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Review Article

Rational design of site-specific artificial metallonucleases for therapeutic applications☆

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ABSTRACT

Artificial metallonucleases (ArMNs) are synthetic nucleases that utilise metals as the catalytic centre for cleaving nucleic acids, with the metal component present in the form of ions, complexes or nanoparticles. ArMNs have been developed as therapeutics for a range of diseases, including genetic disorders, cancer and infectious diseases. Achieving high site-specificity in ArMNs is crucial for their effective application in targeted therapeutic interventions. By employing advanced methodologies, these engineered nucleases can induce targeted modifications in the genome, thereby advancing genetic research, improving disease treatment and fostering biotechnological innovation. This review examines recent advancements over the past 20 years in the design and synthesis of site-specific ArMNs. Illustrated examples are provided to elucidate the underlying principles of their site-specific activity and to highlight their prospective applications. Additionally, strategies for overcoming challenges in enhancing the selectivity of these nucleases are discussed, and anticipated future research directions in this dynamic field are outlined.

1. Introduction

Natural nucleases are enzymes that catalyse the cleavage of nucleotides (e.g. DNA and RNA, DNA: Deoxyribonucleic Acid, RNA: Ribonucleic Acid), playing a vital role in key biological processes including DNA replication [1], recombination [2], and repair [3]. Given their crucial role in these processes, understanding and harnessing their activity for therapeutic applications is of considerable interest. While these natural nucleases excel in efficiency and specificity within biological contexts, they may not demonstrate the desired precision or functionality required for certain advanced biotechnological or therapeutic applications. For instance, some endonucleases like FokI (Flavobacterium

Abbreviations: ArMN, Artificial metallonuclease; DNA, Deoxyribonucleic Acid; RNA, Ribonucleic Acid; EDTA, Ethylenediaminetetraacetic acid; mRNA, messenger RNA; HIV-1, Human Immunodeficiency Virus type 1; N6, 6-position nitrogen atom in adenine rings, the number could also represent other positions; O4, 4-position oxygen atom in thymine rings, the number could also represent other positions; dsDNA, Double-stranded DNA; B-DNA, B-form DNA, a dsDNA structure with a Right-handed double helix; HA, hydrogen bond acceptor; HD, hydrogen bond donor; M, methyl; H, low-polar hydrogen; Zif268, zinc finger binding protein clone 268; ZF, zinc finger; A, adenine; T, thymine; U, uracil; C, cytosine; G, guanine; C^+, protonated cytosine; TFO, triplex-forming oligonucleotide; *B. subtilis*, *Bacillus subtilis*; $tRNA^{His}$, Transfer RNA for Histidine; G4, Guanine quadruplex; TpT, Thymine nucleotide-phosphate backbone-Thymine nucleotide; EDTP, ethylenediamine-N,N,Ń,Ń-tetrakis(methylenephosphonic acid); mer, nucleotide units; nanoenzyme, nanomaterial-based artificial enzyme; AuNP, Gold nanoparticle; PNA, peptide nucleic acid; ARCUT, artificial restriction DNA cutter; pcPNA, *Pseudo*-complementary Peptide Nucleic Acid; TPMA, tris-(2-picolyl)amine; $5N_3$-TPMA, 5-N_3-tris-(2-picolyl)amine; bp, base pairs; pCSanDI-HYG, a closed circular plasmid DNA; FokI, Flavobacterium okeanokoites restriction enzyme I; $CD34^+$, Cluster of Differentiation 34-positive cell; P1G, peptide sequence 1, with glycines modified on the both sides; Sp1(zf23), specificity protein 1 zinc finger 2 and 3; GLI(zf45), glioma-associated oncogene zinc finger 4 and 5; pUC19, a small circular DNA molecule, 2686 base pairs in length; Rev, regulator of expression of virion; RRE, the Rev responsive element; HCV, hepatitis C virus; GGH, peptide sequence: Gly-Gly-His; YrFK, peptide sequence: Tyr-[D-Arg]-Phe-Lys; GGHK, peptide sequence: Gly-Gly-His-Lys; qRT-PCR, Quantitative real-time reverse-transcription polymerase chain reaction; NS5A, a functional protein responsible for HCV viral replication; MDR, multidrug resistance; EGF, epidermal growth factor; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay; IC_{50}, Half Maximal Inhibitory Concentration; OVCAR8, Ovarian Cancer Adriamycin-Resistant 8 cell line; EGFP, enhanced green fluorescent protein; GGHKYKETDL, peptide sequence: Gly-Gly-His-Lys-Tyr-Lys-Glu-Thr-Asp-Leu; LucUbiNeo-ET, an HCV replicon cell line; BABE, (S)-1-(p-bromoacetamidobenzyl)ethylenediaminetetraacetate; NusA, N-utilizing substance A protein; Upf1, an RNA helicase; HER2, human epidermal growth factor receptor 2; DFT, density functional theory.

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okeanokoites restriction enzyme I) only recognise and cleave short, conserved sequences, limiting their versatility [4]. Nucleases such as CRISPR-Cas9 face challenges in *in vivo* delivery due to their large molecular weight [5]. Besides, these natural enzymes often lack the physicochemical stability necessary to withstand harsh environments [6]. Artificial metallonucleases (ArMNs), inspired by natural nucleases, are designed and synthesised to replicate the functions of natural nucleases in cleaving DNA and RNA [7–9]. Currently, considerable efforts are underway not only to achieve the DNA/RNA cleavage with enhanced catalytic activity but also to ensure that these nucleic acids are cleaved with remarkable specificity and selectivity, which holds great promise for therapeutic applications such as gene editing [10,11] and direct disease treatment [12].

The history of ArMNs can be traced back to the 1980 s. Early research focused on designing simple metal complexes that could cleave DNA, demonstrating the feasibility of using metal ions as catalytic centres, such as 1,10-phenanthroline-copper and Fe(II)-EDTA (Ethylenediaminetetraacetic acid) [13]. Over time, advances in ligand design led to the development of more sophisticated metal complexes with improved specificity and activity including aza-crown ether metal complexes [14]. Researchers also explored peptide- and protein-based metallonucleases, aiming to combine DNA-binding domains with metal-binding sites for targeted DNA cleavage, for example, amino-terminal Cu(II)- and Ni(II)-binding (ATCUN) complexes [15]. Recent developments in nanotechnology have enabled the creation of nanoparticle-based metallonucleases with potential applications in gene therapy and biotechnology [16]. Throughout their evolution, ArMNs have become versatile tools for scientific research and therapeutic interventions, fuelled by advancements in coordination chemistry, molecular biology, and nanotechnology.

Despite significant progress in the development of ArMNs, several challenges remain unresolved. Achieving enhanced specificity, particularly in complex genomic environments, remains a major obstacle. Off-target cleavage events pose risks to genomic integrity and can hinder the therapeutic potential of metallonucleases [17,18]. Furthermore, ensuring the stability and activity of these enzymes within biological systems presents another significant challenge [7]. Metallonucleases must retain their catalytic efficiency and specificity amidst the intricate milieu of cellular components. Overcoming these challenges requires collaborative, interdisciplinary efforts across chemistry, biology, and biochemistry, as well as innovative approaches in ligand design, protein engineering, and artificial binding cofactors.

This review will focus on the primary challenge in designing ArMNs: achieving substrate specificity. It will cover the foundational mechanisms underlying specificity, as well as technological advancements and methodologies developed over the past two decades (2004–2024) to achieve the specificity (Fig. 1). In addition, it will highlight the therapeutic applications of these valuable site-specific ArMNs. Moreover, the challenges and future directions for designing site-specific ArMNs with enhanced performance will be discussed in detail.

2. The mechanisms underlying specificity

Nucleic acids contain a variety of functional groups, such as the carbonyl and secondary amine groups present on the pyrimidine of thymine. These functional groups can selectively interact with natural or synthetic compounds, including other nucleic acids, other nucleic acids, thereby facilitating the specific recognition of nucleic acids [19]. For example, single DNA strands can be specifically recognised by complementary sequences through the unique hydrogen bonds between pyrimidines and purines, a process known as Watson-Crick base pairing [20]. Therefore, understanding the interactions between nucleic acids and other natural or synthetic agents is essential for achieving site specificity in ArMNs. This section will focus on the reported categories of targeted nucleic acids and the associated mechanisms employed in designing site-specific ArMNs.

2.1. Single-stranded nucleic acids

In the design of ArMNs, the primary studied single-stranded nucleic acids are single-stranded DNA (ssDNA) and RNA. Both ssDNA and RNA possess the fundamental sequence structures of nucleic acids, where nucleotide units are connected by phosphodiester bonds between the 3′-hydroxyl group of one nucleotide and the 5′-phosphate group of the

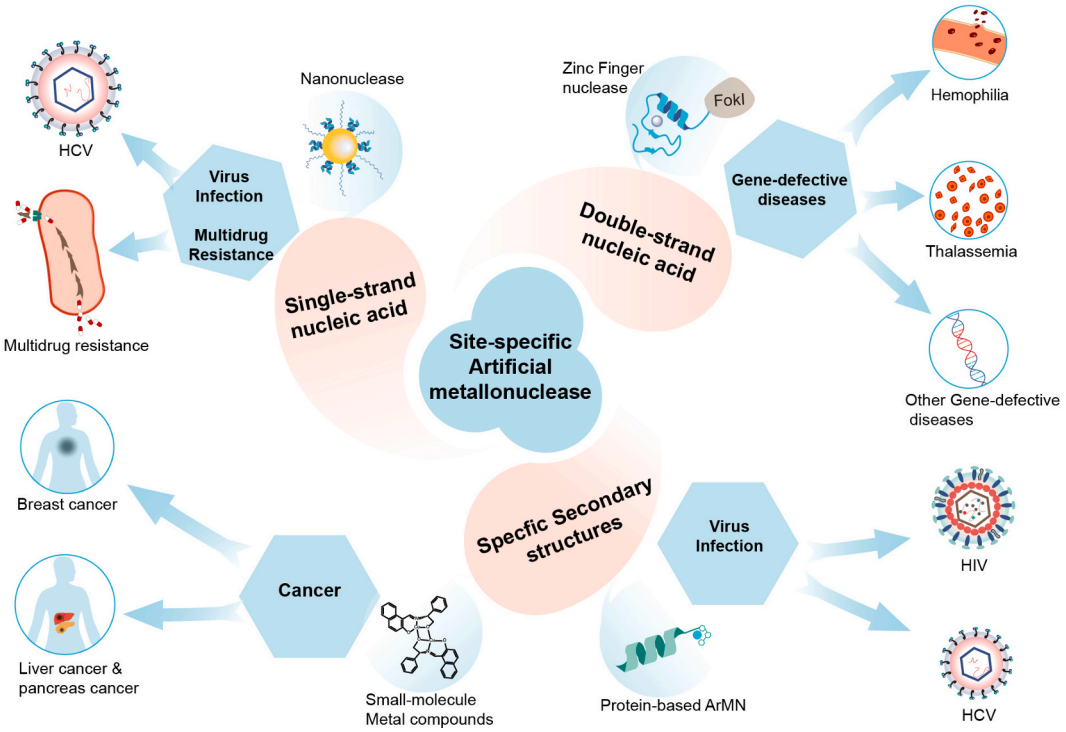

Fig. 1. Overview of the therapeutic application of various site-specific artificial metallonucleases.

subsequent nucleotide (Fig. 2a). The preference for ssDNA in ArMNs design could be attributed to its role of ssDNA in genomic recombination [21], repair [3,22], and replication [1]. RNA is also a key substrate in ArMNs design because it plays a critical role in disease models (e.g. mRNA in the HIV-1 life cycle [23]; mRNA: messenger RNA, HIV-1: Human Immunodeficiency Virus Type 1) and is one of the primary targets for ArMNs [9,12]. To date, the primary mechanism for specific recognition remains Watson-Crick base pairing [24].

Watson-Crick base pairing involves the pairing of adenine–thymine (or uracil) and cytosine-guanine. Through the mechanism, the constituent mononucleotides on a single-stranded DNA or RNA molecule can pair with specific nucleotides from a complementary DNA or RNA sequence, enabling site-specific recognition. This Watson-Crick base pairing is primarily established through hydrogen bonds between pyrimidines and purines of nucleotides (Fig. 2b). In this pairing, adenine pairs with thymine via two hydrogen bonds: one between adenine N6 (6-position nitrogen atom in the adenine ring) and thymine O4 (4-position oxygen atom in the thymine ring), and another from adenine N1 to thymine N3; guanine pairs with cytosine via three hydrogen bonds: one between Guanine O6 and cytosine N4, another between Guanine N1 and cytosine N3, the third between guanine N2 and cytosine O2 [25]. Since Watson-Crick base pairing facilitates the precise recognition of constituent mononucleotides on a single-stranded sequence, this mechanism is widely applied in site-specific recognition, including antisense oligonucleotide [24], and oligonucleotide derivatives [26].

2.2. Double-stranded DNA

Double-stranded DNA (dsDNA) is a primary substrate in the design of site-specific ArMNs due to its critical role in transmitting genetic information [27]. There are three primary forms of dsDNA—A-form DNA, B-form DNA, and Z-form DNA—which are differentiated by their helical geometry, sugar pucker conformations, number of base pairs per turn, and the environmental conditions that favour their formation. B-form DNA (B-DNA)—a right-handed double helix—is the most extensively studied configuration for site-specific cleavage, likely because of its widespread occurrence in mammalian genomes [28]. B-DNA comprises two complementary ssDNAs that form a double-helix *via* Watson-Crick base pairing.

In this secondary structure, the double helix presents a major groove and a minor groove in both flanks of the duplex. Site-specificity can be achieved by recognising the distinct patterns of functional groups on these grooves, such as hydrogen bond acceptor (HA), hydrogen bond donor (HD), methyl (M), and low-polar hydrogen (H). In the major groove, for instance, the cytosine-guanine base pair can present an order of HA-HA-HD-H or H-HD-HA-HA, while the adenine–thymine base pair might show an order of HA-HD-HA-M or M−HA−HD−HA (Fig. 2c, 2d) [27]. These orders and the conformation of double-helix strands provide a crucial foundation for the precise recognition of individual base pairs [29]. In addition to these grooves, the phosphodiester backbone of DNA strands also contributes the recognition due to its unique functional groups, such as phosphate moieties [30].

The first mechanism that leverages these features of dsDNA for site-specificity is the formation of protein-DNA complexes. This mechanism has been well-documented in DNA-protein interactions. For example, the zinc finger binding protein clone 268 (Zif268), a protein with three tandem zinc finger (ZF) binding domains, has been reported to bind target dsDNA strands by recognising both the major groove and the strand backbone [30,31]. Analysis of the crystal structure of the protein-DNA complexes revealed that the ZF protein fits into the major groove of the target dsDNA and subsequently wraps around dsDNA strands. In this process, hydrogen bonds and van der Waals interactions between peptide residues and nucleobases were observed (Fig. 2e), as well as electrostatic interactions between the phosphate backbones of dsDNA and the Zif268 protein. As noted above, this mechanism can be applied to specifically recognise dsDNA through engineered proteins, such as modified ZF [32].

Hoogsteen base pairing is another mechanism used for the recognition of dsDNA. Aside from Watson-Crick base pairing, Hoogsteen base pairing is an alternative pairing that includes adenine–thymine (or adenine–uracil) and protonated cytosine-guanine base pairs. Hoogsteen base pairing forms hydrogen bonds between nucleotides at a distinct set of hydrogen-bonding positions as Fig. 2b shows [33]. In this pairing, the adenine and protonated cytosine bases are flipped, enabling an additional hydrogen bond (adenine N7 to thymine N3, and cytosine N3 to guanine N7 respectively), unlike Watson-Crick base pairing. The distinct hydrogen bonds make Hoogsteen base pairing possible between single strands and duplex DNA. Similar to Watson-Crick base pairing, Hoogsteen base pairing offers precise recognition of DNA sequences, but with weaker affinity [34] and additional requirements, such as acidic conditions (lower pH) for stable C^{+}·GC pairing (Hoogsteen base pairing, a protonated cytosine binds guanine-cytosine Watson-Crick base pair) [35]. These limitations make more conditions be considered in designing Hoogsteen pairing-based ArMNs. Nevertheless, this mechanism is still an appealing tool in ArMNs design, as it can achieve sequence-specific recognition of dsDNA without the need for duplex dissociation. Currently, the most common approach employing Hoogsteen base pairing is *via* triplex-forming oligonucleotides (TFOs, see Section 3.2.2 for details).

2.3. Other specific secondary structures

Another mechanism used for achieving site-specificity is the recognition of nucleic-acid structures and conformations beyond the DNA duplex. In this section, secondary structures of RNA or DNA used for achieving site-specificity will be discussed in this section.

RNA can spontaneously form secondary structures, including elements such as loops and stems [36,37]. These secondary structures give RNA defined structural motifs and enable abundant conformational changes [38,39]. Although these secondary structures may hinder conventional antisense base pairing, conserved structural motifs within RNA allow for site-specific recognition by small molecules and proteins. Indeed, the site-specific recognition and cleavage of *Bacillus subtilis* histidine transfer RNA (*B. subtilis* $tRNA^{His}$) precursor by Fe(II)·Bleomycin (Fig. 2g) have been reported and studied extensively between the 1980 s and 1990 s, the site-specificity of Fe(II)·Bleomycin was attributed to distinct structural features and conformations of nucleic acid rather than the RNA sequence itself [40]. Currently, recognition of RNA secondary structures by ArMNs typically involves conjugation with small molecules [13] or protein-derived binding motifs [7].

In recent years, guanine-quadruplex (G4) DNA has attracted significant attention in biomedical research due to its association with human telomeres, whose shortening is associated with cellular senescence both in cancer cells and normal cells [41]. G4 DNA is a secondary structure formed by single-stranded DNA containing guanine-rich repeats, as shown in Fig. 2f [25,42]. G4 DNA contains guanine quartet planes in which four guanines are stabilised with dense hydrogen bonds and monovalent cations (Normally Na^{+} or K^{+}) [43]. The rigid arrangement of hydrogen bonds limits access to G4 DNA, making it accessible primarily at the N3 position within the minor groove or on the outermost layers of G4 topologies. This feature provides G4 DNA with a greater resistance to direct cleavage [44,45]. However, the planar structure and uniformly distributed charges facilitate recognition by small molecules and metal ions, a primary mechanism exploited in the design of G4 DNA-specific ArMNs. For example, salphen-based metal complexes have been reported to bind G4 DNA directly above its multilayered structures. X-ray crystallography results revealed that the piperidine substituent plays a vital role in stabilising the entire crystal structure (Fig. 2f), indicating that the G4 plane can stably interact with small molecules possessing distinct structural features [46].

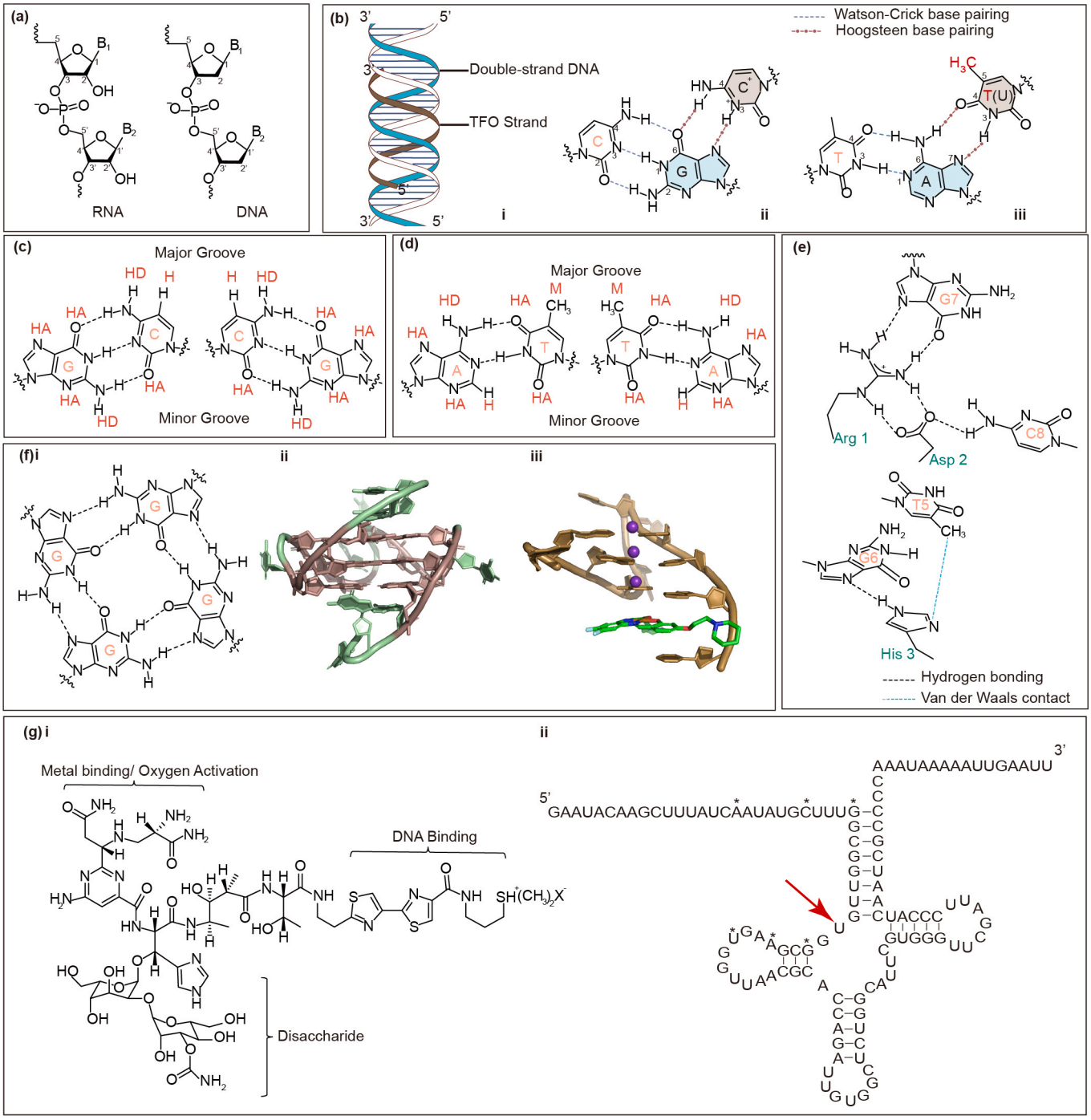

Fig. 2. The main target nucleic acids and mechanisms underlying specificity. (a) Backbone structures of DNA and RNA, with position markers on the ribose ring of RNA and the deoxyribose ring of DNA, B: nucleobase. (b) Duplex DNA, triplex DNA and related base pair interactions. i: Structures of duplex DNA and triplex DNA, where the blue and white represent the duplex structure, and the brown strand indicates the triplex-forming strand. ii: The Watson-Crick base pairing between cytosine and guanine, and Hoogsteen base pairing between guanine and cytosine (protonated cytosine, C^+), C: cytosine, G: guanine, C^+: protonated cytosine; iii: The Watson-Crick base pairing between thymine and adenine, and Hoogsteen base pairing between adenine and thymine, 'A' denotes adenine, 'T' denotes thymine, 'U' denotes uracil, U and T share a similar base structure, but T possesses an additional methyl group at the 5 position (the additional methyl group is labelled with 'CH_3' in red) [33]. (c, d) The major groove of individual G-C base pairs displays an order of HA-HA-HD-H (G-C) and H-HD-HA-HA (C-G), and individual A-T base pairs exhibit an order of HA-HD-HA-M (A-T) and M-HA-HD-HA (T-A) [27]. (e) Hydrogen bonds and van der Waals contacts observed between Zif268 and the target nucleobase [32], where C, G, T refer to nucleobases as described in (b), and His, Asp, Arg denote amino acid residues, with the numbers indicating the order of nucleic acids/amino acids in the corresponding DNA or protein sequences. (f) Structures of Guanine-quadruplex (G4) DNA and G4-salphen complexes. i: Base pairing in G4 DNA [25], ii: a 3D structure of G4 DNA (data obtained from the protein Data Bank, PDB ID: 1XAV and visualised by Pymol 4.6.0, W. L. DeLano, The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, 2002.) [42], iii: a 3D structure of G4 DNA-salphen (PDB ID: 3QSC and visualised by Pymol 4.6.0) [46]. (g) The structure of bleomycin A2, (i) and the B. subtilis $tRNA^{His}$ precursor (ii) [40]. The red arrow indicates the primary cleaving site by bleomycin in the RNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Designed site-specific ArMNs

Based on the mechanisms described above, various methodologies have been developed in recent years to achieve site-specific recognition by ArMNs. In this section, the advances and applications of site-specific methods will be discussed according to their targeted substrates, including single-stranded nucleic acids, dsDNA, and specific nucleic acid secondary structures distinct from dsDNA.

3.1. Designing the site-specificity toward single-stranded nucleic acids

3.1.1. ArMNs targeting ssDNA

In the design of ArMNs, conjugating antisense oligonucleotides is a direct method for recognising ssDNA. Through Watson-Crick base pairing, complementary oligonucleotides can help target DNA sequences. This method was first tried by Komiyama and coworkers by using an ArMN incorporating a 19-mer oligonucleotide and Ce(IV) complex [47]. The iminodiacetate/Ce(IV)-induced hydrolysis of dinucleotide TpT (Thymine nucleotide-phosphate backbone-Thymine nucleotide) further indicated that the oligonucleotide sequence contributes to the delivery of the cleaving centre (the cerium complex).

However, a similar study demonstrated only modest selectivity [48,49], indicating that a precise cleavage requires optimal conditions for oligonucleotides and metal complexes. Subsequently, Suzuki and coworkers reported that within 14–25-mer (nucleotide units) length, 19–21-mer oligonucleotides exhibit an optimal affinity to a 25-mer length ssDNA model [50]. Additionally, Meng and coworkers improved the preparation of cerium complexes using Ce(III) instead of Ce(IV) and developed a more stable cerium complex, Ce(III)- ethylenediamine-N,N,Ń,Ń-tetrakis(methylenephosphonic acid) (EDTP) [51]. Based on these, Komiyama and coworkers designed a new site-specific ArMN for cleaving an 85-mer ssDNA [52]. In this protocol, 20-mer oligonucleotides are modified in the 3′- or 5′- termini with EDTP. Two of these conjugates with different oligonucleotide sequences can recognise the ssDNA sequence forming a 5-mer gap (Fig. 3a). The cleavage is triggered by the additive Ce(III) and selectively occurs within the target site. Aside from the precise recognition of duplex conjugates, another advantage of this protocol is the use of Ce(III)-EDTP. The strong-binding

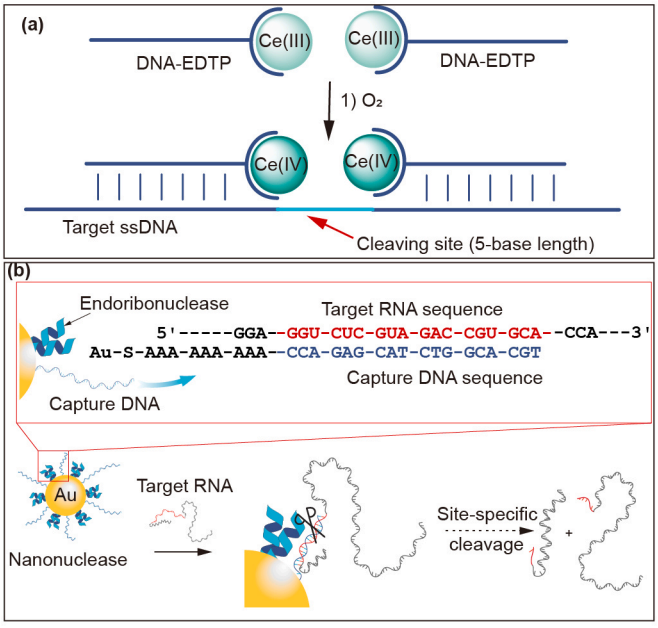

Fig. 3. Site-specific artificial metallonucleases designed for single-stranded nucleic acid. (a) Oligonucleotide-based Ce(III) ArMNs for cutting ssDNA [52]. (b) The AuNP-based nanonuclease targets and cuts RNA sequence [56].

EDTP-Ce(III) requires a much lower equivalent of cerium ions than that of Ce(IV) complexes (2 equiv. vs 100–1000 equiv.), thus avoiding the unwanted off-target cleavage caused by free cerium ions.

3.1.2. ArMNs targeting RNA

The linear RNA sequence is always one of the main substrates for ArMNs. However, the 2′-hydroxy on the ribose ring renders RNA susceptible to the hydrolysis induced by metal ions with strong Lewis acidity [49,53]. The feature makes it difficult to achieve site-specificity when using metals as cleaving centres.

To address this challenge, the emerging nanomaterial-based artificial enzyme (nanozyme) technique offers an alternative strategy. In some nanozymes, metals are utilised as backbones instead of catalytic centres. Because metal nanoparticles (e.g. Gold nanoparticle (AuNP)) possess high stability [54] and high surface-to-volume ratio [55], making them ideal linkers for binding domains and cleavage domains of nucleases. Based on this, Wang and coworkers designed an AuNP-based nanonuclease incorporating non-specific endonuclease and ssDNA [56] (Fig. 3b). The designed single-stranded oligonucleotide enables the complementary recognition to target RNA, then the adjacent endoribonuclease cleaves the captured RNA.

3.2. Designing the site-specificity toward dsDNA

Unlike RNA with its various secondary structures, dsDNA has a relatively regular duplex structure, which makes it appear impossible to achieve site specificity except through recognising the sequence from the DNA duplex. To achieve the recognition of dsDNA, peptide nucleic acid (PNA), TFO, and ZF engineering have been applied in the design of related site-specific ArMNs.

3.2.1. PNA and ARCUT

PNA is a synthetic DNA analogue initially designed for enhanced affinity toward nucleic acid sequences [57]. It is a nucleotide derivative with a neutral polyamide backbone instead of a phosphate one. Because of its neutral backbone, PNA receives less charge repulsion from the negatively charged phosphate backbone of the target DNA strand, thus presenting a high affinity [57]. This property confers PNA with a unique capability: PNA can recognise base-paired target DNA sequences and implement the Watson-Crick base pairing regardless of the hindrance from the parent duplex, which is also named strand invasion [58,59]. Based on this fact, Komiyama and co-workers developed a site-specific cleaving system named ARCUT (artificial restriction DNA cutter). ARCUT utilises Ce(IV)-EDTA as the cleaving element and Pseudo-complementary PNA (pcPNA) as the recognition element. When the DNA double strands are invaded by two 15-mer pcPNA, the binding of laterally shifted base sequences in the target DNA generates two 5-mer single strands [60]. The moderate Lewis acidity of Ce(IV)-EDTA provides a peculiar selectivity toward ssDNA, thus achieving the site-specific cleavage toward the two single strands (Fig. 4a).

3.2.2. TFO and TFO-based ArMNs

TFO is a method using polypyrimidine-oligonucleotide sequences to recognise dsDNA in a parallel orientation [40]. The method and its application can be traced back to the 1980 s [61–63]. However, the preparation of these selective ArMNs was hindered by complicated and labour-intensive protocols, which limited the further development of conjugated oligonucleotides [64]. The advent of a new synthetic approach, click chemistry [65], provides an alternative way to achieve the efficient preparation of TFO-based ArMNs. Based on the technique, a series of ArMNs conjugated to TFO were reported by Kellett and coworkers [66,67]. In these protocols, a 32-mer TFO is modified with a uracil-derived linker and a metal ligand, 5-N3-tris-(2-picolyl)amine (abbreviated as 5 N_3-TPMA), in the middle of the sequence (Fig. 4b). The TFO-based ArMN effectively recognises the target dsDNA fragment, a 113 bp (base pairs) section of green fluorescent protein gene, and

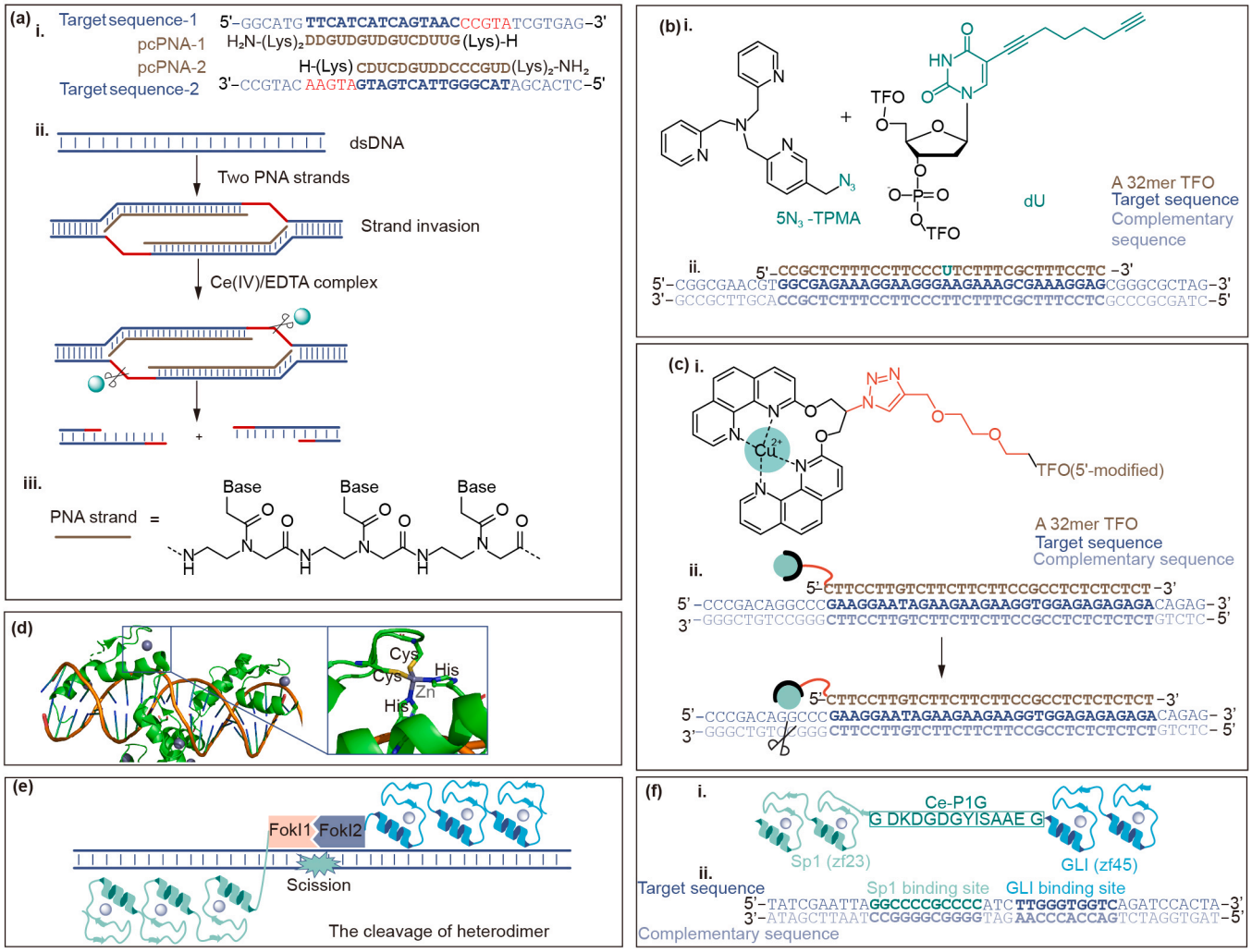

Fig. 4. Site-specific artificial metallonucleases designed for double-stranded DNA (dsDNA). (a) ARCUT nuclease system. i: substrate DNA and binding PNA sequences, ii: the scission process of dsDNA, iii: the backbone structure of PNA sequences [60]. (b) TFO nuclease 1. i: the chelate ligand tris-(2-picolyl)amine (TPMA) and linker (green N3 and carbon chain, linked via click chemistry), ii: modified nucleotide (green U: Uracil in the middle of brown sequence), TFO sequence (brown), and target dsDNA (blue and grey) [66]. (c) TFO nuclease 2. i: full structure of modified metallonuclease and linker. ii: the process of TFO-based site-specific cleavage: the nuclease is 5′- modified TFO strand (brown) and mainly cleaves the target sequence 2–5 bp from the TFO sequence [68]. (d) A cartoon example of zinc finger binding with dsDNA. PDB: 2I13. Visualised by Pymol 4.6.0. (e) The latest ZF-FokI nuclease model, two heterogeneous FokI fused with respective zinc finger sequences select and cleave target DNA sequences, the cleavage will only occur when different FokI nucleases pair [77]. (f) ZF nuclease engineered with Ce(IV)-peptide-linker. i: the zinc-finger nuclease, the P1G sequence is used for chelating Cerium ions, Sp1 and GLI peptide are zinc fingers used for targeting DNA sequences. The cerium ion acts as a cleaving centre. ii: target dsDNA sequence and the binding site of zinc fingers [81]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results in about a 0.10-fold cleavage of the target dsDNA sequence (25 equiv. ArMNs, 1000 equiv. sodium L-ascorbate, 37 °C, 6 h). The cleaving ability and site-specificity of the ArMNs are further analysed through the cleavage of a closed circular plasmid DNA (pCSanDI-HYG) containing the target sequence. In the presence of 50–800 equiv. Sodium L-Ascorbate, the ArMNs provides an effective cleavage toward the supercoiled plasmid (25 equiv. ArMNs, 37 °C, 6 h). In the subsequent study reported by Hocek and coworkers [68], the linkers and modified positions of these TFO-based ArMNs were optimised in detail. Through the footprint experiment of radioactively labelled 98-bp dsDNA, a TFO-ArMNs modified in the 5′-teminus with a polyethylene glycol linker (Fig. 4c) selectively cleaves the target dsDNA sequence 2–5 bp from the recognising TFO sequence (1000 equiv. ascorbate, 1 equiv. ArMNs, 25 °C, 12 h).

3.2.3. *Engineered ZF protein*

In addition to antisense sequences, protein engineering is another powerful method to achieve site-specific recognition of dsDNA. Engineered ZF protein is a significant tool for designing site-specific ArMNs. As noted above, ZF is a representative DNA-binding motif that has been discovered in many transcription factors [69,70]. A typical ZF unit (e.g. Cys_2His_2-type ZF of Zif268) comprising 20–30 amino acid residues could recognise multiple base pairs (3 bp in the same flank of DNA strand for each canonical ZF [30]) of dsDNA specifically (Fig. 4d) [32,71]. This sequence specificity underscores the importance of ZF proteins in site-specific ArMNs design.

Currently, the most frequently studied and reported fused ZF nuclease is ZF-FokI (Flavobacterium okeanokoites restriction enzyme I) [71–73]. (The development of this model before 2005 has been reviewed [74].) ZF-FokI nucleases use the cleaving domain of FokI restriction endonucleases to cleave target DNA through a unique dimer form. The conjugated ZFs on the FokI pair can be flexibly engineered depending on the target DNA sequence. The recognition length of target sequences can be as long as 18 bp when 3 canonical ZFs are fused to each FokI [74]. This feature allows ZF-FokI to achieve a specific cleavage in a complex genetic environment such as human genes [75].

The latest ZF-FokI nuclease model is shown in Fig. 4e, the engineered heterodimeric FokI pair is derived from homologous wild-type FokI for less off-target effect. Ideally, ZF-FokI forms a dimer with different ZF sequences on both sides of the FokI dimer. However, the random dimer formation of homogeneous FokI may generate dimers with the same ZF sequences on both sides, thus leading to an off-target effect [76]. Rebar and coworkers modified the residues on the FokI dimer interface reporting a novel heterogeneous FokI pair, in which the dimer prefers to form using two different FokI monomers rather than two same ones [77]. Through the mutated FokI pair, the engineered ZF-FokI can recognise the target gene with lower off-target effects caused by homodimers. In addition, kinetic studies of ZF and FokI further influenced the design of ZF-FokI. Zandarashvili et al. reported that every ZF unit of ZF protein contributes to the recognition of target DNA unequally [78], certain ZF units are mainly responsible for rapid but nonspecific recognition. Through mutating the residues of ZF, the nonspecific contact can be regulated leading to a more effective recognition of DNA [79]. A similar kinetic factor was demonstrated in ZF-FokI by Rebar and coworkers, and they discovered that the mutated FokI with lower activity exhibits a lower off-target effect in ZF-FokI [80]. Based on these, Rebar and coworkers designed a ZF-FokI model by engineering both the residues of ZF and FokI. The optimised ZF-FokI reduces the off-target effect as high as 3,000-fold compared with the initial model in editing the genes of human $CD34^+$ cells (Cluster of Differentiation 34-positive cells).

In addition to the natural non-specific cleaving domain (e.g. FokI), the artificial cleaving centre has been explored for the conjugation to ZF. Sugiura and coworkers designed a ZF-based ArMN by fusing a cerium-coordinating peptide P1G(GDKDGDGYISAAEG) to two ZF groups, Sp1 (zf23) and GLI(zf45) (Fig. 4f) [81] (P1G: metal binding analogue peptide 1 modified with G amino acid on both sides; GDKDGDGYISAAEG: peptide sequence Gly-Asp-Lys-Asp-Gly-Asp-Gly-Tyr-Ile-Ser-Ala-Ala-Glu-Gly; Sp1(zf23): specificity protein 1 zinc finger 2 and 3; GLI(zf45): glioma-associated oncogene zinc finger 4 and 5). The fused conjugate selectively cleaves the base pairs on the substrate DNA within the DNA-binding site of the two ZF groups (the distance between the two dsDNA binding sites is 3 bp, 37 °C, 5–10 μM Sp1(P1G)GLI, 2 h). The key finding in this protocol is that neither cerium-P1 nor the two ZF groups can cleave the dsDNA alone in the control experiment, indicating that the natural ZF groups may function as more than merely a binding site in ZF-based ArMNs.

3.3. Designing the site-specificity toward specific secondary structures

3.3.1. ArMNs Targeting RNA with secondary structures

RNA with secondary structures is one of the main substrates for site-specific recognition due to the structure and conformation diversity of reported natural RNA [38,39]. Since these structures cannot be recognised directly by complementary sequences, to recognise the RNA secondary structure precisely, there is a high requirement for spatial alignment between binding molecules and targeted RNA. For example, bleomycin Fe(II) can recognise the *B. subtilis* $tRNA^{His}$ precursor selectively, but its site-specificity obviously decreases when cleaving similar RNA substrates [8]. This high requirement makes it difficult to design site-specific ArMNs toward RNA when considering metal ions and their stable ligands. The challenge may explain why similar small-molecule ArMNs are rarely designed after bleomycin Fe(II) was discovered, although there are still bleomycin-homologous molecules reported for RNA recognition [82]. Nevertheless, the studies of bleomycin Fe(II) still give insight into the design of site-specific ArMNs: the recognition of these secondary structures can be achieved by the attachment of effective binding domains [8,9]. This strategy has been applied in designing ArMNs conjugated with peptides.

In the design of ArMNs for RNA recognition, conjugation with peptides has been a main method for achieving site-specificity in recent years [7]. In contrast with small molecules, peptide sequences offer a larger recognition domain across the RNA, allowing for more potential binding positions. For example, in the HIV-1 RNA genome, the Rev responsive element (RRE) mRNA can be recognised by the binding motif of Rev (regulator of expression of virion) protein, namely Rev peptide [83–85]. The peptide specifically recognises RRE stem-loop IIB involving interactions between more than 10 amino residues and 10 nucleotides (Fig. 5a) [86]. The dense binding interactions ensure that the specific recognition by peptides is not easily influenced when the peptide is attached to a cleaving centre [87].

Based on these studies, Cowan and coworkers developed a series of Rev-modified ArMNs and reported the discovery of Fe(II)-EDTA-Rev (Fig. 5b i), an ArMN with a high site-specificity toward RRE stem-loop IIB [88,89]. In this protocol, the Fe(II)-EDTA is attached to the C-terminus of Rev peptide to target the RRE IIB RNA fragment. In the absence of extra redox agents, the conjugate recognises and oxidatively cleaves the G46 nucleotide in the internal bulge of RRE IIB hairpin selectively (Fig. 5b ii). In the presence of ascorbate and peroxide, the main cleaving sites are still restricted within adjacent three nucleotides.

In a similar approach, the same group also succeeded in the preparation of site-specific ArMNs targeting the stem-loop IIB of hepatitis C virus (HCV) RNA [90]. The ArMNs incorporating Cu(II)-GGH (peptide sequence: Gly-Gly-His) in the C-terminus of YrFK (peptide sequence: Tyr-[D-Arg]-Phe-Lys) peptide selectively recognises the HCV RNA fragment and leads to the main cleavage of A15, C18, and U14 nucleotides. The preference of Cu(II)-GGH-YrFK was further studied by using ribosomal RNA as a control group. The results showed that Cu(II)-GGH-YrFK presents a low cleaving ability toward non-target RNA. The same group also reported another similar site-specific ArMN, Cu-GGHKYKETDL (peptide sequence: Gly-Gly-His-Lys-Tyr-Lys-Glu-Thr-Asp-Leu), which targets the stem-loop IV of HCV RNA. Homologous sequences with different peptide lengths were adapted to compare RNA selectivity in this study. The key finding is that the ArMN with a longer peptide does not present an obvious advantage in selectivity or antiviral efficacy over the one with a short peptide, indicating that the peptide length may not be a decisive factor in achieving the precise recognition [91].

3.3.2. ArMNs Targeting G-quadruplex DNA (G4 DNA)

Currently, the metal complex is the main method to specifically recognise G4 DNA in the design of ArMN. This strategy could build based on the studies of the G4 DNA scaffold binding with metal ions and metal complexes. In the research of G4 DNA-based metalloenzyme, G4 DNA was reported to bind various metal ions effectively such as K^+, $NH4^+$, Na^+ [92], Cu^{2+} [93], Ce^{3+} [94]. The feature can be ascribed to the central pocket and multiple-layer structure of G4 DNA [93]. The research on the interaction between G4 DNA and salphen metal complex, as noted in the 2.3 section, demonstrated the affinity of metal complexes toward G4 DNA.

Based on these features of G4 DNA, Cowan and coworkers designed an ArMN by attaching the Cu-GGHK (peptide sequence: Gly-Gly-His-Lys) complex to acridine [95]. The conjugate recognises a 22G4 fragment and cleaves the G4 DNA effectively. The selectivity of the G4-specific ArMN was further enhanced by conjugating the structure of naphthalene diimide instead of acridine (Fig. 5c) [96]. The modified amino-terminal Cu-binding nucleases exhibit ~ 350–510 fold binding affinity toward G4, relative to calf-thymus DNA (a dsDNA). Aside from employing the peptide scaffold, the same group designed binuclear copper nucleases with innovative enantiomeric structures (Fig. 5d) [97]. The metallonucleases selectively cleave G4 DNA and lead to double-stranded cleavage of pUC19 plasmid DNA in a non-random fashion.

4. Therapeutic applications of site-specific ArMNs

In the past two decades, due to their precise cleavage activity toward RNA and DNA, site-specific ArMNs have been widely used in fields such as gene editing and biomedicine, leading to promising developments

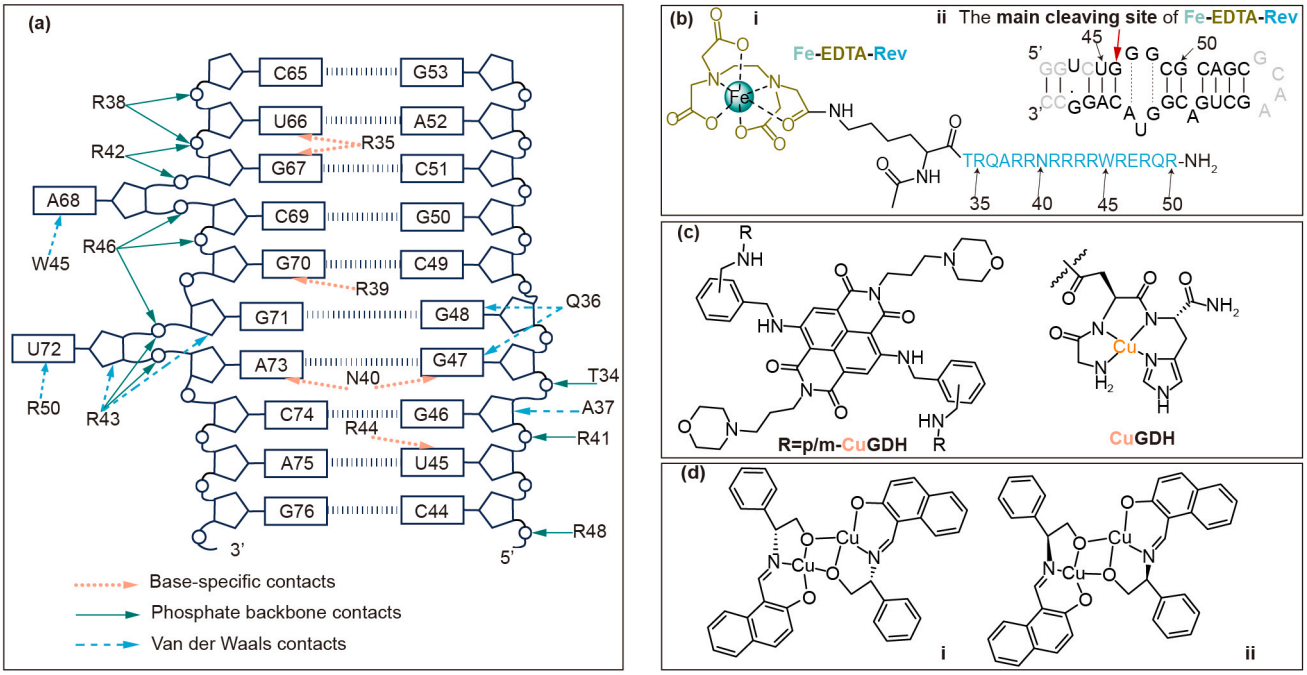

Fig. 5. Site-specific artificial metallonuclease designed for RNA secondary structures (a, b) or Guanine-quadruplex (G4) DNA (c,d). (a) the interactions between Rev peptide (regulator of expression of virion) and RRE (Rev responsive element) RNA secondary structure. The assembly of quadrilaterals (base), pentagons (ribose ring) and circles (phosphate) refers to RNA sequence. A, U, C, G in the quadrilaterals: adenine, uracil, cytosine, guanine; Q, T, A, R, N, W without quadrilaterals: amino acid residues; numbers refer to the position of nucleic acids in the RRE RNA sequence or amino acid residues in Rev peptide sequence. Orange dotted line: Base-specific contacts, green line: Phosphate backbone contacts, blue dashed line: van der Waals contacts [86]. (b) i: the structure of Fe-EDTA-Rev [88]. ii: the main cleaving site of Fe-EDTA-Rev on RNA hairpin. (c) Naphthalene-based G4 metallonucleases, the CuGDH (Cu ions chelated by Gly-Asp-His peptide) active centres are conjugated to the para (p-) or meta (m-) positions on both benzene rings. (d) The structures of the two enantiomeric complexes designed for G4 DNA cleavage adapted from [85,96,97]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

across various stages, including cell research, animal studies, and clinical trials (Table 1).

The application of single-stranded nucleic acid ArMNs is mainly reflected in addressing diseases associated with specific RNA sequences. The AuNP ArMNs designed by Wang et al. (mentioned in section 3.3) was leveraged to treat Huh7-derived human hepatoma cells, resulting in an effective suppression of HCV RNA replication (65 % decrease; measured by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)), as well as the related protein expression (approximately 75 % decrease in NS5A protein expression, measured by Western blot; NS5A is a protein essential for HCV replication). Subsequently, the AuNP ArMN was investigated using an HCV-infected mouse model, demonstrating even greater inhibition of HCV RNA replication *in vivo* compared with in vitro results (average reduction of 99.6 %) [56]. Another significant biomedical application is a protein receptor-binding ligand-modified nanonuclease targeting multidrug resistance (MDR), reported by Wang *et al* [98]. In this protocol, a nanonuclease is fused with Cys-tag epidermal growth factor (EGF) to target MDR mRNA in EGF receptor-expressing cancer cells (Fig. 6c). The assembled nanonuclease performs selective cancer cellular uptake and consequent MDR mRNA cleavage inhibiting the expression of MDR P-glycoprotein. In the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, the nanonuclease-pretreated MDR cells present a 23-fold reduction in the IC_{50} (Half Maximal Inhibitory Concentration) of doxorubicin compared with untreated cells (0.12 nM nanonuclease, Ovarian Cancer Adriamycin-Resistant 8 (OVCAR8) cell line), indicating the substantial efficacy of site-specific nanonuclease in MDR treatment.

Table 1
Designed ArMNs and their application (phases).

No.	Target	Recognition element	Cleavage element	Application/phase
I	ssDNA sequence	Complementary DNA sequence	Ce(III) complex	ssDNA Sequence [52]
II	RNA sequence	Complementary DNA sequence	RNAse	Antibiotics/ animal [56]
III	RNA sequence	Complementary DNA sequence	RNAse	Combating drug resistance/cells [98]
IV	dsDNA sequence	PNA sequence	Ce(IV)-EDTA [60]	Gene editing/cells [106–108]
V	dsDNA sequence	TFO DNA sequence	Cu(II) complex	dsDNA Sequence [66–68]
VI	dsDNA sequence	ZF protein	FokI DNAse	Gene editing/ Clinical Phase II [99–103]
VII	dsDNA sequence	ZF protein	Ce(IV)-peptide	dsDNA sequence [81]
VIII	RNA secondary structures	Virus recognition peptides	Fe(II)/Cu (II) complex	Antivirus/cells [90,91]
IX	G4 DNA	Metal complexes (chelator)	Cu(II) complex	Anticaner/cells [96,97]

The application of dsDNA-based ArMNs mainly focuses on DNA engineering, with one of the most frequently reported examples being ZF-FokI. The ZF nuclease enables various gene-editing approaches (e.g. gene disruption, deletion, correction, and addition, *et al.*) through non-homologous end joining (NHEJ) or homology-directed repair (HDR) [73]. The technique has been used to treat diseases triggered by gene mutations such as haemophilia [99,100], mucopolysaccharidosis [101,102], and several other genetic disorders [103]. The various applications of ZF nucleases [73] and their achievements in clinical trials [104] have been reviewed in detail and will not be discussed here.

ARCUT is another frequently reported dsDNA-based ArMNs system. Relying on its ability to cleavage dsDNA fragments with high precision, this system has been leveraged to selectively target human genomes [105] and facilitate subsequent gene editing [106]. The first ARCUT-

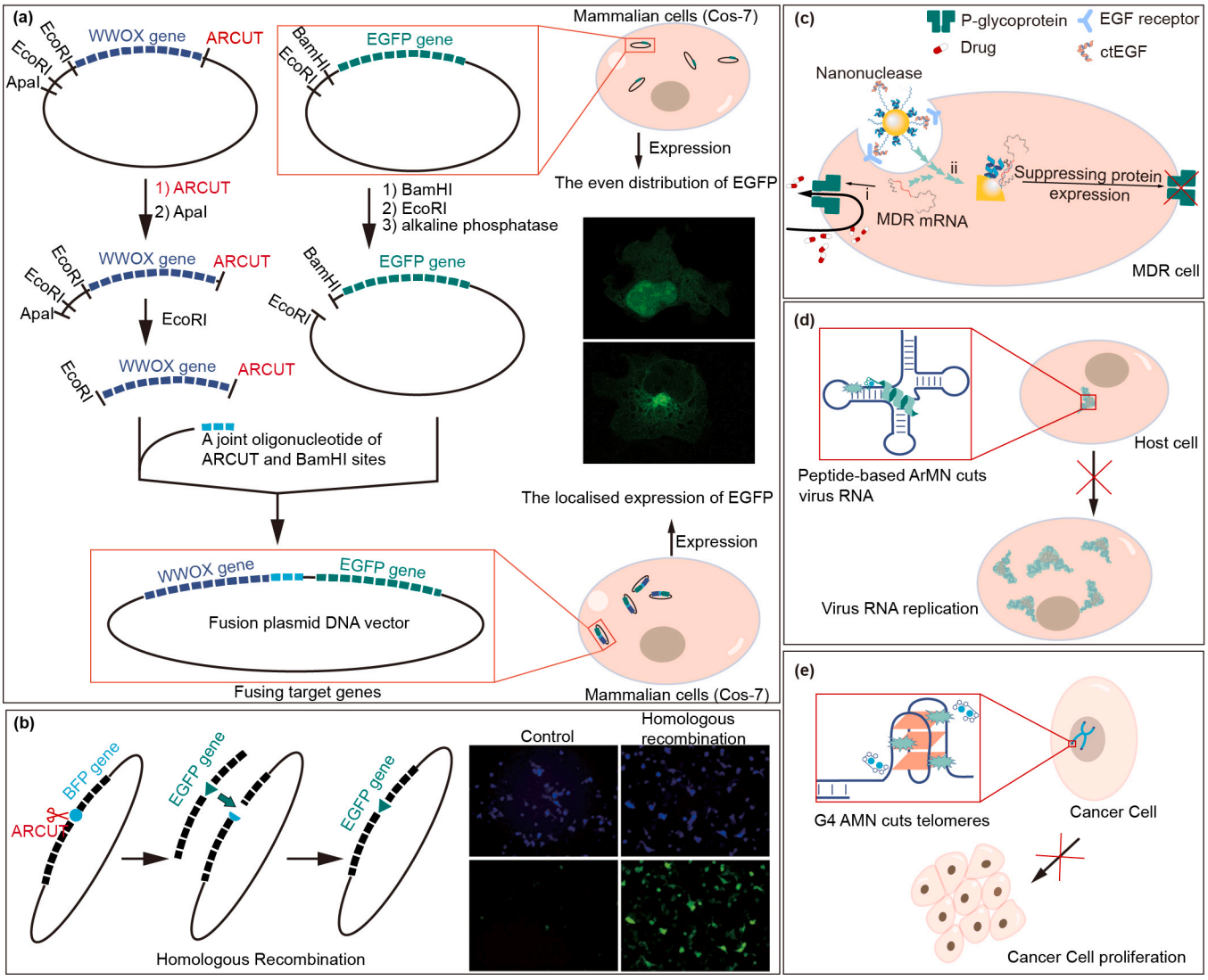

Fig. 6. The application of highlighted site-specific ArMNs: Double-stranded ArMNs (a, b), single-stranded ArMNs (c), RNA secondary-structure ArMNs (d) and G4 DNA ArMNs (e). (a) ARCUT-involved plasmid gene editing for fusing protein [106]. ApaI, EcoRI, BamHI and ARCUT on the plasmid (black circle): related cutting positions of natural endonucleases (ApaI, EcoRI, BamHI) or the artificial metallonuclease (ARCUT). EGFP: enhanced green fluorescent protein that can show fluorescence when expressed in cells. WWOX gene: WW (two tryptophan residues on both sides of the expressed protein)-domain-containing oxidoreductase, this gene is used for localising the expression of EGFP if EGFP is fused to WWOX and expressed successfully. With permission from Wiley. Copyright (2006). (b) ARCUT induced homologous recombination and the expression of mutated gene in 293 T cells [107]. With permission from Royal Society of Chemistry. Copyright (2009). BFP gene: Blue Fluorescent Protein gene, when the plasmid is successfully cut by ARCUT, the homologous recombination will spontaneously repair the double-stranded break based on the homologous EGFP gene and change the gene from BFP to EGFP, leading to the expression of EGFP and the observation of green fluorescence. Blue circle: the specific sequence belonging to BFP gene; Green triangle: the specific sequence belonging to EGFP gene; Dashed lines both on or outside the engineered plasmid DNA (black circles): homologous sequences of BFP and EGFP used for homologous recombination. (c) The ctEGF-modified nanonuclease suppresses multidrug-resistance (MDR) protein expression [98]. i: P-glycoprotein-induced MDR. ii: the process of combating MDR using the ctEGF-modified nanonuclease: The cell will uptake the nanonuclease because of the modified ctEGF protein, then the nanonuclease cuts the mRNA responsible for MDR expression leading to the suppression of MDR. (d) peptide-based ArMNs inhibit the replication of virus RNA through the secondary-structure cleavage [90,91]. (e) G4-targeting ArMNs cleave G4 DNA on the telomeres inhibiting cancer cell proliferation [96,97]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

based gene-editing protocol was reported by Komiyama and coworkers [106]. In this protocol, the target gene, WW-domain-containing oxidoreductase (WWOX), was cut by ARCUT in synergy with other endonucleases within a plasmid substrate, subsequently fusing it with a fluorescent protein (enhanced green fluorescent protein, EGFP) gene. The engineered plasmid vector enabled simultaneous expression of the target protein and fluorescent protein in mammalian cells (Fig. 6a). Subsequently, the same group achieved similar gene editing through homologous recombination (Fig. 6b) [107]. In practical application, the most notable advantage of ARCUT is that it allows straightforward genome manipulation using only two peptide nucleic acid (PNA) strands, instead of complicated restriction enzymes. In addition, this system has shown effective cleavage towards methylated DNA [108]. Considering these features, ARCUT holds significant promise for broader application in gene-editing therapeutics.

ArMNs targeting special secondary structures are mainly applied in antiviral and antitumor research. For antiviral applications, peptide-conjugated ArMNs have been reported to selectively cleave virus mRNAs and exhibit their activity in cell models. For example, the antiviral effects of Cu(II)-GGH-YrFK and Cu-GGHKYKETDL were demonstrated in an HCV replication assay using an HCV replicon cell line (LucUbiNeo-ET) [90,91] (Fig. 6d). In antitumor application, through

their selectivity towards telomeres, the activity of G4 DNA-targeting ArMNs was proved in cancer cells (Fig. 6e). *in vitro* study of Cu-GGHK-acridine ArMNs revealed that cellular senescence in all tested human cancer cell lines (Huh7 – Liver Cancer, MCF7 – Breast Cancer, BxPC3 – pancreatic cancer, LS174T – colon cancer, Caco2 – colon cancer, AsPC1 – pancreatic cancer) could be achieved through the inhibition of cell proliferation induced by these ArMNs [96]. The binuclear-copper ArMNs designed by the same group achieve similar cytotoxicity toward the cell stems of liver cancer (Huh7), breast cancer (MCF7), and pancreatic cancer (Bxpc3) [97]. Notably, G4-targeting ArMNs can kill the cancer cells by cleaving telomeric regions, a feat not yet demonstrated by reported natural nucleases. This suggests that site-specific ArMNs might achieve desired cleavages and possess broader applicability compared with natural nucleases.

In summary, the results described above, achieved using various methods, demonstrate promising potential not only for gene-silencing treatment but also for gene-editing therapies (Fig. 7).

5. Challenges and opportunities

The methodologies described above for designing ArMNs and their applications have significantly influenced life science, especially in therapeutic contexts. Their impressive performance targeting specific nucleic acids provides ample opportunity for *in vivo* applications and even commercial therapeutic development. The challenges that need to be addressed in the therapeutic applications include ensuring bioavailability *in vivo* and achieving highly specific targeting of cell or tissues. These hurdles may be addressed by incorporating strategies from pharmaceutical science, materials science, and computational techniques (Table 2).

5.1. Ensuring bioavailability in vivo

The challenge of bioavailability *in vivo* primarily arises from the degradation and inactivation of site-specific ArMNs in complex biological environments, such as the strongly acidic conditions of gastric fluid, enzymatic degradation, non-specific adsorption onto biological components (biofouling), and interference from abundant metal ions present *in vivo*. To address these stability and bioavailability concerns, several formulation strategies can be utilised, including nanoparticle [108] and microsphere formations [109], pharmaceutical excipients[110], and encapsulation or embedding within protective matrices [111].

Nanoparticle preparation is a formulation approach that transforms drugs into nanometre-scale particles, optionally applying protective or functional coatings, thus enhancing the stability and bioavailability of

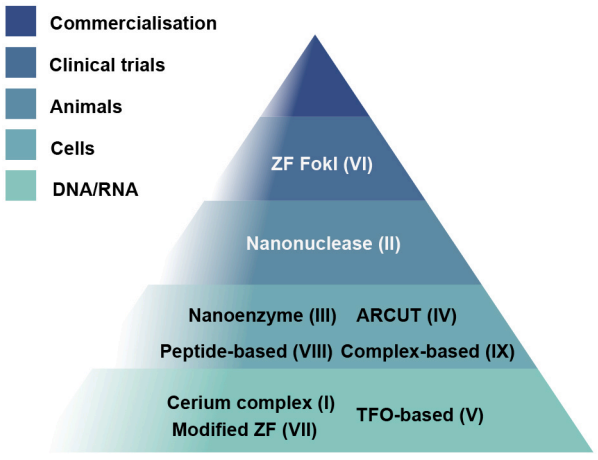

Fig. 7. The pyramid range of ArMNs in the application phase: commercialisation, clinical trials, animals, cells, DNA/RNA model. The numbers behind the ArMNs name correspond to the elucidation number in Table 1.

Table 2
Possible methods to overcome the challenges of ArMNs.

Name	Advantages	Mainly against challenge	Applied area
Nanotechnology	Enhanced stability and bioavailability	Bioavailability & Targeting	*In vivo* pharmacy
Microsphere [109,115,116]	Long-acting effect	Bioavailability & Targeting	*In vivo* pharmacy
SNAC [110,119]	Enhanced bioavailability	Bioavailability	*In vivo* pharmacy
Hydrogel [111,121–126]	Long-acting effect & aqueous environment	Bioavailability	*In vivo* pharmacy
Antibody/peptide-drug conjugates [127–134]	Enhanced delivery & *in vivo* performance	Targeting	Molecular design
High-entropy alloy [135–138]	Stability & potential site specificity	Bioavailability & Targeting	Molecular design
Computational techniques [139–141]	Simulation & rational prediction	Targeting	Molecular design

drugs [112,113]. A classic technique is the nanocrystal approach [114], in which an active pharmaceutical ingredient (API) or an API-adjuvant complex is converted into nanometre-scale crystals. This not only significantly enhances bioavailability but also allows for formulation into various delivery forms resistant to complex *in vivo* conditions. Nanocrystal and other nanoparticle-based preparation methods are particularly promising for small-molecule ArMNs, which require an improved solubility in aqueous environments and protection against degradation in specific physiological conditions, such as gastric acid.

Microspheres are micron-scale particles designed to encapsulate drugs, typically featuring a functional coating that protects the active pharmaceutical ingredient (API) and controls its release [109,115]. In contrast to nanoparticles, microspheres possess a larger core structure, capable of carrying greater quantities of drug molecules or additional cofactors [116]. The scale endows microspheres with controlled-release and long-acting performance in target tissues *in vivo*. These advantages can benefit certain ArMNs designs requiring prolonged action, such as those targeting tumours or serving as antibiotics. Furthermore, the excellent loading capacity of microspheres also provides opportunities to incorporate redox agents that facilitate the activity of site-specific ArMNs, such as ascorbic acid. In addition to tissue delivery, nanoparticle and microsphere formulations can enhance intracellular delivery, which is pivotal for ArMNs. Many ArMNs utilise nucleic acids as targeting elements, which may subject them to the same challenge faced by macromolecules such as RNA drugs: endosomal entrapment. After cellular uptake, drugs risk being sequestered and degraded by endosomes and lysosomes, resulting in diminished efficacy [117]. Endosomal entrapment has raised much concern recently, with nanocarriers emerging as a promising solution. Functionalised nano- or microparticles can enter endosomes and disrupt the endosomal membrane through various stimuli (e.g., pH changes), subsequently achieve endosomal escape and therapeutics [118].

Pharmaceutical excipients incorporating absorption enhancers, such as salcaprozate sodium (SNAC), represent another effective approach for improving bioavailability. SNAC is an absorption enhancer that has been commercially used for the oral administration of peptides [110]. One renowned case is Semaglutide, a diabetes drug that employs SNAC to enhance the gastric absorption of peptide [119]. Studies have demonstrated that SNAC not only acts as an absorption enhancer, facilitating peptide transport across the gastric mucosal cell membrane, but also serves as a pH buffer, counteracting the strongly acidic gastric environment. Given the impressive effectiveness in enhancing peptide bioavailability, SNAC holds significant promise for use in peptide-based ArMNs. Rational adaptation of pharmaceutical excipients can also ensure the clinical safety, accessibility, and adherence of patients [120]. Research in this field could further advance studies in the

commercialisation of ArMNs, making it valuable for both laboratory and industrial applications.

Protective matrices such as hydrogels offer another promising strategy. Hydrogels can deliver therapeutic dosages effectively to targeted tissues [121], especially on skin and mucous tissue [122]. Besides, hydrogel's high-water content makes it an ideal candidate for ArMNs, since most ArMNs require an aqueous medium to perform effective nucleic acid cleavage. Moreover, hydrogels offer several practical advantages, including adjustable mechanical strength, biocompatibility, and sustained release profiles, making them highly versatile for therapeutic applications [111]. The three-dimensional porous network structure of hydrogels allows for the encapsulation of various bioactive agents, such as small molecules, peptides, proteins, and even nanoparticles, protecting these agents from premature degradation and improving their bioavailability. Recent developments in stimuli-responsive hydrogels—such as those sensitive to pH, temperature, or specific biomolecules—further enhance their potential as targeted delivery systems for ArMNs [123–126]. Given these attributes, hydrogel-based delivery systems are highly promising platforms for the future development of effective ArMN-based therapies.

5.2. Achieving highly specific targeting of cells or tissues

Another significant challenge in therapeutic applications of ArMNs is achieving precise targeting of specific cells or tissues. This primarily involves two key aspects: effective delivery of ArMNs to the intended site and ensuring high affinity for the targeted nucleic acid sequences. To address these issues, novel molecular recognition strategies must be developed alongside methods for enhancing nucleic acid binding specificity. One promising strategy is the conjugation of antibodies or peptides with existing metallonucleases, improving their localisation to targeted cells or tissues. Computational techniques, such as structure-based molecular docking, molecular dynamics simulations, and machine learning-driven approaches, can further optimise nucleic acid binding affinity and significantly reduce off-target effects.

For targeted delivery to specific cells or tissues, antibody or peptide conjugates can significantly improve *in vivo* performance. A notable example is the antibody-drug conjugate (ADC) [127,128] called Enhertu, which conjugates cytotoxic topoisomerase I inhibitor with an antibody against human epidermal growth factor receptor 2 (HER2). The ADC effectively target cancer cells, highlighting the effectiveness of antibody-mediated targeting [129]. Although applications of such conjugation methods in ArMN development remain limited, a study by Wang et al. successfully demonstrated their potential in overcoming multidrug resistance in a cellular model [98]. This indicates the considerable promise of antibody or peptide conjugation strategies in ArMN design.

For targeting nucleic acids, feasibility has been demonstrated particularly in designs aimed at virus RNA [88–91]. Further exploration of peptide-based candidates should be encouraged, potentially drawing inspiration from related metallonuclease studies. Indeed, the concept of designing site-specific ArMNs has been widely applied in structural characterisation of nucleic acids, including detailed analysis of DNA/RNA structures and DNA/RNA-protein complexes. Similar to peptide-based strategies, protein-conjugated ArMNs can specifically cleave target nucleic acid sites, with cleavage domains typically restricted to within approximately 10–20 Å, based on the intrinsic properties of metal cleavage centres, such as Fe-based complexes [130]. For example, EDTA-Fe and BABE-Fe (BABE-Fe: Iron (S)-1-(p-bromoacetamidobenzyl) ethylenediaminetetraacetate) have been attached to RNA strands involved in spliceosome-related reactions, the secondary structure can be inferred through mapping the substrate at various stages [130,131] (Fig. 8a). Another interesting application involves molecular probes, where attached ArMNs exhibit cleavage preferences depending on protein conformations, producing characteristic electrophoresis footprints. This feature makes ArMNs highly effective as site-specific probes for analysing protein conformations and protein-nucleic acid interactions [132], such as EDTA-Fe conjugated to NusA (N-utilising substance A) protein [133] (Fig. 8b). Furthermore, similar strategies have been applied in high-throughput screening to characterise protein-RNA binding sites. For example, Green and coworkers modified various amino residues on target protein with BABE-Fe and characterised the interactions between Upf1 (an RNA helicase) and its binding sites [134]. Although these studies were not directly intended for therapeutic ArMN development, the insights gained from protein conformational studies and nucleic acid-binding preferences offer valuable templates for future design of DNA/RNA-specific ArMNs in therapeutic contexts.

Another promising technique for designing ArMNs involves the use

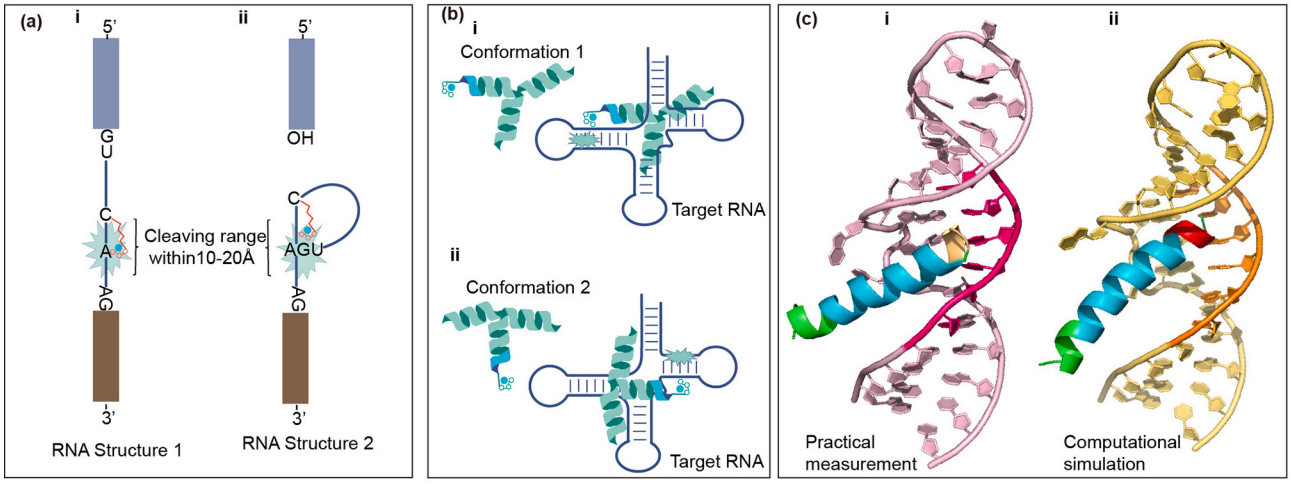

Fig. 8. Techniques that could be used in site-specific design. (a) The analysis of spliceosome synthesis. i, ii: the ArMN is attached to RNA sequences to investigate the change of RNA sequences. When the RNA structures change in different stages of spliceosome synthesis, the cleaving centre will be activated by adding metal ions leading to different RNA mapping. [130,131]. (b) Identifying the conformations of protein using ArMNs. i, ii: The same protein fused with ArMNs will perform different cleaving sites when the protein possesses different conformations [132,133]. (c) Comparing the practical measurement with the computational simulation of peptide-RNA structure. i: the structure of RRE RNA and Rev peptide complexes measured practically (PDB: 1ETF, visualised by Pymol 4.6.0) [86]. ii: the structure of RRE RNA and Rev peptide complexes simulated by AlphaFold 3 [140,141] (visualised by Pymol 4.6.0). The yellow (i)/red (ii) terminus of peptide: the end that has been used for fusing Fe-EDTA and subsequent ArMNs cleavage by Covan and coworkers [88,89]; the hot pink (i)/orange (ii) RNA sequence: the cleaving domain reported by Covan and coworkers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of high-entropy alloys (HEAs) [135], composed of multiple metal elements in defined proportions, resulting in unique catalytic properties [136,137]. Compared with traditional alloys, HEAs maintain mechanical robustness within biological environments while exhibiting enhanced catalytic activity due to greater mobility of metal ions, facilitating various reactions including redox processes. Besides, HEAs formulated with specific elemental ratios have demonstrated site-specific catalytic activity towards substrates such as long-chain alkanes [138]. Given their stability and catalytic specificity under harsh conditions, HEAs represent an ideal platform for functionalisation with site-specific targeting elements and delivering active metal ions crucial for nuclease activity in ArMN design.

Computational simulation represents another valuable complementary approach for site-specific design. Currently, computational simulations are widely employed to assess molecular interactions, ranging from predicting static molecular complex structures (e.g. AlphaFold 3 and docking analysis) to detailed atom-level interactions (e.g. density functional theory (DFT) simulation) [139]. Studies utilising these approaches are increasingly prevalent in molecular design research. A particularly notable computational model is AlphaFold 3, which accurately predicts the static conformations of individual peptide or nucleic acid sequences and complex structures formed between biomolecules, such as protein–nucleic acid complexes [140,141]. For instance, using AlphaFold 3 to model the Rev peptide–RRE RNA interaction yielded a predicted structure closely matching experimental observations [86] (Fig. 8c). Computational simulations can also be used for screening selective elements to minimise off-target effects. Through rational design and in silico modelling, optimised target elements can achieve stable targeting of specific gene fragments, even those with sequence variability due to human genetic diversity [142]. Such optimisation will significantly enhance the feasibility of ArMN-based clinical therapeutics. This groundbreaking technique holds significant promise for large-scale, site-specific molecular design as computational models and analytical methods continue to evolve.

6. Conclusion

In recent decades, significant progress has been achieved in understanding the site-specificity of ArMNs, particularly concerning substrate characteristics, interactions between substrates and binding domains, and design methodologies. These advancements have enabled more precise nucleic acid recognition, substantially broadening the potential application of ArMNs in multiple fields such as biomedicine and gene editing.

In this review, we have examined recent advancements in the design and synthesis of site-specific ArMNs, outlined fundamental principles underlying nucleic acid targeting specificity, and illustrated relevant applications. It is hoped that this review will provide researchers in the field with timely insights, innovative design methodologies, and a comprehensive understanding of site-specific ArMNs, facilitating further exploration and practical innovation. Additionally, by highlighting existing challenges and potential solutions, we aim to facilitate further exploration, stimulate interdisciplinary collaborations, and inspire practical innovations in therapeutic and diagnostic applications.

CRediT authorship contribution statement

Xi Lu: Writing – original draft, Writing – review & editing, Visualization. **Chengchen Zhang:** Writing – review & editing, Conceptualization, Visualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

References

[1] W. Yang, Nucleases: diversity of structure, function and mechanism, Q. Rev. Biophys. 44 (1) (2011) 1–93.
[2] S.C. Kowalczykowski, An Overview of the Molecular Mechanisms of Recombinational DNA Repair, Cold Spring Harb. Perspect. Biol. 7 (11) (2015) 36.
[3] T.M. Marti, O. Fleck, DNA repair nucleases, Cell. Mol. Life Sci. 61 (3) (2004) 336–354.
[4] A. Pingoud, G.G. Wilson, W. Wende, Type II restriction endonucleases-a historical perspective and more, Nucleic Acids Res. 42 (12) (2014) 7489–7527.
[5] V.A. Sioson, M. Kim, J. Joo, Challenges in delivery systems for CRISPR-based genome editing and opportunities of nanomedicine, Biomed. Eng. Lett. 11 (3) (2021) 217–233.
[6] N.E.T. Castillo, et al., Enzyme mimics in-focus: Redefining the catalytic attributes of artificial enzymes for renewable energy production, Int. J. Biol. Macromol. 179 (2021) 80–89.
[7] B.K. Maiti, et al., Designed Metal-ATCUN Derivatives: Redox- and Non-redox-Based Applications Relevant for Chemistry, Biology, and Medicine, Iscience 23 (12) (2020) 38.
[8] S.M. Hecht, Bleomycin: New perspectives on the mechanism of action, J. Nat. Prod. 63 (1) (2000) 158–168.
[9] R.A. Giroux, S.M. Hecht, Characterization of Bleomycin Cleavage Sites in Strongly Bound Hairpin DNAs, J. Am. Chem. Soc. 132 (47) (2010) 16987–16996.
[10] M.L. Maeder, C.A. Gersbach, Genome-editing Technologies for Gene and Cell Therapy, Mol. Ther. 24 (3) (2016) 430–446.
[11] N. Shigi, J. Sumaoka, M. Komiyama, Applications of PNA-Based Artificial Restriction DNA Cutters, Molecules 22 (10) (2017).
[12] J.C. Joyner, J.A. Cowan, Target-directed catalytic metallodrugs, Braz. J. Med. Biol. Res. 46 (6) (2013) 465–485.
[13] D.S. Sigman, A. Mazumder, D.M. Perrin, Chemical Nucleases, Chem. Rev. 93 (6) (1993) 2295–2316.
[14] F.Z. Li, et al., Nucleic acid and phosphoester hydrolytic cleavage catalysed by aza-crown ether metal complexes as synthetic nucleases, Prog. React. Kinet. Mech. 39 (3) (2014) 209–232.
[15] Y. Jin, J.A. Cowan, DNA cleavage by copper-ATCUN complexes. Factors influencing cleavage mechanism and linearization of dsDNA, J. Am. Chem. Soc. 127 (23) (2005) 8408–8415.
[16] Y. Huang, J. Ren, X. Qu, Nanozymes: Classification, Catalytic Mechanisms, Activity Regulation, and Applications, Chem. Rev. 119 (6) (2019) 4357–4412.
[17] A.V. Wright, J.A. Doudna, Protecting genome integrity during CRISPR immune adaptation, Nat. Struct. Mol. Biol. 23 (10) (2016) 876–883.
[18] A.T. Vessoni, et al., Autophagy and genomic integrity, Cell Death Differ. 20 (11) (2013) 1444–1454.
[19] A.C. Cheng, et al., Recognition of nucleic acid bases and base-pairs by hydrogen bonding to amino acid side-chains, J. Mol. Biol. 327 (4) (2003) 781–796.
[20] J.D. Watson, F.H. Crick, Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid, Nature 171 (4356) (1953) 737–738.
[21] D.R. Duckett, et al., Nucleic acid structure and recognition, Biophys. Chem. 68 (1–3) (1997) 53–62.
[22] E.P. Mimitou, L.S. Symington, DNA end resection: Many nucleases make light work, DNA Repair 8 (9) (2009) 983–995.
[23] E. Mailler, et al., The Life-Cycle of the HIV-1 Gag-RNA Complex, Viruses-Basel 8 (9) (2016).
[24] J.H.P. Chan, S.H. Lim, W.S.F. Wong, Antisense oligonucleotides: From design to therapeutic application, Clin. Exp. Pharmacol. Physiol. 33 (5–6) (2006) 533–540.
[25] U. Heinemann, Y. Roske, Symmetry in Nucleic-Acid Double Helices, Symmetry-Basel 12 (2020) 5.
[26] N.T. Thuong, C. Helene, Sequence-specific recognition and modification of double-helical dna by oligonucleotides, Angewandte Chemie-Int. Ed. 32 (5) (1993) 666–690.
[27] Alberts, B., et al., *Molecular Biology of the Cell, Sixth Edition*. 2015. p. 1-1342.
[28] D.M. Spencer, et al., The expression of antibodies to Z-DNA in the blood of patients with systemic lupus erythematosus: Relationship to autoantibodies to B-DNA, Clin. Immunol. 255 (2023).
[29] N.C. Seeman, J.M. Rosenberg, A. Rich, Sequence-specific recognition of double helical nucleic acids by proteins, PNAS 73 (3) (1976) 804–808.
[30] S.A. Wolfe, L. Nekludova, C.O. Pabo, DNA recognition by Cys_2His_2 zinc finger proteins, Annu. Rev. Biophys. Biomol. Struct. 29 (2000) 183–212.
[31] S. Bhaduri, N. Ranjan, D.P. Arya, An overview of recent advances in duplex DNA recognition by small molecules, Beilstein J. Org. Chem. 14 (2018) 1051–1086.
[32] S. Negi, et al., The past, present, and future of artificial zinc finger proteins: design strategies and chemical and biological applications, J. Biol. Inorg. Chem. 28 (3) (2023) 249–261.

[33] E.N. Nikolova, et al., A Historical Account of Hoogsteen Base-Pairs in Duplex DNA, Biopolymers 99 (12) (2013) 955–968.
[34] C. Li, et al., Triplex-forming oligonucleotides as an anti-gene technique for cancer therapy, Front. Pharmacol. 13 (2022).
[35] E.N. Nikolova, et al., Characterizing the Protonation State of Cytosine in Transient G•C Hoogsteen Base Pairs in Duplex DNA, J. Am. Chem. Soc. 135 (18) (2013) 6766–6769.
[36] M. Zuker, D. Sankoff, RNA Secondary Structures and Their Prediction, Bull. Math. Biol. 46 (4) (1984) 591–621.
[37] L.E. Vandivier, et al., The conservation and function of RNA secondary structure in plants, Annu. Rev. Plant Biol. 67 (1) (2016) 463–488.
[38] D.H. Mathews, W.N. Moss, D.H. Turner, Folding and Finding RNA Secondary Structure, Cold Spring Harb. Perspect. Biol. 2 (12) (2010).
[39] M. Wu, I. Tinoco, RNA folding causes secondary structure rearrangement, PNAS 95 (20) (1998) 11555–11560.
[40] S.M. Hecht, RNA degradation by bleomycin, a naturally-occurring bioconjugate, Bioconjugate Chem. 5 (6) (1994) 513–526.
[41] S.M. Kerwin, G-quadruplex DNA as a target for drug design, Curr. Pharm. Des. 6 (4) (2000) 441–471.
[42] G.N. Parkinson, M.P.H. Lee, S. Neidle, Crystal structure of parallel quadruplexes from human telomeric DNA, Nature 417 (6891) (2002) 876–880.
[43] S. Burge, et al., Quadruplex DNA: sequence, topology and structure, Nucleic Acids Res. 34 (19) (2006) 5402–5415.
[44] A.V. Pavlova, et al., Impact of G-Quadruplexes on the Regulation of Genome Integrity, DNA Damage and Repair, Biomolecules 11 (9) (2021).
[45] M. Sauer, K. Paeschke, G-quadruplex unwinding helicases and their function in vivo, Biochem. Soc. Trans. 45 (2017) 1173–1182.
[46] N.H. Campbell, et al., Molecular basis of structure-activity relationships between salphen metal complexes and human telomeric DNA quadruplexes, J. Med. Chem. 55 (1) (2012) 209–222.
[47] M. Komiyama, Sequence-specific and hydrolytic scission of DNA and RNA by lanthanide complex-oligoDNA hybrids, J. Biochem. 118 (4) (1995) 665–670.
[48] F.H. Zelder, A.A. Mokhir, R. Krämer, Sequence selective hydrolysis of linear DNA using conjugates of Zr(IV) complexes and peptide nucleic acids, Inorg. Chem. 42 (26) (2003) 8618–8620.
[49] F. Mancin, P. Scrimin, P. Tecilla, Progress in artificial metallonucleases, Chem. Commun. 48 (45) (2012) 5545–5559.
[50] S. Suzuki, et al., Experimental optimization of probe length to increase the sequence specificity of high-density oligonucleotide microarrays, BMC Genomics 8 (2007).
[51] J.X. Meng, H.J. Wu, D.X. Feng, Fluorimetry determination of trace Ce^{3+} with EDTP, Spectrochimica Acta Part A-Mol. Biomol. Spectrosc. 56 (10) (2000) 1925–1928.
[52] T. Loennberg, et al., Oxidation of an Oligonucleotide-Bound Ce^{III}/Multiphosphonate Complex for Site-Selective DNA Scission, Chem.-A Eur. J. 16 (3) (2010) 855–859.
[53] Z. Yu, J.A. Cowan, Metal complexes promoting catalytic cleavage of nucleic acids - biochemical tools and therapeutics, Curr. Opin. Chem. Biol. 43 (2018) 37–42.
[54] H. Wang, K.W. Wan, X.H. Shi, Recent Advances in Nanozyme Research, Adv. Mater. 31 (45) (2019) 10.
[55] Z.R. Wang, et al., Structure and activity of nanozymes: Inspirations for de novo design of nanozymes, Mater. Today 41 (2020) 81–119.
[56] Z. Wang, et al., Nanoparticle-based artificial RNA silencing machinery for antiviral therapy, PNAS 109 (31) (2012) 12387–12392.
[57] D.R. Corey, Peptide nucleic acids: Expanding the scope of nucleic acid recognition, Trends Biotechnol. 15 (6) (1997) 224–229.
[58] P.E. Nielsen, et al., Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide, Science 254 (5037) (1991) 1497–1500.
[59] J.C. Hanvey, et al., Antisense and antigene properties of peptide nucleic-acids, Science 258 (5087) (1992) 1481–1485.
[60] Y. Yamamoto, et al., Site-selective and hydrolytic two-strand scission of double-stranded DNA using Ce(IV)/EDTA and pseudo-complementary PNA, Nucleic Acids Res. 32 (19) (2004) 7.
[61] H.E. Moser, P.B. Dervan, Sequence-specific cleavage of double helical DNA by triple helix formation, Science 238 (4827) (1987) 645–650.
[62] C.H.B. Chen, D.S. Sigman, Nuclease Activity of 1,10-phenanthroline copper – sequence-specific targeting, PNAS 83 (19) (1986) 7147–7151.
[63] J.C. Francois, et al., Sequence-specific recognition and cleavage of duplex DNA via triple-helix formation by oligonucleotides covalently linked to a phenanthroline copper chelate, PNAS 86 (24) (1989) 9702–9706.
[64] B. McGorman, et al., Enzymatic Synthesis of Chemical Nuclease Triplex-Forming Oligonucleotides with Gene-Silencing Applications, Nucleic Acids Res. 50 (10) (2022) 5467–5481.
[65] H.C. Kolb, M.G. Finn, K.B. Sharpless, Click chemistry: Diverse chemical function from a few good reactions, Angewandte Chemie-Int. Ed. 40 (11) (2001) 2004–2021.
[66] N. Zuin Fantoni, et al., Development of Gene-Targeted Polypyridyl Triplex-Forming Oligonucleotide Hybrids, Chembiochem 21 (24) (2020) 3563–3574.
[67] T. Lauria, et al., A Click Chemistry Approach to Developing Molecularly Targeted DNA Scissors, Chemistry-a European Journal 26 (70) (2020) 16782–16792.
[68] A. Panattoni, et al., Oxidative DNA Cleavage with Clip-Phenanthroline Triplex-Forming Oligonucleotide Hybrids, Chembiochem 21 (7) (2020) 991–1000.
[69] C. Andreini, et al., Counting the zinc-proteins encoded in the human genome, J. Proteome Res. 5 (1) (2006) 196–201.
[70] C. Andreini, et al., Zinc through the three domains of life, J. Proteome Res. 5 (11) (2006) 3173–3178.
[71] H. Kim, J.S. Kim, A guide to genome engineering with programmable nucleases, Nat. Rev. Genet. 15 (5) (2014) 321–334.
[72] Z. Yang, et al., Genome editing and its applications in genetic improvement in aquaculture, Rev. Aquac. 14 (1) (2022) 178–191.
[73] F.D. Urnov, et al., Genome editing with engineered zinc finger nucleases, Nat. Rev. Genet. 11 (9) (2010) 636–646.
[74] M.H. Porteus, D. Carroll, Gene targeting using zinc finger nucleases, Nat. Biotechnol. 23 (8) (2005) 967–973.
[75] Y. Aiba, J. Sumaoka, M. Komiyama, Artificial DNA cutters for DNA manipulation and genome engineering, Chem. Soc. Rev. 40 (12) (2011) 5657–5668.
[76] K. Beumer, et al., Efficient gene targeting in Drosophila with zinc-finger nucleases, Genetics 172 (4) (2006) 2391–2403.
[77] J.C. Miller, et al., An improved zinc-finger nuclease architecture for highly specific genome editing, Nat. Biotechnol. 25 (7) (2007) 778–785.
[78] L. Zandarashvili, et al., Asymmetrical roles of zinc fingers in dynamic DNA-scanning process by the inducible transcription factor Egr-1, PNAS 109 (26) (2012) E1724–E1732.
[79] L. Zandarashvili, et al., Balancing between affinity and speed in target DNA search by zinc-finger proteins via modulation of dynamic conformational ensemble, PNAS 112 (37) (2015) E5142–E5149.
[80] J.C. Miller, et al., Enhancing gene editing specificity by attenuating DNA cleavage kinetics, Nat. Biotechnol. 37 (8) (2019) 945–952.
[81] T. Nakatsukasa, et al., Site-specific DNA cleavage by artificial zinc finger-type nuclease with cerium-binding peptide, Biochem. Biophys. Res. Commun. 330 (1) (2005) 247–252.
[82] M.G. Costales, et al., How We Think about Targeting RNA with Small Molecules, J. Med. Chem. 63 (17) (2020) 8880–8900.
[83] J. Kjems, et al., Specific binding of a basic peptide from HIV-1 rev, EMBO J. 11 (3) (1992) 1119–1129.
[84] R.Y. Tan, et al., RNA recognition by an isolated alpha-helix, Cell 73 (5) (1993) 1031–1040.
[85] R.D. Peterson, J. Feigon, Structural change in Rev responsive element RNA of HIV-1 on binding Rev peptide, J. Mol. Biol. 264 (5) (1996) 863–877.
[86] J.L. Battiste, et al., alpha helix-RNA major groove recognition in an HIV-1 Rev peptide RRE RNA complex, Science 273 (5281) (1996) 1547–1551.
[87] K.A. Lacourciere, J.T. Stivers, J.P. Marino, Mechanism of neomycin and Rev peptide binding to the Rev responsive element of HIV-1 as determined by fluorescence and NMR spectroscopy, Biochemistry 39 (19) (2000) 5630–5641.
[88] J.C. Joyner, J.A. Cowan, Targeted Cleavage of HIV RRE RNA by Rev-Coupled Transition Metal Chelates, J. Am. Chem. Soc. 133 (25) (2011) 9912–9922.
[89] J.C. Joyner, K.D. Keuper, J.A. Cowan, Kinetics and mechanisms of oxidative cleavage of HIV RRE RNA by Rev-coupled transition metal-chelates, Chem. Sci. 4 (4) (2013) 1707–1718.
[90] S. Bradford, J.A. Cowan, Catalytic metallodrugs targeting HCV IRES RNA, Chem. Commun. 48 (25) (2012) 3118–3120.
[91] M.J. Ross, S.S. Bradford, J.A. Cowan, Catalytic metallodrugs based on the LaR2C peptide target HCV SLIV IRES RNA, Dalton Trans. 44 (48) (2015) 20972–20982.
[92] C.R. Geyer, D. Sen, Evidence for the metal-cofactor independence of an RNA phosphodiester-cleaving DNA enzyme, Chem. Biol. 4 (8) (1997) 579–593.
[93] Y. Li, et al., Higher-order human telomeric G-quadruplex DNA metalloenzyme catalyzed Diels-Alder reaction: an unexpected inversion of enantioselectivity modulated by K(+) and NH4(+) ions, Chem. Commun. (Camb) 51 (67) (2015) 13174–13177.
[94] H. Bertrand, et al., The importance of metal geometry in the recognition of G-quadruplex-DNA by metal-terpyridine complexes, Org. Biomol. Chem. 5 (16) (2007) 2555–2559.
[95] Z. Yu, M.L. Han, J.A. Cowan, Toward the Design of a Catalytic Metallodrug: Selective Cleavage of G-Quadruplex Telomeric DNA by an Anticancer Copper-Acridine-ATCUN Complex, Angewandte Chemie-Int. Ed. 54 (6) (2015) 1901–1905.
[96] Z. Yu, et al., Rapid Telomere Reduction in Cancer Cells Induced by G-Quadruplex-Targeting Copper Complexes, J. Med. Chem. 62 (10) (2019) 5040–5048.
[97] S. Parveen, et al., Enantiomeric copper based anticancer agents promoting sequence-selective cleavage of G-quadruplex telomeric DNA and non-random cleavage of plasmid DNA, Metallomics 12 (6) (2020) 988–999.
[98] Z.L. Wang, et al., Biomimetic RNA-Silencing Nanocomplexes: Overcoming Multidrug Resistance in Cancer Cells, Angewandte Chem.-Int. Ed. 53 (7) (2014) 1997–2001.
[99] S.W. Pipe, Gene therapy for hemophilia, Pediatr. Blood Cancer 65 (2) (2018).
[100] J.J. Sung, et al., Restoration of FVIII expression by targeted gene insertion in the FVIII locus in hemophilia A patient-derived iPSCs, Exp. Mol. Med. 51 (4) (2019) 1–9.
[101] P. Zapolnik, A. Pyrkosz, Gene Therapy for Mucopolysaccharidosis Type II-A Review of the Current Possibilities, Int. J. Mol. Sci. 22 (11) (2021).
[102] P. Harmatz, et al., First-in-human in vivo genome editing via AAV-zinc-finger nucleases for mucopolysaccharidosis I/II and hemophilia B, Mol. Ther. 30 (12) (2022) 3587–3600.
[103] M.-S. Kim, A.G. Kini, Engineering and Application of Zinc Finger Proteins and TALEs for Biomedical Research, Mol. Cells 40 (8) (2017) 533–541.
[104] Y. Lu, et al., Improvements of nuclease and nickase gene modification techniques for the treatment of genetic diseases, Front. Genome Ed. 4 (2022) 892769.
[105] K. Ito, N. Shigi, M. Komiyama, An artificial restriction DNA cutter for site-selective gene insertion in human cells, Chem. Commun. 49 (60) (2013) 6764–6766.
[106] Y. Yamamoto, et al., Chemical-reaction-based site-selective DNA cutter for PCR-free gene manipulation, Chembiochem 7 (4) (2006) 673–677.

[107] H. Katada, et al., Homologous recombination in human cells using artificial restriction DNA cutter, Chem. Commun. 43 (2009) 6545–6547.
[108] J.-M. Zhou, et al., Genetic recombination without using either restriction enzyme or PCR, Nucleic Acids Symp. Ser. 2005 (49) (2004) 259–260.
[109] D.D. Liu, et al., Potential applications of drug delivery technologies against radiation enteritis, Expert Opin. Drug Deliv. 20 (4) (2023) 435–455.
[110] N. Kommineni, et al., SNAC for Enhanced Oral Bioavailability: An Updated Review, Pharm. Res. 40 (3) (2023) 633–650.
[111] A.C. Daly, et al., Hydrogel microparticles for biomedical applications, Nat. Rev. Mater. 5 (1) (2020) 20–43.
[112] D.Y. Chen, et al., Nanoparticle drug delivery systems for synergistic delivery of tumor therapy, Front. Pharmacol. 14 (2023).
[113] W.W. Gao, et al., Nanoparticle-based local antimicrobial drug delivery, Adv. Drug Deliv. Rev. 127 (2018) 46–57.
[114] T.L. Chang, et al., Nanocrystal technology for drug formulation and delivery, Front. Chem. Sci. Eng. 9 (1) (2015) 1–14.
[115] X.Y. Wang, et al., Dual-Responsive Microsphere Based on Natural Sunflower Pollen for Hemostasis and Repair in Inflammatory Bowel Disease, ACS Appl. Mater. Interfaces 16 (24) (2024) 30658–30670.
[116] Y.T. Wang, et al., Mucoadhesive nanocrystal-in-microspheres with high drug loading capacity for bioavailability enhancement of silybin, Colloids Surf. B-Biointerfaces 198 (2021).
[117] S. Chatterjee, et al., Endosomal escape: a bottleneck for LNP-mediated therapeutics, Proc. Natl. Acad. Sci. U.S.A. 121 (11) (2024).
[118] A. Ahmad, et al., Endolysosomal trapping of therapeutics and endosomal escape strategies, Drug Discov. Today 29 (8) (2024).
[119] S.T. Buckley, et al., Transcellular stomach absorption of a derivatized glucagon-like peptide-1 receptor agonist, Sci. Transl. Med. 10 (467) (2018).
[120] C.S. Higano, J. Hafron, Adherence With Oral Anticancer Therapies: Clinical Trial vs Real-world Experiences With a Focus on Prostate Cancer, J. Urol. 209 (3) (2023) 485–493.
[121] X.Y. Zhang, et al., Chitosan-based self-healing hydrogel dressing for wound healing, Adv. Colloid Interface Sci. 332 (2024).
[122] Y.X. Pan, et al., Effects of a semi-interpenetrating network hydrogel loaded with oridonin and DNase-I on the healing of chemoradiotherapy-induced oral mucositis, Biomater. Sci. 12 (17) (2024) 4452–4470.
[123] S. Graham, P.F. Marina, A. Blencowe, Thermoresponsive polysaccharides and their thermoreversible physical hydrogel networks, Carbohydr. Polym. 207 (2019) 143–159.
[124] Y.N. Fan, et al., Advances in Smart-Response Hydrogels for Skin Wound Repair, Polymers 16 (19) (2024).
[125] Z.W. Zhou, et al., Targeted modulation of intestinal epithelial regeneration and immune response in ulcerative colitis using dual-targeting bilirubin nanoparticles, Theranostics 14 (2) (2024) 528–546.
[126] Z.G. Li, et al., Magnetic-responsive hydrogels: From strategic design to biomedical applications, J. Control. Release 335 (2021) 541–556.
[127] C. Dumontet, et al., Antibody-drug conjugates come of age in oncology, Nat. Rev. Drug Discov. 22 (8) (2023) 641–661.
[128] S.M. Swain, M. Shastry, E. Hamilton, Targeting HER2-positive breast cancer: advances and future directions, Nat. Rev. Drug Discov. 22 (2) (2023) 101–126.
[129] S.J. Keam, Trastuzumab Deruxtecan: First Approval, Drugs 80 (5) (2020) 501–508.
[130] C.S. Grewal, O.A. Kent, A.M. MacMillan, Radical probing of spliceosome assembly, Methods 125 (2017) 16–24.
[131] O.A. Kent, A.M. MacMillan, Early organization of pre-mRNA during spliceosome assembly, Nat. Struct. Biol. 9 (8) (2002) 576–581.
[132] T. James, et al., Determining the Architecture of a Protein-DNA Complex by Combining FeBABE Cleavage Analyses, 3-D Printed Structures, and the ICM Molsoft Program, Methods Mol. Biol. (Clifton N.J.) 1334 (2015) 29–40.
[133] S. Mishra, R. Sen, N protein from lambdoid phages transforms NusA into an antiterminator by modulating NusA-RNA polymerase flap domain interactions, Nucleic Acids Res. 43 (12) (2015) 5744–5758.
[134] A.P. Schuller, et al., Directed hydroxyl radical probing reveals Upf1 binding to the 80S ribosomal E site rRNA at the L1 stalk, Nucleic Acids Res. 46 (4) (2018) 2060–2073.
[135] G.H. Cao, et al., Liquid metal for high-entropy alloy nanoparticles synthesis, Nature 619 (7968) (2023) 73–77.
[136] Y. Xin, et al., High-Entropy Alloys as a Platform for Catalysis: Progress, Challenges, and Opportunities, ACS Catal. 10 (19) (2020) 11280–11306.
[137] M.A. Rahim, et al., Low-temperature liquid platinum catalyst, Nat. Chem. 14 (8) (2022) 935–941.
[138] J.M. Tang, et al., Dynamic configurations of metallic atoms in the liquid state for selective propylene synthesis, Nat. Nanotechnol. 19 (3) (2024).
[139] S.K. Niazi, Z. Mariam, Computer-Aided Drug Design and Drug Discovery: A Prospective Analysis, Pharmaceuticals 17 (1) (2024).
[140] M. Varadi, et al., AlphaFold Protein Structure Database in 2024: providing structure coverage for over 214 million protein sequences, Nucleic Acids Res. 52 (D1) (2023) D368–D375.
[141] J. Jumper, et al., Highly accurate protein structure prediction with AlphaFold, Nature 596 (7873) (2021) 583–589.
[142] S. Cancellieri, et al., Human genetic diversity alters off-target outcomes of therapeutic gene editing, Nat. Genet. 55 (1) (2023) 34.