CD) and Raman signals (Fig. 3A) [135]. AuNPs modified with 6-fluorescein-phosphorous amide were separated from GO after miR-21 was hybridized with the probe, and the Raman signal decreased proportionally with the increase in the concentration of miR-21 in the cell. This imaging strategy exhibited good stability and target specificity. The limit of detection for miRNA-21 was 0.03 amol ng^{-1} RNA, which provides a reference for the detection and design of nucleic acid targets with low expression levels in living cells.

3.2. *In situ signal amplification strategies*

The conventional nucleic acid probe design is based on one-to-one signal transduction, in which a probe binds to a target and is activated to produce a detectable signal. This design is suitable for highly expressed targets but is limited for low-abundance targets such as miRNA. To improve the sensitivity and specificity of a probe, signal amplification strategies were applied to enhance the signal from "one-to-one" to "one-to-many" imaging, which allows trace determination of the probe in living cells with low-abundance targets. HCR, CHA, and entropy-driven DNA catalysis (EDC) are classic nonenzymatic isothermal signal amplification technologies. These strategies are mainly based on the toehold-mediated strand displacement (TMSD) reaction, which is simple in design and highly stable. The DNA enzyme amplification strategy has strong signal amplification ability and does not require protein enzymes or the removal of certain unbound enzymes for detection. These strategies are the common signal amplification techniques used in living cell nucleic acid probes [136].

3.2.1. *Hybridization chain reaction (HCR)*

In 2004, Dirks and Pierce proposed the HCR [137], which uses two complementary metastable DNA hairpins with short single-stranded sticky ends to store potential energy. Dynamically, the two chains captured by the hairpin structure coexist in a meta-stable state in the absence of an initiator. When the initiator (target chain) is introduced, it participates in the TMSD reaction to open the first hairpin. This forms a new single-chain structure domain that serves as the second initiator, which reacts with and opens the second hairpin,

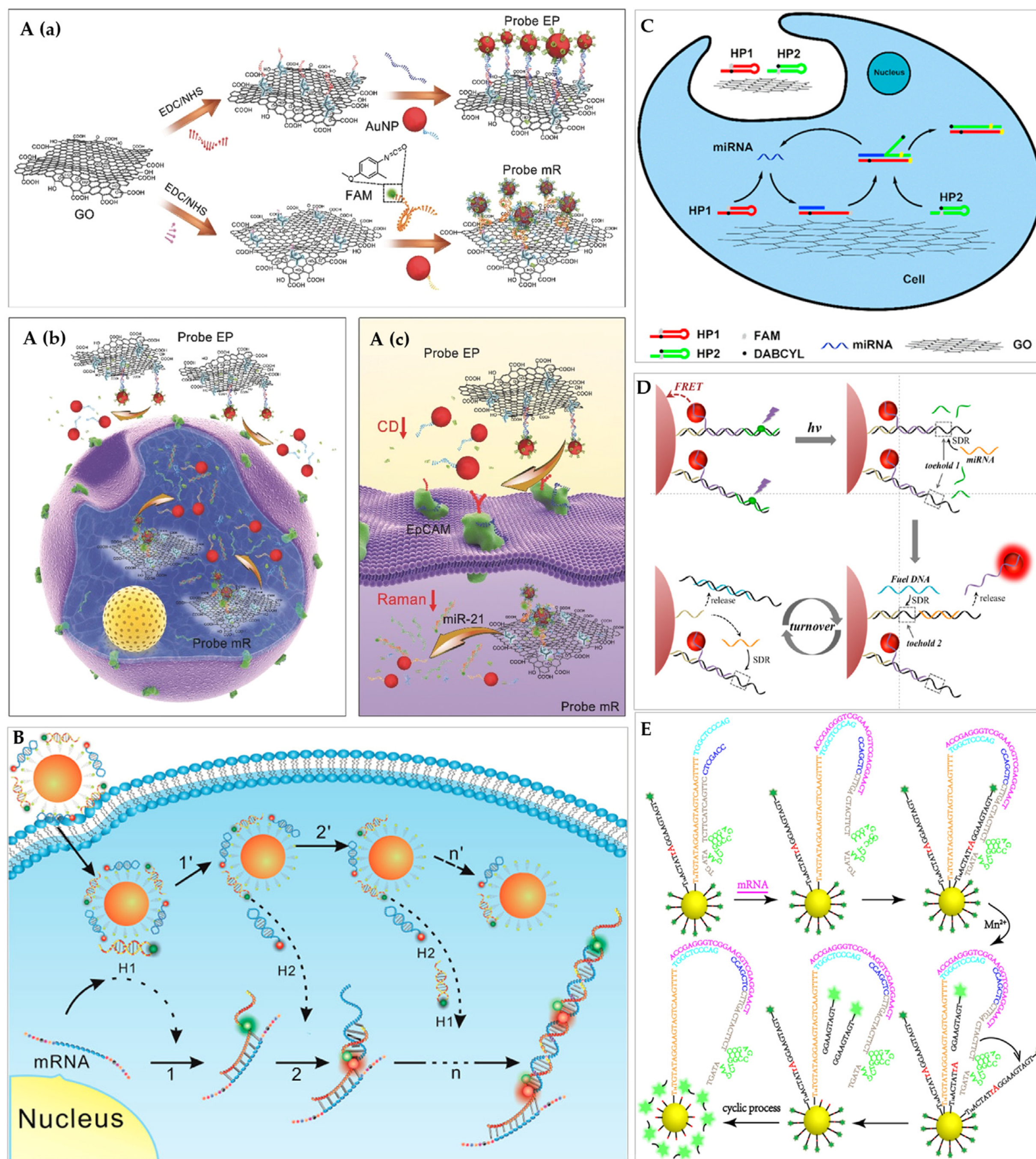

Fig. 3 – (A) GO–Au system for EpCAM and miR-21 detection by CD and Raman signals: (a) The fabrication routes for probes. (b) The probes were used for dual detection of two types of targets. (c) The probe mR is used for detection of miR-21 by the Raman signal. Reproduced with permission [135]. Copyright 2019, American Chemical Society. (B) AuNP delivers HCR hairpins into cancer cells to recognize mRNA imaging. Reproduced with permission [133]. Copyright 2015, American Chemical Society. (C) GO-based CHA hairpin probe for imaging miR-21 in cancer cells. Reproduced with permission [138]. Copyright 2016, Elsevier. (D) The system achieves significant signal amplification by designing a two-step chain displacement reaction. Reproduced with permission [140]. Copyright 2018, American Chemical Society. (E) DNA enzyme motor realizes signal amplification in living cells. Reproduced with permission [141]. Copyright 2022, Springer Nature.

resulting in another single-chain domain. This cascade reaction continues and cross-opens the two hairpins until they are depleted, generating a DNA polymer nanowire. Donor or receptor fluorescence is often incorporated into the hairpin structures to produce detectable FRET signals. Wu et al. [133] prepared AuNPs coated with cationic peptides to target survivin mRNA in cancer cells, the AuNPs delivered the HCR hairpin into the cells, where the hairpin recognized survivin mRNA, and triggered a cascade reaction that produced FRET signals (Fig. 3B).

3.2.2. *Catalytic hairpin assembly (CHA)*

Similar to HCR, CHA uses two complementary metastable hairpins to store potential energy. The target nucleic acid, such as miRNA, hybridizes with the toehold region of one hairpin, thereby opening the hairpin structure and allowing it to hybridize with the second hairpin. The target chain is shifted, the next reaction is initiated, and the two hairpins produce a nucleic acid double chain with fluorescent groups. This target-chain recovery strategy can significantly amplify the signals of low-abundance targets. Liu et al. [138] developed a GO-based CHA hairpin probe for imaging miR-21 in cancer cells. When the probe enters the cell and comes into contact with miR-21, hairpin probe 1 (HP1) with a fluorescein amidite (FAM) fluorophore at the end combines with miR-21 and exposes the single-stranded tail, which facilitates hybridization with another hairpin chain (HP2) to produce double-stranded DNA (dsDNA) with a fluorophore and release miR-21. Moreover, GO can accumulate CHA products and enrich the fluorescence signals (Fig. 3C).

3.2.3. *Entropy-driven DNA catalysis (EDC)*

The EDC process is based on a toehold exchange reaction driven by the entropy gain of released molecules and does not involve hairpin structures. A typical EDC system generally contains a three-chain complex and a fuel chain. When the catalyst (target gene) chain appears, it combines with the three-chain complex; the signal and catalyst chain are released through multiple chain displacement, and the target catalyzes the reaction again to produce multiple signal outputs [139]. Shen et al. [140] designed a AuNP QD complex to target miRNA-21 in cells. miR-21 can release quantum dots through a two-step chain replacement reaction to achieve significant signal amplification (Fig. 3D).

3.2.4. *DNA enzymes*

As a DNA sequence that catalyzes various reactions such as RNA cleavage, DNAzyme can be used as an excellent signal amplifier for the enzyme-free and highly sensitive detection of various targets. Therefore, several DNAzyme-based amplification and sensing platforms have been developed for this purpose. Liu [141] proposed a DNA-Au nanomachine based on a toehold-mediated chain shift reaction (TSDR) driven by a DNAzyme and triggered by survivin mRNA. This DNA-Au nanomachine comprised dozens of FAM-labeled RNA–DNA substrates and several DNase double strands blocker. The blocking strand contained two functional segments, which were used to complement survivin mRNA and inhibit DNAzyme. In the presence of survivin mRNA, the blocking chain combined with survivin mRNA, and the DNAzyme motor chain activated the nanomachine. Survivin mRNA serves as a bridge between the blocking chain and the motor of the DNAzyme, triggering the activity of the DNAzyme motor and enabling it to walk independently along a Au-based three-dimensional (3D) orbit. Each walking step cleaved the substrate chain and released FAM-labeled DNA fragments from the AuNPs to restore the fluorescence quenched by the AuNPs (Fig. 3E).

4. Delivery unit

One of the most important characteristics of live cell nucleic acid probes used for detection is their ability to penetrate cells effectively and maintain stability in the cellular environment. Many methods have been used for probe delivery, such as electroporation. Adapters for transmembrane penetration have also been developed. In recent years, many studies have used different nanomaterials in the delivery unit to develop probes with good stability, high target specificity, and image-sensing capabilities in complex physiological environments. Different nanomaterials have various physical and chemical properties and specific advantages.

4.1. *Internalized aptamers*

Adapters screened by Cell-SELEX can specifically bind to membrane proteins, and some can be internalized into cells through the translocation of target proteins to achieve specific intracellular delivery.

The aptamer AS1411, which exhibits specific binding to nucleolar proteins overexpressed on the cell membrane of many cancers, can be effectively internalized by cancer cells based on the shuttling behavior of nucleolar proteins between the cytoplasm and the nucleus. Qiu et al. [49] developed a self-delivering double-stranded DNA probe targeting MnSOD mRNA in MCF-7 cells. One strand was an MB-targeting mRNA, and the other strand was AS1411. These were used as two photocleavable (PC) linkers for MB-targeting cell delivery. The choline phosphate (CP) chain was inserted into two light-cutting connectors. After entering the cell, the CP chain was cut, and MB was released under the action of a UV pulse to achieve targeted imaging (Fig. 4A).

Li et al. [64] developed an aptamer-functionalized MB (Apt-Tri-MB) probe, designed as a rigid triangular skeleton of DNA to resist nuclease degradation, in which the MB chain was used for target recognition. Additionally, the probe was introduced into cells using the AS1411 aptamer. The probe enabled the targeted imaging of miR-21 and let-7a in MCF-7 cells (Fig. 4B).

MUC1 is a transmembrane glycoprotein that is typically overexpressed in tumor cells. Kim et al. developed a multivalent carrier-free aptamer-RNA-based probe (CF probe) to detect miR-34a in MCF-7 and T47D cells [115]. The probe consisted of a complementary oligonucleotide chain of miR-34a and a complementary short RNA chain containing a MUC1 aptamer with fluorescence and quenching groups. This aptamer attached to MUC1 on the plasma membrane and promoted its internalization via reticulin-mediated endocytosis. Upon entry into the cell, the short RNA chain with

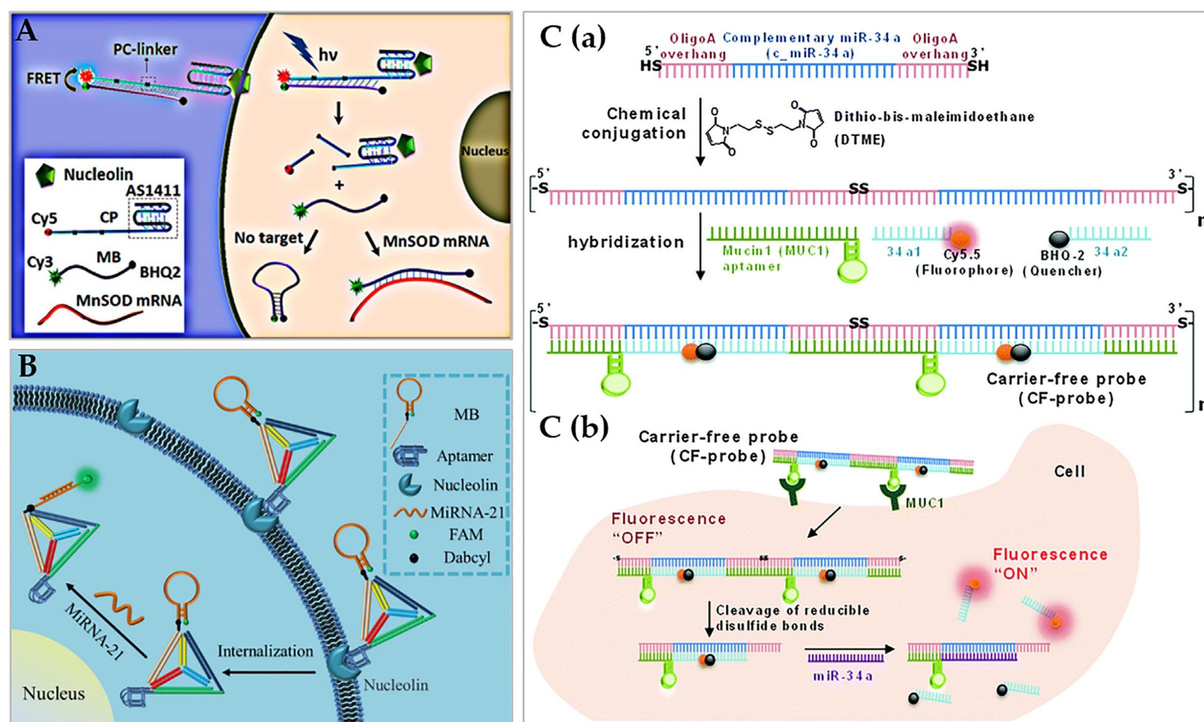

Fig. 4 – (A) Self-delivery double-stranded DNA probe via AS1411. Reproduced with permission [49]. Copyright 2013, American Chemical Society. (B) Apt-Tri-MB probe targeted miRNAs in MCF-7 cells through AS1411. Reproduced with permission [64]. Copyright 2020, American Chemical Society. (C) Self-delivery probe targeting intracellular miRNA based on MUC1 aptamer. (a) The preparation of CF-probes. (b) cellular uptake and intracellular processing. Reproduced with permission [115]. Copyright 2015, Royal Society of Chemistry.

miR-34a was recognized and fell off, generating a fluorescence signal (Fig. 4C).

4.2. *Organic nanomaterials*

4.2.1. *DNA nanostructure*

Recently, probes based on DNA nanostructures have received considerable attention. Currently, there are many types of nucleic acid probes, such as 1D DNA nanowires [142], Y-shaped scaffolds [143], DNA nanotubes [144], DNA origami [145], DNA tetrahedral nanostructures [146], DNA polyhedral nanostructures (including cubes [28], octahedra [147], and other polyhedral structures), 3D DNA origami [148], and DNA nanoflowers [149]. DNA nanostructures play various roles, such as bolstering the biological stability of the probe, augmenting the efficiency of cell uptake, accelerating the reaction rate, and amplifying the signal output. DNA nanostructures have great advantages in living cell applications, and some interesting designs for cell-delivery function have been reported.

Tetrahedral DNA nanostructures (TDNs) can penetrate the cell membrane independently of transfection reagents. Using single-particle tracking, Liang et al. [150] demonstrated that TDNs rely on cave proteins for rapid internalization. Hu et al. constructed a dendritic layered DNA structure by the self-assembly of five identical TDNs [151]. The nucleic acid dendrimer framework showed good intracellular delivery, high biocompatibility, and stability in the cellular environment.

Wang et al. [70] developed a DNA nanolantern-based CHA (NLC) probe for the simultaneous detection of mi21 and let7 in living cells and mice (Fig. 5A). The nanostructure formed by this NLC probe exhibited good membrane permeability, effective internalization by cells without the aid of any transfection reagents, and enhanced imaging of the target cells and mice via CHA technology.

4.2.2. *Cationic liposomes*

Lipids and lipid-derived nanoparticles represent a large category of delivery systems. Liposomes have been used to encapsulate mRNA since the late 1970s. For example, Dimitriadis formulated a monolayered liposome to deliver the rabbit globin's mRNA to the isolated mice [152]. In 1989, Malone et al. developed a cationic liposome that transported luciferase mRNA into human, rat and mouse cells [153]. Cationic liposomes are among the most commonly used delivery carriers for nanomaterials. In general, the probe is encapsulated in liposomal particles to achieve intracellular internalization and has good nuclease resistance. Recently, Alshehri et al. [26] demonstrated that cationic liposomes could achieve intracellular internalization and probe release, mainly via a trellising-dependent pathway and possibly by exocytosis. Han et al. [154] constructed a liposome-MB coated with microRNA MB for the detection and imaging of miR-181a,

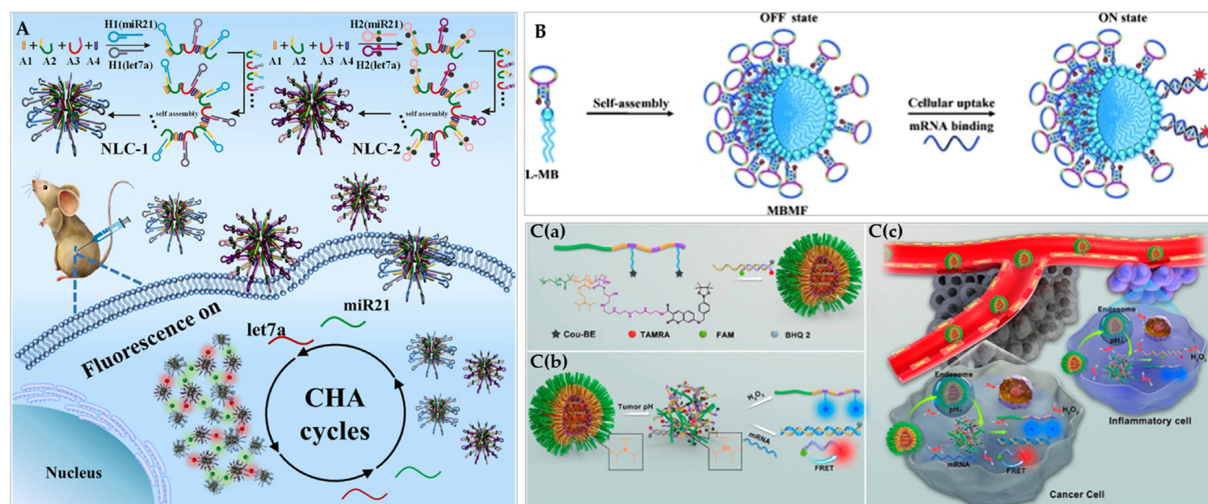

Fig. 5 – (A) Schematic diagram of design and work flow of NLC probe. Reproduced with permission [70]. Copyright 2021, Elsevier. (B) Design and working diagram of MB micelle flare (MBMF). Reproduced with permission [37]. Copyright 2013, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) Schematic diagram of probe imaging in inflammatory and cancer cells. Reproduced with permission [54]. Copyright 2019, American Chemical Society.

miR-21, and miR-31 in living cells. Fu et al. [45] developed an antisense oligonucleotide chain coated with liposomes and labeled it with a radionuclide (99mTc) to target MDM2 mRNA in MCF2 cells, and molecular imaging of living xenograft tumor mice was performed using a single-photon emission computerized tomography (SPECT) scanner.

4.2.3. *Micelles*

Micelles are also used to improve the delivery efficiency and stability of probes, owing to their good biocompatibility and nuclease resistance. Zhang [44] reported the design of an anti-MDR1 MB-based micelle-doxorubicin (a-MBM-DOX) nanosystem that achieved target imaging and inhibition of the MDR1 gene in OVCAR8/ADR cancer cells and delivered DOX, integrating tumor diagnosis and treatment. Chen et al. [37] proposed an MB micelle flare (MBMF) for mRNA detection and gene therapy. MBMFs can be used for intracellular imaging of c-raf-1 mRNA (Fig. 5B).

4.2.4. *Polymer nanoparticles*

Recently, polymer nanoparticles have been extensively used for the delivery of living cells because of their advantageous biocompatibility, biodegradability, and nontoxicity [155–157]. Moreover, they have a stable structure and useful protective effect on delivery. Various biological molecules, such as proteins, nucleic acids, peptides, and phospholipids, can be chemically coupled or physically embedded in polymer nanoparticles owing to their different structures [158].

Poly (lactic-co-glycolic acid) (PLGA) is an extensively studied nanocarrier with a high transfection efficiency and low toxicity. Malik et al. [27] constructed a short cationic PNA probe delivered by PLGA-mediated cells to achieve specific targeting and inhibition of miR-155 in DLBCL cells.

Adinolfi et al. [52,159] designed core-shell polymethylmethacrylate nanoparticles (PMMA-NPs) as carriers for oligodeoxynucleotide MBs to target survivin mRNA in A549 cells for fluorescence imaging. The nanoparticles consisted of a hydrophobic PMMA core covalently functionalized with fluorescein, and an outer hydrophilic shell modified with a primary amine group and a quaternary ammonium salt. The PMMA-NPs exhibited high biocompatibility, extremely low cytotoxicity, and biological inertness along with low synthesis costs.

More importantly, Guan et al. constructed a fluorescent cocktail strategy to better distinguish tumors, inflammation, and normal cells [54]. They constructed two fluorescent probes (an H_2O_2 probe and a tumor-related mRNA probe) and formed nanoparticles in pure water with a pH-responsive amphiphilic polymer-wrapped probe (Fig. 5C). Normal and abnormal cells are distinguished using H_2O_2 probes. When the polymer enters a tumor or inflammatory cell, it is converted into a hydrophilic polymer because its pH is lower than that of pKa, and the nanoparticles decompose and release the DNA probe. The DNA probe prevents inherent interference based on the FRET principle using tumor-related survivin mRNA as the target to further distinguish tumor cells from inflammatory cells.

4.3. *Gold nanoparticles*

AuNPs are likely the most commonly used carriers for nucleic acid delivery in living cells. In 1996, Mirkin et al. reported spherical nucleic acids (SNAs) composed of a gold particle core and DNA shell [160]. SNAs have unique properties, including cellular uptake without a transfection reagent, enhanced binding affinity with the complementary sequence, minimal immunogenicity, and reduced nuclease degradation tendency [161].

Nanoflares were first developed in 2007 (Fig. 6A) [30]. Nanoflares are composed of a gold core, and their recognition chain contains approximately 20 base pairs that complement

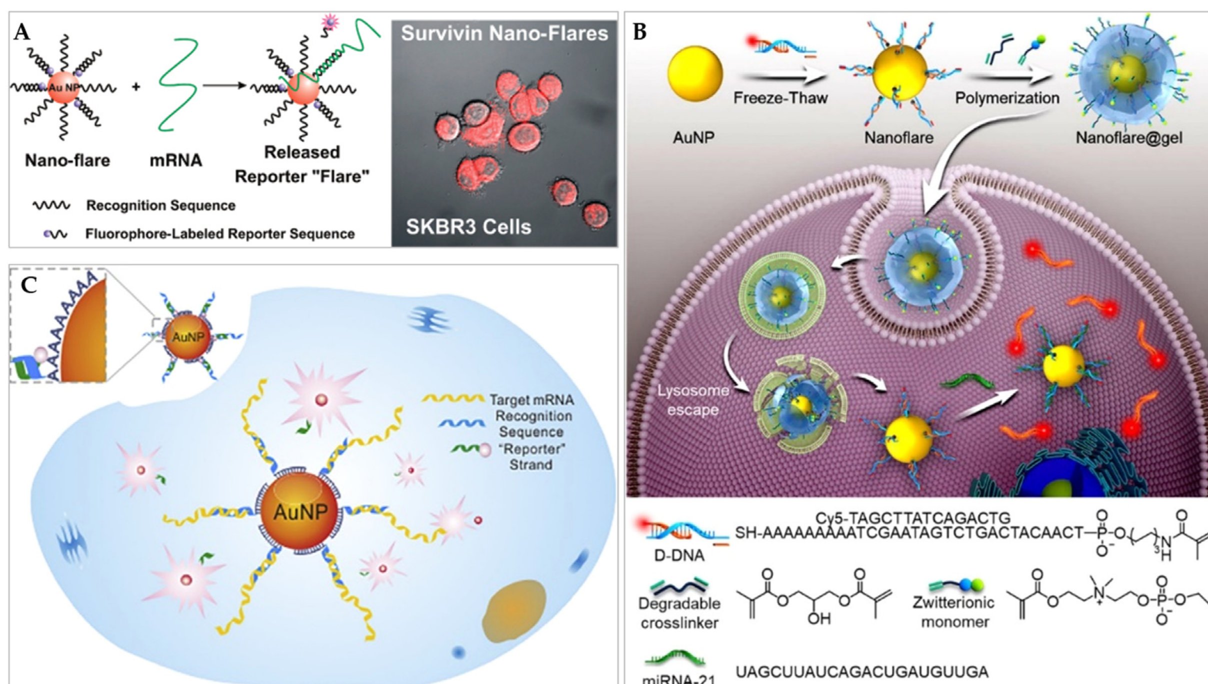

Fig. 6 – (A) Design and workflow of NanoFlares. Reproduced with permission [30]. Copyright 2007, American Chemical Society. (B) "Trojan Horse" protected nanoflare loaded nucleic acid probe to realize intracellular miR-21 imaging. Reproduced with permission [162]. Copyright 2022, American Chemical Society. (C) The design of FSNA probe. Reproduced with permission [36]. Copyright 2018, Elsevier.

the target mRNA. These recognition chains hybridize with flare chains labeled with short fluorophores (approximately eight complementary base pairs). The short distance between the AuNPs and fluorophores quenches the fluorescence signal. When the mRNA target is present and combines with the recognition chain, the flare chain is replaced, the fluorophore and AuNPs separate, and fluorescence is initiated. Nanoflares are the only probes that combine cell transfection, enzyme protection, and RNA detection and quantification.

Recently, research based on AuNPs has remained very popular. Nanoflares can enter many cell lines rapidly and in large quantities, but most are easily trapped in the body and transferred to lysozymes with high levels of glutathione (GSH) and nuclease. GSH replaces the DNA chain on the surface of AuNPs, and nuclease degrades the free DNA chain, generating false-positive signals. Wu et al. [162] developed a "Trojan Horse"-protected nanoflare (nanoflare@gels) to deliver nucleic acid probes into cancer cells. This "Trojan Horse" is a gel shell composed of zwitterionic CPs and acid degradation crosslinkers, which are polymerized *in situ* around the nanotorch nucleus. CPs can promote the nanoflare@gels in cells. After entering the cell, the "Trojan Horse" rapidly degrades in the lysosome, triggering a proton sponge effect, which results in the instantaneous release of the nanoflare into the cytoplasm. This approach achieved high-fidelity and quantitative targeted imaging of miR-21 in HeLa and MCF-7 cells and distinguished cell lines with different miR-21 expression levels (Fig. 6B).

Sun et al. [39] developed an LNA-functionalized gold nanoflare probe for mRNA in cancer cells, which enabled targeted imaging and the downregulation of the hTERT mRNA and transplanted telomerase activity, thus inhibiting cancer cell growth.

Kyriazi et al. designed an innovative AuNP dimer with multiple synergic functions within a cellular environment [163]. This dimer is formed by covalent linking using the copper-free click chemistry method. Moreover, it can be used to simultaneously detect two types of mRNA and has strong anti-nuclease activity. In this case, oligonucleotide chains targeting keratin 8 and vimentin mRNAs were present on the surface of the nanoparticles.

Fluorescent spherical nucleic acid (FSNA) probes based on AuNPs have been widely studied due to their good sensitivity, delivery efficiency, and low cytotoxicity. Zhu et al. [36] designed and synthesized a polyadenine (polyA)-mediated FSNA probe based on AuNPs. The length of the PolyA tail was used to precisely adjust the surface density of the nanoprobe. Compared to naked probe nanoparticles, it achieved a faster response speed and higher sensitivity in targeting intracellular mRNA (Fig. 6C).

4.4. *Graphene oxide*

GO is a two-dimensional (2D) nanomaterial that has been extensively used in the biomedical field owing to its desirable biocompatibility, cellular uptake, fluorescence quenching ability, and abundant modifiable sites [164,165]. As a delivery carrier, GO often binds nucleic acid probes via π–π interactions. Ratajczak et al. [55] developed an optical biosensor system based on the self-delivery of a GO-carrier

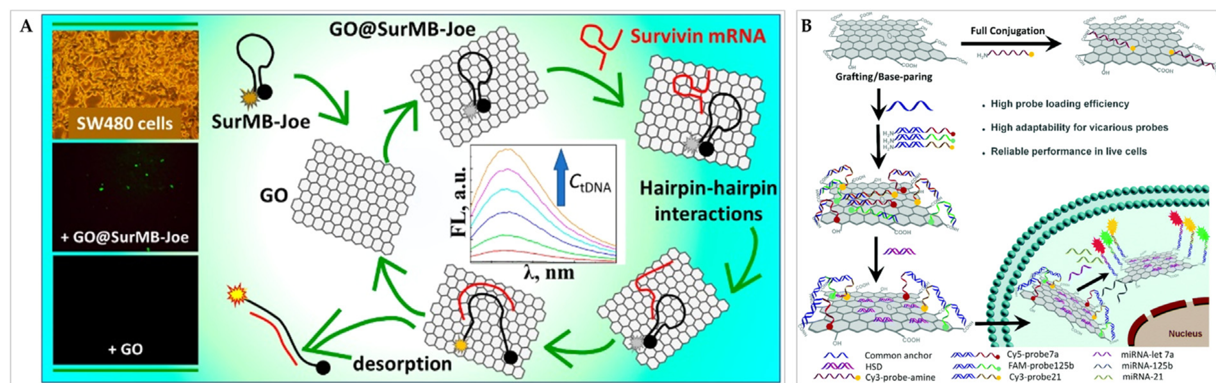

Fig. 7 – (A) An optical biosensor system based on the self-delivery of a GO carrier MB to CRC cells. Reproduced with permission [55]. Copyright 2018, the authors; (B) A GO-DNA nanodelivery system based on chemical coupling. Reproduced with permission [29]. Copyright 2018, Royal Society of Chemistry.

MB to colorectal cancer (CRC) cells, and the MB targeted survivin mRNA in cells and enabled fluorescence imaging (Fig. 7A).

However, this nanosystem, which is based on physical adsorption, is expected to present specificity problems when applied in cells or *in vivo*. Moreover, the competitive desorption effect of biological molecules (including proteins and nucleic acids) in cell culture media or blood will reduce delivery efficiency [166]. Yu et al. [29] fabricated a GO-DNA nanodelivery system based on chemical coupling and achieved simultaneous detection of multiple miRNAs in multiple cancer cells. Additionally, they developed a grafting/base-pairing method. The pre-annealed amino termination anchor sequences of different dye-labeled probes were grafted onto GO via N-hydroxysuccinimide/N-ethyl-N′-(3-(dimethylamino)propyl)carbodiimide (NHS/EDC) amide coupling (Fig. 7B). This nanosystem can be effectively delivered into cells, where is exhibits high stability and nuclease resistance, and enables detection of the dynamic expression of multiple miRNAs.

4.5. *Joint application of nanomaterials*

4.5.1. *PDA coating*

Polydopamine (PDA) is a biocompatible polymer. PDA coatings achieve extraordinary adhesion on almost any substrate surface, couple biomolecules via catecholamine and amino functional groups, and quench the fluorophores close to its surface.

Zheng et al. [62] developed multifunctional nucleic acid probe nanoparticles based on PDA-coated AuNPs to achieve self-delivery and target miR-21 in cancer cells. In the design of the nucleic acid probe, PDA is deposited on the surface of AuNPs by self-polymerization under weakly alkaline conditions to form PDA-functionalized AuNPs (AuNPs@PDA), which combine with a fluorescein isothiocyanate (FITC)-labeled double-stranded nucleic acid probe to quench its fluorescence. The resulting nanoprobe has good stability and high loading. After entering the cell, the targeted miR-21 achieves fluorescence imaging via the strand displacement reaction (Fig. 8A).

PDA-coated hairpin DNA with adsorbed fluorophore markers has been demonstrated to be useful for analyzing various microRNAs. Liu et al. [116] developed a flower-like nanocarrier based on polydopamine-modified AuNF (DA-AuNF). This multipronged vector can specifically target multiple signals for miRNA-21 in HeLa cells based on CHA, which improves the detection sensitivity of microRNAs (Fig. 8B).

Jiang et al. [34] developed a 2D black phosphorus (BP) nanosheet modified by calcium cation-doped PDA (BP@PDA). The incorporation of calcium cations and PDA coatings endows this nanoplatform with better stability, photothermal performance, and target binding ability than bare BP, reducing the occurrence of false positive signals. This nanoplatform achieves the specific recognition and fluorescence imaging of c-myc mRNA in HepG2. (Fig. 8C).

4.5.2. *GO and AuNPs*

Notably, the development of a new type of AuNP and GO nanocomposite probe (AuNP/GO probe) that simultaneously precursor and mature miRNAs in living cells and enables fluorescence imaging has been reported [63]. The AuNPs were used to image the precursor miR21, while the GO probe used HCR technology to target mature miRNA for imaging, including miR-21, let7, and their corresponding precursors (Fig. 8D).

4.5.3. *DNA nanostructures and internalized aptamers*

Dong et al. developed a DNA mini-hexahedron (DMH) that achieved intracellular delivery, release, and targeting of multiple miRNAs in the cell [58]. DMH self-assembles from six single-stranded oligonucleotide chains, one containing the AS1411 sequence for specific uptake and two fluorescent-labeled detection probes encapsulated in two DMH edges with quenching groups by partial complementary hybridization. This study compared the delivery ability of DMH with and without AS1411, and the delivery ability of DMH with the AS1411 sequence to cancer and normal cells. Based on these two comparisons, it was confirmed that a simple DMH structure can achieve self-delivery into cells, while the AS1411 aptamer has effective cell-specific targeting ability,

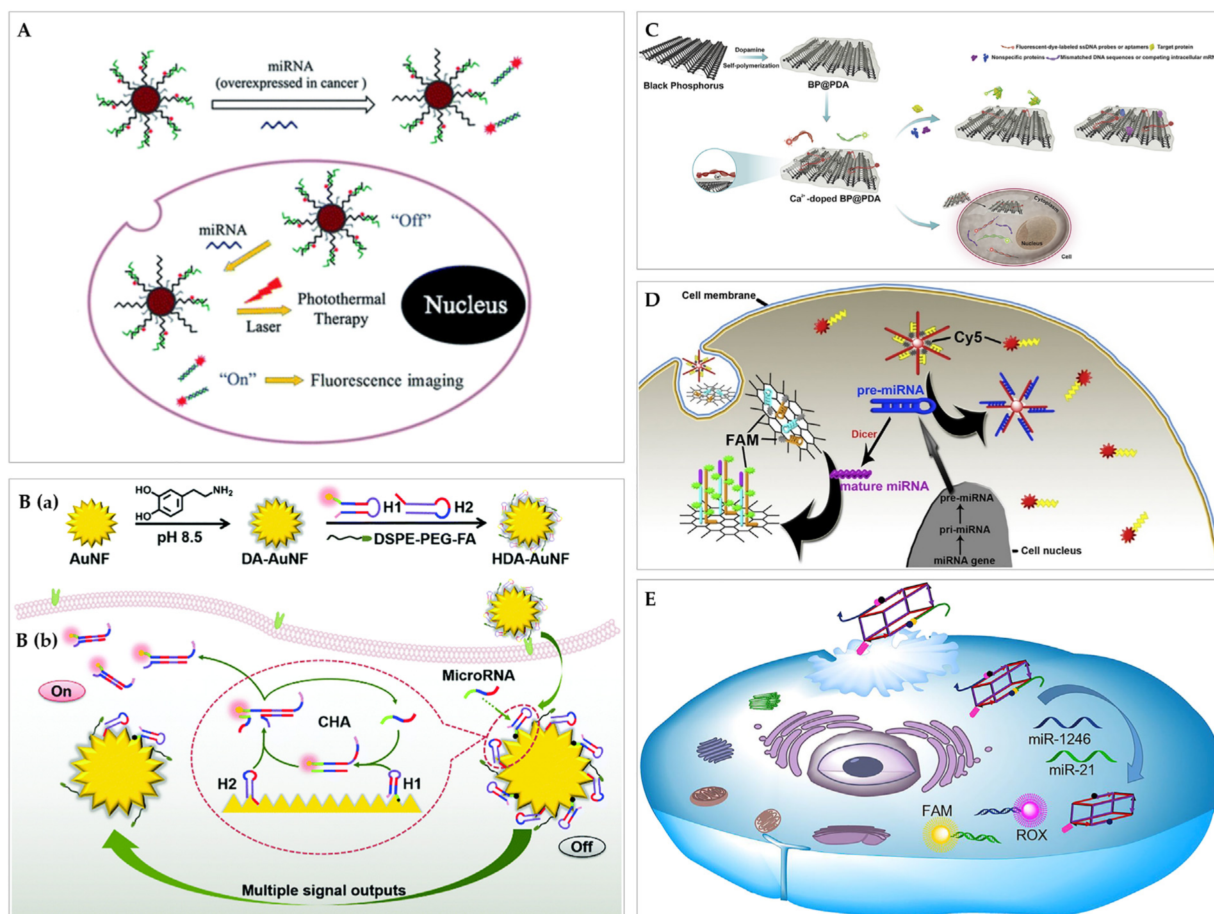

Fig. 8 – (A) Multifunctional nanoparticles formed by AuNP coated with PDA [62]. (B) Schematic illustration of assembly and imaging of DA-AUNF nanoplatform. Reproduced with permission [116]. Copyright 2018, Royal Society of Chemistry. (C) PDA-modified 2D black phosphorus nanosheet carries nucleic acid probe to realize cell self-delivery and imaging mRNA. Reproduced with permission [34]. Copyright 2020, Elsevier. (D) AuNP/GO probe realizes self-delivery of living cells and imaging miRNA. Reproduced with permission [63]. Copyright 2018, Elsevier. (E) DNA mini-hexahedron and AS1411 sequence jointly deliver probes into cells to target miRNA. Reproduced with permission [58]. Copyright 2018, American Chemical Society.

and the combination of the two components further enhances probe self-delivery via receptor-targeted enrichment. After entering the cell, a single-chain suspension is used to achieve target recognition by shifting the probe from the edge and generating the corresponding fluorescence for detection. The nanostructure achieved the simultaneous detection of miRNA-1246 and miRNA-21, and it exhibited good nuclease resistance (Fig. 8E).

5. Conclusion and outlook

Recently, the early diagnosis and personalized treatment of cancer have become a focus of research. The design and discussion of nucleic acid probes targeting tumor markers have been ongoing for decades. Nanomaterial-loaded nucleic acid probes have been used to develop a high-quality diagnostic platform for nucleic acid targets in living cells with appropriate biocompatibility, nuclease resistance, specificity, and high signal transmission ability. However, there are still many challenges to overcome before these probes can be used in the clinical environment. Based on this review, we propose guidelines to optimize and analyze them individually.

(1) **Target selection:** For the past ten years, nucleic acid probes have been expected to be very useful for tumor biomarker detection and to facilitate early cancer diagnosis. Related designs and research have emerged, resulting in endless opportunities. Several studies have focused on probe design to enhance stability, optimize cell delivery, reduce background signals, and improve sensitivity, which are crucial. However, researchers often focus on the selection of specific targets, often mRNA or miRNA markers, such as survivin mRNA and miR-21, which have been extensively investigated. These tumor markers, which have been demonstrated by many studies to be highly expressed in various cancers, can facilitate early cancer diagnosis; however, there is more innovative research to be conducted on the selection of targeted nucleic acid chains. Different tumors, different

subtypes of the same tumor, and even different development stages of the same type of tumor have specific DNA or RNA, and there have been many reports on probe designs that can simultaneously identify multiple targets. The application of this information for developing the targeting units of probes, designing more cancer-specific and personalized targets, and improving cancer recognition are prospective research areas for nucleic acid probes that are worth considering and exploring in the future. Moreover, this approach could significantly expand the scope of applications for living cell probes.

(2) **Joint application of nanomaterials:** Regarding the live cell delivery of probes, the excellent performance of nanomaterials has attracted considerable attention. Most diagnostic probes use a nanoplatform to achieve cell internalization. Different nanomaterials have their own advantages and disadvantages, which are not discussed in detail here. Recently, some studies have combined two or more nanomaterials to construct diagnostic platforms with improved functions, and this approach is worthy of recognition and further development. The joint construction and application of the advantages of different nanomaterials will compensate for the shortcomings of single materials to develop a diagnostic platform with strong specificity, high stability, high biocompatibility, high sensitivity, and low toxicity, and to achieve more specific cancer-targeting strategies.

(3) **Bioimaging:** The complex cellular physiological environment makes it challenging to achieve satisfactory specificity, sensitivity, and signal duration of imaging, which requires an accurate distinction between background and reporting signals, the prevention of false-positive signals, and high resistance to nucleases. Fluorescence imaging is the simplest and the most commonly used imaging technique. In addition, signal amplification strategies can enable the effective measurement of low-abundance targets. The design of fluorophores with strong photostability, photobleaching resistance, acceptable biocompatibility, and low toxicity remains an active research field. However, some of the major problems associated with fluorescence imaging are its poor quantification and limited sensitivity. Because many RNAs exist in both normal and cancer cells, improving the quantitative *in situ* monitoring of target expression content is worth further consideration.

(4) **More clinical research:** Living cells have a complex physiological environment, but their components *in vitro* remain relatively simple compared to the situation *in vivo*. Probes that can be applied to cells grown in culture media are not necessarily applicable to the internal environment. First, it is necessary to determine whether the target site is available after intravenous injection, and whether the probe can escape the immune system and achieve tolerance in the blood environment. Second, it is necessary to consider the configuration of imaging equipment and the safety of imaging. Third, the biological toxicity and metabolic pathway of the probe must be considered. Although probes are designed to have low toxicity, enrichment effects are inevitable. A notable and feasible research prospect is the design of biodegradable or targeted probes. Here, we have summarized the recent applications of probes targeting nucleic acid molecules combined with nanomaterials in cancer cells and detailed the design of the probe based on the target, delivery, and imaging units. Subsequently, we focus on a nanomaterial-mediated probe diagnostic platform that can be delivered into the cell, classify such probes according to the material properties and describe representative examples. Currently, nucleic acid probes for early cancer imaging in living cells still encounter numerous challenges; however, their potential is limitless.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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