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Review Article

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Applications of DNA tetrahedron nanostructure in cancer diagnosis and anticancer drugs delivery

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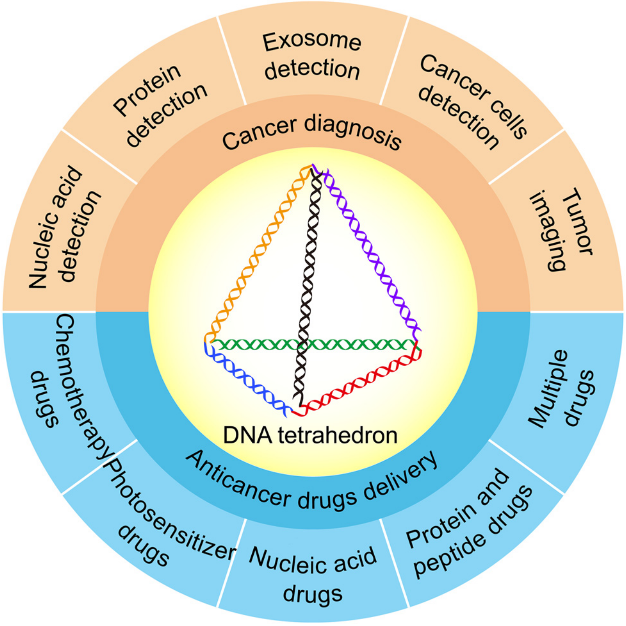
Abstract: DNA nanostructures constructed under the guidance of DNA nanotechnology have developed rapidly for the last two decades, standing at the forefront in the biomedical field. Among them, DNA tetrahedron nanostructure (DTN) has emerged as one of the most representative DNA nanostructures. DTN was easily formed by one-step annealing of four single-stranded DNA. Due to its unique advantages such as simple and stable structural composition, high synthesis efficiency, uniform nanometer size, high programmability, and good biocompatibility, DTN has been widely used in biological detection, biological imaging, drug delivery, and other fields, and shows a great potential. Especially in the detection of cancer-related biomarkers and the delivery of anticancer drugs, nano-platforms based on DTN has achieved great success. In this review, we focus on the applications of DTN in cancer diagnosis and therapy, as well as the challenges and prospects.

Keywords: DNA tetrahedron, cancer diagnosis, anticancer drugs delivery



Graphical abstract

DNA tetrahedron-based cancer diagnosis and anticancer drugs delivery

1 Introduction

Cancer is the second leading cause of human death worldwide (after cardiovascular disease) and increasingly affects people's health and life expectancy [1–3]. Therefore, early diagnosis and timely treatment have become extremely essential to improve the prognosis of cancer patients. Traditional diagnostic techniques have relied on a combination of tumor imaging and histopathological examinations, including molecular testing, imaging, and immunohistochemistry, with an inherent limitation of low diagnostic accuracy [4]. Furthermore, these techniques are invasive and could have some adverse consequences and risks of trauma. To address these issues, a number of non-invasive and sensitive methods have been developed, including liquid biopsy, which could be detected at an early stage by sampling circulating biomarkers [5–8]. Cancer biomarkers (DNA, RNA, proteins, lipids, metabolites, *etc.*) could intuitively quantify specific cancer states. Detection of

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these biomarkers is of great significance for identifying different clinical stages of cancer patients and formulating precise treatment strategies. However, the detection sensitivity using cancer biomarkers is still a challenge.

Combination of surgical resection with chemotherapy and/or radiation therapy remains the mainstream treatment for human cancer. However, chemotherapy drugs have toxic side effects on normal cells, when they systemically kill tumor cells [9]. In order to more accurately target tumors and reduce toxic side effects, anticancer drug delivery systems have been intensely investigated [10,11]. A variety of nanomaterials have been used for anticancer drug delivery, including high-molecular polymer nanoparticles [12], lipid nanomaterials [13,14], nanoemulsions [15], dendrimers [16], carbon nanomaterials [17], metals, and magnetic nanomaterials [18,19]. These carriers could effectively improve drug solubility, entrapment efficiency, half-life, and drug uptake by cancer cells. However, they still have some intrinsic drawbacks involving potential cytotoxicity [20–22] and difficulty in degrading *in vivo* [23,24]. These safety concerns limit their use in the body.

In recent years, the rapid development of DNA nanotechnology has provided new ideas for the diagnosis and treatment of cancer. In 1980s, Seeman pioneered the field of DNA nanotechnology [25–29], using DNA to self-assemble nanostructures, which changed the traditional role of DNA. Since then, DNA nanotechnology has developed rapidly, with investigators building 2D and 3D structures of various sizes or shapes [30–36]. For the past few decades, DNA nanostructures in combination with functional molecules have been widely used in chemical biology [37–40], analytical chemistry [41–43], medicine [44–48], and material science [49–51]. Among these nanostructures, DNA tetrahedron nanostructure (DTN) is a classic and popular 3D framework, originally reported by Turberfield's group [52,53]. Four DNA oligonucleotide strands are mixed in a buffer in equimolar ratio, and could be efficiently assembled into DTN in a few minutes by only one step of annealing. In addition, Mao *et al.* invented the double-bundle DNA tetrahedron [54]. The DTNs have been shown to possess several advantages, including natural biocompatibility and biodegradability, structural stability, unparalleled programmability, functional diversity, and ease of cellular internalization. DNA tetrahedron have been widely used in biomedical field [55–61]. The DTN has emerged as a promising diagnostic and therapeutic nanoplatform, showing a great potential in both early diagnosis and treatment of cancer.

In this review, we will summarize recent progress in DTN in building functional and smart nanoplatforms for cancer diagnosis and therapy. We will also introduce the application of DTN in cancer diagnosis from the detection of small molecules, proteins, tumor cells and their applications in imaging. Furthermore, we will review the application of DTN in the drug delivery, including chemotherapeutic agents, photosensitizers, CpG oligonucleotides, aptamers, siRNA, *etc.* Finally, we will discuss future challenges and give some thoughts on the development prospects of cancer diagnosis and delivery systems based on DTN.

2 Cancer diagnosis based on DTN

The detection using biomarkers plays an important role in the diagnosis and treatment of diseases. In the past two decades, DTN has been widely used in cancer detection by chemical modification or ingenious sequence design, which could be employed to construct efficient analytical platforms for biomarkers.

2.1 Nucleic acid detection

2.1.1 MicroRNAs (miRNAs)

MiRNAs are important biomarkers for the early diagnosis of cancer [62–64], but their detection sensitivity remains challenging due to their short length and low abundance [65].

In 2010, Fan *et al.* developed the first electrochemical DNA (E-DNA) sensor platform based on 3D DNA nanostructure through DTN in order to improve the sensitivity and specificity of detection [66]. This tetrahedron-structured probe (TSP) has thiol groups at three vertices, which could easily and firmly anchor the DNA tetrahedron to the Au surface, and a ssDNA probe strand extends from the other vertex. Importantly, this TSP could be held on the Au surface in a highly ordered upright orientation only by its rigidity. Compared with ssDNA probe-based sensors, this TSP-based DNA sensor has 25–100-fold increase in single-base mismatches recognition factor. In 2012 , by employing this TSP-based platform, they developed an ultrasensitive electrochemical miRNA sensor (EMRS) for reliable quantitative detection of attomolar (<1,000 copies) miRNAs with high sequence specificity [67]. EMRS exhibits a high single-base mismatch discrimination ability and effectively discriminates closely related sequences in the human let-7 sequence family. More importantly, EMRS showed excellent performance in detecting the expression level of miR-21 in esophageal squamous cell carcinoma clinical samples. Fan *et al.* further constructed DNA tetrahedron-based microarrays by covalently coupling (amine-aldehyde interaction) DNA tetrahedron onto glass substrates [68]. Good log-linear range for miRNA (let-7a) detection from 10 fM to 100 pM, limit of detection (LOD) was 10 fM. In addition, Huang *et al.* designed a pyramid-shaped probe using DNA tetrahedron as a scaffold with an overhanging stem-loop structure [69]. The stem-loop structure has two regions, one of which hybridizes to the miRNA target and the other attaches to the signaling probe. Finally, the avidin-modified horse radish peroxidase is attached to generate the enzymatic signal. Compared to the traditional sandwich assay, this design is suitable to detect the short miRNA targets, for example it could detect as low as 1 fM target of miRNA-141.

To improve the detection sensitivity, Zuo's group combined hybridization chain reaction (HCR) [70,71] amplification with TSP-based platform [72]. Through the synergetic effect of tetrahedron nanostructure and HCR, the detection sensitivity is greatly improved to 100 aM for DNA detection and 10 aM for miRNA detection. Another group reported an ultrasensitive miRNA detection method based on analyte-triggered gold nanoparticle (AuNP) localization and HCR double amplification [73], which allows us to detect target miRNA at limit of 2 aM. In addition, Yang and colleagues fabricated an electrochemiluminescence (ECL)-electrochemical (EC) hybrid biosensor for ultrasensitive detection of miRNA-133a [74]. Two $[Ru(bpy)_3]^{2+}$-labeled hairpins were used as ECL probes and HCR fuel strands. The sensor could achieve ultrasensitive detection of miRNA-133a with a detection limit of 12.17 aM.

Amplification strategies using certain enzymes could also improve detection sensitivity. For example, Miao *et al.* developed a DTN-based biosensor that combines strand displacement and miRNA recycling [75]. The signal probe was labeled with silver nanoparticle (AgNP) and using strand displacement polymerization, more AgNPs were fabricated on the electrode surface in the presence of the target miRNA and Klenow fragment, which provides intensive EC signals from solid-state Ag/AgCl reaction. The biosensor exhibits high sensitivity with a LOD as low as 0.4 fM.

Rolling circle amplification (RCA) [76,77] is an enzyme-based isothermal amplification technique that has been used in numerous biosensors, allowing the detection of miRNAs with high specificity and sensitivity. Miao *et al.* constructed an ultrasensitive analysis platform for miRNA detection using RCA strategy [78]. Probe DTNs on the electrode surface are used to recognize the target miRNA, and subsequent primer probes trigger RCA. AuNP-labeled signal probes that hybridize to RCA products provide significant EC signals with LOD as low as 50 aM.

Several other strategies have also been used to improve the specificity and sensitivity of detection of miRNA. Chai *et al.* reported an EC biosensor that could simultaneously detect two miRNAs [79] (Figure 1a). On the basis of the original TSP, a novel multifunctional DNA circle capture probe was added, which could detect miRNA-21 and miRNA-155 at the same time. This biosensor showed a wide linearity ranging from 0.1 fM to 10 nM with LOD of 18.9 aM for miRNA-21 and 39.6 aM for miRNA-155. In addition, Song *et al.* designed a novel carbon-based EC biosensor by combining TSP and screen-printed carbon electrodes [80]. The three vertices of the TSP contain three amine groups, which were immobilized on the carbon working electrode by the reaction of amino and carboxyl groups, and the other vertex contains an extended DNA probe. The LOD for miRNA-141 was 10 aM. Moreover, Yang *et al.* developed a novel EC DTN sensor using an inverted DTN as a functional probe, which was coupled to the gold electrode surface only through the thiol group at one vertex [81]. The LOD was as low as 1 fM. Mu *et al.* reported a proposed plasmonic biosensor based on DTN carriers and surface-enhanced infrared absorption (SEIRA) device for label-free and ultrasensitive detection of miR-155 [82]. This biosensor uses metamaterial perfect absorber forming Fabry–Perot optical cavity to provide up to 1,000 fold near field intensity enhancement over the miR-155 fingerprint spectral bands, with a high sensitivity of 1.162% pm^{-1} and excellent LOD of 100×10^{-15} m. Importantly, they demonstrated that using DTN as a scaffold could greatly improve the LOD of SEIRA-based biosensor, which is about 5,000 times lower than using ssDNA as probes and lower than fluorescence detection methods about 100 times. In order to improve the signal-to-noise ratio of the detection, Yang *et al.* reported a DNA tetrahedron reversion signal-gain strategy *via* background-to-signal conversion for ultrasensitive and highly specific electrical detection of miRNAs in blood [83]. By using an enzymatically catalyzed deposition model of a DNA-targeting conducting molecules (polyaniline; PANI), they observed the highest signal contribution per unit area for highly charged 3D DNA tetrahedron probe (DTP), relative to 2D polyA probe and the lowest 1D ssDNA probe. Using this efficient signal transduction design, miRNAs detection increases sensitivity up to 0.29 fM. More importantly, this high-performance sensor allows generalized sandwich detection of tumor-associated miRNAs in complex biological matrices and further differentiates tumor patients from normal individuals. Miao *et al.* reported a nanomachine that performs DNA walking on DTNs for ultra-sensitive detection of miRNAs [84]. The target miRNA triggered the assembly of triplex-forming oligonucleotide, a DNA walker strand that moves around DNA tetrahedron, and cleaves track strands to generate an EC signal, which was amplified over multiple cycles with an LOD as low as 4.9 aM.

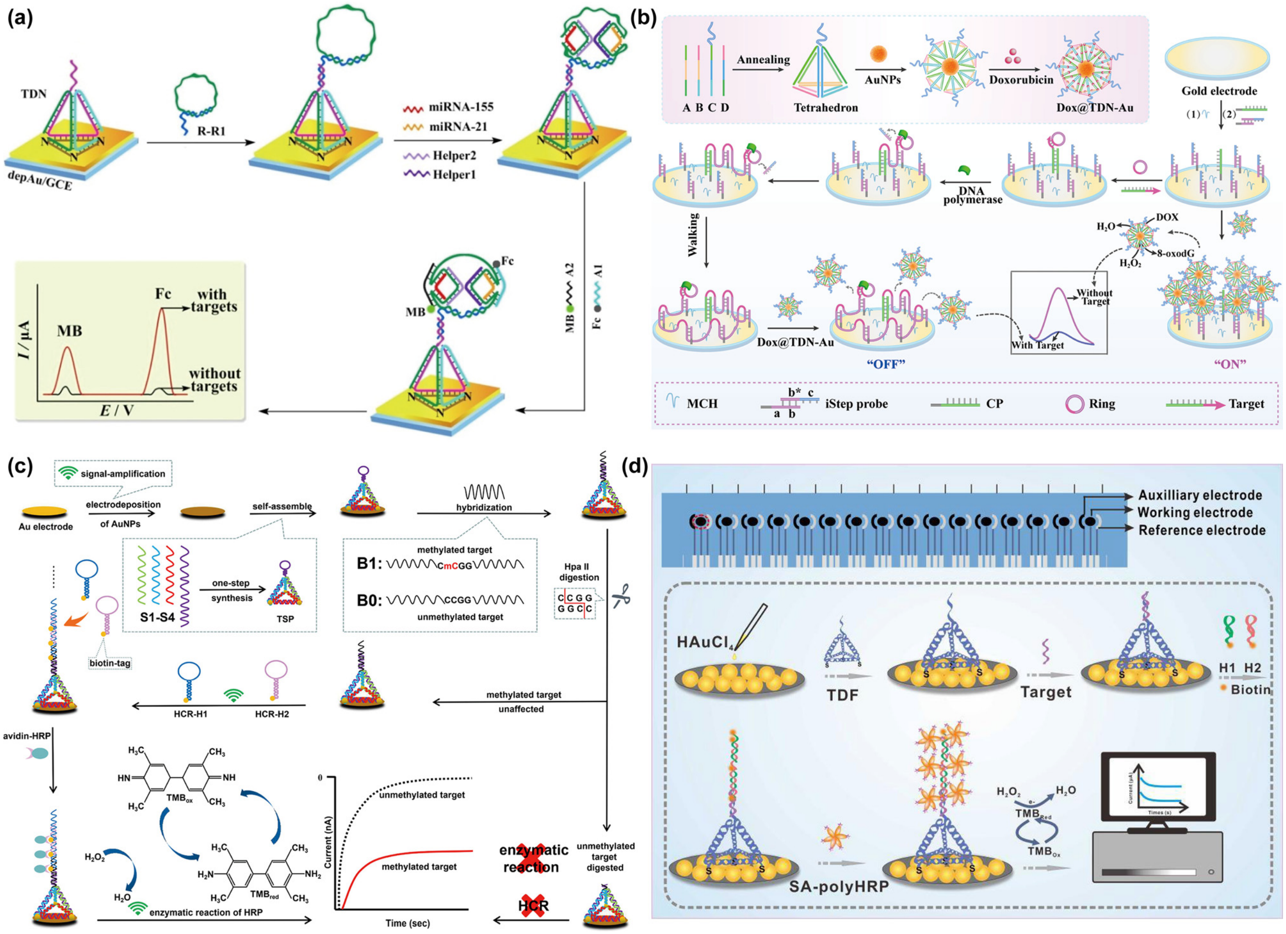

Figure 1: DNA tetrahedron-based EC biosensors for nucleic acid biomarkers detection. (a) Novel DNA circle capture probe for simultaneous detection of two miRNAs [79] Copyright 2020, Elsevier. (b) Target-driven rolling walker based EC biosensor for ctDNA detection [87] Copyright 2020, Elsevier. (c) HCR coupled with DNA tetrahedron for methylated DNA detection [96] Copyright 2019, American Chemical Society. (d) DNA tetrahedron-modified AuNPs combined with HCR signal amplification for cfDNA detection [101] Copyright 2022, MDPI.

2.1.2 Circulating tumor DNA (ctDNA)

ctDNA is a single or double strand gene fragments derived from tumor or circulating tumor cells (CTCs) and subsequently is released into peripheral blood [85]. Studies have shown that ctDNA is a potential tumor biomarker [86], which has received increasing attention in cancer assessment and is considered as a promising clinical detection substance. The conventional methods of ctDNA detection are polymerase chain reaction and DNA sequencing, but these detection techniques could be interfered by chemical substances, resulting in inaccurate results, long detection time, cumbersome process, and high cost. Therefore, the development of inexpensive, rapid, and highly sensitive ctDNA detection approaches remains a major challenge.

In order to solve the above problems, several DNA tetrahedron-based ctDNA detection platforms have been developed. For example, in 2020, Chen and colleagues reported a high-power rolling walker for sensitive ctDNA detection based on RCA and tagging of doxorubicin (DOX) @DNA tetrahedron functionalized AuNPs [87] (Figure 1b). This biosensor has high sensitivity for target detection with LOD as low as 0.29 fM. In the same year, Zhang *et al.* fabricated a convenient and rapid EC platform based on screen-printed electrodes decorated by DTNs [88]. This method accurately detected two major epidermal growth factor receptor (EGFR) mutations (19del, L858R) at around 30 pg ctDNA in plasma. In addition, they accurately genotyped the EGFR mutation profiles in 13 patients with non-small cell lung carcinoma (NSCLC), and the results were in good agreement with those from commercial genetic testing. In 2022, Liu's group developed an EC biosensor that could rapidly detect ctDNA using DTNs and red blood cells [89]. Red blood cells and DNA tetrahedrons could simultaneously capture ctDNA which forms a sandwich structure through complementary base pairing. Methylene blue (MB) was used as the signal probe with LOD of 0.66 fM.

2.1.3 Methylated DNA

DNA methylation is an important epigenetic modification that is widespread in mammals [90]. Aberrant DNA methylation is directly linked to cancer pathogenesis [91,92]. Many studies have shown that methylated DNA could be released into the circulation at different stages of the tumor, and this cell-free DNA could be used as a biomarker for cancer detection [92,93]. However, tumor-associated methylated DNA constitutes only a small fraction of the total DNA in complex clinical samples such as plasma, and its accurate and rapid detection remain a major challenge.

Wang *et al.* reported a DTN-based biosensor for the detection of circulating methylated DNA using a sequential discrimination–amplification strategy [94]. This biosensor integrates dual sequence recognition process and cascades signal amplification process to identify as few as one methylated DNA molecule in the presence of a 1,000-fold excess of unmethylated alleles, showing high sensitivity. Furthermore, they achieved successful detection of tumor-associated DNA methylation in 200 μL of plasma from lung cancer patients. Liu *et al.* developed a highly sensitive E-DNA biosensor for the detection of methylated DNA based on DTN using the RCA strategy [95]. Due to the enhanced EC signal generated by DNAzymes with RCA signal amplification, this biosensor could sensitively detect target methylated DNA, the LOD reaches 0.1 fM with the detection range of 10^{-15}–10^{-9} M. In 2019, Zheng *et al.* constructed an EC biosensor for methylated DNA detection based on DNA TSP [96] (Figure 1c). The tetrahedral overhanging stem-loop structure could hybridize to a target sequence with a 5′-CCGG-3′ palindrome followed by a methylation-specific restriction endonuclease (*Hpa*II) cleavage procedure. Methylated DNA sequence could block cleavage, and unmethylated DNA sequence was cleared by cleavage. Through HCR and horseradish peroxidase (HRP) amplification, a distinguishable signal could finally be obtained, which sensitively detects the different methylation states of the target sequence. This design showed a broad dynamic range from 1 aM to 1 pM with LOD lower than 1 aM.

2.1.4 Cell-free DNA (cfDNA)

Tumor-associated cfDNA [97] is a dynamic biomarker derived from different release mechanisms, such as necrosis, apoptosis, and active release from cancer cells, *etc.* cfDNA could be used for early diagnosis of cancer [98,99], but its content in blood and other body fluids is extremely low, so it is important to develop a specific and sensitive analytical platform for the detection of cfDNA.

Zuo *et al.* used DNA tetrahedron as valence-controlled signal amplifiers with high modularity for ultrasensitive detection of tumor-associated cfDNA (ALU115) [100]. This method enhanced sensitivity by 3–5 orders of magnitude and improved dynamic range. In addition, Mi's group reported an ultrasensitive cfDNA EC biosensor based on DNA tetrahedron-modified AuNPs combined with HCR signal amplification [101] (Figure 1d). They programmed three sizes of DNA tetrahedron (TDF-26, TDF-16, TDF-7) to detect representative cfDNA (breast cancer susceptibility gene 1, BRCA-1) closely associated with breast cancer. TDF-26 achieved an optimal LOD of 1 aM with a linear range of 1 aM to 1 pM. Importantly, they also detected BRCA-1 in minimal serum samples, demonstrating the potential use of this sensor in clinical research.

2.2 Protein detection

Cancer-related protein biomarkers are of great significance in the early detection and clinical treatment of cancer [102]. DNA tetrahedron-based biosensors are also widely used to detect protein biomarkers.

Prostate cancer (Pca) is one of the most common malignant tumors in men worldwide [103]. Prostate-specific antigen (PSA) is a gold standard serum biomarker for early diagnosis of Pca [104]. Zuo *et al.* developed an EC immunobiosensor for PSA detection using TSPs conjugated antibodies [105]. They found that the detection sensitivity was largely dependent on the nanoscale spacing of the immobilized antibodies, and used AuNPs to further amplify the sensing signal, achieved an extremely low LOD of 1 pg/mL. Fan *et al.* fabricated DNA tetrahedron-based multifunctional detection microarrays by covalently coupling DNA tetrahedron onto glass substrates [68]. The LOD of the PSA was 40 pg/mL. Furthermore, 1.84 ng/mL PSA and 2.95 ng/mL α-fetoprotein were clearly detected in human serum. Miao *et al.* fabricated a sensitive PSA detection method based on DNA tetrahedron and exonuclease T (Exo T)-aided cyclic cleavage [106]. This method was linear from 0.5 pg/mL to 50 ng/mL, with a LOD as low as 0.15 pg/mL, and successfully determined PSA in human serum samples. Subsequently, based on the original method, they constructed an EC aptamer sensor for PSA detection using AgNPs as signal reporters [107] (Figure 2a). This sensor

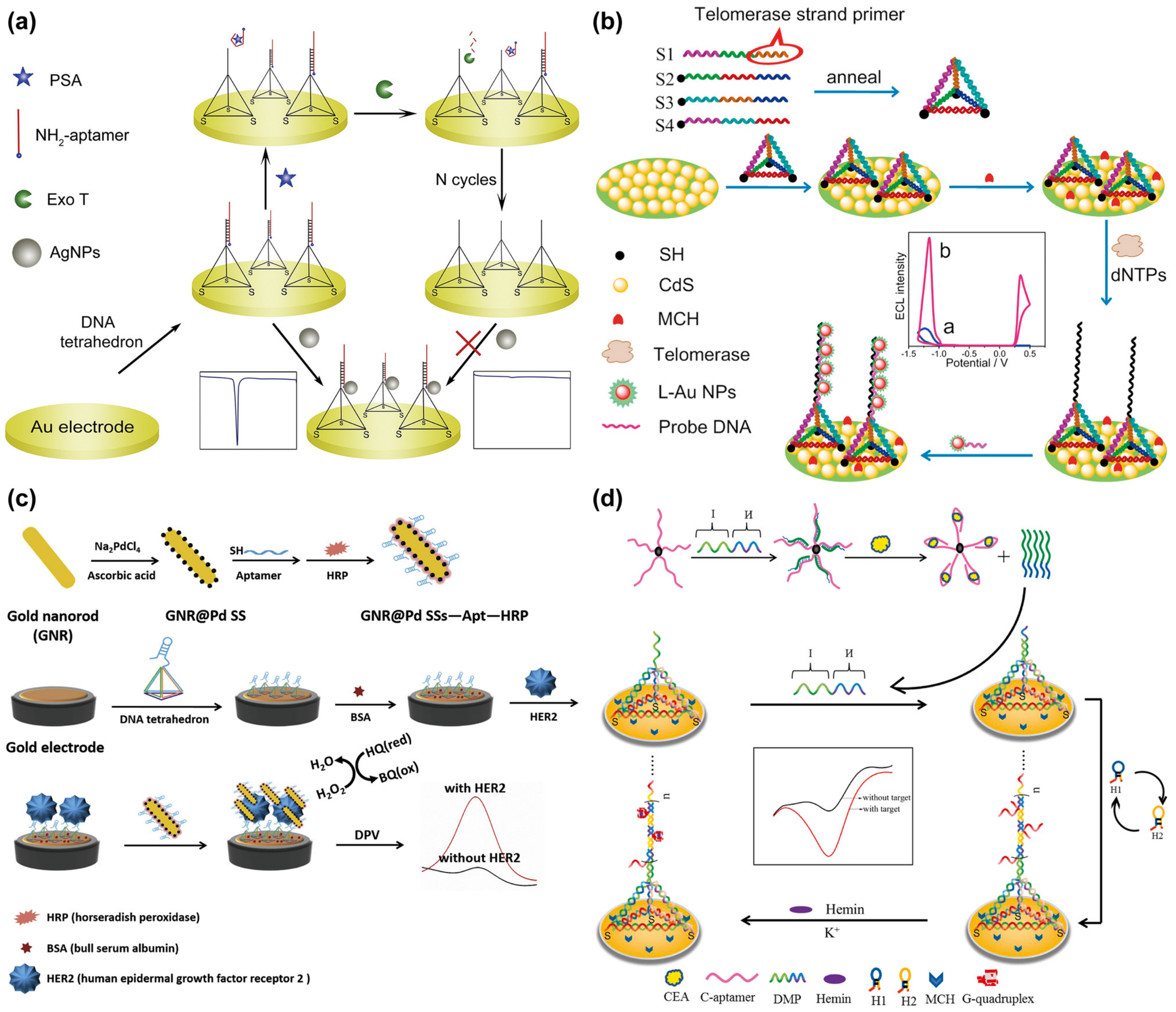

Figure 2: DNA tetrahedron-based EC biosensors for protein biomarkers detection. (a) DNA tetrahedron-aptamer probe combined with Exo T for PSA detection [107] Copyright 2018, Elsevier. (b) A SPR-based ECL sensor for telomerase detection [114] Copyright 2017, Elsevier. (c) Three-dimensional DNA tetrahedron biosensor for HER2 effective detection [117] Copyright 2019, Elsevier. (d) DTPs combined with HCR strategy for CEA detection [121] Copyright 2019, Elsevier.

exhibits high responsive sensitivity with a linear range of 1 pg/mL to 160 ng/mL and LOD was further down to 0.11 pg/mL. In 2021, Dai *et al.* functionalized molybdenum disulfide (MoS_2) field-effect transistor using DNA tetrahedron and biotin-streptavidin (B-SA) conjugation and constructed an environmentally friendly biosensor for the specific detection of PSA [108]. The LOD of PSA in clinically relevant serum samples was 1 fg/mL. In the same year, Mi *et al.* developed a novel microfluidic–electrochemical (μFEC) system based on microfluidic chips and DNA tetrahedron biosensor for rapid and automated detection of PSA [109]. After optimizing the condition, they obtained a nice linear range (1–100 ng/mL) and detection limit (0.2 ng/mL) in less than 25 min. Furthermore, the system successfully detected PSA in real clinical samples.

Telomerase has an important role in tumorigenesis because it enables cancer cells to bypass the telomere-dependent cell death pathway and divide indefinitely [110]. Therefore, accurate detection of telomerase activity is of great significance for the early diagnosis and treatment of cancer [111]. Liu and colleagues, reported an EC telomerase biosensor based on a spired DNA tetrahedron telomere strand primer [112]. They added a double-stranded segment between the DNA tetrahedron and the telomere strand primer, which resulted in a higher signal-to-noise ratio compared to conventional DNA TSP. The LOD reached 10 HeLa cells. Liu *et al.* invented a label-free ultrasensitive telomerase detection method *via* multiple telomeric hemin/G-quadruplex triggered PANI deposition and DNA tetrahedron structure (DTS) signal modulation [113]. By optimizing the DTS size, they found that DTS (37 base pairs per side) had the best sensitivity for telomerase detection. The detection dynamic range of this strategy was 5–5,000 HeLa cells, and the LOD was 1 HeLa cell. Xu *et al.* reported a surface plasmon resonance (SPR)-based ECL sensor for the detection of telomerase [114] (Figure 2b), using DNA tetrahedron scaffolds as connectors between quantum dots (QDs) and metal nanoparticles. This sensor could be used to detect telomerase activity in different cancer cells. Shen *et al.* reported a method based on DNA tetrahedron nanomachine with doxorubicin-spherical nucleic acid tags for sensitive determination of telomerase activity [115]. The analytical range extends from 10 to 1.0×10^4 HeLa cells/mL with a lower LOD of 2 cells/mL. In addition, they assessed the differences in telomerase activity between different types of cancer cells. Importantly, the sensing system was also used to detect cancer cell telomerase activity in accumulated normal cells with satisfactory results. Li *et al.* designed a tetrahedral DNA nanostructure with hairpin-shaped DNA probes rich in cytosine bases (for the synthesis of silver nanoclusters) at four vertices for telomerase detection [116]. The introduction of telomerase could quickly “turn on” the fluorescence of silver nanoclusters to achieve accurate detection of telomerase activity with an LOD as low as 1 cell.

Several other cancer-related protein biomarkers were also sensitively detected by DNA tetrahedron-based biosensors. Zhou *et al.* developed a DTN biosensor for sensitive detection of human EGFR2(or HER2) [117] (Figure 2c). The biosensor used the bioconjugate of gold nanorod@Pd superstructures–aptamer–horseradish peroxidase as the signal probe to realize signal amplification. The DNA tetrahedron-HER2-nanoprobes sandwich-type structure was established on the surface of the gold electrode surface by virtue of the aptamer extending from the DNA tetrahedron to recognize and capture HER2. LOD of HER2 was as low as 0.15 ng/mL with a wide range of linearity from 10 to 200 ng/mL. Chen *et al.* constructed a sandwich-type EC aptasensor to detect HER2 by combination of DTN-aptamer as recognition probes and flower-like nanozyme/HRP, which was used as signal probe [118]. A broad linear range was from 0.1 to 100 ng/mL and the LOD was 0.08 ng/mL. By combing the advantages of the TSP platform and DNA-bridged antibody immobilization, a highly sensitive and regenerative EC immunosensor was constructed for the detection of tumor-necrosis-factor alpha (TNF-α) [119]. The detection range of this sensor was from 100 pg/mL to 5 ng/mL. Ding *et al.* reported a graphene-DNA tetrahedron-AuNP-modified gold disk electrode for highly sensitive detection of dihydronicotinamide adenine dinucleotide (NADH) [120]. LOD of NADH was 1 fM. Ye’s group reported a label-free and double recognition–amplification strategy for carcinoembryonic antigen (CEA) detection [121] (Figure 2d). Another strategy was based on a newly designed dual-function messenger probe combined with DTPs and HCR, this method enabled the detection of CEA at low concentration with LOD as 18.2 fg/mL and linearity range from 0.0001 to 50 ng/mL. Mi *et al.* reported a biosensor based on DNA TSP and polyA mediated AuNPs for sensitive detection of breast cancer susceptibility gene [122]. LOD was 0.1 fM, linear range was from 1 fM to 1 nM. Fang *et al.* reported a 3D DNA tetrahedron nanomaterial (MoS_2@Fe_3O_4 @AuNPs@DNA tetrahedron @Antibody) for enrichment of the cancer biomarker heat shock protein 90α (HSP90α) in human plasma [123]. Detection dynamic range was from 20 to 480 ng/g.

2.3 Exosome detection

Exosomes are extracellular vesicles secreted by cells with a diameter ranging from about 40 to 160 nm and carry a variety of biomolecules, including proteins, DNA, RNA, *etc.* [124]. Exosomes are involved in information exchange between cells and are considered to be important biomarkers for cancer development and metastasis [125,126]. Therefore, the development of effective cancer-related exosomes detection strategies is very important for cancer diagnosis.

E-DNA sensor platforms based on DNA TSPs were widely used for the detection of exosomes. Tan *et al.* reported an aptasensor for direct capture and detection of hepatocyte exosomes by incorporating aptamers into DTNs [127] (Figure 3a). Directed immobilization of aptamers significantly improved accessibility to suspended exosomes, and the DNA tetrahedron-assisted aptasensor detected exosomes 100 times more sensitive than the single-stranded aptamer-functionalized aptasensor. Wang *et al.* demonstrated a novel SPR strategy to specifically detect exosomes [128]. Two different aptamers could specifically recognize and capture liver cancer (SMMC-7721) exosomes, and polydopamine-functionalized gold nanoparticle-assisted signal amplification. This assay showed excellent specificity and stability for SMMC-7721 exosomes and could be used for 50% human serum analysis. Yang *et al.* constructed a novel DTP sensor [81]. Different from traditional DNA tetrahedron probes, inverted DTPs were used, with a thiol group at one vertex for anchoring on the gold surface, and the remaining three vertices for capturing

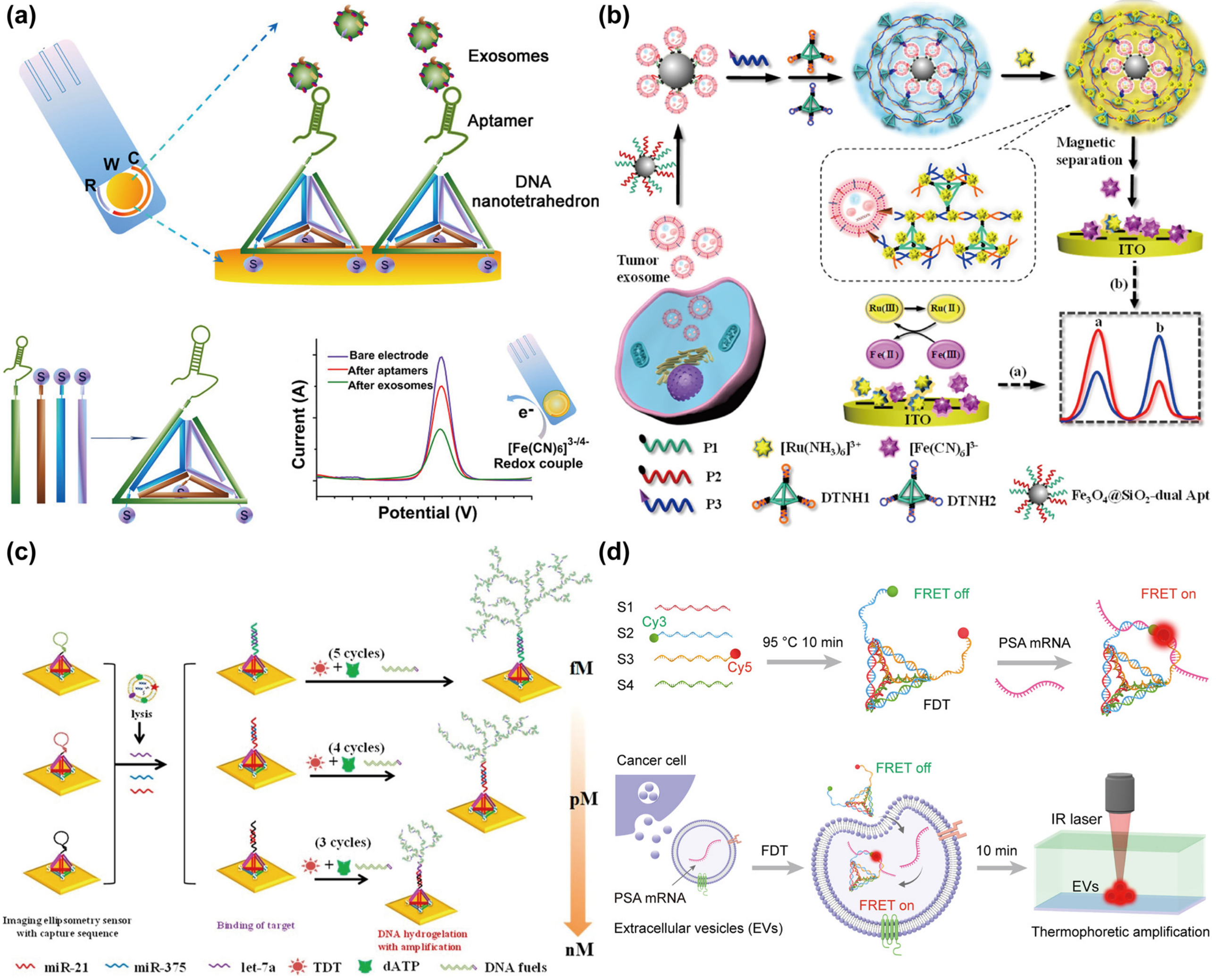

Figure 3: DNA tetrahedron-based exosomes detection. (a) The DNA nanotetrahedron-assisted EC aptasensor for cancerous exosome detection [127] Copyright 2017, American Chemical Society. (b) Schematic illustration of the ratiometric immobilization-free EC sensing system for tumor exosome detection [130] Copyright 2021, American Chemical Society. (c) Imaging ellipsometry biosensor based on DNA hydrogelation for exosomal miRNA detection [133] Copyright 2020, American Chemical Society. (d) The DTTA for *in situ* detection of extracellular vesicle mRNA [138] Copyright 2021, Elsevier.

exosomes. The exosomes' LOD of this sensor was 5.32×10^4 exosomes/mL. Wang *et al.* reported a triboelectric sensor based on DNA tetrahedron modification for capture and ultrasensitive detection of exosomes [129]. This is the first time that DTN is introduced into the triboelectric nanogenerator system for selective capture of exosomes. Electron transfer between the abundant amino groups from exosomes and the tribo-materials produces a measured signal that enables direct quantification without any signal amplification with the LOD as low as 3 exosomes/μL.

Through DNA tetrahedron carrying specific aptamers and specific recognition of tumor exosomes, some immobilization-free EC sensing methods were used for the direct capture, enrichment, and detection of tumor exosomes. Li *et al.* reported a ratiometric immobilization-free EC sensing method based on a dual-aptamer recognition system and hyperbranched DNA superstructure signal amplification strategy for the direct capture and detection of tumor exosomes [130] (Figure 3b). This method successfully achieved highly sensitive and selective detection of tumor exosomes with LOD of 3.0×10^4 particles/mL. More importantly, this method could directly detect tumor exosomes in complex sample culture media and human serum samples. Ye *et al.* developed an artificial enzymatic cascade amplification strategy based on mucin 1 (MUC1) aptamer and switchable DNA tetrahedron (SDT) scaffolds for exosome detection in MCF-7 breast cancer cell line [131]. MUC1-positive exosomes could trigger the self-assembly of HRPmimicking DNAzymes and bring DNAzymes and glucose oxidase (GOx) into spatial proximity. MCF-7 exosomes were detected with high sensitivity at LOD of 1.51×10^5 particles/mL and could be distinguished from other breast cancer cell-derived exosomes. In a recent study, Li's group used DTNs as "nanoweights" to enrich target exosomes only by low-speed and short-time centrifugation [132]. Two DTNs were designed, each carrying specific aptamers for exosomes biotarget identification. One DTN served as "nanoweights" that triggered cross-linking of multiple target exosomes, thus enabling low-speed and short-time centrifugation for enrichment. Another DTN further narrows down the target exosome subtype and initiated the HCR for signal amplification. The LOD was as low as 1.8×10^2 particles/μL. When compared to enzyme linked immunosorbent assay and fluorescence assays, the sensitivity of this method was increased by 1,000-fold and 10-fold, respectively.

Sensitive detection of exosomal miRNAs (Exo-miRs) could help to further improve the accuracy of cancer diagnosis. Yang *et al.* constructed an imaging ellipsometry sensor based on DNA hydrogel for sensitive detection of Exo-miRs [133] (Figure 3c). By changing the number of cycles of DNA hydrogel amplification, the exosomal miRNA detection range of this method was tunable from fM to nM, with LOD of 0.2 fM for let-7a, 10 fM for miR-375, and 40 pM for miR-21. Yang *et al.* reported an EC sensor based on supercharged DNA tetrahedron nanolabel for ultrasensitive detection of Exo-miRs [134]. This intrinsic supercharge of DNA tetrahedron adsorbed a large number of electroactive molecules, and using of peptide nucleic acids instead of DNA probes minimized background signals, which greatly improved the detection sensitivity with the LOD at 34 aM. Ding *et al.* developed a surface plasmon resonance imaging biosensor for ultrasensitive detection of NSCLC associated Exo-miRs through the synergy between DNA tetrahedron and Au-on-Ag heterostructure [135]. With an LOD as low as 1.68 fM, this biosensor could simultaneously detect four Exo-miRs in a single clinical sample and accurately distinguished healthy individuals from NSCLC patients. Qiu *et al.* reported multifunctional DNA tetrahedron assisted catalytic hairpin assembly EC biosensor for sensitive detection of Exo-miRs [136]. This biosensor could detect Exo-miRs as low as 7.2 aM in 30 min. More importantly, by detecting four tumor-associated Exo-miRs in a breast cancer cohort, this method could diagnose breast cancer with high efficiency and sensitivity.

DNA tetrahedron was an ideal nanocarrier, which could be transported into exosomes, detect Exo-miRs *in situ*, and avoid damage to exosomes. Luo *et al.* reported a fLuorescent Intracellular-Guided Hairpin-Tetrahedron nanoprobe that *in situ* visualized miRNAs inside exosomes [137]. This strategy uses DNA tetrahedron to facilitate exosomes penetration without cargo extraction by damaging exosomal membranes. Sun *et al.* developed a DNA tetrahedron-based thermophoretic assay (DTTA) for *in situ* sensitive detection of EV mRNA [138] (Figure 3d). Without the need for RNA extraction and enzymatic amplification, this method achieved a LOD of 14 aM for detection of exosomal PSA miRNAs. More importantly, the DTTA could discriminate between PCa and benign prostatic hyperplasia.

2.4 Cancer cells detection

CTCs are tumor cells that have sloughed off the tumor and extravasate into blood, and are directly related to the metastasis and spread of cancer [139,140]. CTCs are considered to be important biomarkers of cancer, and the detection of CTCs is of great significance for the early diagnosis of cancer and the assessment of recurrence and metastasis [7,141]. However, the concentration of CTCs is extremely low compared to other cells in the blood, high detection sensitivity and specificity of CTCs is still challenging.

In 2014, Zuo *et al.* used the E-DNA sensor platform based on DNA tetrahedron nanostructure for the first time to detect cancer cells simply, sensitively and specifically [142] (Figure 4a). They redesigned a multi-branched HCR (mHCR) to produce long products with multiple biotins for signal amplification and multiple branched arms for multivalent binding. This detection platform has a LOD of 4 cancer cells. Based on the DNA tetrahedron EC sensor, Miao *et al.* introduced Cys-Arg-Gly-Asp-Ser-modified AgNPs as a signal source for the selective detection of cancer cells [143]. This method detected cancer cells from 10^4 to 10^7 cells/mL. Zhang *et al.* reported a sensitive HepG2 cellular biosensor platform [144] (Figure 4b). DNA tetrahedron, HepG2 cells, and MIL-101(Fe) (a typical type of Fe-MOF)@AuNPs form a sandwich-like structure with the help of aptamers, and the signal was amplified by HCR. Therefore, this sensor detected HepG2 cells with high selectivity and high sensitivity, and LOD of 5 cells/mL. Chen *et al.* demonstrated an EC cell sensor with specific recognition signal amplification based on dual aptamers and hybrid nanoprobes for highly selective detection of MCF-7 cells [145]. DNA tetrahedron-cell-nanoprobe sandwich structure was constructed on the surface of gold electrode under the interaction between two aptamers (AS1411, MUC1) in MCF-7 cells. This sensor had a LOD as low as 6 cells/mL. In addition, this was the first time that metal organic framework PCN-224 was introduced as nanocarrier. In 2020, Li *et al.* reported an enzyme-free EC method for specific capture, sensitive detection, and efficient release of CTCs *via* DTNs combined with HCR [146]. With the

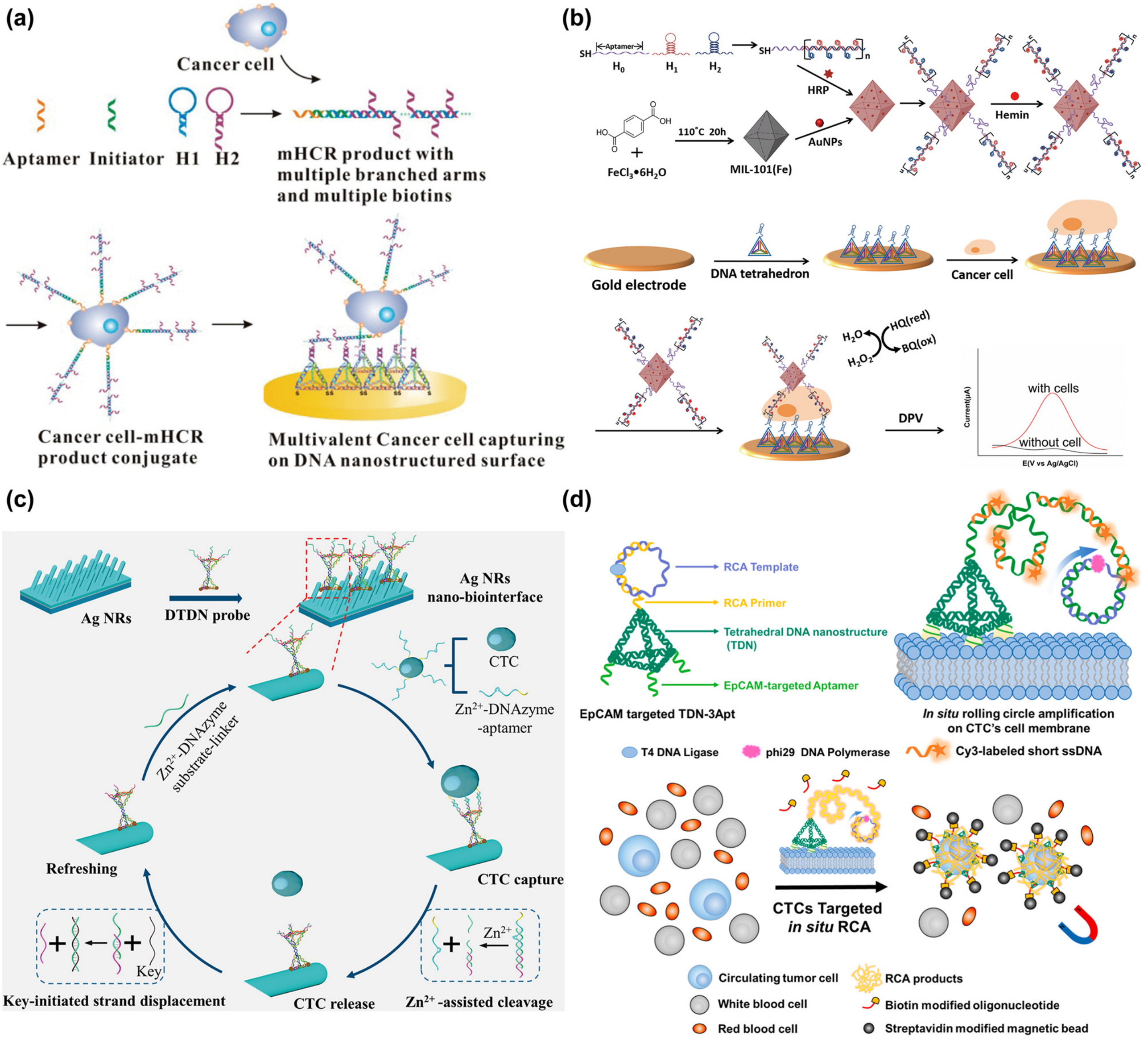

Figure 4: DNA tetrahedron-based cancer cells detection. (a) DNA tetrahedron biosensor combined with HCR for cancer cells detection [142] Copyright 2014, American Chemical Society. (b) Functional hybrid nanoprobe EC aptasensor for HepG2 cancer cells detection [144] Copyright 2018, Elsevier. (c) Double-DTN-probe-functionalized Ag NRs nanobiointerface for CTCs capture and nondestructive release [149] Copyright 2022, American Chemical Society. (d) A DNA tetrahedron-aptamer complex-mediated RCA strategy for the detection of CTCs with low EpCAM expression [153] Copyright 2022, Elsevier.

excellent signal amplification ability of HCR, this method detected MCF-7 cells from 10 to 10^4 cells/mL with an ultralow LOD of 3 cells/mL. More importantly, the captured CTCs could be released through the strand displacement reaction, which greatly reduced the damage to cell function and structural integrity. Mi *et al.* developed an EC biosensor based on double DNA tetrahedron for ultrasensitive detection and release of CTCs [147]. An upright DNA tetrahedron was used as a rigid scaffold for modification onto the gold electrode surface, and the three vertex strands of an inverted DNA tetrahedron was hybridized with aptamers for the capture of CTCs. Furthermore, the captured CTCs could be efficiently released by benzonase nuclease with little damage to cells. The LOD of this sensor was 1 cells/mL, the release efficiency reached 88.1–97.6%, and the viability of the released cells reached up to 98%. In a recent study, Li *et al.* reported a biosensor for CTCs detection based on DNA tetrahedron and a polyvalent aptamer network [148]. Polyvalent capture probes generated by amplification (RCA) reactions could greatly improve the capture efficiency of CTCs. Importantly, a detectable EC signal could be obtained through target CTCs-induced allostery of DNA hairpins without the need of additional signaling probe. The LOD of this sensor was 23 cells/mL with the detection range of 10^2 to 5×10^4 cell/mL.

In addition to traditional gold electrodes, some novel substrates combined with DTN have also been developed for the capture and detection of tumor cells. For example, Wang *et al.* reported a novel nanobiointerface for efficient capture, high-sensitivity detection, and nondestructive release of CTCs [149] (Figure 4c). This nanobiointerface consists of two parts: a substrate of silver nanorods (AgNRs) array fabricated by oblique angle deposition, and multivalent double-DTPs. After optimization of the condition, the nanobiointerface could capture 90.2% of SGC-7901 cells in PBS, and 93.4% of the cells that were released by Zn^{2+}-assisted DNAzyme cleavage, and the survival rate of CTCs after being released was about 98.0%, with LOD at 1 cell. Importantly, they used this nanobiointerface for the detection of CTCs in blood, and obtained a relatively good result with 83.8% capture efficiency and 91.2% release efficiency. Mi *et al.* combined amino-modified DNA tetrahedron, aptamer-triggered HCR (apt-HCR), and herringbone channel (HB) chip to construct a microfluidic system (T-μFS) for the detection of CTCs [150]. Amino-modified DNA tetrahedron could be immobilized on the glass substrate of the HB chip and hybridized with multiple branched arms of the apt-HCR products for efficient capture of CTCs. Benzonase nuclease-specific degradation removed DNA tetrahedron and promoted the release of CTCs. The capture efficiency, release efficiency, and cell viability of this T-μFS for CTCs were over 90%.

In addition, immobilized-free EC cytosensor based on aptamer-functionalized DTNs have also been widely used for ultrasensitive detection of tumor cells. In 2019, Fan *et al.* reported a DTN-programmed approach to topologically engineer receptor-ligand interactions on cell membrane [151]. Based on the four vertices of DNA tetrahedron being capable of binding to aptamers by hybridization, they synthesized DNA tetrahedrons functionalized with 1–3 aptamers targeting epithelial cell adhesion molecule (EpCAM, over-expressed on the membrane of tumor cells). DNA tetrahedron with three aptamers (2-simplex) showed about 19-fold enhanced binding affinity and prevented cellular endocytosis, and captured CTCs with high efficiency. Further, Li *et al.* demonstrated label-free homogeneous EC strategy based on multiaptamer-functionalized DNA tetrahedron nanostructures (Apt-DTNs) for ultrasensitive detection of cancer cells [152]. They prepared four Apt-DTNs with different aptamer valences that could bind to membrane proteins on the surface of target cells, and were easily separated from solution by centrifuging cancer cells and their surface-bound DNA tetrahedron. This strategy enabled sensitive and selective detection of cancer cells. Zhang *et al.* used DNA tetrahedron in combination with RCA reaction for efficient enrichment of CTCs [153] (Figure 4d). Three vertices of the DNA tetrahedron were modified with EpCAM targeted aptamers, which recognizes and binds to target cells. Subsequently, the RCA product hybridizes with the biotin-modified DNA capture strand, and the target cells are rapidly separated using streptavidin-modified magnetic beads. This method improved the capture efficiency in EpCAM-low-expressing breast cancer cells. Cai *et al.* reported a fluorescent detection method based on DNA tetrahedron and Cas13a signal amplification for the detection of tumor cells [154]. The three vertices of the DNA tetrahedron were modified with cholesterol groups, which could anchor on the lipid bilayer of tumor cells, and binding of target DNA to another vertex of the DNA tetrahedron activated the Cas13a signaling amplification mechanism. The LOD for this method was 1 cell/mL.

2.5 Tumor imaging

Identifying tumor is critical for cancer diagnosis and treatment in clinical applications [155,156]. Among the numerous imaging modalities, optical imaging is widely used in laboratory-based biomedical research due to its convenience and low cost. However, fluorescent molecules used in optical imaging have limited their clinical application because of their low water solubility and low permeability. DNA tetrahedron has excellent biocompatibility, biodegradability, non-toxicity, and easy endocytosis by mammalian cells, and can be coupled with different molecules, which make up for some deficiencies of optical imaging. Therefore, it can be widely used in tumor imaging.

Sentinel lymph nodes (SLNs) are the first lymph nodes that cancer cells reach after traversing the lymphatic vessels from the primary tumor. Thus, SLNs imaging is critical for accurate staging of cancer. Ahn *et al.* designed Cy5 fluorophore-labeled DNA tetrahedron and used it for SLNs imaging [157]. The experimental results demonstrated that DNA tetrahedron was efficiently imaged in SLNs and had extended retention times at nodes compared to linear DNA probes.

However, as an *in vivo* imaging platform, the *in vivo* stability, *in vivo* pharmacokinetics, and biodistribution properties of DNA tetrahedron had not been thoroughly investigated. To address these issues, Wang *et al.* developed multi-armed DNA tetrahedron nanostructures (TDNs) for

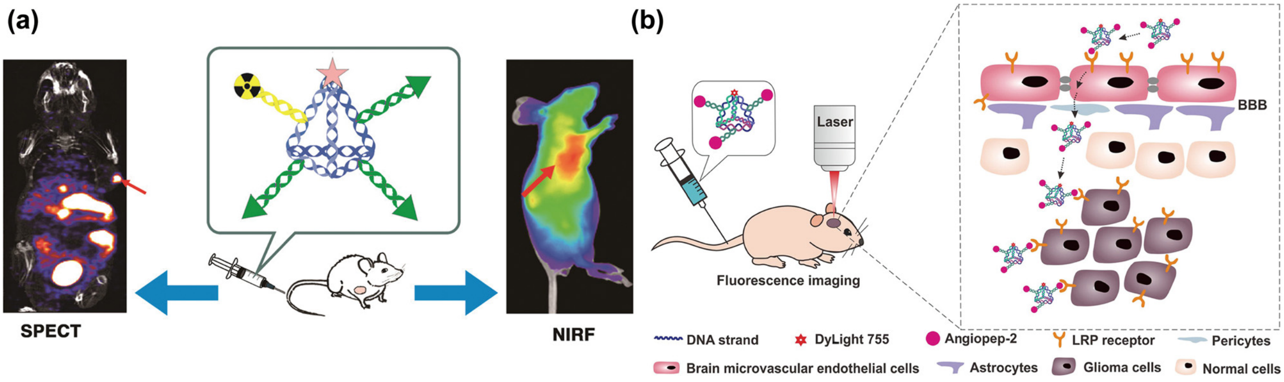

Figure 5: DNA tetrahedron-based tumor imaging. (a) Multiple-armed DTNs for tumor imaging [158] Copyright 2016, American Chemical Society. (b) Targeted imaging of brain tumors with DTP [159] Copyright 2018, American Chemical Society.

dual modality *in vivo* imaging using near-infrared (NIR) fluorescence and single-photon emission computed tomography (SPECT) [158] (Figure 5a). They site-specifically anchored fixed amounts of NIR dyes (Dylight-755), radiolabels (^{99m}Tc), and folic acid (FA) groups to DNA tetrahedron for imaging the tumor in tumor-bearing mice. They used both NIR and SPECT modalities to recognize noninvasive tumor-targeted imaging in tumor-bearing mice. Furthermore, they found that the presence of the arm strands in TDNs significantly enhanced their *in vitro* stability, keeping them intact in serum for at least 12 h, and that TDNs circulated in the blood system twice as long as double-stranded DNA.

Developing imaging probes for crossing the blood–brain barrier (BBB) has been a major challenge. Fan *et al.* reported an angiopep-2 (ANG)-modified DNA tetrahedron for targeted imaging of brain tumors [159] (Figure 5b). ANG-modified TDN remained intact in serum for at least 12 h, and ANG modification potently enhanced cellular uptake of TDN in brain capillary endothelial cells and U87 malignant glioma (U87MG) cells. Importantly, ANG-TDNs could cross the BBB in both *in vitro* and *in vivo* models, and strong fluorescence imaging was shown in U87MG xenograft in nude mice.

Fluorescence signal amplification at the tumor site facilitates more sensitive and accurate *in vivo* tumor imaging. Lin *et al.* reported a DNA tetrahedron-enhanced HCR probe (T-probe) system for rapid and accurate imaging of tumor-associated biomarkers *in vivo* [160]. The tumor cells overexpressing miRNA21 and nucleolin were selected as targets. The T-probe system could be simply synthesized in two steps, and the results from *in vitro* live cell studies showed the selectivity of the probe for target cells, as well as the ultrasensitive detection of target biomarkers. Furthermore, this T-probe system exhibited enhanced serum stability and biocompatibility. Most importantly, T-probe system exhibited excellent *in vivo* fluorescence within 15 s through peripheral-tumor injection and within 10 min through tail-vein injection, and reliable signal localization and tumor amplification were achieved in tumor-bearing mice.

3 Drug delivery based on DTN

Due to the excellent programmability and biocompatibility, and low biotoxicity of DTNs, they are ideal drug carriers for overcoming obstacles in cancer therapy. In recent years, various DNA tetrahedron-based drug delivery nanosystems have been reported for cancer therapy.

3.1 Chemotherapy drugs

Chemotherapy drugs can be loaded onto DTN through three main methods: intercalation, electrostatic interaction, and chemical modification.

3.1.1 Intercalation

Doxorubicin (DOX), an anthracycline drug, has long been one of the most commonly used drugs in cancer treatment. DOX slows or stops the growth of cancer cells by intercalating and interacting with DNA, inhibiting the synthesis of biological macromolecules [161,162]. DOX has spectral antitumor activity and effectively kills cancer cells; DOX also harms normal cells and causes toxicity to the majority of non-cancerous organs [163]. These toxic side effects limit the use of conventional DOX in clinical treatment, especially for patients with advanced cancer who require

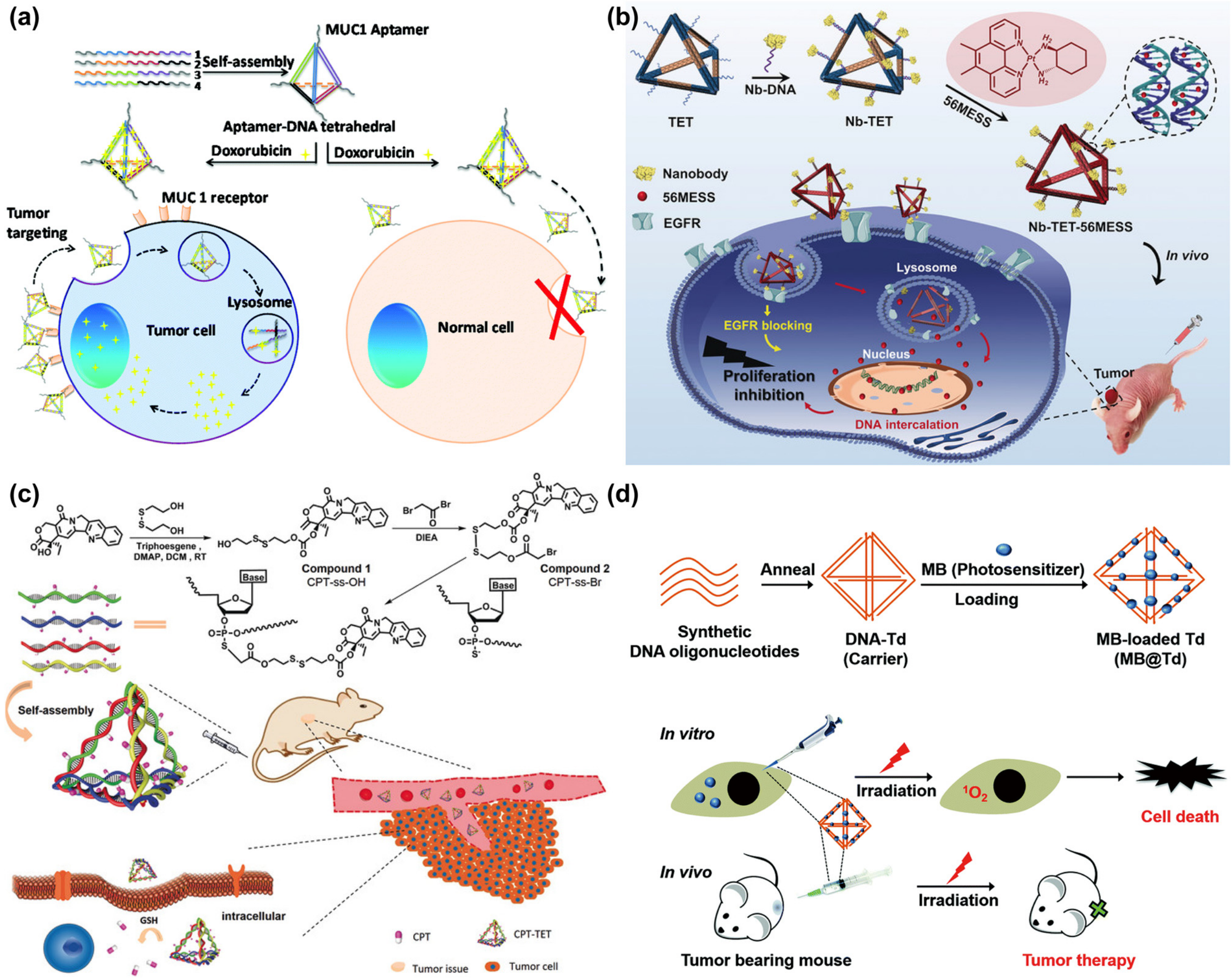

Figure 6: DNA tetrahedron-based chemotherapy drugs and photosensitizers delivery systems. (a) Multivalent aptamer-modified DNA tetrahedron for DOX delivery [174] Copyright 2019, Royal Society of Chemistry. (b) Double-bundle DNA tetrahedron for targeted platinum drug delivery [184] Copyright 2019, John Wiley and Sons. (c) CPT-grafted DNA tetrahedron to inhibit tumor growth [194] Copyright 2019, John Wiley and Sons. (d) MB-loaded DNA tetrahedron for *in vivo* photodynamic therapy [206] Copyright 2016, Royal Society of Chemistry.

increased doses [164,165]. Therefore, it is highly desirable to develop drug delivery systems for DOX, which mitigate adverse side effects and improve delivery efficiency. DOX can intercalate in the double helix of DNA, thus, DTN can be used as carriers for DOX delivery.

In 2013, Ahn *et al.* used DNA tetrahedron to deliver DOX for the first time, which effectively inhibited the growth of multidrug resistance (MDR) breast cancer [166]. Their study showed that DOX loaded on DNA tetrahedron, when compared with free DOX, not only accumulated more in cells but also could bypass the efflux pathway of MDR cells, and thus effectively inhibited the growth of MDR cells. Subsequently, to resolve the poor serum stability of native D-DNA, mirror DTNs were constructed using left-handed DNA (L-DNA) for tumor-specific delivery of DOX [167]. The experimental results showed that L-DNA tetrahedron was more stable and easier to be taken up by cells than D-DNA tetrahedron, thus greatly improving the anti-tumor efficacy of DOX. In addition to conventional intravenous administration, the DNA tetrahedron drug delivery system could also be used for transdermal administration. Xu *et al.* found that DOX-loaded DNA tetrahedron reached a 350–400 μm underneath mouse skin and effectively inhibited melanoma growth by topical administration [168].

Targeting specific cells is the first prerequisite for drug delivery. Although delivery of DOX *via* DNA tetrahedron (DNA tetrahedron-DOX) was more accessible to cells than free DOX, it is necessary to further improve the targeting of DNA tetrahedron-DOX in order to increase the specificity of DOX uptake and reduce systemic toxicity. DNA tetrahedron not only has the ability to carry DOX, but also can take functional groups, such as small molecules, aptamers and peptides, monoclonal antibody *etc.* DNA tetrahedron, *via* chemical modification or base-complementary pairing interactions, coupled with these functional groups can target cancer cells through ligand-receptor recognition strategy.

Aptamers are single-stranded DNA or RNA molecules that can form certain secondary and tertiary structures with high affinity and strong specificity of binding to target ligand [169]. Dai *et al.* used MUC1 aptamer-modified DNA tetrahedron to deliver DOX [170]. MUC1 aptamer-modified DNA tetrahedron selectively and preferentially transported DOX to MUC1-positive MCF-7 breast cancer cells and reduced DOX uptake by MUC1-negative cells. Lin *et al.* constructed a sgc8c aptamer-modified DNA tetrahedron to specifically deliver DOX to protein tyrosine kinase 7-overexpressing cells [171]. Gint4.T is an aptamer capable of recognizing platelet-derived growth factor receptor β (PDGFRβ) on tumor cells. Cheng *et al.* used Gint4.T-modified DNA tetrahedron for loading DOX to inhibit glioma cell proliferation by targeting PDGFRβ [172]. Dual or multiple targeting can increase the specificity of drug delivery. Jiang *et al.* employed MUC1 (specific to MUC1) and AS1411 (specific to nucleolin) aptamers co-modified DNA tetrahedron for DOX delivery, which further enhanced DOX specific uptake and its efficacy [173]. Qi *et al.* reported 1–3 MUC1-aptamers modified DNA tetrahedron for DOX delivery for breast cancer treatment [174] (Figure 6a). Studies have shown that the number of aptamers on DNA tetrahedron had a significant effect on its cellular uptake efficiency, and the multivalent modified aptamers significantly enhanced the uptake efficiency in MCF-7 tumor cells, with the decrease in the uptake efficiency of normal cells. In a recent study, Ding *et al.* reported a covalently bound branched DNA aptamer cluster-modified DNA tetrahedron nanoplatform for efficient delivery of DOX [175]. Branched aptamer cluster-modified DNA tetrahedron showed highly targeted cellular uptake, significantly and selectively inhibited tumor cell proliferation upon loading with DOX.

FA can bind with high affinity to folate receptor (FR) which overexpressed on the surface of human cancer cells. Therefore, FA-modified drug delivery systems can be used for targeted therapy. Yang *et al.* modified DNA tetrahedron with FA to deliver DOX, leading to specific induction of apoptosis in HT-29 colon cancer cells [176]. Both DOX and FA were conjugated to the 3′-OH of ssDNA *via* click chemistry. Sun *et al.* used SL2B aptamer and FA dual-targeted modified DNA tetrahedron for DOX delivery to combat colorectal cancer [177]. Dual-targeting functionalized DNA tetrahedron could be simultaneously recognized by vascular endothelial growth factor (VEGF) and FRs on the surface of HT-29 cancer cells. They demonstrated that this DNA tetrahedron delivery system caused sufficient inhibition of HT-29 cells at a much lower DOX concentration.

The tumor-penetrating peptide can specifically penetrate tumor blood vessels and tumor tissue, so tumor-penetrating peptide can be used to deliver drugs into tumors. He *et al.* used tumor-penetrating peptide-modified DNA tetrahedron (p-TDN) for targeted DOX delivery to inhibit brain tumor cell growth [178]. Compared with DNA tetrahedron and double-helical DNA, the tumor cell uptake rate of the tumor-penetrating peptide-modified DNA tetrahedron was greatly increased, and the growth inhibitory effect on U87MG cells could be significantly enhanced. Li and colleagues reported a precise quantity of KLA peptide-modified DNA tetrahedron nano-delivery platform to deliver DOX to mitochondria to enhance apoptosis [179]. DNA tetrahedron modified with 3 KLA peptides showed the strongest effects on cellular uptake, mitochondrial targeting, programmed apoptosis pathway activation, and *in vitro* anticancer efficacy after DOX loading. Lin *et al.* used iRGD-modified DNA tetrahedron for DOX-targeted delivery to triple-negative breast cancer [180]. iRGD is a tumor penetrating peptide consisting of 9 amino acids (CRGDKGPDC), in which the RGD sequence and the CendR motif are used for tumor targeting and tissue penetration, respectively [181]. The novel complex nanoparticle formed by iRGD-modified DNA tetrahedron loaded with DOX showed excellent deep penetration and drug accumulation in three-dimensional multicellular tumor spheroids. Furthermore, this nanoparticle exhibited excellent antitumor and antiangiogenic activity in the 4T1 subcutaneous breast tumor model.

DNA tetrahedron can also form a targeted drug delivery system with monoclonal antibodies. Leong *et al.* used cetuximab, an antibody specifically targeting EGFR, to modified DNA tetrahedron for targeted delivery of DOX [182]. Three vertices of the DNA tetrahedron were conjugated with cetuximab, which maximized the chance of binding to the EGFR of cancer cells and achieved a high targeting effect, which translated into a higher cancer cell killing effect.

Platinum drugs are widely used clinically as first-line chemotherapy drugs for treatment of various cancers [183]. Ding *et al.* developed a DNA nanoplatform for targeted delivery of platinum drugs based on double-bundle DNA tetrahedron [184] (Figure 6b). The platinum-based drug (56MESS) was loaded onto the double-stranded DNA tetrahedron by intercalating 56MESS into the DNA double helix. To achieve targeted delivery, an anti-EGFR nanobody was coupled to DNA and assembled on the double-stranded DNA tetrahedron. They found that this DNA nanoplatform exhibited superior targeting EGFR-overexpressing cells and highly potent antitumor activity without observable systemic side effects.

Metal complexes play an important role in clinical cancer therapy. Chen *et al.* used DNA tetrahedron as nano-delivery vehicles for ruthenium polypyridyl complexes to achieve enhanced tumor delivery and nullified systemic toxicity [185]. Furthermore, they successfully delivered anticancer metal complexes ($[Ir(ppy)_2phen]^+$ PF_6) by using MUC1 and AS1411 double aptamer-modified DNA tetrahedron, and enhanced the therapeutic effect in glioma [186].

3.1.2 Electrostatic interaction

Paclitaxel (PTX), a natural anticancer drug, has been widely used in the clinical treatment of various malignancies including cancer of breast, ovary, head and neck, and lung [187]. Lin *et al.* used electrostatic interaction to load PTX onto DNA tetrahedron nanostructures (TDNs) for overcoming drug-resistant lung cancer [188]. The results showed that PTX/TDN could be transported into and out of the cells through caveo-dependent and exocytotic pathways, thereby bypassed the drug efflux pump and enhanced the intracellular drug accumulation. They showed that PTX/TDN could significantly inhibit P-glycoprotein expression, and thus it was very promising to overcome the drug resistance in lung cancer. Cai *et al.* used GMT8 and Gint4.T aptamers modified DNA tetrahedron to deliver PTX for targeted therapy of glioblastoma multiforme (GBM) [189]. This delivery system considerably inhibited the GBM cell proliferation, migration, and invasion, and induced apoptosis in U87MG cells.

Temozolomide (TMZ), an alkylating agent that penetrates the BBB, is the gold standard therapy for GBM, anaplastic astrocytoma, and anaplastic oligoastrocytoma [190,191]. Lin *et al.* used two aptamers (AS1411 and GS24) modified DNA tetrahedron assembled with TMZ to form nanoparticles for the treatment of GBM [192]. This nanoparticle could penetrate the BBB and be taken up by GBM cells, resulting in enhancement of TMZ lethal effect on GBM. More importantly, compared with free TMZ, the nanoparticle not only killed TMZ-sensitive cells, but also overcame TMZ-resistance by consuming O6-methylguanine-DNA-methyltransferase.

3.1.3 Chemical modification

Camptothecin (CPT), a cytotoxic quinoline alkaloid that inhibits DNA topoisomerase, is also widely used in cancer therapy [193]. Zhang *et al.* used phosphorothioate DNA to graft CPT and assemble into DNA tetrahedron (CPT-TET) to construct a precise and controllable drug delivery system for tumor therapy [194] (Figure 6c). By adjusting the number and sites of grafted CPT on the DNA backbone, the drug loading capacity could be increased while ensuring the water solubility of the drug delivery system. CPT-TET exhibited more efficient cellular uptake and higher cytotoxicity, and inhibited tumor growth more effectively in tumor-bearing mouse model.

Maytansine (DM1) is a microtubule inhibitor that binds to β-Tubulin and effectively limits the aggregation or dissociation of tubulin [195]. The anti-tumor effect of DM1 is 100–1,000 times over other anti-microtubule drugs [196,197]. Lin *et al.* modified the HER2-targeted aptamer to one vertex of the DNA tetrahedron and loaded DM1 to the other three vertices by chemical conjugation, which forms a novel DNA tetrahedron-based drug delivery vehicle (HTD) for the treatment of HER2-positive breast cancer [198]. Subsequently, they used red blood cell membrane in combination with traditional liposomes to develop a tumor-targeting and pH-sensitive hybrid membrane that encapsulated HTD in biomimetic camouflage, and assembled to form the final hybrid delivery system. This delivery system exhibited extended half-life *in vivo* and enhanced antitumor efficacy.

5-Fluorouracil (5-FU) is a pyrimidine analog, which exerts its anticancer effects by inhibiting thymidylate synthase and incorporating its metabolites into RNA and DNA, and is widely used in the treatment of various cancers [199]. Lin *et al.* reported DNA tetrahedron-based nanomedicine modified with 5-FU and AS1411 [200]. This nanomedicine exhibited enhanced therapeutic effect on breast cancer cells (MCF7 cell line) without negative side effect on normal mammary cells (MCF10A cell line). Zhang *et al.* used DNA tetrahedron as a carrier for the first time to deliver 5-fluoro-2′-deoxyuridine oligomers [201]. In addition, DNA tetrahedron was modified with cholesterol to enhanced cellular uptake. This delivery system exhibited enhanced cytotoxicity. 2′-Deoxy-5-fluorouridine (floxuridine) is a clinically used fluoropyrimidine drug, catabolized in the body to form 5-FU [202]. Zhu *et al.* incorporated floxuridine into DNA strands by solid-phase synthesis and further assembled into DNA tetrahedron to construct a DNA trojan Horses drug delivery system [203]. DNA trojan Horses with a tetrahedron structure exhibited superior anticancer ability both *in vitro* and *in vivo* when compared to free floxuridine.

3.2 Photodynamic therapy (PDT) drugs

PDT is a cancer treatment method based on photosensitizers (PS) that convert oxygen into reactive oxygen species

(ROS) to induce toxic death of cancer cells [204]. Hundreds of PS have been used in PDT clinically or preclinically [205]. However, most PS have poor water solubility, low cell/tissue permeability, and weak tumor specificity, resulting in limited PDT efficacy. As a completely water-soluble nanomaterial, DNA tetrahedron can also specifically target tumors. Therefore, using DNA tetrahedron as a delivery vehicle for PS will overcome some of the inherent shortcomings of PS and improve their therapeutic effect.

In 2016, Ahn *et al.* used DNA tetrahedron as a delivery vehicle for the photosensitizer MB for PDT [206] (Figure 6d). The results showed that each DNA tetrahedron could be loaded with 16 MB, and the delivery system had decent photodynamic therapy effect *in vitro* and *in vivo*.

Heptamethine cyanine IR-780 is a photosensitizer with effective photothermal (PTT) and photodynamic (PDT) response [207]. Meng *et al.* used DNA tetrahedron (Td) to develop nanoparticles of uniform size (224 nm) with IR-870 (IR780@Td) *via* electrostatic interaction and π–π stacking [208]. Compared with IR-780, IR780@Td showed better water solubility and photostability, and the PTT/PDT efficiency was significantly improved *in vitro* and *in vivo*. Under NIR laser irradiation, intravenous injection of IR780@Td advanced tumor imaging and enhanced anti-tumor effects.

Tang *et al.* used DNA tetrahedron to deliver an AIE-active natural PS, palmatine hydrochloride (PaH), for fluorescence imaging and PDT [209]. Compared with free PaH, DNA tetrahedron combined with PaH provided a 7.9-fold higher ROS generation efficiency, and greatly improved the PDT efficiency. In addition, they also found that the binding ratio of DNA tetrahedron to PaH was 1:4.

To further improve the selectivity of PS, Lin *et al.* used MUCl aptamer-modified DNA tetrahedron as the carrier for selective delivery of 5,10,15,20-tetra-(*N*-methyl-4-pyridyl) porphine (TMPyP4) to MUCl-positive tumor cells [210]. This PS delivery system promoted the production of ROS as well as cytotoxicity in MUC1-positive cells, without significantly killing MUC1-negative cells.

3.3 Nucleic acid drugs

3.3.1 Cytosine-phosphate-guanine (CpG)

Unmethylated CpG motifs are ubiquitous in bacteria but uncommon in vertebrate genomic DNA [211]. Oligodeoxynucleotides (ODNs) containing CpG motifs activate host defense mechanism in mammals, leading to innate and adaptive immune response [212]. CpG motifs can be specifically recognized by Toll-like-receptor 9 (TLR9) expressed by dendritic cells, B cells, macrophages, *etc.*, and stimulate these cells to produce pro-inflammatory cytokines, such as TNF-α, interleukin-6 (IL-6), *etc.* [213]. Therefore, CpG is a safe and effective immune adjuvant in basic research and clinical trials, and widely used in cancer immunotherapy [214]. However, free CpG oligonucleotides are difficult to be taken up by cells and easily degraded by nucleases, thus requiring a suitable carrier for the delivery of CpG oligonucleotides.

As DNA, CpG sequences can be easily incorporated into DTN to improve their stability and specific cellular uptake rate. Fan *et al.* used DNA tetrahedron as carrier to load and deliver multivalent CpG motifs [215] (Figure 7a). The results of this study showed that the DNA tetrahedron nanocomposite was resistant to nuclease degradation and remained substantially intact in fetal bovine serum (FBS) and cells for 24 and 8 h, respectively. CpG-functionalized DNA tetrahedron could enter macrophage-like RAW264.7 cells noninvasively and efficiently without the help of transfection agent. Intracellularly, CpG was recognized by TLR9, and stimulated high-level secretion of various pro-inflammatory cytokines, including TNF-α, IL-6, and IL-12. The secretion amount of pro-inflammatory cytokines was positively correlated with the number of CpG motifs carried on DNA tetrahedron.

3.3.2 Small-interfering RNA (siRNA)

siRNA is double-stranded non-coding RNA of 21–23 base pairs in length [216]. siRNA either induces mRNA degradation or prevents mRNA translation by targeting specific complementary mRNA [217]. Therefore, siRNA was proved to be a powerful molecule for silencing target genes through RNA interference pathway [218]. Since many cancers are caused by aberrant expression of related genes, and since siRNA can effectively silence target genes, the delivery of synthetic siRNA into cancer cells can effectively treat cancer [219]. However, siRNAs are easily degraded by a variety of nucleases in blood, and their negatively charged nature makes them unable to penetrate cell membranes effectively, resulting in low cellular uptake. In addition, the small size of siRNA makes it easy to be rapidly cleared by the kidney [220,221]. To this end, it is necessary to find a suitable delivery vehicle to escort the siRNA to the target cells. DTNs not only have the advantages of resistance to enzymatic degradation and efficient cellular uptake, but also easily form complex with siRNA through complementary base pairing. Therefore, DTNs are widely used to deliver siRNA for treatment of cancer.

In 2012, Anderson *et al.* first used DNA tetrahedron to deliver siRNA into cells and silence target genes in tumors

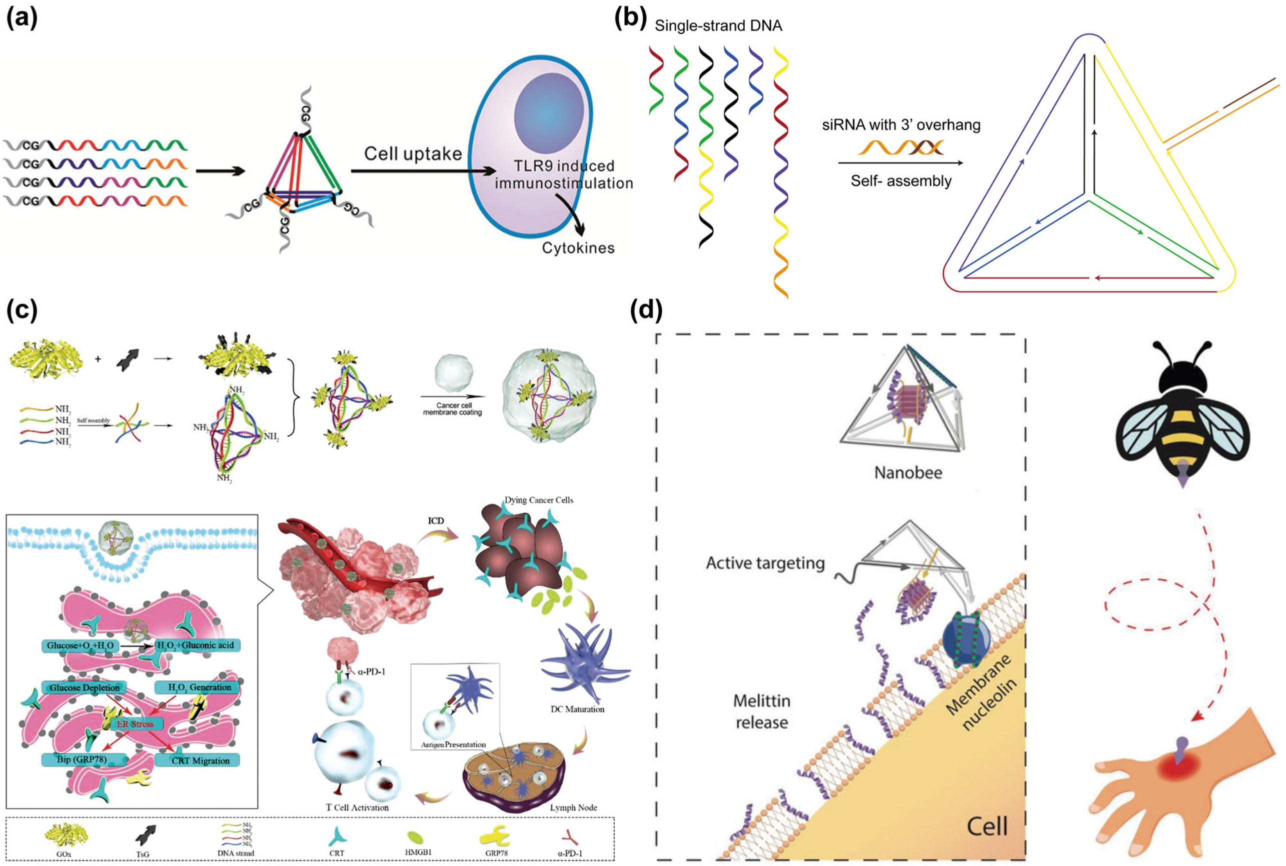

Figure 7: DNA tetrahedron-based nucleic acid, protein, and peptide drugs delivery systems. (a) DNA tetrahedron for noninvasive intracellular delivery of CpG and its immunostimulatory effect [215] Copyright 2011, American Chemical Society. (b) DNA tetrahedron for targeted *in vivo* siRNA delivery [222] Copyright 2012, Springer Nature. (c) Preparation and application of DNA tetrahedron nanoregulator for enhanced cancer immunotherapy [249] Copyright 2020, John Wiley and Sons. (d) The DNA tetrahedron-based "nanobee" for active targeting therapy [252] Copyright 2020, John Wiley and Sons.

[222] (Figure 7b). They found that for optimal delivery of siRNA to cells, at least three FA molecules were required per DNA tetrahedron, and that gene silencing occurs only when folate was in the proper spatial orientation. In addition, siRNA loaded on DNA tetrahedron showed a longer blood circulation time ($t1/2 \approx 24.2$ min) compared to parent siRNA ($t1/2 \approx 6$ min). Based on this finding, Cheng *et al.* reported that AS1411 aptamer-modified DNA tetrahedron could efficiently deliver siRNA to glioma cells and induce apoptosis [223]. Lin *et al.* also used AS1411 aptamer-modified DNA tetrahedron to deliver anti-Braf siRNA (siBraf) to silence the target gene in malignant melanoma cells [224]. They found that siBraf delivered by DNA tetrahedron exhibited more efficient cleavage of Braf mRNA than free siBraf. Qi *et al.* developed a series of DNA tetrahedron nanocages by conjugating different amounts of i-motif and therapeutic siRNA at the four vertices of the DNA tetrahedron [225]. The experimental results showed that the DNA tetrahedron nanocages loaded with i-motif and siRNA at the same time escaped from endosomal degradation and successfully interfered with EGFR expression in tumor cells, and the combination of two i-motifs and two siRNAs simultaneously loaded on one DNA tetrahedron nanocage had the best tumor growth inhibition efficiency. In addition, Zhu *et al.* used DNA tetrahedron as a vehicle to deliver copper transporter 1 (CTR1) mRNA-targeted siRNA into pancreatic cancer cells to prevent copper uptake, thereby significantly inhibited cancer progression [226].

DNA tetrahedron is highly programmable. Therefore, two different siRNAs can be simultaneously loaded and delivered into cancer cells using DNA tetrahedron to synergistically treat cancer. For example, Han *et al.* used FA molecule-modified DNA tetrahedron as a vehicle to simultaneous delivery siRNAs that target programmed death ligand 1 (PD-L1) and subtilisin-kexin type 9 (PCSK9) for synergistic treatment of colorectal cancer [227]. Their findings showed that this DNA tetrahedron-based co-delivery system of siRNAs not only reactivates T cells inhibited by

PD-L1 but also effectively silences the PCSK9 expression *in vitro*. Importantly, this co-delivery system showed excellent tumor accumulation and extraordinary therapeutic efficacy in CT26 colon cancer mice without toxic side effects.

Although siRNAs are a potent therapeutic tool to silence disease-causing mRNAs, their *in vivo* efficacy may be compromised due to the lack of target tissue specificity. The researchers constructed a siRNA delivery platform that could target specific organs by changing the size of the DNA tetrahedron and assembling the tetrahedron using DNA modified with different sugar backbones. Ahn *et al.* designed and assembled DNA tetrahedron with 20 base pairs on each side that could be specifically distributed into the liver upon systemic administration [228]. They found that when siRNA was delivered using this tetrahedron, the siRNA could preferentially accumulate in the liver and downregulate the ApoB1 protein. In the same year, they prepared four small-sized DNA tetrahedrons by self-assembly of oligonucleotides modified with different sugar backbones and screened them to develop a kidney-targeted delivery platform for siRNA [229]. After intravenous injection of the four DNA tetrahedrons into mice, their biodistribution was analyzed using *in vivo* imaging and ESI-MS. They found that the L-DNA tetrahedron was most preferentially localized to the kidney. Subsequently, they used L-DNA tetrahedron to successfully deliver siRNA into kidney cells where it downregulated the target genes.

Typically, siRNA delivery by DNA tetrahedron was achieved by extension of sticky ends. This method was simple to synthesize and it greatly improved the cellular uptake rate of siRNA. However, siRNA was exposed outside the DNA tetrahedron and easily degraded by nucleases during the delivery process, and the intracellular release of siRNA was uncontrolled. To address these issues, Cai *et al.* constructed a closed tetrahedron nanobox based on DNA tetrahedron, and siRNA could be encapsulated in the nanobox [230]. And one edge of the nanobox was tuned to a C-rich sequence that could be converted into a four-stranded i-motif structure at pH 5.0, resulting in the disassembly of the tetrahedron structure to release the siRNA. They found that this fully encapsulated loading method significantly protected siRNA from nuclease and serum degradation, and silenced target genes well both *in vitro* and *in vivo*.

DTN not only serves as carrier, but also could be further assembled into high-order nanostructures for siRNA delivery. Zhang *et al.* used DNA tetrahedron as building blocks to combine with functional siRNA assembly to form a novel DNA nanogel for siRNA delivery [231]. By embedding functional siRNA inside, the nanogel successfully protected the siRNA from degradation and delivered it into cells to achieve target gene silencing. Their study showed potential noncationic vectors for RNA delivery for cancer therapy.

3.3.3 DNAzyme

DNAzymes are single-stranded oligonucleotides with enzymatic activity that catalyzes a variety of reactions, including DNA and RNA cleavage, ligation, and phosphorylation [232]. The activities of some DNAzymes depend on unique cofactors (such as metal ions and proteins) and specific sequences; therefore, DNAzymes are widely used in biosensing and gene silencing therapy [233,234]. However, the negatively charged nature of DNAzymes makes them poorly permeable into cell membrane, and thus efficient delivery of DNAzymes into cells remains challenging.

DNA tetrahedron is considered a promising DNAzyme delivery system that can achieve excellent cell permeability without transfection agents. Lin *et al.* added Dz13 (a DNAzyme that cleaves the mRNA of c-Jun and suppresses the growth of squamous cell carcinomas) sequence directly to the 5′ end of a single-stranded DNA and further assembled to form Dz13-modified DNA tetrahedron nanostructures (TDN–Dz13) [235]. Compared with free Dz13, TDN–Dz13 exhibited high cellular uptake and efficiently cleaved target c-Jun mRNA intracellularly leading to inhibition of cell proliferation.

3.3.4 Antisense oligonucleotides (ASO)

ASOs are chemically synthesized oligonucleotides, typically 12–30 nucleotides in length, that are complementary to target mRNA sequences and induce gene silencing [236]. Therefore, ASO can be used for regulation of gene expression and has great potential as a gene therapy agent for malignant tumors. Unfortunately, ASO also suffers from the inherent shortcomings of nucleic acid drugs, *i.e.*, unmodified ASO is rapidly degraded in biological matrices and/or filtered by the kidneys with poor cell membrane permeability [237,238]. Thus, it is necessary to construct an efficient ASO delivery system to overcome these limitations.

Ding *et al.* first reported using a double-bundle DNA tetrahedron to deliver ASO (silencing proto-oncogene *c-raf*) into live cells [239]. Unlike conventional DNA tetrahedron, double-bundle DNA tetrahedron allowed to carry more ASO leading to increase in the effective concentration. Furthermore, the disulfide-containing ASO was located on the inner surface of the double-stranded DNA tetrahedron, which was effectively resistant to enzymatic degradation, and the double-stranded DNA tetrahedron was further functionalized with a nuclear localization signal (NLS) peptide to improve ASO targeting. The results showed that this ASO delivery system effectively reduced the expression levels of *c-raf* mRNA and protein.

3.4 Protein and peptide drugs

The vast majority of cancers are directly related to the abnormal expression or functional mutation of certain proteins. Therefore, protein-based cancer therapies have received increasing attention due to the highly specific activity and low toxicity of therapeutic proteins [240,241]. However, the protein itself is easily inactivated by enzymatic degradation and difficult to penetrate cell membrane, which limits its application [242,243]. Researchers have developed a series of DNA origami nanostructure-based carriers to escort proteins to target cellular locations [39,244–247]. Recently, DNA tetrahedron nanocarriers have also been explored for protein delivery.

Ahn *et al.* reported a streptavidin-mirror DNA tetrahedron (STV-Td) hybrid platform for intracellular delivery of various enzymes [248]. Biotin-modified enzyme was loaded onto STV-Td *via* high affinity between B-SA. Importantly, they successfully inhibited tumor growth in a mouse tumor model using STV-Td to deliver caspase-3 (CASP3).

Tang *et al.* developed a functionalized DNA tetrahedron nanoregulator for specifically triggering endoplasmic reticulum (ER) stress to enhance cancer immunotherapy [249] (Figure 7c). Functional (GOx was loaded onto the four vertices of the DNA tetrahedron, and was further wrapped by the cancer cell membrane to form the nanoregulator. When nanoregulator accumulated in the ER, the catalytic reaction initiated by GOx could consume glucose and generated H_2O_2, to prompt cells undergoing immunogenic cell death (ICD). In combination with immune checkpoint inhibitor (α-PD-1), this nanomodulator showed significant inhibitory effects on breast cancer and melanoma.

Xiang *et al.* used aptamer-modified DNA tetrahedron to encapsulate protein drug (RNase A) for targeted delivery into cancer cells [250]. T4 DNA ligase-treated and sealed DNA tetrahedron effectively protected protein drugs from degradation, and aptamer-modified DNA tetrahedron further improved the delivery specificity and cellular uptake efficiency of the protein drugs. Furthermore, high concentrations of glutathione in cancer cells could specifically cleave disulfide bonds to release RNase A, leading to apoptosis in specific cancer cells.

Ke *et al.* reported a DNA tetrahedron-based enzymatic cascade strategy for synergistic cancer therapy *in vivo* [251]. They precisely assembled GOx and HRP simultaneously on DNA tetrahedron. This nanocomposite was efficiently taken up by cells, and GOx could effectively deplete glucose for tumor starvation therapy and increased local hydrogen peroxide concentration. Moreover, HRP catalyzed Indol-3-acetic acid (IAA) in the presence of H_2O_2, to generate free radicals (IAA) leading to tumor cell apoptosis. The spatial confinement of DNA tetrahedron enhanced the transport efficiency of intermediate metabolites between enzyme cascades. This strategy demonstrated effective synergistic cancer therapy both *in vitro* and *in vivo*.

Lin *et al.* demonstrated a melittin-loaded DTN for active targeted therapy of melanoma as a “nanobee” [252] (Figure 7d). They found that upon exposure to the target protein on the cell membrane, the framework of the DNA tetrahedron underwent a conformational change, which resulted in the selective release of melittin from the exoskeleton, a selective lethal effect, and enabled the active nanobee to target tumor cells. At the same concentration of melittin, nanobee exhibited higher and more selective cytotoxicity to target cells when compared with free melittin. Furthermore, a combination of experimental screening and molecular dynamic analysis confirmed that melittin was completely encapsulated by the DNA tetrahedron, which was believed to be the basis of the minimization of off-target effects of the nanobee.

3.5 Multiple drugs

Accumulating evidence indicates that single therapy no longer meets the needs of a majority of cancer treatment, and combination therapy with multiple drugs has become a promising therapeutic strategy, which may require a multifunctional drug delivery system. Chemotherapy drugs can be loaded onto DNA tetrahedron by intercalating into the DNA double helix, chemical coupling, directly adding to the DNA strand, or by hybridizing with the DNA strand. In addition, DNA tetrahedron not only is non-toxic with reasonable biocompatibility, but can also simultaneously load and deliver different drugs. Thus, DNA tetrahedron is an ideal carrier for building a multifunctional drug delivery system.

3.5.1 Chemo-gene therapy

Combination of DOX and nucleic acid drugs has been loaded with and delivered by DNA tetrahedron for treatment of cancer. Zhong *et al.* used aptamer-modified DNA tetrahedron to co-deliver DOX and DNAzyme for targeted

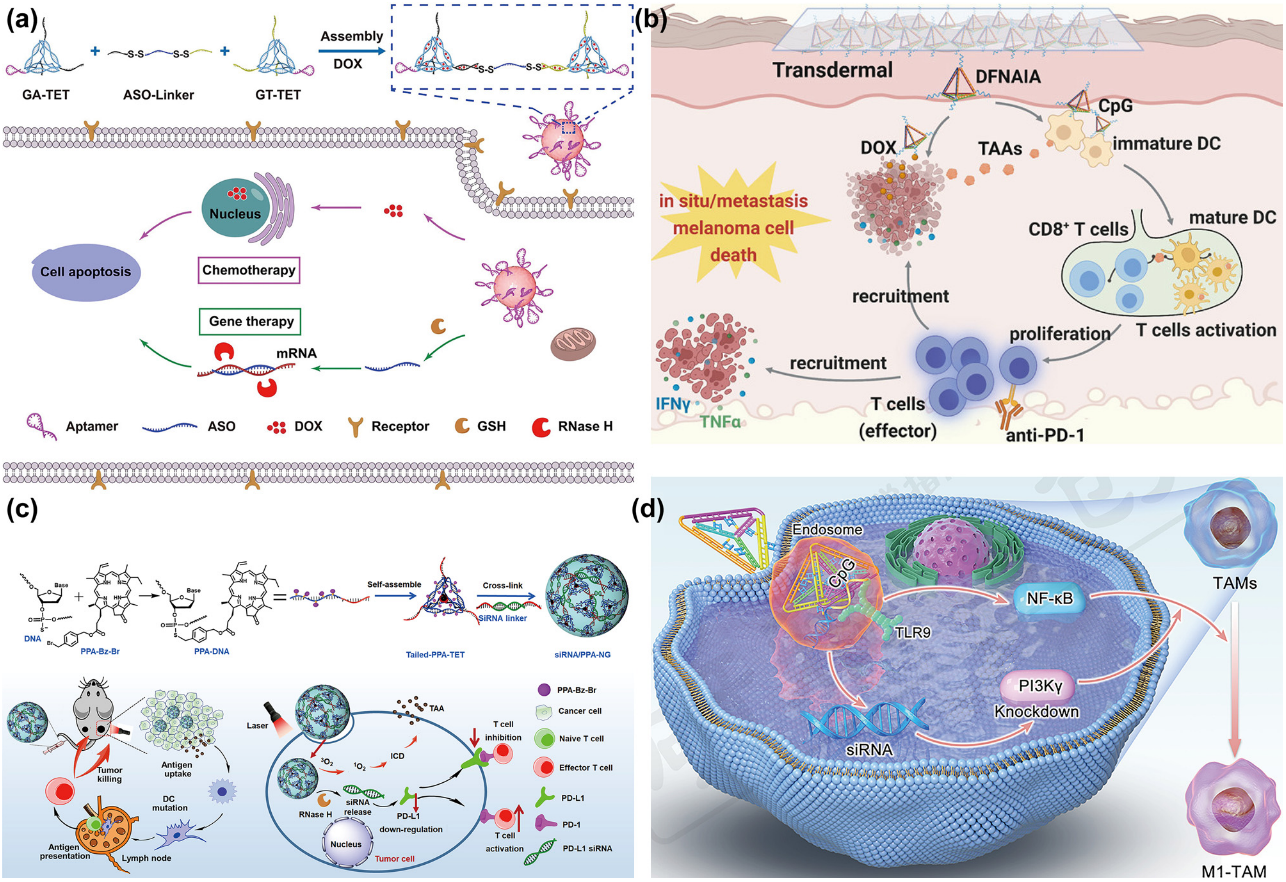

Figure 8: DNA tetrahedron-based multiple drugs delivery systems. (a) An aptamer-modified DNA tetrahedron-based nanogel for efficient combined chemo-gene therapy of multidrug-resistant tumors [254] Copyright 2021, American Chemical Society. (b) DNA tetrahedron loaded with DOX and CpG for transdermal delivery treatment of malignant melanoma based on chemoimmunotherapy [258] Copyright 2022, American Chemical Society. (c) Copackaging photosensitizer and PD-L1 siRNA in DNA nanogel for synergistic cancer gene-photodynamic therapy [262] Copyright 2022, American Association for the Advancement of Science. (d) Delivery of CpG and siRNA by DNA tetrahedron for gene-immunotherapy [268] Copyright 2022, Elsevier.

and synergistic chemo-gene therapy [253]. The aptamer and DNAzyme extended from the two vertices of the DNA tetrahedron, and one edge of the DNA tetrahedron was an i-motif sequence that could release DOX with better pH response. Compared with a single agent treatment, this nanocarrier system could greatly improve the therapeutic effect in cancer cells.

Ding *et al.* used aptamer-modified DNA tetrahedron to co-assemble with ASO to form DNA nanogels which then loaded with DOX, leading to the development of a combined chemo/gene therapy for multidrug-resistant tumor [254] (Figure 8a).

A recent study used DNA tetrahedron to co-load DOX and ASO, combined with gold nanoclusters (AuNCs), to self-assemble to form a programmed disassembly nanohybrid delivery system for activated tumor imaging and combination gene-chemotherapy [255]. They connected three strands of the DNA tetrahedron with the ASO targeting TK1 mRNA through disulfide bonds, and inserted DOX into the double helix of the DNA tetrahedron. Subsequently, the AuNCs were electrostatically adsorbed on the DNA tetrahedron. Within the cells, legumain could hydrolyze the ligands of AuNCs, thereby activating cancer imaging; and high concentrations of glutathione could cleave disulfide bonds to release ASO to silence TK1 mRNA. This nanohybrid delivery system showed antitumor activity both *in vitro* and *in vivo*.

3.5.2 Chemoimmunotherapy

Cancer chemoimmunotherapy is a combination of chemotherapy and immunotherapy that can exert a synergistic effect to improve anti-tumor efficiency [256]. Li's group used DNA tetrahedron as a carrier to co-deliver the chemotherapy drug DOX and the immune adjuvant CpG for synergistic cancer therapy [257]. They found that this strategy enhanced the immunostimulatory effects of CpG while preserving the tumor-killing activity of DOX, indicating synergistic anti-tumor effects as compared with monotherapy. Liu *et al.* employed DNA tetrahedron to co-deliver DOX and CpG for the treatment of malignant melanoma [258] (Figure 8b). DNA tetrahedron had excellent skin penetration, and thus the DNA tetrahedron could bring DOX and CpG deeper into tumor tissue than free drugs *via* transdermal delivery, resulting in significant tumor suppressive effect. In addition, when combined with PD1 antibody, the therapeutic effect was further enhanced, and greatly prolonged the survival time of animals. Importantly, superior antitumor immune responses against distant tumors were also observed on bilateral tumor models due to the induced systemic immune responses. Using a similar assembly strategy, Lin *et al.* also used DNA tetrahedron to simultaneously load DOX and CpG to facilitate ICD-related chemo-immunotherapy [259]. In this study, DOX acted both as a chemotherapeutic agent and ICD inducer, whereas CpG immunostimulatory properties could facilitate DOX-induced immunotherapy. Therefore, this delivery system showed excellent antitumor effect and immune activation. In addition, their synergistic effect was further enhanced when co-administered with PD-L1.

3.5.3 Chemo-photodynamic therapy

Ye *et al.* reported a peptide/DNA nanocomplex for targeted co-delivery of chemotherapy drugs and photosensitizers to cancer cells [260]. The AS1411 aptamer extends from the three vertices of the DNA tetrahedron, and the G-quadruplex structure formed by AS1411 binds to the photosensitizer (TMPyP4). After DOX was loaded on the DNA tetrahedron, the synthesized polypeptide cationic polymer mPEG-PAsp (TECH) wrapped the DNA tetrahedron to form the final nanostructure composite. They found that the nanocomposite was efficiently taken up by HeLa cells and escaped from lysosome efficiently. Under 660 nm laser irradiation, TMPyP4 induced the upregulation of intracellular ROS, which in combination with DOX effectively inhibited HeLa cell growth, and showed excellent chemo-photodynamic synergistic anticancer activity.

3.5.4 Gene-photodynamic therapy

Ding *et al.* constructed a multifunctional DNA nanocarrier based on double-bundle DNA tetrahedron [261]. The vector can be loaded with nucleic acid drugs (ASO for gene therapy) and protein drugs (KillerRed for photodynamic therapy) to effectively regulate gene expression and inhibit cancer cell proliferation. This functional DNA nanocarrier could effectively penetrate the cell membrane and subsequently transported the drug into the subcellular organelles (mitochondria and nucleus), where the drug efficiently silenced the gene expression and significantly inhibited the cancer cell growth.

The chemical composition and pharmacodynamics of small-molecule drugs and macromolecular biologics are fundamentally different, so it remains challenging to load these two drugs into suitable nanocarriers for synergistic cancer therapy. Zhang *et al.* successfully chemically grafted a pheophytin A (PPA) photosensitizer to ssDNA pre-designed with phosphorothioate modification sites, and the four DNA strands grafted with PPA were assembled into DNA tetrahedron [262] (Figure 8c). The DNA tetrahedron was further hybridized with (PD-L1) siRNA containing cohesive ends to form siRNA and PPA co-loaded DNA nanogels. With dual therapeutic agents inside, DNA nanogels could photodynamically kill tumor cells and induced significant ICD. By silencing PD-L1 mRNA in tumor cells, this strategy also significantly promoted anti-tumor immune responses which synergistically enhanced anti-tumor efficacy. In addition, the results of hematoxylin–eosin staining showed that the DNA nanogels was nontoxic to other tissues and organs, and exhibited excellent biosafety and biocompatibility.

3.5.5 Gene-immunotherapy

Tumor-associated macrophages (TAMs) are the macrophages recruited from circulating monocytes to tumors and play key roles in tumor growth, invasion, and metastasis [263,264]. According to different activation modes, TAMs are divided into M1 and M2 phenotype macrophages [265]. M1 macrophages secrete proinflammatory cytokines, which can activate immune responses and have tumor-killing ability [266]. In contrast, M2 macrophages secrete anti-inflammatory cytokines that suppress adaptive immunity and promote tumor growth and progression [266]. Unfortunately, macrophages within the tumor microenvironment (TME) often behave as M2 macrophages. Therefore, repolarization of M2 macrophages to M1 macrophages is a promising therapeutic strategy in human malignancy.

Tian *et al.* used DNA tetrahedron to simultaneously delivered siRNA and CpG (DNA tetrahedron-siRNA/CpG) to covert M2 into M1 macrophages and thus release pro-inflammatory cytokines to attack tumors [267]. The results showed that DNA tetrahedron-siRNA/CpG stimulated excellent macrophage uptake efficiency *in vitro*, CpG and siRNA conferred the ability to reprogram macrophages, *i.e.*, M2 macrophages were repolarized into M1 macrophages. Furthermore, this delivery system possessed parallel delivery capability and induced striking apoptosis and necrosis of tumor cells. Importantly, in the 4T1 xenograft tumor-bearing mouse model, injection of DNA tetrahedron-siRNA/CpG into the 4T1 xenograft tumor-bearing mice effectively and consistently inhibited tumor growth with low adverse effects on major organs. Chen *et al.* also used DNA tetrahedron to co-deliver siRNA and CpG [268] (Figure 8d), which efficiently reprogrammed TAMs to the M1 phenotype and elicited significant antitumor immune responses. Furthermore, excellent antitumor efficacy was demonstrated in a breast cancer xenograft mouse model without significant systemic side effects.

3.6 DNA tetrahedron-based hybrid delivery systems

The hybrid drug delivery system formed by the combination of DTN and other nanomaterials is also widely used for the delivery of anticancer drugs. Hybrid drug delivery systems, which can combine the advantages of several different nanomaterials, may be one of the most promising applications in the near future.

3.6.1 High-molecular polymers

He *et al.* used redox-responsive polyethyleneimine (disulfide cross-linked PEI, PSP) to coat DOX-loaded DNA tetrahedron, a novel nanocomplexes (PSP/TDNs@DOX NCs) [269] (Figure 9a). PSP/TDNs@DOX NCs enhanced its serum stability by preventing enzymatic degradation, allowed DOX to deeply penetrate tumor cell/tissue, and overcame multidrug-resistant cancer.

Su *et al.* reported a hybrid carrier composed of cationic conjugated polymers (CCPs) and DNA tetrahedron for targeted co-delivery of DOX and ASO (G3139) [270]. They found that CCP combined with DNA tetrahedron to create a unique drug-carrying pocket, which was favorable for DOX loading. The combination therapy of DOX and G3139 delivered by this hybrid delivery system exhibited excellent anticancer activity *in vitro* and *in vivo*.

3.6.2 Liposomes

Shi *et al.* used pH-sensitive liposomes to coat DOX-loaded DNA tetrahedron to construct a programmatic liposome-based nanostructured drugs (LNSDs) for drug-resistant tumor therapy [271] (Figure 9b). LNSD could effectively reduce DOX leakage during delivery, and significantly improved antitumor efficacy in drug-resistant breast tumor-bearing mice when compared with DOX and liposome-based DOX.

Deng *et al.* constructed a dendrimer based on DNA tetrahedron, then loaded pH-sensitive Dox-MPK prodrugs, and finally coated liposomes and macrophage membranes in sequence, fabricated an intelligent cell-like nano-prodrug (Dox-MPK@MDL) by hierarchical self-assembly [272]. The results showed that Dox-MPK@MDL specifically targeted lung metastases of breast cancer through the biomimetic metastasis homing effect, and intelligently triggered the release of DOX at metastatic cancer cells, thereby significantly inhibiting lung metastasis.

3.6.3 Metal nanomaterials

Willner *et al.* reported hybrid nanostructures, which was composed of pH- and miRNA-responsive DNA tetrahedron/metal–organic frameworks (MOFs), as delivery vehicles for fluorescent dyes and chemotherapies [273]. This hybrid nanostructure locked by DNA tetrahedron gates, the pH- and miRNA-stimulation responses acted as keys to unlocked the hybrid system and released the drug. The hybrid nanostructures combined the high loading capacity of drugs in the porous MOFs and the effective cell permeation properties of the DNA tetrahedron. Subsequently, Willner *et al.* reported a similar study, in which the hybrid nanostructures were ATP- and VEGF-responsive [274].

Meng *et al.* constructed a size-controllable nanoparticle system based on DNA tetrahedron nanostructures (TDN) and gold nanoparticles (AuNPs) for deep tumor-penetration therapy [275] (Figure 9c). This system programmatically disassembled and released DOX as pH decreased from normal tissues to the TME and cancer intracellular regions.

He *et al.* reported a nanocomposite based on DOX-loaded DNA tetrahedron and gold nanorods (GNRs) for chemo-photothermal therapy [276] (Figure 9d). The results showed that the AS1411 aptamer-modified DNA tetrahedra loaded with DOX was further compressed by PEI-coated GNRs, which allows us to achieve photothermal function and lysosome escape. The nanocomposite induced severe apoptosis and necrosis of cancer cells under NIR irradiation, and the synergistic effect of photothermal and chemotherapy showed excellent cancer cell killing effect.

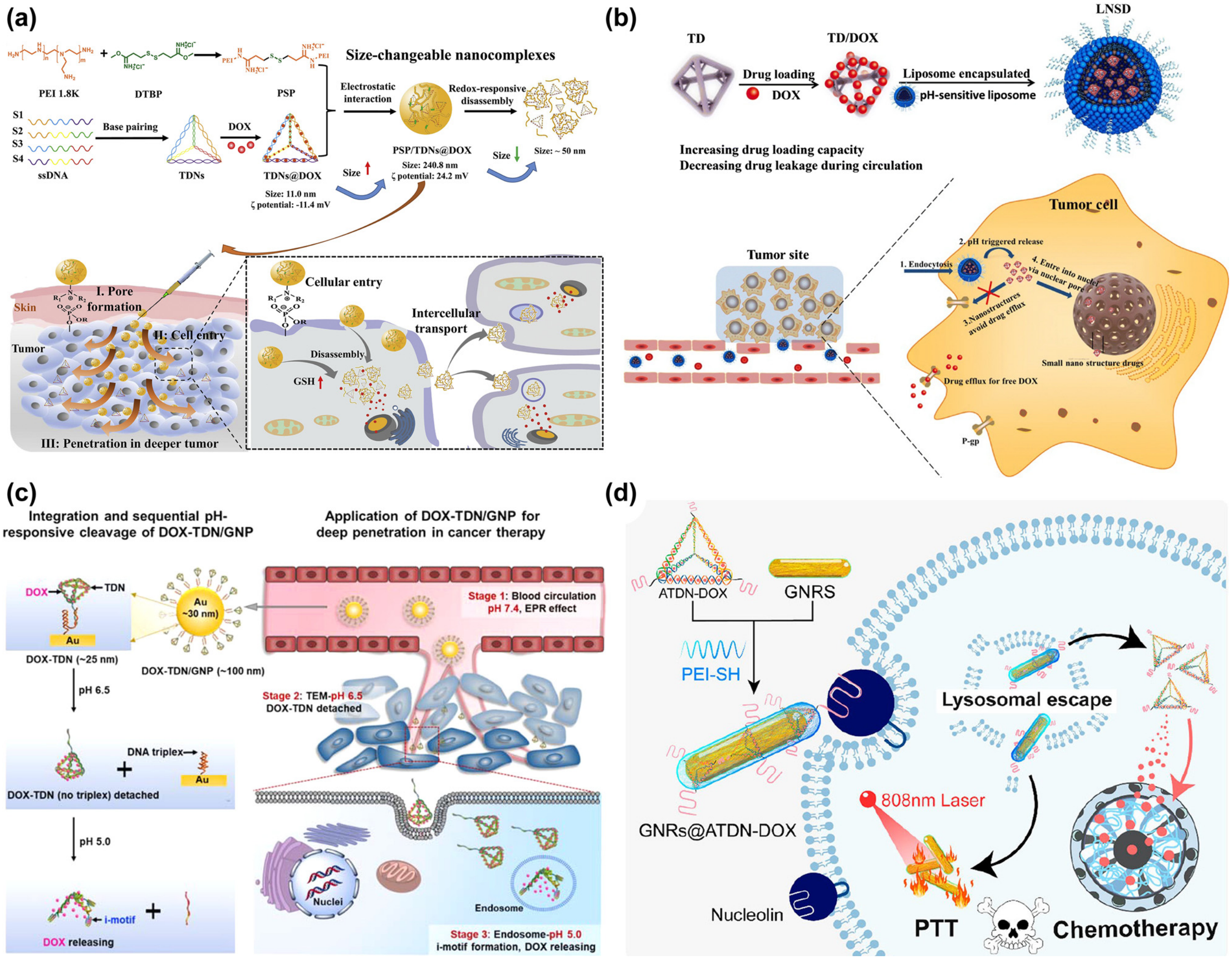

Figure 9: DNA tetrahedron-based hybrid delivery systems. (a) Redox-responsive polyethyleneimine/DNA tetrahedron/DOX nanocomplexes for deep cell/tissue penetration to overcome multidrug resistance [269] Copyright 2021, Elsevier. (b) A pH-sensitive liposome-based nanostructured DOX for drug resistance tumor treatment [271] Copyright 2019, Springer Nature. (c) Structure of DOX-TDN/GNP and the pH-triggered cleavage of the nanoformulation for deep tumor-penetrating anticancer therapy [275] Copyright 2022, American Chemical Society. (d) DNA tetrahedron/GNRs composites for chemo-photothermal therapy [276] Copyright 2022, Oxford University Press.

4 Challenges and prospects

In the past decade, DTN-based biomedical applications have made incredible progress in the field of cancer therapy. Among them, the applications of diagnosis and drug delivery have been discussed in this review. While DNA tetrahedrons are ideal carriers, there are still many challenges that need to be resolved in order to translate them into clinic. The stability of DNA tetrahedron in a physiological environment, the interaction between DNA tetrahedron and cells, and the pharmacokinetics of DNA tetrahedron are the major hurdles that DNA tetrahedron face in the field of cancer therapy. We discuss these challenges in detail below.

4.1 Stability

The stability of DTNs in the physiological environment is the most basic prerequisite for their use as cancer therapeutic carriers. Nucleases that degrade DNA are present in almost all types of tissues and body fluids, posing a serious threat to the application of DNA tetrahedron in cancer diagnosis and treatment. Therefore, a number of previous studies have evaluated the stability of DNA tetrahedron in the presence of selected nucleases or serum-containing media.

Keum and Bermudez analyzed the stability of DNA tetrahedron when incubated with various enzymes by gel electrophoresis [277]. They found that in the presence of DNase I or 10% FBS, both DNA tetrahedra were much more stable than DNA double helices. In 2011, Turberfield *et al.* simultaneously labeled DNA tetrahedron with Cy3 and Cy5, and used fluorescence resonance energy transfer to explore its intracellular stability [278]. The findings indicated that the DNA tetrahedron remained largely intact inside cells for at least 48 h. In addition, Fan *et al.* found that the addition of sidearms on the DNA tetrahedron did not affect its stability [158]. Lin *et al.* synthesized six DNA tetrahedron of different sizes (T7, T13, T17, T21, T26, and T37) and explored their stability in adipose stem cell lysates or 10% FBS [279]. They observed that T7 was very unstable in 10% FBS or cell lysates, and the others remained structurally intact for 8 h in 10% FBS and 12 h in cell lysates.

Recently, some new technical means have been employed to explore the stability of DNA tetrahedron in the presence of nucleases and serum. Langlois and Clark investigated the stability of DNA tetrahedron used size exclusion chromatography and found that DNA tetrahedron exhibited half-lives of 23.9 h and 10.1 h in 20 and 50% diluted human serum, respectively [280]. Medintz *et al.* directly microinjected DNA structures into cells, bypassed endocytic uptake, and assessed their stability in living cells [281]. The results of energy transfer loss monitored by fluorescence microscopy showed that the DNA crosshair was rapidly degraded within 20 min, while the DNA tetrahedron remained intact for at least 1 h after injection.

Magnesium ion (Mg^{2+}) is another factor that affects the stability of DNA tetrahedron. Perrault *et al.* found that low Mg^{2+} concentrations reduced the stability of DNA nanostructures in cell culture media [282]. Similar to other DNA nanostructures, DNA tetrahedron was assembled using millimolar Mg^{2+} to counteract the electrostatic repulsion between DNA helices [283]. However, in physiological environments such as blood or serum, Mg^{2+} concentrations are much lower [282]. Although monovalent cations such as Na^+ or K^+ in physiological environments could also stabilize DNA tetrahedron or other DNA nanostructures, they were much less efficient than Mg^{2+}, and required much higher concentrations than physiological conditions [284–287].

DNA tetrahedron has been used in *in vivo* experiments, including intravenous injection into mice, and can be transported to tumor sites through the bloodstream with sufficient stability [46,58,288]. But as drug delivery vehicle, it is necessary to further design DNA tetrahedron to have optimal stability under physiological conditions. Ahn *et al.* used L-DNA assembly to form mirror DNA tetrahedron (L-DNA tetrahedron), and they found that L-DNA tetrahedron exhibited better serum stability than natural D-DNA-constructed DNA tetrahedron (D-DNA tetrahedron) both *in vitro* and *in vivo* [167]. In addition, the *in vivo* half-life of L-DNA tetrahedron was also much longer than that of D-DNA tetrahedron. Lin *et al.* employed PEI to form nanocomplexes with DNA tetrahedron, which improved stability of DNA tetrahedron through the protection of PEI [289]. However, PEI has obvious cytotoxicity, and to solve this problem, Cai *et al.* used PEGylated protamine to create nanocomplexes with DNA tetrahedron through electrostatic interaction [290]. PEGylated protamine effectively slowed the clearance and degradation of DNA tetrahedron *in vivo*, making the them more stable in circulation. Su *et al.* developed a hybrid material composed of DNA tetrahedron and CCPs [270]. Their results showed that CPP could act as a shell to “protect” DNA tetrahedron. While bare tetrahedral DNA was rapidly degraded within 12 h in the nuclease cocktail and 100% serum, DNA tetrahedron-CCP was not significantly degraded for up to 24 h in the nuclease cocktail and 100% serum. In addition, Qian *et al.* used polyamines instead of Mg^{2+} to mediate the assembly of DNA tetrahedron [291]. Comparing with Mg^{2+}-assembled DNA tetrahedron, polyamine-assembled DNA tetrahedron exhibited higher serum stability. Therefore, extensive research on structural design and modification has significantly improved the stability of DNA tetrahedron and enhanced their potential use in biological environments.

4.2 Cell internalization

The polyanionic nature of DNA creates strong electrostatic repulsion between the DNA and the negatively charged cell membrane. Thus, unmodified DNA oligonucleotides alone cannot be efficiently internalized by cells without the aid of transfection agents. However, back in 2011, DNA tetrahedron was found to easily enter many different types of cells without using transfection agents [215,278]. Because DNA tetrahedrons are negatively charged and hydrophilic, cells are less likely to absorb them passively. Inspired by the way of viruses entering cells at the nanoscale [292], DNA tetrahedron was believed to be internalized in the cells through endocytosis. Currently, there are four distinct pathways of endocytosis in cells: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin- and caveolin-independent endocytosis [293–295]. Fan *et al.* used a single particle tracking method to monitor the entry and transport pathways of DNA tetrahedron in living cells [296]. Accumulated fluorescence imaging and biochemical experiments revealed that the entry of DNA tetrahedron into cells was an energy-dependent endocytosis process. After the DNA tetrahedron interacted with the cell membrane, it was rapidly internalized through the caveolin-mediated pathway.

Although it enters cells better than oligonucleotides, DNA tetrahedron had been modified with targeting ligands such as aptamers [175], FA [176], tumor-penetrating peptides [178], and monoclonal antibodies [182] in order to further improve its uptake efficiency and specificity, and reduce its systemic toxicity. Cell membrane receptors overexpressing in cancer cells are also expressed in normal cells, thus targeting a single receptor must result in nonspecific drug uptake, leading to increased toxicity to normal cells and reduced anticancer efficacy. Therefore, dual or multiple targeting may increase the specificity of drug delivery. For example, Jiang *et al.* used MUC1 and AS1411 aptamers co-modified DNA tetrahedron, and confirmed that bifunctional DNA tetrahedron could minimize toxic side effects and cope with drug resistance [173].

As mentioned above, the endocytic pathway of the DNA tetrahedron leads to its entry into the lysosome [296]. In the acidic environment of lysosome, some antitumor drugs cannot reach the target site and may be decomposed, and the function of drug molecules will be diminished. Therefore, in order to maximize the efficacy of antitumor drugs, DNA tetrahedron and their loaded drugs need to escape from the lysosome for high-precision targeting at subcellular resolution. This is crucial for the broad application of DNA tetrahedron in cancer therapy. Fan's group functionalized DNA tetrahedron with the NLS peptide that endowed it with nuclear targeting ability. This strategy helped the DNA tetrahedron to escape from the lysosome and facilitated its accumulation in the nucleus [296]. Li *et al.* modified DNA tetrahedron with precise amounts of KLA peptides to help DNA tetrahedron escape from lysosomes and achieve mitochondrial targeting [179]. In addition, the nanocomplexes that was formed by some cationic polymers and DNA tetrahedron also achieved lysosome escape [260,289,290]. Therefore, in order to better deliver anticancer drugs and achieve functional diversity, more elaborate design of DTS and introduction of more stimulatory elements are needed to ensure its escape from lysosomes.

4.3 Pharmacokinetics

DNA tetrahedron is widely used in the diagnosis and treatment of cancer. However, the current research on the pharmacokinetics of DNA tetrahedron, such as circulation, distribution, and metabolism *in vivo*, is not deep enough. Cancer occurs in most organs of the human body [3]. Previous studies have shown that size and shape, surface charge and properties, material composition, and route of administration of delivery system can all affect the localization of nanoparticles in specific organs [44]. The size of DNA tetrahedron commonly used to construct delivery systems is less than 10 nm, which is mainly excreted by the kidney with toxic side effects on the kidney [158,167], and also is unfavorable for the accumulation of DNA tetrahedron in cancer tissues. Therefore, in order to better deliver drugs and target specific organs, it is necessary to further investigate on the material composition, size, and surface charge of DNA tetrahedron.

Furthermore, the interaction of DNA tetrahedron nanoparticles with proteins needs to be studied more systematically. In fact, nanoparticles in biological fluids are adsorbed by proteins to form protein corona that can significantly affect their stability and integrity [297]. Protein corona composition have been shown to alter the therapeutic properties of various nanoparticles [298,299]. Protein corona and their effects on DNA tetrahedron-based drug delivery systems remain largely unexplored. Ahn *et al.* prepared four DNA tetrahedron by self-assembly of oligonucleotides modified with different sugar backbones, and they found that these four DNA tetrahedron had significantly different types and quantities of protein adsorption [229]. For this reason, the elucidation of the properties of protein coronas needs to be considered when constructing a drug delivery system using DNA tetrahedron.

Biosecurity is another concern. While DNA is biodegradable and biocompatible, things can change when they are assembled into DNA tetrahedron. For this reason, before DNA tetrahedrons are used in clinical applications, their potential immunostimulatory properties must be systematically investigated. At the same time, more in-depth studies are needed to determine whether DNA tetrahedron can lead to deleterious genetic recombination.

The investigations in terms of pharmacokinetics and biosafety will provide strong guidance for the better use of DNA tetrahedron in cancer therapy.

4.4 Production cost

In addition to the above challenges, production cost is a considerable challenge. For practical biomedical applications, gram-scale functional DTNs of high purity are required. Once these DTNs are produced on a large scale, purification after assembly is an important step before their use in specific applications. To meet this challenge, a variety of strategies for purifying DNA nanostructures have been developed, including ultrafiltration [300], gel purification [301,302], rate-zonal centrifugation [303], polyethylene glycol precipitation [304], size-exclusion chromatography [305], magnetic bead capture [306], fast protein liquid chromatography [300] and ultra-high performance liquid chromatography [307], *etc.* However, these strategies are tedious, time-consuming, or have considerable sample loss, requiring the development of methods with higher separation efficiencies.

The ssDNA currently assembled into DNA tetrahedron is mainly from commercial solid-phase synthesis, and the cost of constructing functional DNA tetrahedra is much higher than that of traditional polymer and lipid nanomaterials. Dietz *et al.* reported a low-cost and scalable method for the large-scale production of ssDNA of almost arbitrary sequence and length by using bacteriophage [308]. Recently, Gu *et al.* presented the paired-end cutting assisted by DNAzymes (PECAN), which enables the large-scale production of ssDNA of any sequence and any length (60–7,000 nt) with single-base precision, at milligram to gram yields, and with a purity greater than 98.5% [309]. Once industrialized by ton-scale fermentation, PECAN can provide ssDNA synthesis in gram level at significantly reduced cost of 0.2$/mg, more than three orders of magnitude cheaper than chemical synthesis. With the continuous innovation of ssDNA preparation methods, the cost of assembling functional DNA tetrahedrons will also continue to decrease.

5 Conclusion

Overall, DTNs have shown unlimited energy in the field of cancer therapy. The great advantage of DTNs lies in the versatility of DNA tetrahedron themselves. DNA tetrahedron can be adapted to the loading of a variety of drug molecules through intercalation, chemical coupling, electrostatic adsorption, and base complementation. These functionalized DTNs can respond to various cellular signals and have combinatorial strategies and multiple drug release mechanisms. In addition, the ever-growing DNA reaction machinery allows the translation of biological and chemical signals through structural reorganization of oligonucleotides. These functions will enable DTNs to become independent nano-systems for the detection of various biomolecules, such as proteins, nucleic acids, *etc.*, which are key indicators for the diagnosis of various cancers. Also, we have reason to believe that DTNs will become one of the most important tools in cancer therapy.

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