Review

Functional Xeno Nucleic Acids for Biomedical Application

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Functional nucleic acids(FNAs) refer to a type of oligonucleotides with functions over the traditional genetic roles of nucleic acids, which have been widely applied in screening, sensing and imaging fields. However, the potential application of FNAs in biomedical field is still restricted by the unsatisfactory stability, biocompatibility, biodistribution and immunity of natural nucleic acids(DNA/RNA). Xeno nucleic acids(XNAs) are a kind of nucleic acid analogues with chemically modified sugar groups that possess improved biological properties, including improved biological stability, increased binding affinity, reduced immune responses, and enhanced cell penetration or tissue specificity. In the last two decades, scientists have made great progress in the research of functional xeno nucleic acids, which makes it an emerging attractive biomedical application material. In this review, we summarized the design of functional xeno nucleic acids and their applications in the biomedical field.

Keywords Functional nucleic acid; Xeno nucleic acid; Chemical modification; Biomedical application

1 Introduction

Deoxyribonucleic acid(DNA) and ribonucleic acid(RNA) are two kinds of natural nucleic acids[1]. They exist in lots of living organisms and are highly important in many biological processes[2,3]. Functional nucleic acids(FNAs) are the kind of nucleic acids that have specific functions beyond traditional genetic roles[4,5]. In the past three decades, scientists have made great efforts on FNAs, and have a deep understanding of their molecular structures and biological functions[6]. FNAs, such as aptamers, nucleic acid enzymes(NAzymes) and molecular beacons(MBs) have been widely applied in screening, sensing, and imaging fields[7−10]. However, the potential application of FNAs in the biomedical field is still restricted arising from the intrinsic properties of natural nucleic acids(DNA/RNA), such as stability, biocompatibility, biodistribution, and immunity[11].

Chemical modification is a vital method to develop useful nucleic acid with additional properties[12−14]. So far, it has been used to synthesize and investigate nucleic acid analogues with modifications at nucleotide backbone, bases and sugar[15]. Nucleobase modification can regulate the strength and specificity of base-pairing, and phosphodiester backbone modification can improve pharmacokinetic properties and nuclease resistance. Sugar moiety modification has beneficial effects on various properties of natural nucleic acid, including duplex-forming ability, nuclease resistance, and toxicity in cells and animals[12]. Xeno nucleic acids(XNAs) first appeared in 2009 in a theoretical paper on nucleic acid polymers[16]. It refers to the nucleic acids that differ from deoxyribose and ribose in chemical backbone motif. By comparison with natural DNA and RNA, XNAs possess desirable biological properties for biomedical application, such as increased biological stability, enhanced binding affinity, improved tissue specificity, and reduced immune responses[12,17]. In recent years, numerous works have used XNA to improve the performance of FNAs, and functional xeno nucleic acids have been applied to varied biomedical fields. In this review, we will focus on the design concept and biomedical application of functional xeno nucleic acids in the past two decades.

2 Brief Introduction of XNAs

XNAs refer to a class of nucleic acid analogues with sugar moiety modification(Fig.1)[15], which significantly enlarged the functions of nucleic acids in biotechnology and nanomedicine fields by altering the inherent properties of natural DNA/RNA[18]. For example, a kind of RNA that adds a methyl group in the 2′-hydroxy of native ribosel is named 2′-*O*-methyl(2′-OMe) RNA. 2′-*O*-Methylation nucleosides and CH_3I can synthesize the 2′-OMe ribonucleosides of A, T and C in the presence of Ag_2O, and 2′,3′-*cis*-diol and diazomethane can synthesize the 2′-OMe ribonucleosides of G. Then, these 2′-OMe ribonucleosides can be converted to the protected 2′-OMe ribonucleoside-3′-phosphates for synthesizing 2′-OMe RNA[19]. The 2′-deoxy-2′-fluoro(2′-F) RNA refers to the modified nucleic acid that 2′-hydroxyl on the sugar is replaced by fluorine. There are different groups that have reported the methods to synthesize 2′-fluoroadenosine(2′-F-A), 2′-fluorouridine(2′-F-U), 2′-fluorocytidine(2′-F-C) and 2′-fluoroguanosine(2′-F-G)[20]. And, Eckstein *et al.*[21] realized the successful introduction of 2′-F-U, 2′-F-C, and 2′-F-G into the hammerhead ribozyme. 2′-Deoxy-2′-fluoroarabinonucleic acid

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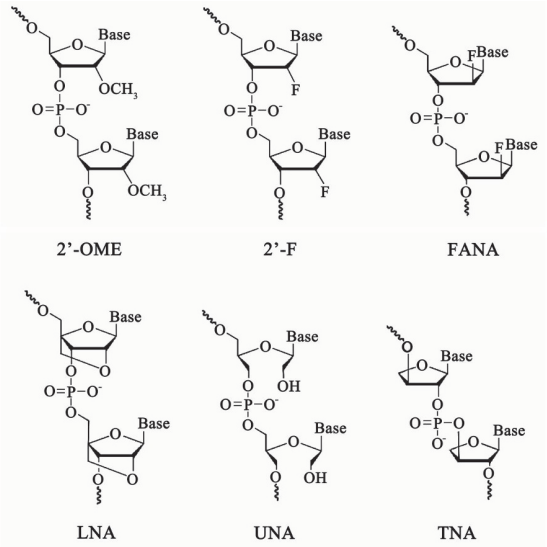

Fig.1 Selected examples of XNA: 2′-*O*-methyl(2′-OMe) RNA, 2′-deoxy-2′-fluoro(2′-F) RNA, 2′-deoxy-2′-fluoroarabinonucleic acid(FANA), locked nucleic acid(LNA), unlocked nucleic acid(UNA), and threose nucleic acid(TNA)

(FANA) is an RNA analogue, in which the ribose ring has been replaced by a 2′-fluoroarabinose moiety. The sugar adopts a C2′/O4′-endo conformation much like DNA, in stark contrast to the C3′-endo conformation seen by both F-RNA and RNA. Until now, several mature synthetic methods of FANA have been reported by different laboratories, and put into commercial production[22]. Bridged nucleic acids(BNA) or locked nucleic acids(LNA) are a type of RNA, in which a methylene bridge is added between the 2′-oxygen and 4′-carbon on ribose sugar and locks in the C3′-endo(RNA-like) conformation. There are two methods to synthesize LNA monomers: linear or convergent strategy[23,24]. Then through a commercial DNA synthesizer, LNA phosphoramidites can be easily inserted into oligonucleotides with standard reagents and coupling protocols. Unlocked nucleic acid(UNA) is synthesized with O5′-dimethoxytrityl protected ribonucleosides as the starting material. The C2′—C3′ bond is cleaved by treatment with sodium periodate followed by reduction with sodium borohydride, followed by selective 2′-hydroxy benzoylation and standard O3′-phosphitylation. Then, UNA nucleotides can be incorporated into either DNA or RNA oligonucleotides using standard automated synthesis procedures and UNA phosphoramidites[25]. (3′,2′)-*α*-*L*-Threose nucleic acid, also called as threose nucleic acid(TNA), is an analogue of RNA, in which the natural ribose sugar is replaced by an unnatural threose sugar. The phosphoric acid group is connected with the carbon at the 2′ and 3′ positions of the threose ring by phosphodiester linkages to form the five-atom backbone repeating unit of TNA[26,27]. Until now, TNA nucleosides, phosphoramidites, triphosphates and analogues can be robustly and safely synthesized according to previous reports[28,29], and TNA oligonucleotides can be easily synthesized using Kod-RI polymerases[30,31].

3 Biomedical Application of Functional Xeno Nucleic Acids

In the last two decades, extensive efforts have been made to developing biomedical applications of functional xeno nucleic acids and resulted in many significant and exciting publications. In this section, we summarize the biological application of several functional xeno nucleic acids, including XNA-based aptamers, XNA-based enzymes(XNAzymes) and XNA-based MBs.

3.1 XNA-based Aptamers

3.1.1 Design of XNA-based Aptamers

Aptamers refer to a type of single stranded nucleic acid molecules screened by systematic evolution of ligands by exponential enrichment(SELEX) technology that can bind to targets with high selectivity and affinity. Compared with protein-based antibody, the *in-vitro* selected aptamers possess many incomparable merits, including conformational flexibility, molecular stability, target affinity, and binding specificity[32]. Since their development, aptamers have gradually become an ideal class of molecular recognition probes. It has been widely applied in environmental detection and biosensing fields. However, due to the low nuclease resistance of natural DNA/RNA aptamers, its application in the biomedical field is still seriously limited. One effective approach to this problem is to replace the traditional nucleic acid substrates with XNAs to enhance the resistance to nucleases and escape the detection of immune system. As shown in Fig.2, two general strategies have been developed to synthesize XNAs-based aptamers, including post-SELEX and SELEX[33]. In the post-SELEX method, XNAs are introduced into aptamers selected by SELEX technique to obtain the XNA-based aptamers. It is post-SELEX method that developed the first aptamer drug(Macugen®, pegaptanib sodium injection) for age-related macular degeneration therapy[34]. In addition, there is a key point in the preparation of XNA-based aptamers by post-SELEX method that chooses the appropriate decoration positions so as not to decrease activity. The other SELEX strategy is to use the XNA-containing libraries to obtain the XNA-based aptamers. For example, Pastor *et al*.[35] constructed a 2′F-pyrimidines modified oligonucleotide library to select CD40-RNA aptamers. In this method, the XNA-based aptamers can be obtained directly from the library on the existence of polymerase mutants that recognize XNAs.

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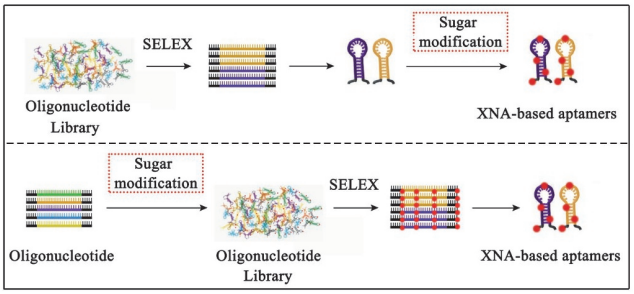

Fig.2 Schematic illustration of the post-SELEX and SELEX methods for developing XNAs-based aptamers

So far, XNA-based aptamers are capable of targeting hen-egg lysozyme, human thrombin, human vascular endothelial growth factor[36], human immunodeficiency virus(HIV)-reverse transcriptase and HIV *trans*-activating response(TAR) RNA element[37−39], human neutrophil elastase[40] and coronavirus SARS-CoV-2[41], etc.

3.1.2 Biomedical Application of XNA-based Aptamers

Recently, XNA-based aptamers showed massive potential in various biomedical applications. Until now, multiple species of XNA-based aptamers have been selected for drug delivery and cancer therapy. Fattal *et al*.[42] reported a 2′-F-modified RNA aptamer for binding CD44 protein and CD44-expressing cells, which exhibited high specificity and affinity. And it was surprisingly found that the conjugation of PEGylated liposomes to anti-CD44 aptamers could further improve the binding affinity[43]. Subsequently, basing on liposomes conjugated anti-CD44 aptamers, an siRNA delivery nanoplatform was constructed[Fig.3(A)][44]. In CD44 high-expressed triple-negative breast cancer cells, it could efficiently target CD44 protein to realize robust gene silencing both *in vitro* and *in vivo*. Another XNA-based aptamer, named apt69.T, is a 2′-fluoro-pyrimidine modified aptamer that selected by Franciscis group[45] to bind B cell maturation antigen-expressing myeloma cells[Fig.3(B)]. It could not only inhibit the nuclear factor kB pathway of APRIL-dependent *in vitro*, but also demonstrate high potential against tumor cells by conjugating to anti-mir-222 and mir-137. Therefore, apt69.T could act as a powerful tool for direct targeting and delivery of therapeutics to B cell maturation antigen expressing myeloma cells.

Another example of an *in vitro* selected XNA aptamer is a TNA aptamer reported by Yu *et al*.[46], which could bind PD-L1 protein with nanomolar affinities to effectively inhibit the interaction of PD-1 and PD-L1 *in vitro*. After the injection of this TNA aptamer into the colon cancer xenograft mouse model, it was gradually accumulated at the tumour site and remarkably inhibited the growth of tumour *in vivo*. Recently, a

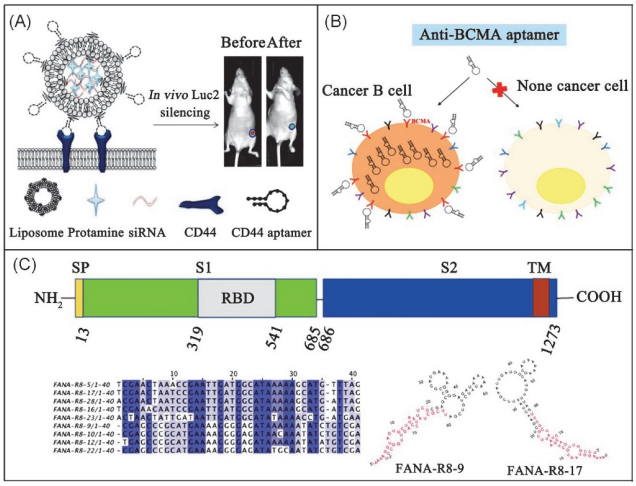

Fig.3 2′-F-modified RNA aptamer-based nanoplatform for gene silencing(A), model of Apt69 aptamer binding B cell maturation antigen-expressing myeloma cells(B) and FANA aptamer binding the SARS-CoV-2 S protein receptor-binding domain to block ACE2 binding(C)

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FANA aptamer that bound the SARS-CoV-2 S protein receptor-binding domain(RBD) was selected by DeStefano group[41], which could effectively inhibit the interactions of RBD and ACE2 receptor[Fig.3(C)]. The inhibition efficiency was well matched to neutralizing antibodies in binding RBD. Overall, this work established a universal principle to discover aptamers as virus inhibitors.

3.2 XNAzymes

3.2.1 Design of XNAzymes

Nucleic acid enzymes(NAzymes) play an important role in promoting sequence-specific RNA cleavage[47,48]. So far, both ribozymes and deoxyribozymes(RNAzymes and DNAzymes) with high catalytic activity have been produced except the general natural RNAs catalytic motifs, such as group I intron, hairpin and hammerhead. These RNAzymes and DNAzymes perform catalytic reactions with great precision due to the flexible binding and discrimination of nucleic acid substrates *via* Watson-Crick-based interactions. Unfortunately, the biological application of nucleic acid enzymes is seriously limited by the intrinsic nucleic acid stability *in vitro*, which are easily digested by nuclease. Here, XNAzymes could overcome this problem by enhancing nuclease resistance and enabling chemical reactions not accessible to their natural counterparts. XNAzymes refer to a type of DNAzymes/ribozymes containing XNAs, which could function as a nuclease, or a ligase in degradation reactions[49]. Holliger and colleagues[50] reported the original four XNAzymes(FANAzyme, ANAzyme,

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HNAzyme, and CeNAzyme) that derived from XNAs with four different backbone chemistries. XNAzymes are divided into three types according to their different functions, in which XNA ligase XNAzymes have the XNA-XNA ligase activity, RNA ligase XNAzymes possess the RNA-RNA ligase activity, and RNA endonuclease XNAzymes are capable of cleaving RNA substrates. These facts clearly exhibit that XNAzymes, like DNAzymes and RNAzymes, possess the capacity to catalyze chemical reactions on oligonucleotide substrates[12].

3.2.2 Biomedical Application of XNAzymes

Although XNAzymes obtained by *in vitro* selection demonstrated certain catalytic activity, it remained unknown if XNAzymes could take work in the biomedical application. Many attempts had been made to get a definite answer for this question. Yu *et al*.[51] designed a TNA catalyst named Tz1[Fig.4(A)], which exhibited strong sequence selectivity towards a mutant epidermal growth factor receptor(EGFR) messenger RNA associated with drug resistance. It showed a strong application potential in selectively silencing the expression of mutant EGFR gene in non-small cell lung cancer cell lines. This report demonstrated that TNA could serve as a potential RNA cell competitor or progenitor. In addition, TZ1 could selectively knockdown intracellular RNA, which also showed that functional threose nucleic acids could be further applied to the future biomedical field.

XNAzymes have made rapid progress as their excellent performance in the biomedical application. In particular, XNAzyme 10-23, a new version of the DNAzyme 10-23 with XNA-modified nucleotide, with robust intracellular RNA cleavage capabilities make it an attractive candidate as a therapeutic gene silencing reagent. One of the XNAzyme 10-23 reported by Spitale and colleagues[52] could selectively target a type of mutation, which related to a deleterious glycine-to-valine substitution in the twelfth amino acid(G12V) of the Kirsten rat sarcoma viral oncogene homologue(KRAS) [Fig.4(B)]. Experimental results showed that XNAzyme 10-23 could selectively knockdown G12V gene and achieved more than 50% reduction of G12V transcripts, while the wild-type transcripts remained minimal off-targeting in adenocarcinoma cells line containing both G12Ve and wild-type KRAS alleles. As a result, the expression of phosphorylated MEK, a key downstream effector of the oncogenic KRAS signaling pathway, was significantly inhibited. In addition, XNAzymes are also an appropriate candidate for point-of-care diagnostic testing in nucleic acid detection. Here, combining XNAzyme 10-23 with an analyte preamplification strategy, Chaput *et al*.[53] constructed a multicomponent XNA-based nucleic acid detection platform, which could realize the highly sensitive

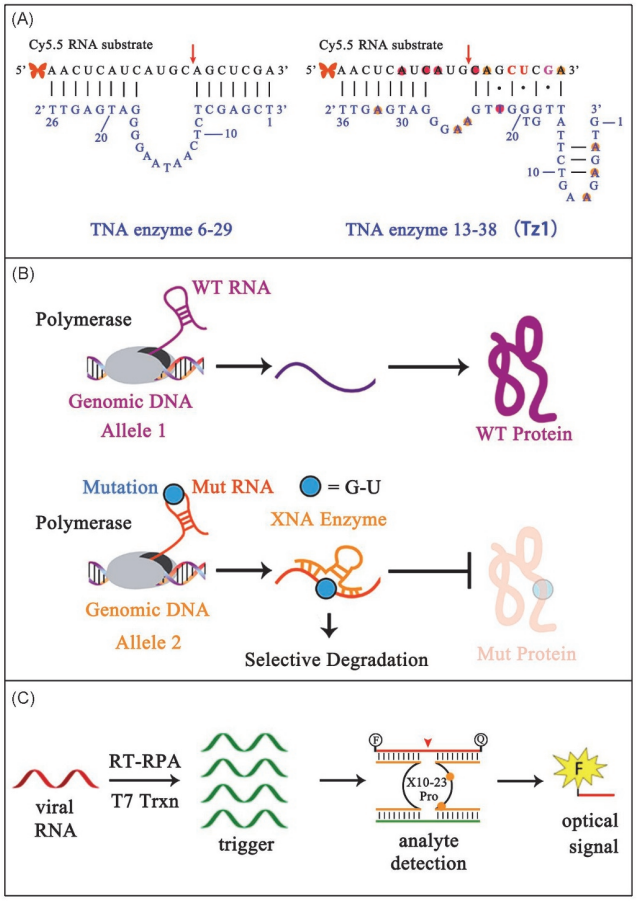

Fig.4 TNA enzymes catalyzing RNA cleavage(A), XNAzyme 10-23 targeting mutations in KRAS(B) and XNAzyme-mediated nucleic acid detection(C)

(B) Reprinted with permission from Ref.[52], Copyright 2021, American Chemical Society; (C) reprinted with permission from Ref.[53], Copyright 2021, American Chemical Society.

detection of the viral pathogen responsible for COVID-19 [Fig.4(C)]. This XNA-based nucleic acid platform could balance the RNA substrates binding with products release *via* finely adjusted XNA chemotypes, thus enhancing gene silencing activity in the cellular environments. As the parent 10-23 DNAzyme originally described by Mokany and coworkers[54], XNAzyme 10-23 conversed into a split enzyme could generate a multicomponent optical sensor, which was capable of producing an output signal according to the existence of the input target sequences. This RNA-encoded viral nucleic acid analyte reporter(REVEALR) platform achieved ultrasensitive detection of the target with a low detection limit(*ca.* 10 copies/μL) *via* the traditional paper-based lateral flow and fluorescence readout modalities.

3.3 XNA-based MBs

3.3.1 Design of XNA-based MBs

MBs refer to the oligonucleotides labeled on both ends. They

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have a stable stem-loop structure, in which the quenching group is adjacent to the reporting dye(chromophore). In the absence of a target DNA/RNA sequence, the fluorescence of chromophore is quenched *via* transferring energy to a proximate quencher[55−57]. While, after hybridization with targets, the fluorophores are spatially separated through the MBs conformational changes, resulting in fluorescence generation[58]. MBs exhibit the outstanding sensitivity and selectivity in the biomedical field due to their unique stem-loop structure thermodynamic stability, fluorophore-quencher pair diversity and efficient inherent signal conversion capacity[59,60]. However, native MBs show the poor target binding affinity and nuclease degradation ability[61]. As a solution, XNAs-based artificial MBs can be selected as a suitable candidate to enhance the target binding affinity and nuclease degradation ability[62,63].

XNA-based MBs usually have three nucleic acid recognizing modes. In the first mode, the sequence recognition element-XNAs are connected to the fluorescent dye(reporter group) to form the light-up probes. These probes possess very low fluorescence in the solution on account of the reporter dye intrinsic properties, while in the presence of the target, XNA combines with the target to induce the interaction between the nucleic bases and dye, causing the light-up probes to fluoresce brightly[Fig.5(A)]. The second XNA-based MBs are a type of oligonucleotide with a stem-loop structure and doubly end-labeled fluorophores. Specifically, MBs possess two complementary stem sequences, in which one end is labeled with a fluorescent reporter dye and the other one is labeled with a fluorescent quencher molecule. In this stem-loop conformation, the reporter fluorophore of MBs was effectively quenched. In the presence of the target, the stem-loop hairpin structure of XNAs is opened, which separates the reporter dye and the quencher molecule and results in produced fluorescence[Fig.5(B)]. The third MBs for nucleic acid detection are based on fluorescence resonance energy transfer(FRET). FRET refers to the process that the energy of an excited dye donor transfers to a ground-state dye acceptor through a nonradioactive process of long-range dipole-dipole interactions. Generally, FRET-based MBs are consisted of an acceptor dye, a donor dye and a complementary XNA sequence toward the target nucleic acid. In the absence of the target, the MBs show weak fluorescence under the excitation of donor absorption due to the separated acceptor and donor. However, in the presence of the target, the MBs hybridize with the target nucleic acid sequence, resulting in the change of MB conformation, shortening the distance between the receptor and the donor, and producing fluorescence[Fig.5(C)].

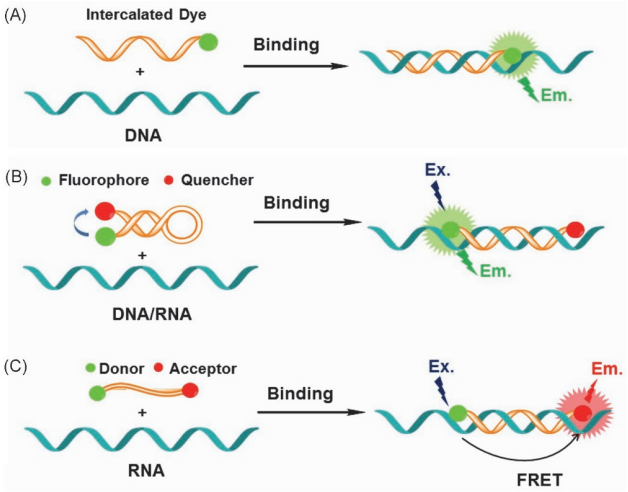

Fig.5 Schematic diagram of three recognizing modes of XNAs-based MBs

(A) The intercalated dye of probe can fold back and intercalate between the formed Watson-Crick base pairs or serve as a base surrogate that is forced to intercalate adjacent to the expected mutation site; (B) upon hybridization with a complementary sequence, the stem-loop hairpin structure of MB opens, which separates the reporter dye and the quencher and results in an increased fluorescence intensity; (C) in the single-stranded state, the donor and the acceptor are separated from each other. When the probe encounters a target RNA, the MB undergoes a spontaneous conformational reorganization that forces the stem together, leading to a FRET signal change. Reprinted with permission from Ref.[62], Copyright 2013, Theranostics.

3.3.2 Biomedical Application of XNA-based MBs

At present, even if MB fluorescent sensing has made significant progress in cancer diagnosis and therapy, it cannot detect the cancer-relevant targets due to poor stability *in vivo*. The XNAs-based MBs have enhanced nuclease resistance, which can perfectly avoid this problem. Combining LNA-MBs and LNA modified primers, Tallini *et al*.[64] developed a real time RT-PCR technology for the monitoring of hepatitis C virus(HCV) viral load in plasma and serum samples. It not only did not have the HCV genome heterogeneity, but also cloud use different beacon probes and short primers to amplify in the same reaction tube for internal RNA control. Because the short-length LNA primers could not form hairpin loop structures, their PCR efficiency was up to almost 100%. This detection platform achieved a high sensitivity of 50 IU/mL. In addition, this platform could be used as a useful means to monitor disease progression by screening a large number of samples and measuring viral loads due to its high specificity and robustness in a wide range of genotype detection. Subsequently, a variety of LNA-MBs-based biosensors with high sensitivity and specificity have been developed. For example, Mak *et al*.[65] reported a single-probe strip test for the rapid and sensitive detection of miRNA-21 mimics[Fig.6(A)]. This design combined the elements of thiol and biotin, DNA/LNA oligonucleotide probes(molecular beacons-MB), and gold nanoparticles(AuNPs), which was able to regulate the accessibility of the biotin group to specifically trigger the interaction of the boilable with the streptavidin test zone only in the presence of the miRNA-21 mimics. The response of the

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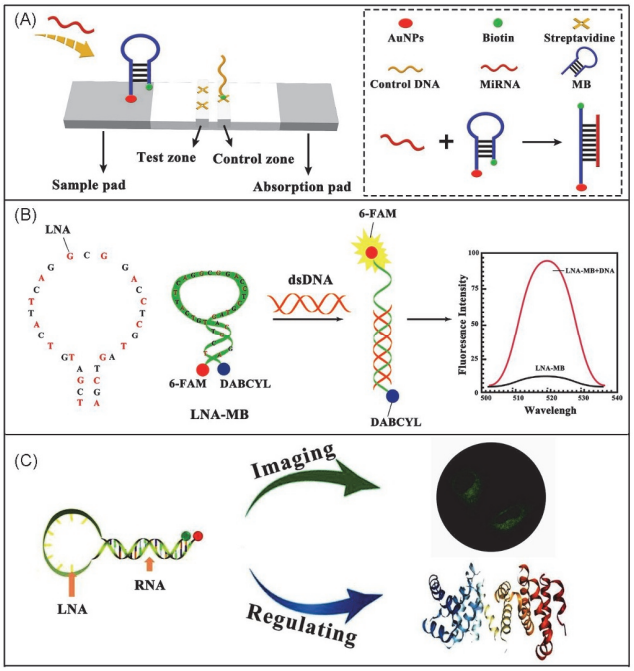

Fig.6 Schematic diagram of miRNAs quantitative analysis(A), structure of LNA-MBs and schematic illustration of DNA detection with LNA-MBs(B) and LNA-MBs for miRNA imaging and regulating(C)

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strip test showed a wide linear range(0.5—20 nmol/L) and a low detection limit(115 pmol/L). Importantly, the proposed strip test demonstrated the potential for point-of-care clinical applications. In addition, based on novel LNA MBs, Ju *et al*.[66] developed a simple method to detect the ring finger domains1(UHRF1) DNA and ubiquitin-like proteins extracted from breast cancer serum samples[Fig.6(B)]. These LNA-MBs were consisted of a 4-mer stem with DNA/LNA alternating bases and a 27-mer loop, which significantly enhanced their detection stability and efficiency in biofluids. Meanwhile, the method displayed the high sensitivity and selectivity to UHRF1 DNA, the dynamic range was 3 orders of magnitude, and the detection limit was as low as 11 nmol/L(S/N=3). Very recently, new progress has been made in the development of molecular beacons to realize the imaging the cancer-relevant targets *in situ*. Xu *et al*.[67] developed novel versatile LNA-MBs for disturbing dicer-mediated cleavage process and intracellular precursor miRNAs(pre-miRNAs) imaging [Fig.6(C)]. These smart LNA-MBs conducted targeted recognition reaction with pre-miRNA to promote the probe conformational changes, block the dicer cleavage site, inhibit the cleavage process, and then achieve the miRNA expression down-regulation. Simultaneously, the FRET between the fluorophores of doubly end-labeled in LNA-MBs was broken by the target recognition reaction, which further induced the relevant fluorescence signals changes, realizing the *in situ* imaging analysis of pre-miRNA and inhibition events.

4 Conclusions and Perspectives

Nucleic acid biomedical research has penetrated into all aspects, including the development of biological tools, diagnostic methods and unique drugs. Compared to natural nucleic acids, XNAs exhibit excellent advantages, such as stability, biocompatibility, biodistribution, and immunity, and thus XNAs as an upgraded version of nucleic acids have been synthesized and applied in varied biomedical fields over recent years. The great advances in XNA molecular and biomedicine technologies have paved the way for functional xeno nucleic acids.

However, there are still some challenges that should be solved before functional xeno nucleic acids becoming an available research tool, such as optimization of delivery, selectivity, reproducibility, and long-term safety. First, it is a urgent issue to test the candidates of functional xeno nucleic acids and take every appropriate control for achieving their successful biomedical application. Second, because the current biomedical researches depend on a vast range of dosage functional xeno nucleic acids, it is urgent to design an excellent delivery system to realize their efficient uptake in cells(*i.e.*, maximizing efficacy while minimizing dosage). Third, the precise and large-scale manufacture of long XNAs is still a difficult issue at present, although short- to medium-length XNAs are available in most biomedical fields. Fourth, the combination of functional xeno nucleic acids and other molecular technologies is also a mainstream direction of future development. For example, CRISPR/Cas systems, which are powerful tools with a series of advantages, have been applied to many new technologies of epigenetic regulation, transcriptional activation/repression, and genome editing. Unfortunately, several problems including the low efficiency of gene disruption, *in vivo* instability and drug carriers in the field of drug delivery systems(DDS) technology limit their practical application seriously. Introducing functional xeno nucleic acids at appropriate positions of guide RNA is a potential way to improve the inherent properties of CRISPR system.

Briefly, future research should focus on the strategies to develop high performance functional xeno nucleic acids with lower cost, higher yield and higher fidelity to meet the emerging biomedical requirements, such as bioanalysis and personalized medicine.

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Conflicts of Interest

The authors declare no conflicts of interest.

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