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RNA-cleaving DNAzymes for accurate biosensing and gene therapy

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RNA-cleaving DNAzymes, which are single-stranded catalytic DNA, have attracted considerable attention in bioanalysis and biomedical applications because of their high stability, high catalytic activity, easy synthesis, easy functionalization, and modification. By incorporating these DNAzymes with amplification systems, the sensing platforms can be used to detect a series of targets with high sensitivity and selectivity. In addition, these DNAyzmes possess therapeutic potential by cutting the mRNA in cells and viruses to regulate the expression of the corresponding proteins. This review systematically summarizes the applications of RNA-cleaving DNAzymes in recent years, explaining the uniqueness and superiority of RNA-cleaving DNAzymes in biosensing and gene therapy. Finally, this review extends the discussion to the challenges and perspectives of applying RNA-cleaving DNAzyme as a diagnostic and therapeutic agent. This review provides the researchers with valuable suggestions and promotes the development of DNAzymes for accurate analysis, early diagnosis, and effective therapy in medicine and their broader applications beyond biomedicine.

1. Introduction

Accurate analysis of disease-related markers is critically essential for early diagnosis and making a treatment plan.[1,2] In recent years, the appearance of some new biological detection and therapeutic technologies has improved the quality of life of patients and provided a chance for survival. However, some actual obstacles still hinder the practical applications of these technologies. Taking cancer as an example, some existing diagnostic techniques including biopsy,[3] molecular detection,[4] and bioimaging technology[5] have some insurmountable obstacles to providing convincing results for patients. Besides, apart from surgery and chemotherapy, gene therapy is an emerging technology with high security, while low delivery efficiency and poor targeting ability still hinder its widespread application.[6] Although researchers have made many attempts

to overcome these problems, current progress could not meet the demands for accurate diagnosis and efficient therapy.

DNAzymes, which can integrate diagnosis and therapy, have attracted researchers' attention to overcome these obstacles. DNAzymes are generated by *in vitro* screening technologies, and they have specific catalytic functions under the effect of cofactors. Since the first RNA-cleaving DNAzyme was discovered in 1994, an increasing number of DNAzymes with different structures and functions have been reported.[7] Among these, the 10–23 type and 8–17 type DNAzymes are the most extensively investigated due to their advantage of cleaving targeted substrate RNA.[8–12] Specifically, the recognition arms of DNAzymes combine with their substrates by Watson–Crick base pairing to cleave them by the catalytic core of DNAzymes with the help of cofactors. The working principle of the 10–23 type RNA-cleaving DNAzyme is shown in Fig. 1. With excellent RNA cleavage capability, these DNAzymes have been widely recognized in biosensing and gene therapy.

The significant advantages of high stability, high catalytic activity, easy synthesis, easy functionalization, and modification endow DNAzymes with enormous potential in disease diagnosis and therapy, especially when they are integrated with nanosystems.[13] The catalytic activity of DNAzymes can be activated only with the participation of cofactors, such as Pb^{2+}, Mg^{2+}, and Cu^{2+}.[9] By integration into special detection systems, DNAzymes have been widely employed for sensing metal ions, nucleic acids, proteins, and enzymes.[14] However, the ligand–receptor interaction mode is not able to satisfy the demands

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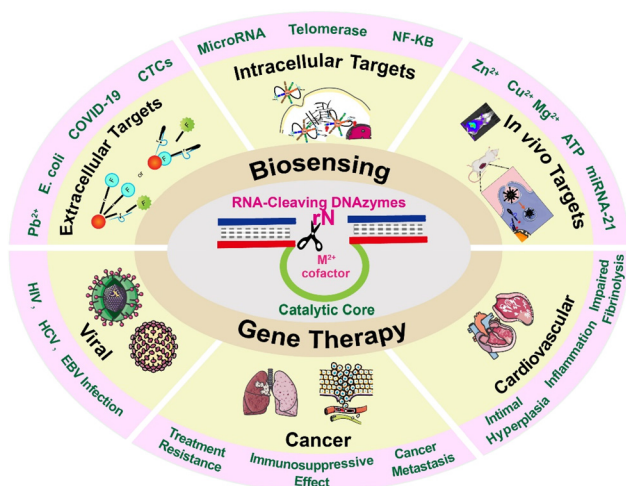†These authors contributed equally to this work.

Fig. 1 Schematic illustration of DNAzyme mediated RNA cleavage and its applications for biosensing and gene therapy.

for highly sensitive detection of low concentration targets, where signal amplification technologies are necessary to perform the analysis. Encouragingly, a series of amplification systems were developed to incorporate with DNAzymes for achieving sensitive detection. For example, the simple incorporation of DNAzymes with rolling circle amplification (RCA) technology endows the assay system with a more powerful signal amplification ability, thus greatly lowering the detection limits.[15] Besides, based on the RNA cleaving characteristics,

these DNAyzmes exhibit therapeutic potential by cutting the mRNA in cells and viruses to regulate the expression of the corresponding proteins. Compared to other gene therapy tools, RNA-cleaving DNAzymes show more stability and silencing effects.[16] Based on these points, this review summarized the recent progress of RNA-cleaving DNAzymes in biosensing and gene therapy and systematically introduced the advantages of DNAzymes in intracellular, extracellular, and *in vivo* sensing, as well as in the gene therapy of cancers, cardiovascular diseases, and viral infections. The wide use of RNA-cleaving DNAzymes proves that these small nucleic acids can serve as efficient tools for the development of biological detection platforms and new gene therapy drugs for viral infections, tumors, and other diseases.

2. DNAzyme-based biosensing

As one functional nucleic acid, DNAzymes with RNA cleaving properties have remarkable advantages, such as low cost, robust stability, and high catalytic efficiency.[17] A large number of DNAzyme-based biosensing platforms have been exploited to address the shortcomings of limited sensing capability and sensitivity. DNAzymes can be introduced into the sensing system to detect different targets, such as metal ions,[18–20] small molecules, proteins,[21,22] microRNAs,[23] other biomarkers,[24,25] *etc.* These DNAzyme-based biosensors have been applied in different situations such as extracellular, intracellular, and *in vivo* sensing.[26–28] Table 1 summarizes the

Table 1 RNA-cleaving DNAzyme-based biosensing

Application	Target	Sample type	LOD	Ref.
Extracellular	Pb^{2+}	All-sealed paper	0.004 nM	31
		Drinking water	0.96 ppb	35
	UO_2^{2+}	Environmental water sample	2.6 nM	32
	Cu^{2+}	Serum	3 nM	43
	Hg^{2+}	$[Fe(CN)_6]^{3-/4-}$ solution	23 fM	38
		Tap water	3.58 nM	37
	Tetracycline	Milk	4.411 nM	45
	Ca^{2+}	Serum	3.8×10^{-6} M	44
	E. coli	Milk	250 CFU mL^{-1}	47
		Apple juice	1.57 CFU mL^{-1}	48
	Cysteine	Human urine	2 nM	53
	Thrombin	Serum	4.5 pM	52
	COVID-19	Nasopharyngeal swab	0.96 copy per μL	51
	CTCs	Peripheral blood	1 cell per mL	54
Intracellular	MicroRNA	MCF-7 and HeLa cells	19.4 pM	61
		HeLa cells	—	60
		A549, H520 cells	10^2–10^3 cells per mL	58
		A549 cells	—	59
	Survivin mRNA	HeLa cells	1 pM	62
	ATP	MCF-7 cells	—	64 and 65
		4T1 cells	—	66
	NF-κB	HeLa cells	1.8 pM	69
	Telomerase	HeLa cells	7 cells per mL	67
	UDG	HeLa cells	8.71×10^{-6} (U mL^{-1})	72
	UDG	LO2, MCF-7, and HepG2	0.23 (mU mL^{-1})	25
In vivo	Zn^{2+}	Zebrafish	—	74
	Zn^{2+}	MCF-7 tumor-bearing mice	—	36, 73 and 75
	MicroRNA	MCF-7 tumor-bearing mice	1.8×10^{-11} M	80
			—	32 and 81

recent progress in RNA-cleaving DNAzyme-based biosensing systems.

2.1 Extracellular biosensing

DNAzyme-based biosensors have been widely used to detect food contaminants,[29] pathogens,[18] metal ions,[30–32] and various small molecules.[33,34] Compared with conventional detection methods, RNA-cleaving DNAzyme-based biosensors have demonstrated superior stability and accuracy. Due to the diversity of molecular targets, DNAzyme-based sensing systems could be applied to different areas, such as food safety, environmental monitoring,[35] and clinical diagnostics.[36] For example, Yuan *et al.* reported a DNA nanomachine as a super signal amplifier with a simple and efficient "Crab Claw" shape for the ultra-sensitive electrochemical detection of mercury ions (Hg^{2+})[37] (Fig. 2A). The electrochemical sensor

has a low detection limit of 3.58 fM for Hg^{2+}. It provided a simple, high-detection sensitivity, and rapid method for detecting heavy metal ions in environmental pollutants. Similarly, Chai *et al.* designed a bifunctional DNAzyme-based electrochemical biosensor with two detection pathways to test Hg^{2+} in the environment.[38] Compared with the conventional single pathway, this strategy was effective at avoiding false positive signals, and providing highly reliable and sensitive data, thus successfully circumventing the shortcomings of previous proportional electrochemical biosensors (Fig. 2B). Lead ions (Pb^{2+}), as one of the most toxic metallic pollutants, can cause serious damage to the human body when lead ion contaminated water is consumed. However, the detection methods developed in the past few decades were limited to well-trained operators.[39] Thus, Guo *et al.* developed a fast and precise Pb^{2+} sensing nanoplatform based on DNAzyme–AuNP
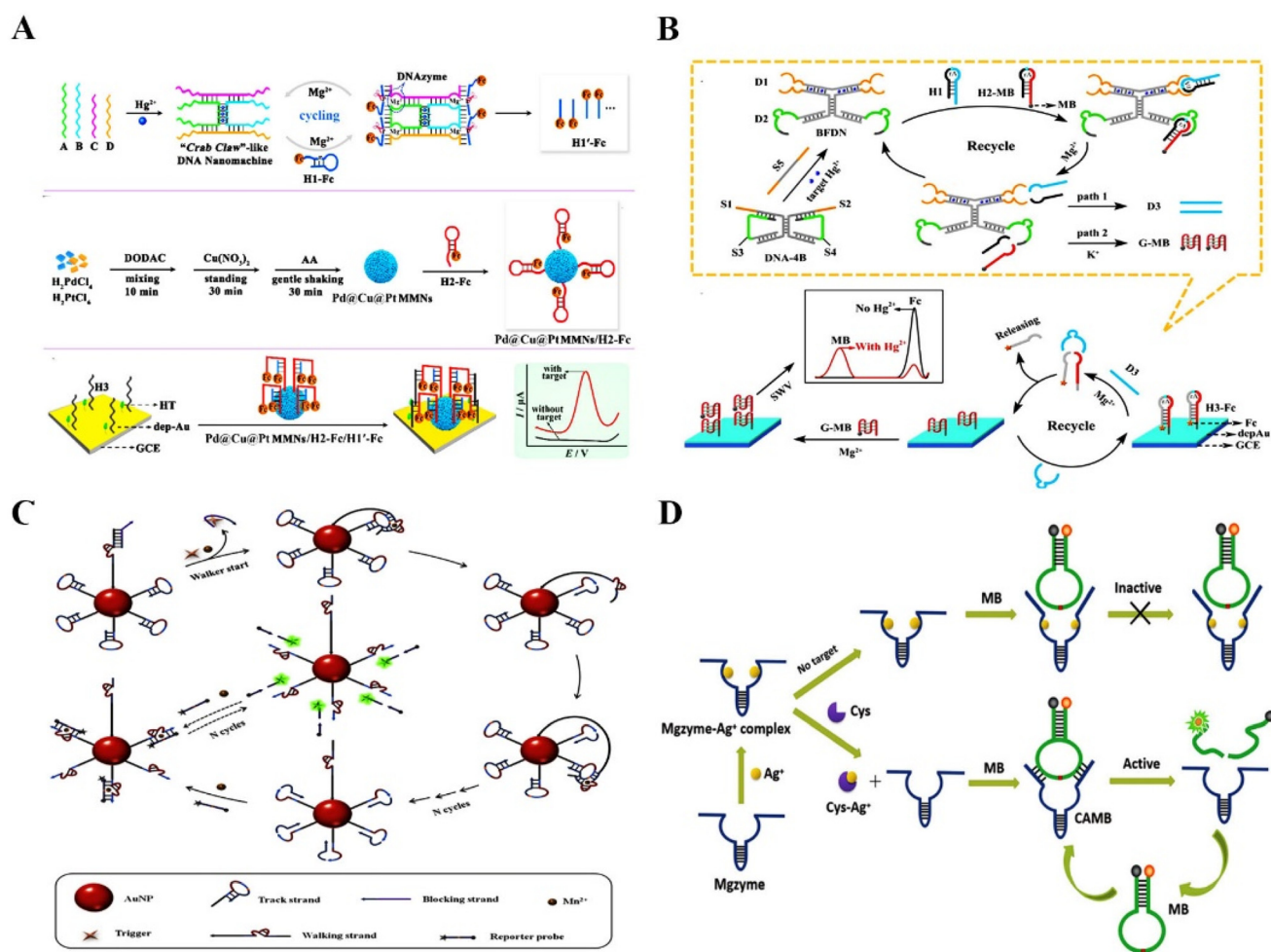
Fig. 2 The application of RNA-cleaving DNAzymes in extracellular sensing. (A) A DNA nanomachine as a super signal amplifier with a "Crab Claw" shape for the ultra-sensitive electrochemical detection of Hg^{2+}.[37] This figure has been reproduced from ref. 37 with permission from American Chemical Society, copyright 2021. (B) A bifunctional DNAzyme electrochemical biosensor with two detection pathways for the detection of Hg^{2+} in the environment.[38] This figure has been reproduced from ref. 38 with permission from American Chemical Society, copyright 2019. (C) Accurate identification of mutant pathogens based on programmable DNAzymes and targeted nucleic acid sequences.[52] This figure has been reproduced from ref. 52 with permission from Elsevier B.V. All rights reserved, copyright 2021. (D) A turn-on fluorescent biosensor based on the competitively binding silver ion on DNAzymes for amplified Cys detection in human urine samples.[53] This figure has been reproduced from ref. 53 with permission from Royal Society of Chemistry, copyright 2019.

probes for highly selective and sensitive detection of Pb^{2+}.[40] Huang *et al.* reported an electrochemical DNAzyme based biosensing platform to detect Pb^{2+} in spiked real-world samples.[41] In brief, these methods are simple, fast, and highly sensitive and offer potential platforms for detecting metal ions in real water samples.

Food-borne diseases have a significant impact on human health worldwide. Bacterial contamination and excessive metal ion-containing edibles are the main culprits, which means that developing rapid and reliable detection strategies is essential to prevent the occurrence of such diseases.[42,43] Xiong *et al.* proposed an electrochemical biosensor using glass carbon electrodes modified with nitrogen-doped graphene, gold nanoparticles, and DNAzymes to detect the concentration of Ca^{2+} in the serum of dairy cows.[44] The developed biosensor exhibited an ultrasensitive sensing ability with a detection limit of 3.8×10^{-6} M, which presented a high potential to diagnose subclinical hypocalcemia. Sun *et al.* developed a simple and low-cost fluorescence signal-on sensing strategy based on DNAzymes to detect tetracycline in milk (detection limit of 4.411 nM).[45] *Escherichia coli* (*E. coli*) is a food-borne pathogen with widespread sources and low infectious doses from contaminated milk, water, and other routes, which seriously threatens human health.[46] Nonspecific binding is one of the severe challenges in food detection. Didar *et al.* reported a species-specific protein activatable DNAzyme probe to detect *E. coli* in milk.[47] The probe significantly improved the performance of DNAzyme, showing excellent detection ability for *E. coli* in milk, with a detection limit as low as 250 CFU mL^{-1} in less than one hour. Xu *et al.* also constructed a fluorescence sensor based on the *E. coli*-specific protein activatable RNA-cleaving DNAzymes to detect *E. coli* in drinking water and apple juice samples (detection limit of 1.57 CFU mL^{-1}).[48] All of the above reports have proved the potential of the RNA-cleaving DNAzyme-based sensing platforms in detecting different analytes with high selectivity and sensitivity. In addition to analyzing food contaminants, rapid, high-speed, and acute detection of disease-related biomarkers in biological samples is vital for identifying and diagnosing.[49] However, current Watson–Crick hybridization-based assays have false positives because of the cross-reactivity of wild-type sequences.[50] Huang *et al.* developed a method for the accurate identification of mutant pathogens based on a programmable DNAzyme and targeted nucleic acid sequences.[51] The DNAzyme has site-specific cleavage capability, enabling the highly specific identification of single nucleotide variants (SNVs). To increase the detection sensitivity, T7 RNA polymerase-mediated transcriptional amplification was designed. This rapid, simple, highly sensitive biosensor could detect SNVs and COVID-19 variants with a detection limit of 0.96 copy per μL. For another disease sensing, Jiang *et al.* reported a DNAzyme walker-induced cascade signal amplification strategy for sensitively detecting disease-associated protein thrombin with a detection limit of 4.5 pM[52] (Fig. 2C). Furthermore, Xie *et al.* presented a turn-on fluorescent biosensor for amplified cysteine (Cys) detection in human urine samples based on the competitive binding of silver ions with DNAzymes[53] (Fig. 2D). Song *et al.* proposed a ratiometric surface-enhanced Raman spectroscopy (SERS)-based method through the circulating tumor cell (CTC)-triggered DNA walker with the assistance of Zn^{2+}-specific DNAzymes for highly sensitive detection of CTCs.[54] Hence, the biosensor has a very low background fluorescence and significantly increased fluorescence intensity, with a detection limit of 2 nM. In fact, RNA-cleaving DNAzymes are suitable to serve as sensing tools for specific testing of a large number of analytes, thus DNAzyme-based sensing strategies have been applied to overcome the deficiencies of clinical detection.

2.2 Intracellular biosensing

DNAzymes are ideal for metal-ion binding because of their vital role in the formation of the catalytic core in single-stranded DNA.[14] Although RNA-cleaving DNAzymes have been revealed to be promising tools for sensing extensive analytes, the lack of metal cofactors is an important challenge for intracellular analysis.[55] Technically, intrinsic intracellular cofactors are not sufficient for the efficient formation of the catalytic structural domain for the cleavage of substrates. It has been shown that metal cofactor-doped nanoparticles can be used to activate DNAzymes and increase their intracellular catalytic activity.[56]

Several vital reviews have confirmed the functions of miRNAs during disease progression, and the potential to serve as a reliable biomarker for disease diagnosis.[57,58] To overcome the above barriers, Chu *et al.* reported DNAzyme logic circuits using Cu/ZIF-8 nanoparticles (NPs) to image multiple microRNAs (miRNAs) in living cells[59] (Fig. 3A). Cu^{2+} could be efficiently released from the pH-responsive Cu/ZIF-8 NPs to serve as a cofactor, which enabled the Cu^{2+}–DNAzyme logic circuits to respond to multiple endogenous miRNAs. Fig. 3B shows great potential of the DNAzyme logic circuits to detect microRNAs. Dong *et al.* also reported a DNAzyme-based self-driven intracellular sensor consisting of ZIF-8 and DNAzymes for microRNA detection (Fig. 3C).[60] The DNAzymes are driven by Zn^{2+} to cleave the substrate strand for fluorescently imaging intercellular miRNA. Similarly, Li *et al.* investigated a light-activated walker consisting of a dissociable ZIF-8 for ensuring sufficient internal cofactors (Zn^{2+}) to facilitate the detection of miRNA in living cells.[61] Li *et al.* presented a photo-cage and cofactor self-supported DNAzyme biosensing platform to detect intracellular mRNA with high selectivity and sensitivity.[62] The newly proposed biosensor has good analytical performance for a model tumor biomarker (survivin mRNA) with a low detection limit in the pM level (Fig. 3D). Thus, RNA-cleaving DNAzymes have high sensitivity, excellent specificity, and high biostability in sensing miRNAs in living cells by introducing additional cofactors.

In addition to microRNA, adenosine triphosphate (ATP), which is the direct source of energy, plays a vital role in multiple physiological activities.[63] DNAzyme-based sensors have emerged as a potential tool for detecting ATP in cancer cells due to their differential expression in cancer cells and normal
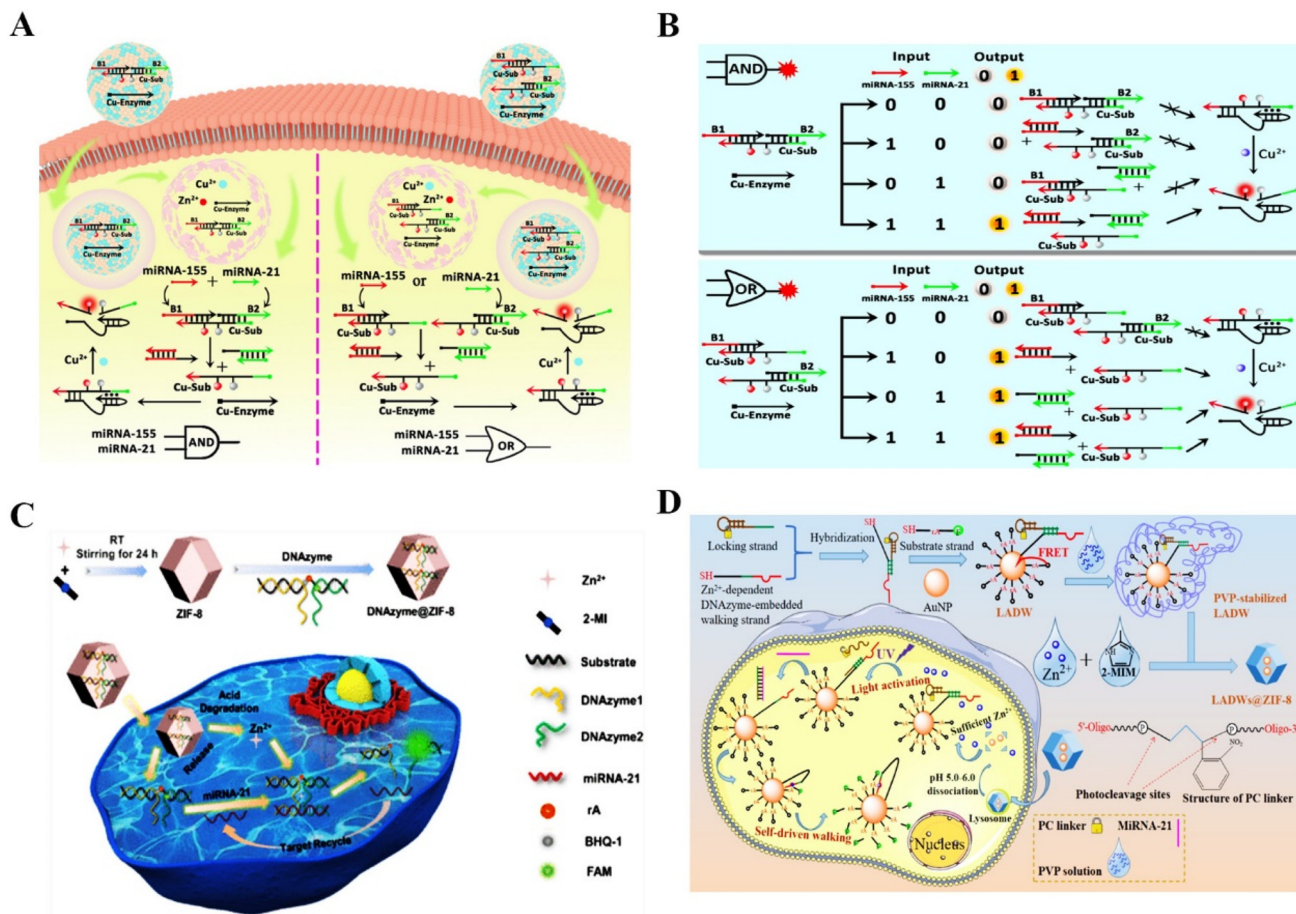
Fig. 3 The application of RNA-cleaving DNAzymes in intracellular sensing. (A) DNAzyme logic circuits for the detection of multiple miRNAs in living cells.[59] This figure has been reproduced from ref. 59 with permission from American Chemical Society, copyright 2021. (B) The schematic illustration of Cu^{2+}–DNAzyme-powered YES logic gate for detecting the miRNAs input in A549 cells and HeLa cells.[59] This figure has been adapted from ref. 59 with permission from American Chemical Society, copyright 2021. (C) A self-driven intracellular sensor consisted of ZIF-8 and DNAzymes for microRNA detection.[60] This figure has been adapted from ref. 60 with permission from American Chemical Society, copyright 2022. (D) A photo-caged and cofactor self-supported 3D DNAzyme biosensing platform for the detection of intracellular mRNA.[62] This figure has been reproduced from ref. 62 with permission from American Chemical Society, copyright 2021.

cells. Liu *et al.* proposed an ATP-sensing platform by combining MnO$_2$ nanoparticles with a DNA amplifier. The platform could provide adequate Mn^{2+} cofactors degraded by GSH for precise biosensing of ATP in living cells.[64] This novel Mn^{2+}-dependent DNAzyme biosensor could detect low-abundance of endogenous ATP in cancer cells. Dai *et al.* also reported an ATP/miRNA sensing dual-locked nanosensor using a honeycomb MnO$_2$ nanosponge (hMNS) to image ATP in cancer cells.[65] The GSH-degradable MnO$_2$ could supply rich Mn^{2+} for accurate DNAzyme cleavage (Fig. 4A). Similarly, Liu *et al.* reported a cascade catalytic amplification strategy using MnO$_2$ nanosponge and DNAzyme for fluorescence detection of ATP.[66] Stimulated by overexpressed GSH in cancer cells, the nanoprobe released the cofactor Mn^{2+}, which activated substrate-specific cleavage and generated an optical signal (Fig. 4B). This nanoprobe exhibited satisfactory biosafety and could detect ATP specifically within cancer cells. The above works provided some reliable methods for ATP imaging in cancer cells and opened up a new approach for high-precision disease diagnosis.

Furthermore, the detection of protein biomarkers plays a critical role in early diagnosis.[67] Nuclear Factor Kappa-B (NF-κB) is a vital mediator of inflammatory pathways and can act as a biomarker for inflammation related disease diagnosis.[21,68] However, the low expression of NF-κB in several cell lines limits its recognition. Huang *et al.* designed a novel biodegradable DNAzyme nanoprobe with cofactor self-supporting properties for detecting the protein in HeLa cells. The Zn^{2+}-dependent DNAzyme nanoprobe showed high-sensitivity in the determination of NF-κB with a low detection limit,[69] providing a promising method for efficient imaging of low-abundance biomolecules in living cells. α-Fetoprotein (AFP), a major serum protein produced by the adult hepatic cell, is of great significance for clinical tumor diagnosis.[22] Xiang *et al.* reported a fluorescent probe to this cancer biomarkers.[70] As a result, the metal ion-dependent DNAzymes had homogeneous and sensitive detection ability with a limit of 1.8 pM. Moreover, aberrant uracil-DNA glycosylases (UDG) might cause many diseases such as cancers, thus UDG can act as an avail-
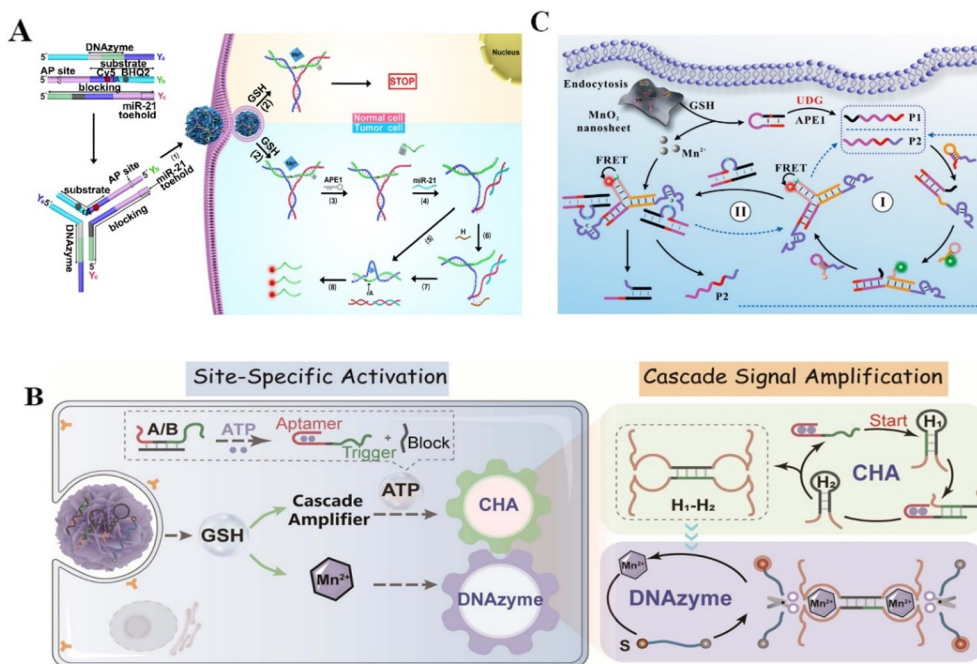
Fig. 4 RNA-cleaving DNAzyme-based biosensors for the detection of other biomarkers.[65] (A) This figure has been adapted from ref. 65 with permission from American Chemical Society, copyright 2022. (B) The detection of ATP.[66] This figure has been reproduced from ref. 66 with permission from American Chemical Society, copyright 2021. (C) The detection of UDG.[25] This figure has been reproduced from ref. 25 with permission from American Chemical Society, Copyright 2021.

able biomarker for clinical disease diagnosis. Accurately detecting UDG in living cells is vital for clarifying DNA repair pathways and discovering anti-cancer drugs.[71] Zhang *et al.* constructed an entropy-driven dumbbell-shaped DNAzyme assembly circuit for imaging UDG in living cells and distinguishing cancerous cells from normal cells.[72] Targeting UDG to excise damaged uracil released the trigger to form two catalytically active DNAzyme units. The Mg^{2+}–DNAzyme units enhanced the fluorescence signal for detecting intracellular UDG activity, providing a simple and reliable method for biological research and clinical diagnosis. Liu *et al.* also constructed a DNAzyme nanosystem for imaging intracellular UDG by combining DNAzyme with MnO_2 nanosheets.[25] In this strategy, intracellular GSH triggered the release of Mn^{2+} from MnO_2 nanosheets, acting as a cofactor to promote catalytic cleavage of substrates (Fig. 4C). The nanosystem realized UDG detection in living cells with enhanced sensitivity (LOD 0.23 mU mL^{-1}). Although DNAzyme-based biosensing is in the primary stage of experimental research, all of the above studies revealed the excellent efficiency of RNA-cleaving DNAzymes in enhanced biosensing and provided a valuable strategy for cellular-level clinical diagnosis and prognosis.
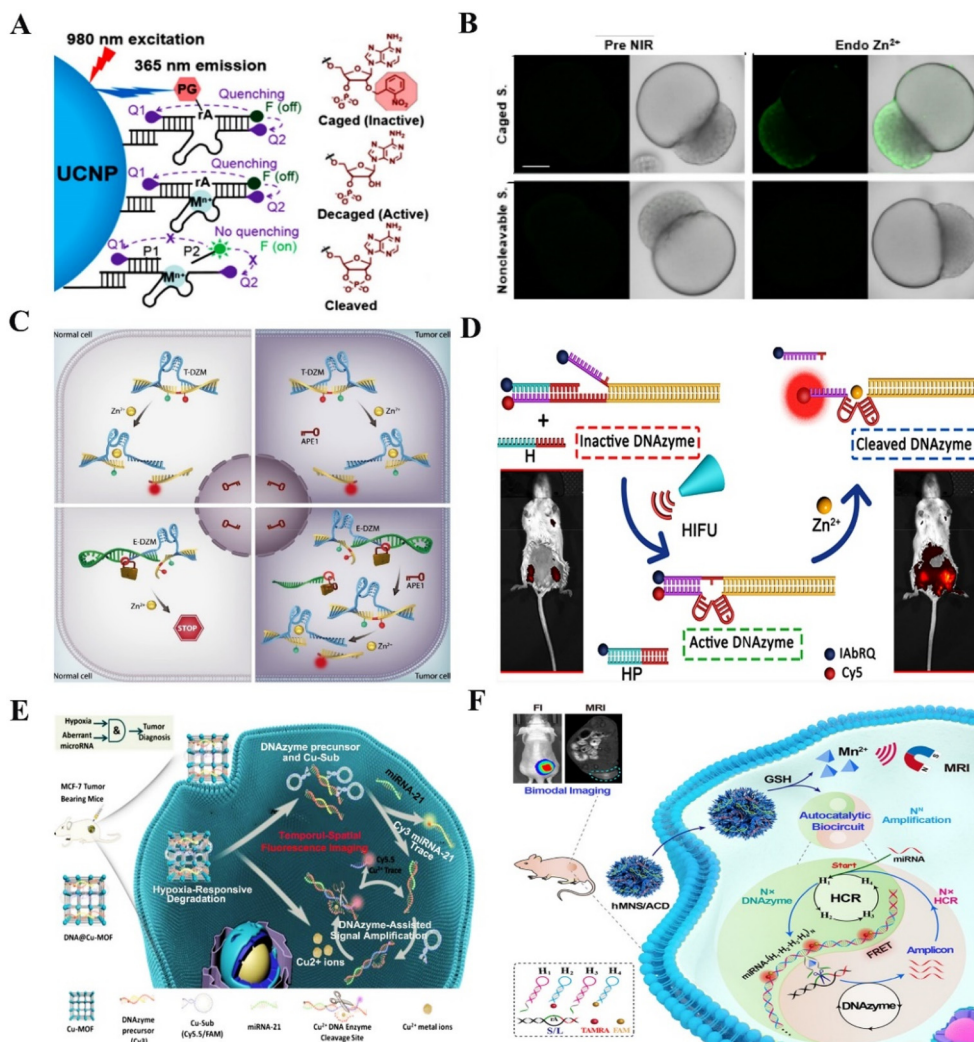
2.3 *In vivo* biosensing

Although there have been many successful examples of using mRNA-cleaving DNAzyme for sensing diverse targets under different conditions, the applications of these DNAzyme-based probes were restricted by the inefficient *in vivo* accumulations or insufficient stability of the probes. Fortunately, the recently

developed nanoprobes have great potential in solving these obstacles due to their self-protection effect and targeting ability.

Acute heavy metal ion poisoning or long-term ingestion would cause serious pathological changes, thus understanding the spatial-temporal distribution and the role of metal ions in biological systems is particularly important.[73] Lu *et al.* reported a Zn^{2+}-specific near-infrared (NIR) activatable DNAzyme nanoprobe which allowed real-time metal ion tracking of early zebrafish embryos[74] (Fig. 5A). As shown in (Fig. 5B), the fluorescence signal inside the zebrafish embryo was significantly enhanced after NIR irradiation, while the same non-cleavable structure showed no fluorescence enhancement. Furthermore, Li *et al.* proposed an enzyme-activatable DNAzyme sensor for *in vivo* differentiating metal ion signaling[36] (Fig. 5C). Compared to the conventional DNAzyme sensors, the blocking sequence contained a basic site that could be cut by cancer-specific enzymes, thus selectively restoring the inhibited ability of metal ion sensing in cancer cells. Lu *et al.* reported a Zn^{2+}-selective DNAzyme probe with a protector strand to block the formation of the catalytic domain, the Zn^{2+}-specific fluorescent imaging has been verified by the new DNAzyme based probe in mice (Fig. 5D).[75]

MiRNAs have been implicated in the progression of many diseases and have emerged as biomarkers for diagnosis and therapy.[76] The aberrant expression of miRNA in combination with tumor microenvironment (TME) characteristics has been extensively investigated for precise tumor imaging.[77,78] Although several assays have been applied to zebrafish embryos, the fluorescent signals were dependent on the transparency of

Fig. 5 RNA-cleaving DNAzyme-based biosensors for *in vivo* sensing. (A) Zn^{2+}-specific NIR activatable DNAzyme nanoprobe for real-time metal ion tracking of early zebrafish embryos.[74] This figure has been reproduced from ref. 74 with permission from American Chemical Society, copyright 2018. (B) The fluorescence signal inside the zebrafish embryo.[74] This figure has been reproduced from ref. 74 with permission from American Chemical Society, copyright 2018. (C) Enzyme-activatable DNAzyme sensor for *in vivo* detection of the metal ion.[36] This figure has been reproduced from ref. 36 with permission from Wiley-VCH GmbH, Copyright 2022. (D) Noninvasive and spatiotemporal control of DNAzyme-based imaging of metal ions *in vivo*.[75] This figure has been reproduced from ref. 75 with permission from American Chemical Society, Copyright 2022. (E) The multi-functional nanosystem contains a copper metal–organic framework (Cu-MOF) and DNAzymes for detecting miRNA *in vivo*.[30] This figure has been adapted from ref. 30 with permission from Elsevier B.V. All rights reserved, copyright 2021. (F) An autocatalytic DNAzymes biosensor for microRNA imaging *in vivo*.[81] This figure has been reproduced from ref. 81 with permission from Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Copyright 2020.

early fish larvae, their application was limited to other non-transparent animal models, where the sensitivity and detection limits were disappointing.[79] To overcome this barrier, Zhang *et al.* created a multifunctional nanosystem containing Cu-MOF and DNAzymes for *in vivo* miRNA detection[30] (Fig. 5E). Under hypoxic conditions, the DNA@Cu-MOF nanosystem released Cu^{2+}, DNAzyme precursors, and substrate strands, with a fluorescent signal recovery for miRNA detection. Similarly, Yang *et al.* developed nanodevices for intracellular and *in vivo* miRNA detection and manual control of drug release based on upconversion nanoparticles (UCNPs).[80] The nanodevices allowed amplified signal for miRNA-21 detection with a lower detection

limit of 1.81×10^{-11} M. Furthermore, Wang *et al.* constructed an autocatalytic DNAzyme amplification bio-scaffold for *in vivo* microRNA imaging.[81] This versatile scaffold not only transported DNA probes but also provided DNAzymes with abundant cofactors (Fig. 5F).

3. RNA-cleaving DNAzymes for gene therapy

Gene therapy is a technique that alters the host's gene to treat diseases. Gene therapy works through several mechanisms,

one of which is to inactivate disease-causing genes. RNA-cleaving DNAzyme is a gene therapy tool that offers a "knockdown" approach to produce gene inactivation, which is a powerful tool for performing sequence-specific RNA cutting. In biological systems, gene inactivation by DNAzymes can be carried out at the mRNA level, and then result in the expression inhibition of an unwanted protein. Following the first study of DNAzyme-based targeting inhibition of the Bcr–Abl oncogene in acute lymphoblastic leukemia, DNAzyme has been successfully applied to gene therapy in many clinical diseases related to gene mutation, especially in cancer, cardiovascular disease, and viral infection (Table 2).

3.1 DNAzyme-mediated gene therapy in cancer

The progression of cancers involves multiple steps and multiple gene mutations, which induce abnormal signaling pathways.[82] In this process, some signaling pathways that determine cell growth, division, and proliferation are improperly activated, while some apoptosis related signaling pathways are blocked, ultimately leading to the occurrence of cancers.[83] Although a series of gene therapy strategies targeting these abnormal genes have acquired significant breakthrough in cancer treatment, metastasis, immunosuppression, and treat-

ment resistance is still the main restriction to cure cancers. Given the significant advantages of DNAzymes, the gene intervention strategy using RNA-cleaving DNAzymes is expected to be a promising alternative for solving the above problems in cancer gene therapy.

3.1.1 Reverse treatment resistance. Treatment resistance, defined by increased tolerance of cancer cells against chemotherapy, radiation therapy, and thermal therapy, has been the main predictor of cancer mortality.[84] A variety of reasons contribute to treatment resistance, including multi-drug resistance, apoptosis suppression, specific genetic or epigenetic alterations, enhancing DNA repair in cancer cells, and so on.[85–87] Therefore, exploiting the RNA-cleaving DNAzymes to target the treatment resistance-related genes may be a promising therapeutic strategy to enhance the efficacy of cancer therapy. As a kind of innate or acquired resistance, chemoresistance is the main cause of the clinical failure of chemotherapy.[88] One of the chemoresistance mechanisms is the overexpression of multidrug resistance (MDR) genes.[89] Among their expressed productions, a large superfamily of transporters named ATP-binding cassette (ABC) transporters, such as breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), and multidrug-resistance protein (MRP), can pump out

Table 2 RNA-cleaving DNAzyme-based gene therapy

Disease	Target	Outcome	Ref.
Cancer	C-jun	Block MRP1 transcription, reverse MDR	95
		Block angiogenesis, enhance cell-mediate immunity	123
		Downregulate MMP-2 and MMP-9, inhibit cell proliferation, migration, and invasion	147
	P-gp	Reverse MDR	92
	MDR1	Reverse MDR	93
	HSP70	Overcome thermoresistance	101 and 102
	Survivin	Promote apoptosis, overcome therapeutic resistance	103, 109 and 110
	EGR-1	Growth inhibition, enhance PDT therapy	16 and 111
		Prevent tumor angiogenesis, promote apoptosis	136
	ATG5	Enhance autophagy suppression	113
	PD-L1	Promote T cell activation, reinforce tumor immune microenvironment	119 and 120
	β-Catenin	Improve DCs infiltration and maturation, T cell activation, enhance adaptive immune response	123
	VEGFR2	Inhibit angiogenesis	135
	IGF-II	Inhibit angiogenesis	137
	Twist	Suppresses EMT, inhibit tumor metastasis	27
	Aurora kinase A	Suppresses EMT, inhibit tumor metastasis	143
	MMP-9	Prevent ECM degradation	149
Cardiovascular diseases	EGR-1	Suppresses VSMC proliferation, inhibit neointima formation	155, 156, 158–160 and 170
		Inhibit inflammation, restore cardiac function	169 and 171
	C-jun	Inhibit VSMC proliferation, inhibit intimal hyperplasia	157 and 161
	PAI-1	Promote neovascularization, reduce fibrin deposition, inhibited VSMC proliferation and migration, prevent neointima formation	166–168
Viral infection	HIV-1 integrase	Inhibit viral replication	173
	HIV-1 CCR5	Decrease fusion and internalization	177
	HIV-1 CXCR-4	Decrease fusion and internalization	178
	HCV NS5B	Inhibit HCV RNA replication and translation	193
	HCV NS3	Inhibit the virus replication	194
	HCV IRES	Inhibit HCV RNA replication and translation	192, 193 and 195
	EBV LMP1	Inhibit cell proliferation, promote apoptosis	183 and 184
		Promote apoptosis, enhance radiosensitivity	185 and 187–191
		Induces DNA damage, causes cell cycle arrest	186
		Inhibit tumor angiogenesis	182

chemotherapy drugs, and reduce the accumulation of drugs in cells.[90,91] Therefore, designing MDR-associated ABC transporters targeting DNAzyme is a reliable treatment strategy for reversing MDR. In this aspect, our laboratory constructed a P-gp targeting DNAzyme nanoflowers by the RCA strategy to reverse the MDR of breast cancer. The nanoflowers have better biodegradability than the traditional nucleic acid nanocarriers. The constructed nanoflowers improved the sensitivity of doxorubicin-resistant breast cancer cells (MCF-7/MDR) towards Dox and reversed the P-gp-mediated MDR by silencing the P-gp gene[92] (Fig. 6A). Zokaei *et al.* used chitosan/cyclodextrin/tripo-

lyphosphate nanocarriers to deliver mRNA-cleaving DNAzyme targeting multi-drug resistance gene-1 (MDR1) into MCF-7/MDR cells. The results showed that the nanoplatform could downregulate MDR1 mRNA and sensitize MCF-7/MDR cells to Dox.[93] In addition to directly targeting MDR-associated ABC transporters, MDR can also be reversed indirectly by interfering with the expression of other regulators, such as the c-jun protein. Several studies have shown that c-jun, as an important transcription factor, upregulates the expression of ABC transporters such as ABCB1, ABCG2, and ABCC1, leading to increased drug resistance to chemotherapy in tumor cells.[94]
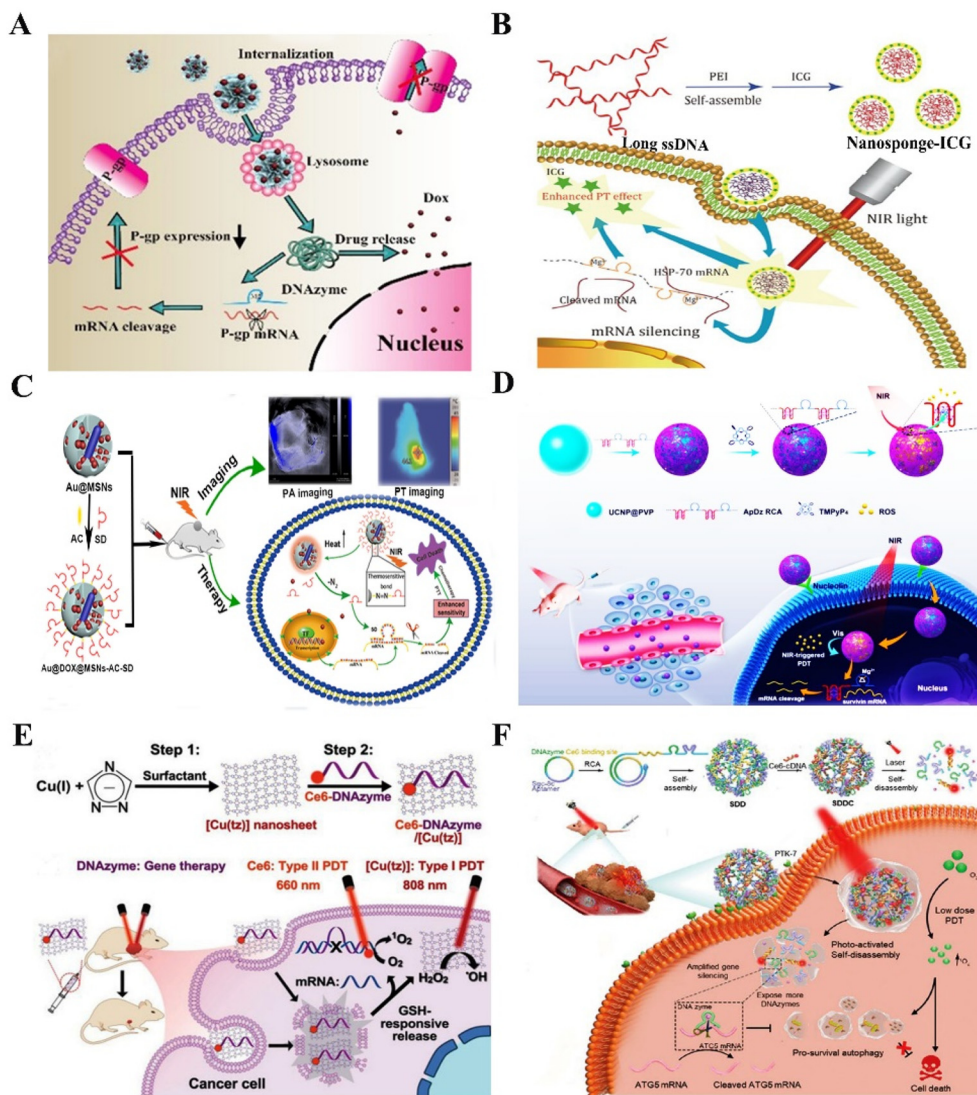
Fig. 6 The DNAzyme-based nanotherapeutic platform for reversing treatment resistance. (A) The DNAzymes NFs targeting P-gp for reversing tumor MDR.[92] This figure has been reproduced from ref. 92 with permission from Elsevier, copyright 2021. (B) The nanosponges targeting HSP70 for overcoming thermoresistance.[101] This figure has been reproduced from ref. 101 with permission from the author(s), copyright 2020. (C) The nanoplatform targeting survivin for enhanced PTT therapy[103] This figure has been reproduced from ref. 103 with permission from the Royal Society of Chemistry, copyright 2018. (D) The DNAzyme-assisted upconversion nanoplatform for enhanced PDT.[109] This figure has been reproduced from ref. 109 with permission from American Chemical Society, copyright 2020. (E) The multifunctional nanoplatform for reversing treatment resistance *via* DNAzyme-mediated EGR-1 gene silencing.[112] This figure has been reproduced from ref. 112 with permission from Wiley-VCH GmbH, copyright 2021. (F) Multifunctional DNA nanoflower for enhanced PDT by DNAzyme-mediated ATG5 gene silencing.[113] This figure has been reproduced from ref. 113 with permission from Wiley-VCH GmbH, copyright 2021.

Sun *et al.* constructed functionalized mesoporous silica nanoparticles (MSNs) to co-deliver Dox and c-jun targeting DNAzymes for overcoming prostate cancer drug resistance and metastasis. The results showed that the co-delivery system effectively increased intracellular Dox concentration in Dox-resistant PC-3 cells, due to RNA-cleaving DNAzyme-mediated downregulation of c-jun expression and subsequent inhibition of MRP1 expression.[95] In a word, although MDR is a thorny problem in cancer chemotherapy, RNA-cleaving DNAzymes show a good synergistic effect by the silencing of drug-resistant genes, showing great application potential in reversing the MDR.

Thermoresistance is an inescapable problem during the hyperthermia-based treatments of cancer, principally involving photothermal therapy (PTT) and magnetic hyperthermia.[96] Although they are promising anticancer strategies to ablate tumors, the accompanied thermoresistance greatly restricts their clinical application.[97] Several studies proposed that heat shock proteins (HSPs), especially HSP70 and HSP90, are the key defense molecules of tumor thermoresistance and endow cancer cells with high resistance to heat.[98–100] Based on this, our laboratory constructed an HSP70 targeting DNAzyme-based nanoplatform by assembling polyetherimide (PEI), indocyanine green (ICG), and multivalent DNAzymes for reversing thermoresistance in PTT. The nanoplatform presented great gene silencing ability and excellent performance in photothermal conversion, which would sensitize MCF-7 cells to hyperthermia by downregulating the overexpression of HSP70[101] (Fig. 6B). In another work, Xi *et al.* synthesized a MnO_2-coated polydopamine ($PDA@MnO_2$) nanoplatform to deliver HSP70 targeting DNAzymes for enhancing PPT efficiency.[102] These works provide promising gene therapy platforms for enhanced PTT *via* silencing HSP genes. In addition, apoptosis suppressor genes are involved in the occurrence, development, prognosis, and treatment resistance of cancers. Targeting apoptosis suppressor genes will be an effective way to reverse thermoresistance. For example, our laboratory synthesized a NIR-light activated nanoplatform using MSN-coated gold nanorods for delivering survivin targeting DNAzymes. The nanoplatform sensitized triple-negative breast cancer cells to heat by silencing the survivin gene[103] (Fig. 6C).

As one emerging tumor treatment strategy, photodynamic therapy (PDT) has been applied in clinical practice. By employing a photosensitizer (PS) and a light source, reactive oxygen species (ROS) are generated to kill the cancer cells. During this process, cancer cells developed various resistance mechanisms to protect themselves from being damaged by ROS, known as PDT resistance.[104–106] Abnormal expression of stress-response-related genes, alteration of apoptosis pathways and autophagy, and increased repair of DNA contribute to PDT resistance.[107] For example, survivin, an inhibitor of apoptosis (IAP) family protein, is involved in PDT resistance and acts as a potential target for improving PDT.[108] Inspired by this point, our laboratory constructed an upconversion PDT nanoplatform by adsorbing a long single-stranded DNA (ssDNA) on the upconversion nanoparticles for precise PDT, which could be trig-gered by NIR light to generate ROS for enhancing apoptosis of cancer cells. The ssDNA with multiple aptamer AS1411 and survivin targeting DNAzymes endowed the upconversion nanoplatform with high recognition, drug loading, and gene silencing capacity[109] (Fig. 6D). Yi *et al.* developed an enzyme-activatable nanodevice by adsorbing a DNA duplex probe onto MnO_2 nanosheets, which endowed the nanodevice with telomerase-triggered Ce6 signal "turn-on" and survivin mRNA silencing ability for enhanced PDT.[110] Early-response genes upregulated after PDT-mediated oxidative stress encoded many transcription factors such as EGR-1. A GSH-responsive MnO_2 nanoplatform was constructed to deliver TB and EGR-1 targeting DNAzymes for enhanced PDT. The result showed that the nanoplatforms could effectively inhibit the growth of tumors *in vivo* and *in vitro* by the combination of PDT and gene therapy.[111] To improve the efficacy and safety of gene silencing, Liu *et al.* synthesized ultrathin 2D nanosheets of Cu(I) 1,2,4-triazolate coordination polymers (CP) to deliver EGR-1 targeting DNAzymes. The 2D CP nanosheets not only have the ability of GSH-responsive DNAzyme release to ensure the DNAzymes with high gene silencing efficiency and stability but also have the performance of photosensitizers. The results showed that 2D CP nanosheets significantly inhibited tumor growth by up to 88.0% in the MCF-7 tumor-bearing mice, indicating the nanoplatforms with the predominant anticancer effect by combining of DNAzyme-based gene therapy and PDT[112] (Fig. 6E). In addition, the stimulation of autophagy in apoptosis-competent cells increases cellular resistance to PDT. In light of this problem, Shi *et al.* synthesized a photoactivated DNA nanoflower (DNF), which consisted of a tumor-targeting Sgc8c aptamer and autophagy-related-genes targeting DNAzymes, and was modified with PS (Ce6). The results confirmed that DNF improved the therapeutic efficiency of low-dose PDT by inhibiting the expression of autophagy gene ATG5 mRNA[113] (Fig. 6F). In view of the abovementioned DNAzyme assisted gene silencing strategy, DNAzyme-based nanoplatforms have represented a promising breakthrough in overcoming PDT resistance.

3.1.2 Overcoming the immunosuppressive effect. Immunotherapy has become the fourth major cancer treatment method after surgery, radiotherapy, and chemotherapy. In the past decade, although significant breakthroughs have been made in immunotherapy, including CAR-T, immune checkpoint inhibitors, vaccines, oncolytic viruses, *etc.*, immunosuppression is a big challenge during cancer immunotherapy.[114,115] Programmed cell death protein 1 (PD-1) and its ligand PD-L1 are important immunoregulatory signaling molecules. The PD-1/PD-L1 signaling pathway can affect the activation of T lymphocytes, and play a vital role in the formation of the local tumor immunosuppressive microenvironment. Studies have shown that the blocking PD-1/PD-L1 signaling pathway could overcome the suppression of immune cells and reactivate cell-mediated immune response.[116–118] The latest research of RNA-cleaving DNAzymes aiming at targeting PD-1/PD-L1 has yielded some promising outcomes. Interestingly, to improve the poor immune response in the

tumor, Liu *et al.* developed Mn^{2+}/Fe^{3+} hybrid metal–phenolic networks (MPNs) loaded with anti-PD-L1 DNAzymes for amplified tumor ferroptosis-immunotherapy. In this study, the nanosystem demonstrated excellent synergistic effects including ferroptosis, PTT, immunogenic cell death (ICD), and Mn^{2+}-activated gene silencing of DNAzymes. The results showed that the nanoplatform achieved a higher level of mature DC infiltration, enhanced intratumoral recruitment of both $CD4^+$ T cells and $CD8^+$ T cells, and increased cytokine excretion (IFN-γ, TNF-α, and IL-6), resulting in strong antitumor immunity[119] (Fig. 7A). Guo *et al.* designed a hybrid nanocarrier with a PLGA core and a MOF shell *via* Fe^{3+}/Mn^{2+}–tannic acid coordination to deliver DPCPX (an adenosine A1 receptor inhibitor) and anti-PD-L1 DNAzyme for reversing the negative effect of adenosine A1 receptor-targeted immunometabolism modulation. The nanosystem showed an obvious ability to downregulate PD-L1 mRNA for promoting $CD8^+$ T lymphocyte infiltration and activation.[120] Furthermore, An *et al.* designed an immunotherapeutic platform composed of a calcium core, aptamer–DNAzyme conjugate (cAD), and a hydrophilic shell (DSPE-PEG$_{2000}$) for boosting tumor immunotherapy. This nanoplatform has multifunctional characteristics of Ca^{2+} interference-mediated TME reprogramming and Ca^{2+}-activated

PD-L1 silencing. The result showed that the nanoplatform not only prevented primary tumor development and lung metastasis but also offered a long-lasting immunological memory to avoid tumor recurrence[121] (Fig. 7B). Du *et al.* used hollow-MnO_2 nanoparticles to co-deliver glycyrrhizic acid and Ce6-labeled-DNAzyme. The nanosystem specifically silenced the PD-L1 gene, which could facilitate T cell activation, simultaneously resulting in immunogenic cell death[122] (Fig. 7C). As the most effective antigen-presenting cells (APCs), DCs are closely related to T cell activation and immunotherapy. To improve tumor antigen presentation and DC infiltration, Zhu *et al.* fabricated a nanovaccine composed of a polyserotonin core and a MOF shell *via* TA/Mn^{2+} coordination for β-catenin targeting DNAzyme delivery. DNAzyme-mediated β-catenin mRNA silencing improved DC infiltration and maturation, resulting in better T cell activation and enhancement of adaptive immune response[123] (Fig. 7D). In another study, Cai *et al.* demonstrated that Dz13 (a DNAzyme-targeting c-jun mRNA) could inhibit squamous cell carcinoma growth involving acquired cell-mediated immunity *via* an increased percentage of $CD8^+$ and $CD4^+$ cells in tumors.[124] A subsequent study by Cai, also demonstrated that Dz13 could prevent tumor recurrence through adaptive immunity.[125] All these studies high-
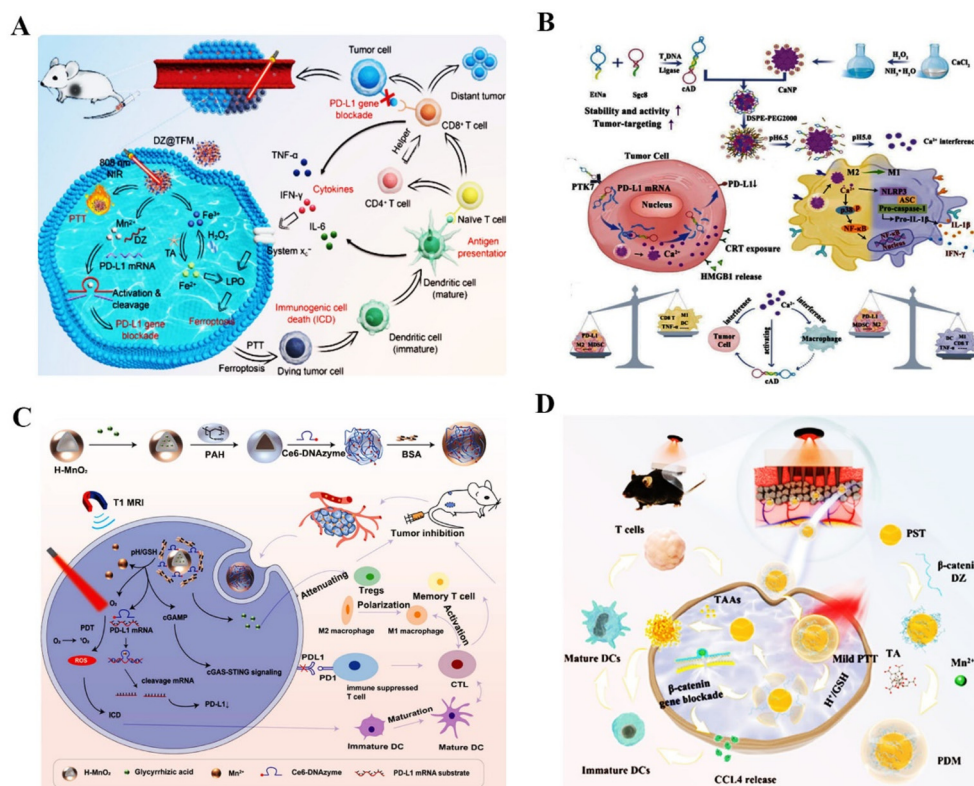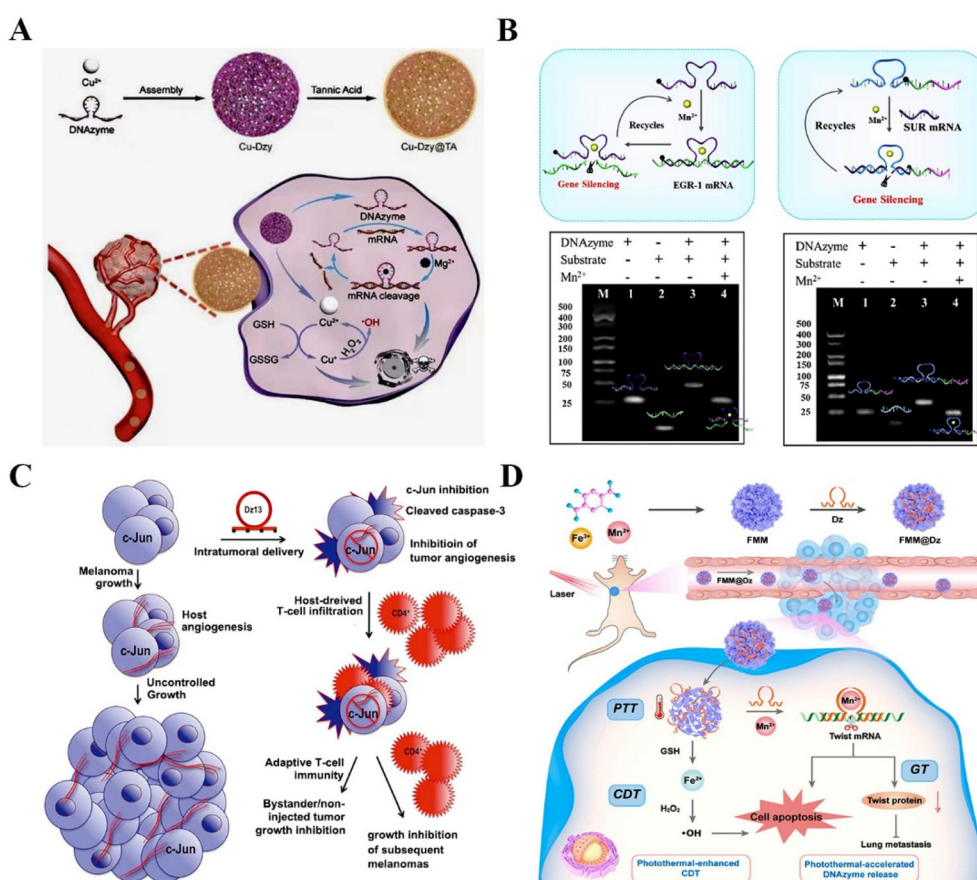

Fig. 7 DNAzyme-based nanotherapeutic platform in tumor immunotherapy therapy. (A) DZ@TFM for PTT-enhanced cyclically amplified tumor ferroptosis-immunotherapy.[117] This figure has been adapted from ref. 117 with permission from Advanced Healthcare Materials, Copyright 2021. (B) A bioactive nanoplatform for boosting tumor immunotherapy.[71] This figure has been adapted from ref. 71 with permission from Advanced Functional Materials, copyright 2022. (C) The nanosystem for synergistic antitumor therapy of PDT and immunotherapy.[119] This figure has been adapted from ref. 119 with permission from Small, copyright 2022. (D) β-Catenin targeting DNAzymes promotes CCL4 excretion and DCs infiltration.[120] This figure has been reproduced from ref. 120 with permission from Research, copyright 2022.

lighted the benefits of combining mRNA-cleaving DNAzymes *via* multifunctional nanomedicine to enhance cancer immunotherapy.

3.1.3 Inhibition of cancer metastasis. Metastasis, the most dangerous stage of tumor progression, is the main cause of death.[126] Although significant advances have been made in the past decades to reveal the underlying mechanisms and therapeutic targets of cancer metastasis, the inhibition of cancer metastasis has not yet achieved the desired outcome in the clinic. Previous research has demonstrated that angiogenesis, epithelial–mesenchymal transformation (EMT), over-expression of metastasis or invasion-related genes, and destruction of the extracellular matrix (ECM) are implicated in the process of metastasis, and they are considered to be promising targets for gene silencing.[127–129]

Firstly, angiogenesis is one of the essential processes in cancer metastasis. The development of new blood vessels will provide the tumor with enough nutrition and oxygen to support its growth and spread.[130] The essential transcription factors that control tumor angiogenesis and promote metastasis of cancer cells in this process include VEGFR,[131] c-jun,[132] EGR-1,[133] and IGF-II.[134] For example, Liu *et al.* synthesized self-assembled Cu–DNAzyme nanohybrids to co-deliver VEGFR2 targeting DNAzymes and Cu^{2+} into cancer cells for chemodynamic therapy (CDT) and gene therapy-based combined therapy. This nanoplatform has ultra-high DNAzyme loading and gene silencing capacity, resulting in effective inhibition of the VEGFR2 expression for amplified anti-tumor effects[135] (Fig. 8A). In another study, Peng *et al.* proposed the MnO_2–PEI–DNAzyme (MnPD) nanoplatform for self-sufficient anti-angiogenesis and pro-apoptosis combined gene therapy. The dual DNAzyme enabled the downregulation of EGR-1 and survivin mRNAs, effectively inhibiting angiogenesis and promoting cell apoptosis[136] (Fig. 8B). Cai *et al.* demonstrated that the c-jun-targeting DNAzymes could reduce intratumoral vascularity in human primary and metastatic melanoma and prevent tumor recurrence and metastasis through adaptive immunity[125] (Fig. 8C). Furthermore, insulin-

Fig. 8 DNAzyme-based therapeutics for inhibiting cancer metastasis. (A) DNAzyme-based nanoplatform targeting VEGFR2 for enhanced tumor treatment.[135] This figure has been reproduced from ref. 135 with permission from Wiley-VCH GmbH, copyright 2021. (B) A versatile nanocomposite with dual gene silencing properties for efficient gene therapy.[136] This figure has been reproduced from ref. 136 with permission from Elsevier B.V. All rights reserved, copyright 2022. (C) The targeting c-jun DNAzyme for reducing intratumoral vascularity.[137] This figure has been reproduced from ref. 137 with permission from Macmillan Publishers Limited, part of Springer Nature, copyright 2018. (D) The DNAzyme-assisted Fe/Mn-based MOF for improved CDT *via* DNAzyme-mediated c-jun gene silencing.[6] This figure has been reproduced from ref. 6 with permission from American Chemical Society, copyright 2022.

like growth factor II (IGF-II), a mitogenic peptide, directly promoted angiogenesis in cancer. IGF-II gene targeting DNAzymes could inhibit the invasion, movement, and migration of liver cancer cells.[137] All of the above reports demonstrated that angiogenesis-related gene targeting DNAzyme is an alternative tool to inhibit cancer metastasis.

EMT is a crucial step in the process of tumor metastasis, which endows cancer cells with metastatic properties by promoting mobility, invasion, and resistance to apoptosis.[138] EMT is mediated by some transcription factors, such as Twist,[139] Slug,[140] and ZEB.[141] Among these factors, Twist is verified to be crucial in more invasive human tumors.[142] Zhao et al. modified the Fe/Mn-based metal–organic framework (FMM) with DNAzymes to realize improved Twist gene silencing for inhibiting tumor growth and metastasis. The FMM nanoplates not only promoted the efficient delivery and controlled release of DNAzymes but also provided the essential cofactors for DNAzymes to improve catalytic efficiency. The results showed that the nanoplatform is a promising combined gene/chemodynamic therapy system for metastatic cancer therapy[6] (Fig. 8D). Another example is Aurora-A kinase, which mediates MAPK-induced metastases via the activation of EMT. Xing et al. constructed the N-acetyl-L-leucine-polyethylenimine to deliver Aurora-A kinase targeting DNAzymes into PC-3 cells. The nanocomplex markedly inhibited the proliferation and migration of human prostate cancer.[143]

Furthermore, the ECM, one of the major components of tumors, plays multiple key roles in tumor progression, such as mechanical support and regulation of the tumor microenvironment.[144] The interaction between cancer cells and the tumor microenvironment (TME) often leads to abnormal ECM, resulting in abnormal mechanical mechanotransduction and further tumor deterioration, even metastasis.[145] Several reports suggested that matrix metalloproteinases (MMPs) are involved in the physiological and pathological degradation of the ECM, which is a remarkable feature of metastasis.[146,147] Therefore, ECM normalization can serve as a powerful adjuvant for defeating cancer metastasis. Nowadays, DNAzyme-based nanoplatforms targeting ECMs have become a promising strategy to achieve precise, efficient, and safe strategies for inhibiting cancer metastasis and improving poor patient outcomes. Yang et al. developed a tumor-targeting PAMAM derivative to deliver DNAzymes Dz13 for inhibiting cell proliferation, migration, and invasion through the silencing of the c-jun mRNA and subsequently reduced the expression of MMP-2 and MMP-9.[148] A similar study reported by Hallett et al. indicated that targeting and silencing of the MMP-9 gene by anti-MMP-9 DNAzyme could inhibit breast cancer cell invasion and metastasis.[149] Taken together, these findings demonstrated the remarkable efficiency of DNAzymes in controlling key pathways associated with cancer metastasis and poor patient outcome.

3.2 DNAzyme-mediated gene therapy in cardiovascular diseases

Gene therapy has shown great promise in treating inherited and acquired cardiovascular diseases in the past decade.

Current studies have found that cardiovascular disease involved complex pathological mechanisms, including intimal hyperplasia,[150] impaired fibrinolysis,[151] and inflammation.[152] Thus, multiple genes can be used as potential targets for gene therapy of cardiovascular diseases. At present, DNAzyme-mediated gene silencing has brought about significant changes in the treatment of cardiovascular diseases.[153,154]

Restenosis induced by intimal hyperplasia seriously restricts the therapeutic effect of cardiovascular diseases. It is a restorative response to local vascular injury and remodeling mediated by multiple cytokines.[155,156] The proliferation and migration of vascular smooth muscle cells (VSMCs) play a master regulatory role in the pathological mechanism of vascular intimal hyperplasia.[157] Research has shown that early growth response-1 (Egr-1) was rapidly upregulated in VSMCs and endothelial cells after pathologic stimulation including ischemia and ischemia-reperfusion injury, resulting in VSMC proliferation and intimal thickening.[158] ED5, a 10–23 DNAzyme that specifically cleaved Egr-1 mRNA, was the first to prove the potential therapeutic efficacy of DNAzyme in vivo.[158] Similarly, ED5 also inhibited Egr-1 expression and neointima formation following permanent ligation of rat common carotid arteries.[156] Another research on ED5 confirmed that it was able to inhibit VSMC proliferation by reducing Egr-1 and proliferating cell nuclear antigen (PCNA) protein expression, indicating that ED5 was an efficient gene therapy approach to prevent restenosis following percutaneous coronary intervention (PCI).[155] Furthermore, Lowe et al. first used Egr-1 cleaving DNAzyme to inhibit in-stent restenosis in pig coronary arteries, demonstrating that DNAzyme could specifically inhibit EGR-1 protein expression, and block VSMC proliferation and regrowth after injury.[159] In addition, EGR-1 cleaving DNAzymes could also prevent stenosis and occlusion of autogenous vein graft in vivo by inhibiting VSMC proliferation and intimal hyperplasia.[160] In addition to EGR-1, c-jun is associated with VSMC proliferation and intimal hyperplasia.[161] Khachigian and his colleagues successfully used a DNAzyme (Dz13) to inhibit vein graft stenosis after autogenous end-side transplantation and coronary artery bypass grafting.[157,162] All these findings demonstrate that catalytic DNAzymes targeting vital regulatory genes have therapeutic prospects in the clinical management of restenosis.

Furthermore, impaired fibrinolysis is a critical cause of atherothrombotic cardiovascular disease. Plasma plasminogen activator inhibitor (PAI)-1, a major inhibitor of the fibrinolytic system, increases in many clinical pathological states that are associated with a high risk of cardiovascular diseases, such as atherosclerosis, coronary heart disease, angina, and myocardial infarction. Elevated levels of PAI-1 could cause excessive blocking of tissue-plasminogen activator, resulting in decreased clot breakdown and eventually a detrimental blood clot.[163–165] Witkowski et al. designed a PAI-1 DNAzyme to treat the lumen of the artery after balloon injury. The PAI-1 DNAzyme not only reduced fibrin deposition both in the arterial lumen and arterial media but also inhibited VSMC proliferation and migration to inhibit the formation of neointimal

tissue, successfully preventing post-angioplasty neointima formation.[166] Elevated levels of PAI-1 are also associated with the inhibition of tissue microvasculature growth *via* reduced plasmin-dependent matrix degradation, direct pro-apoptotic effects on pre-formed endothelium, and migration of endothelial progenitors.[167] In this case, several researchers have confirmed that reducing cardiac PAI-1 activity by PAI-1 targeting DNAzymes may enhance myocardial neovascularization and improve functional recovery.[168]

Additionally, several studies have provided powerful evidence that inflammation represents a high-risk factor for the development and progression of cardiovascular diseases. In terms of the molecular mechanism of cardiovascular risk and inflammation, emerging research has demonstrated the role of EGR-1 as an important link between these pathological conditions. Some research groups exploited whether the inhibition of Egr-1 in intracoronary could reduce infarct size after myocardial ischemia-reperfusion by regulating its downstream effector molecules. Bhindi *et al.* delivered human EGR-1 targeting DNAzymes into intracoronary region in the ischemia-reperfusion (IR) model of pig. They found that DNAzymes reduced the infarct size and improved cardiac functional recovery at the initiation of ischemia-reperfusion (IR) by modulating its downstream effector molecules, such as ICAM-1, TNF-α, PAI-1, and TF.[169] Similarly, Bhindi *et al.* also found that DNAzymes could reduce myocardial infarct size following IR in rats.[170] In their further research, Bhindi and his colleague demonstrated that EGR-1 targeting DNAzymes had a protective effect against myocardial IR injury through IL-6-dependent JAK–STAT signaling.[171] All these results clearly showed the potential of DNAzyme in attenuating the inflammation of damaged tissue to promote cardiac function.

3.3 DNAzyme-mediated gene therapy in viral infection

Viruses are important pathogens that threaten human health, which are essentially composed of nucleic acid (DNA or RNA) and a protein coat. Nucleic acids are the critical components that constitute the virus genome and provide the genetic information for virus proliferation, heredity, and mutation. Gene-targeting strategies are being developed to silence the key virus genes, where DNAzyme can act as viral mRNA cleavage tools to cause the downregulation of viral proteins. Thus, DNAzymes have been validated in a variety of viral diseases, such as human immunodeficiency virus (HIV) infection, Epstein-Barr virus (EBV), hepatitis B virus (HBV), and hepatitis C virus (HCV).

3.3.1 HIV infection. HIV is classified into the retrovirus family, and antiretroviral drugs are currently employed as the main medicines for the treatment of HIV infection.[172] Although antiretroviral therapy can inhibit the replication of the virus for a period of time, HIV infection cannot be completely eliminated. DNAzyme-based gene therapy can destroy specific targets of viruses to inhibit the invasion of viruses and interfere with viral nucleic acid replication, gene transcription, and translational and post-translational modification.[12] For example, HIV integrase is an enzyme responsible for integrating the proviral genome into the human genome and is a prerequisite for HIV replication. Inhibition of the expression of HIV-1 integrase by 10–23 DNAzyme could inhibit viral replication.[173] Additionally, HIV-1 requires CCR-5 and CXCR-4 chemokine receptors for fusion and internalization into host cells.[174–176] Banerjea *et al.* used DNAzymes to silence host cell receptor CCR-5 and CXCR-4 mRNA, resulting in decreased fusion of cell membranes.[177,178] The result demonstrated that these DNAzymes had the function of interfering with the expression of the chemokine receptors and decreasing viral internalization.

3.3.2 EBV infection. EBV, a double-stranded DNA herpesvirus, is usually carried lifelong as an asymptomatic infection. EBV has been thought to be associated with the development of several malignancies, such as Burkitt's lymphoma, Hodgkin's disease, and undifferentiated nasopharyngeal carcinoma.[179,180] Latent membrane protein 1 (LMP1), a key oncoprotein of EBV, is closely associated with various changes in EBV-associated cancers, including nasopharyngeal carcinoma (NPC). This protein might be responsible for apoptosis inhibition, cell proliferation, immortalization and transformation promotion, and metastasis of cancer cells.[181,182] Therefore, suppressing the expression of LMP1 by RNA-cleaving DNAzyme might provide a novel strategy for combating EBV-related diseases. Promising evidence has verified that EBV-encoded LMP1 targeting DNAzyme could inhibit LMP1 protein expression, reduce cell proliferation, promote cancer cell apoptosis, and enhance the radiosensitivity of nasopharyngeal carcinoma by regulating multiple signaling pathways, including NF-κB and JNKs/c-jun signaling pathways.[183–190] More importantly, Cao *et al.*, for the first time, carried out the preclinical study of DNAzyme (DZ1) on NPC patients.[191] The data confirmed that DNAzyme treatment was safe and well-tolerated to serve as an adjuvant for radiotherapy of NPC.

3.3.3 HCV infection. Hepatitis C virus (HCV) is a positive-stranded RNA virus with an approximately 9.5 kb linear genome whose replication was mediated by viral RNA polymerase.[192] The coding region of HCV consisted of four structural proteins (core protein C, envelope proteins E1 and E2, and P7) and six non-structural proteins (NS2, NS3, S4A, NS4B, NS5A, and NS5B).[193] An internal ribosome entry site (IRES) within the mRNA 5′-noncoding region mediates the initiation and controls the translation process of the HCV genome. Moreover, the HCV NS3 protein with serine protease and helicase activity is responsible for the post-translational processing of viral proteins. Therefore, IRES and HCV NS3 are currently the main targets of antiviral gene therapy. Ryoo *et al.* presented an iron oxide nanoparticle-assisted functional DNAzyme system for the treatment of HCV by silencing the HCV NS3 gene.[194] The iron oxide-based DNAzyme delivery system can be tracked through non-invasive magnetic resonance imaging. To overcome the drawback of poor stability of DNAzyme, 2′-*C*-methyl and 2′-*O*-methyl-modified 10–23 DNAzymes were designed for targeting the 5′-IRES region of HCV.[195] Moreover, DNAzymes can also inhibit viral RNA translation and replication by cleaving other related target RNAs of HCV.[192,193]

In addition, DNAzyme-mediated gene therapy has also been used for other types of viral infections, including respiratory syncytial virus (RSV),[196,197] influenza A virus,[198,199] and HBV,[200,201] indicating that DNAzyme-based gene therapy is an effective approach for treating a variety of viral infections.

4. Conclusions and outlook

Collectively, exciting progress has already been made in the area of RNA-cleaving DNAzymes. This review systematically summarized the applications of RNA-cleaving DNAzymes in recent years, explaining the uniqueness and superiority of RNA cleaving-DNAzymes in biosensing and gene therapy. RNA-cleaving DNAzymes can cleave RNAs in a sequence-specific manner, which is key to accurate biosensing and gene therapy. Due to the excellent advantages such as high stability, high catalytic activity, easy synthesis, easy functionalization, and modification, the RNA-cleaving DNAyzmes can be incorporated into different biosensors to specifically identify metal ions, neutral molecules, bacteria, and other cofactors. Furthermore, DNAzymes have prominent merits, such as no permanent influence on the genome, no immune responses and significant cytotoxicity, and multiple turnover rates as well as suitability for large-scale rapid production. However, the current application of DNAzyme-based biosensing systems focuses on the laboratory environment, and *in vivo* detection is still limited by many biological factors. To make DNAzymes more applicable in practical detection, it is necessary to improve the anti-interference ability of the detection system and improve stability *in vivo*, and more in-depth research is urgently required to solve the above problems. In addition, based on the characteristics of cutting specific sites of RNA, RNA-cleaving DNAzymes have been used to destroy pathogenic RNA in cells or viruses with potential therapeutic effects. Although DNAzymes have a similar function to antisense oligonucleotides, siRNA, and other nucleic acid drugs, they have incomparable properties. DNAzymes have higher gene silencing ability compared to antisense oligonucleotides. Moreover, they have higher stability and lower synthesis cost than siRNA and work independently of the RNA-induced silencing complex (RISC). However, inefficient delivery is an essential problem that hinders the widespread use of DNAzyme-based gene therapy. Nowadays with the rapid development of nanotechnology, new exciting studies for self-supported multifunctional nano-delivery systems to promote the delivery efficiency of DNAzymes are underway. Subsequently, the effectiveness of DNAzymes should be taken into consideration in the future, including the binding efficiency with their targets, the cellular uptake efficiency and stability, and the concentration of local divalent cations that may affect the effectiveness of the final treatment of DNAzyme. In addition, more diseases should be taken into account for RNA-cleaving DNAzyme research. In the last five years, although the use of RNA-cleaving DNAzymes in gene therapy has been explored extensively, most of the diseases being studied are cancers. Therefore, the application of DNAzymes should not be limited to cancer treatment, and wider disease research should be carried out. In fact, the gene therapy of DNAzymes in related diseases is still active in the scientific research stage, and there is still a long distance to clinical application. Many scientific problems need to be further explored and solved, including how to transport DNAzymes from the administration site to the blood circulation, how to overcome the physiological barrier in the body to reach the target organ, how to enter the cells, and how to improve the activity of DNAzymes *in vivo*. Therefore, we firmly believe that with the in-depth study and the development of novel efficient delivery systems, DNAzyme will certainly play a greater role in diagnostics, enzymology, molecular biology, and other research fields as well as gene therapy in diverse diseases.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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