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Peptide nucleic acid (PNA) and its applications in chemical biology, diagnostics, and therapeutics

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Peptide nucleic acid (PNA) stands as one of the most successful artificial oligonucleotide mimetics. Salient features include the stability of hybridization complexes (either as duplexes or triplexes), metabolic stability, and ease of chemical modifications. These features have enabled important applications such as antisense agents, gene editing, nucleic acid sensing and as a platform to program the assembly of PNA-tagged molecules. Here, we review recent advances in these areas.

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Introduction

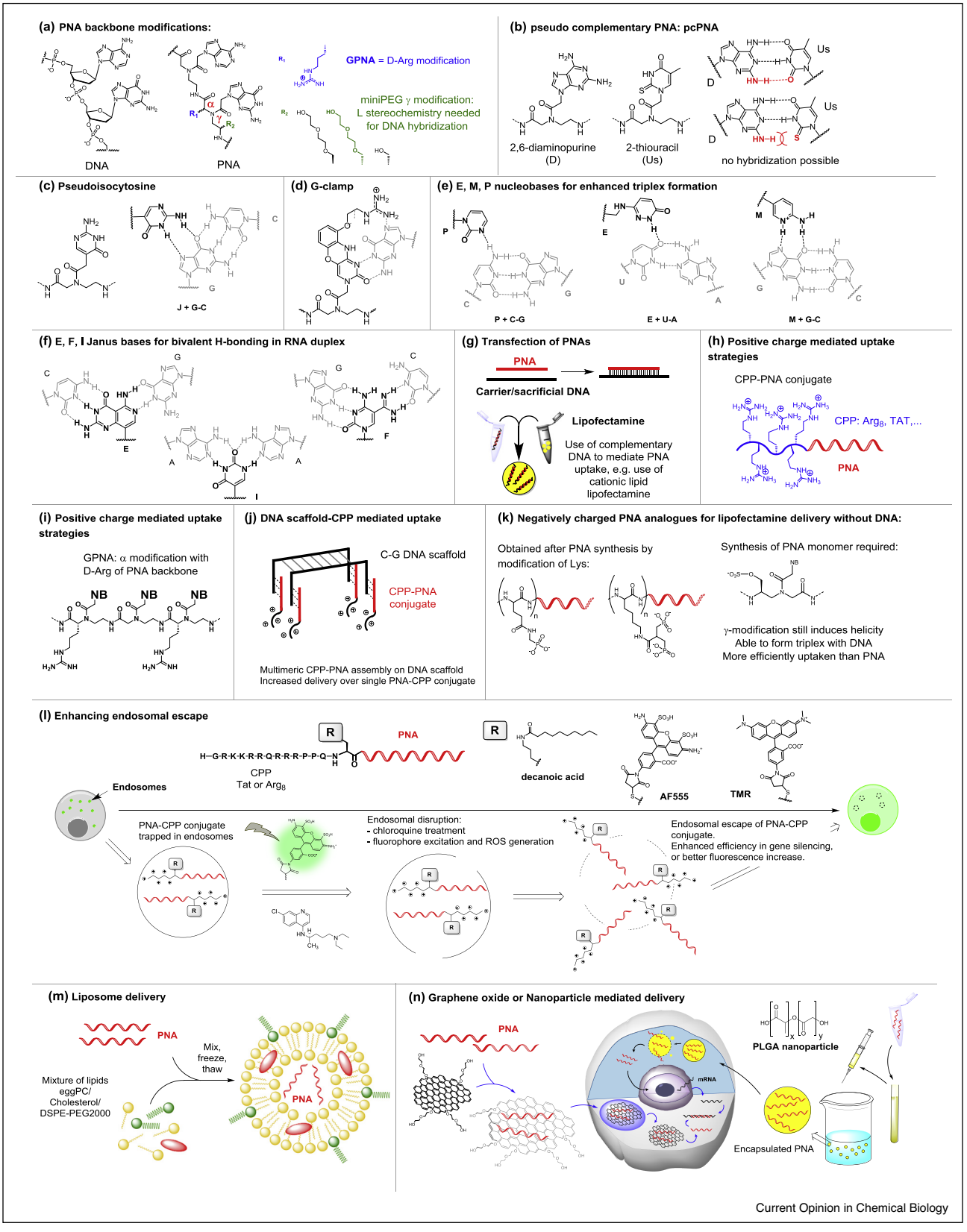
Peptide nucleic acid (PNA) is an artificial oligonucleotide mimetic with a peptidic backbone in lieu of a phosphoribosyl backbone. As such, it combines the properties of both peptides and nucleic acids [1–3]. Remarkably, it forms more stable duplexes with DNA or RNA than either of the DNA or RNA homoduplexes and is metabolically stable. These advantageous features instigated tremendous interest in PNAs as antisense and antigene agents. However, unmodified PNAs have poor cellular permeability, which has curtailed early efforts in this direction. Since its original report, numerous modifications to the original backbone and nucleobases have been investigated as a means to improve its physicochemical properties, cellular permeability, duplex stability and to conjugate ligands or other functional molecules for diverse applications. While significant parallel advances have also been made with other nucleic acid platforms such as morpholinos and LNAs, PNAs remain very attractive for their ability to hybridize with high affinity and sequence specificity to target sequences [4]. These features have enabled a number of applications beyond antisense and antigene properties, including nucleic acid sensing, templated ligand display and gene editing.

Modifications

The neutral backbone of PNA entails that hybridization interactions (hydrogen bonding and base stacking) are not partially offset by repulsive electrostatic interactions as is the case with phosphoribosyl backbones. This feature allows more versatile conditions for hybridization and facilitates their strand invasion into dsDNA. However, the flipside is poorer aqueous solubility and a tendency to aggregate. The peptidic nature of the PNA backbone lends itself to modifications and a large array of substituents have been explored, [5] in addition to conformationally constrained PNA [6]. Substituents at the alpha (α)-position are well tolerated; however, enhanced hybridization properties are achieved with gamma (γ)-substituents (Figure 1a) [7••]. Chirality at the γ-position induces a helicity that is beneficial to DNA and RNA hybridization for the *L*-stereoisomer while precluding hybridization to DNA and RNA in the *D*-stereochemistry. Nonetheless, *D*-γ-PNA can still hybridize with achiral PNA or *D*-γ-PNA, providing an orthogonal dimension to program hybridizations and assemblies in a biological context [8•]. The utility of this hybridization orthogonality has recently been harnessed in PNA-based circuit [9•,10].

Modifications at the γ-position have been used to fine-tune physicochemical properties (e.g. mini-PEGs) [11•] and to conjugate ligands [12]. The versatility of modifications at the γ-position of PNA addresses many of the unmodified PNA backbone's shortcomings. In parallel, significant progress has also been achieved with designed nucleobases. An elegant example of this is the development of pseudo-complementary PNA in which 2,6-diaminopurine (D) and 2-thiouracil (Us) are used as surrogates for A and T, respectively, which can still hybridize a target sequence but cannot self-complement (Figure 1b) [13–15]. Similarly, pseudoisocytosine (J) has been used to favor triplex formation at neutral pH (Figure 1c) [16•]. This technology facilitates the design of PNAs that form stable invasion complexes in genomic DNA. Nucleobases have also been designed to enhance hybridization stability. A prominent example is the G-clamp, which significantly enhances a G-C base pairing (Figure 1d) [17]. More recently, several nucleobases have also been developed to favor triplex formation with

Figure 1



Structure of PNA and representative substituent and novel nucleobases; technologies for cellular delivery.

dsRNA, notably, 2-aminopyridine (M), 2-pyrimidinone (P) and 3-oxo-2,3-dihydropyridazine (E) that extend the design of triplexes to any dsRNA sequence (Figure 1e) [18,19•]. Another recent advance in nucleobase design is the design of Janus bases capable of bifacial hybridization for targeting dsDNA or dsRNA under physiological conditions (Figure 1f), [20,21] thereby providing an inroad for the design of PNAs that target the secondary and tertiary structures of nucleic acid biopolymers.

Cellular delivery

The challenges associated with the cellular permeability of higher-molecular-weight molecules are well recognized and most PNA oligomers used have a molecular weight (3–6 KDa), above the threshold of straight diffusion across a cellular membrane. Despite their charge neutrality and overall lipophilic character, unmodified PNA oligomers are essentially not taken up by cells *in vitro* and are rapidly cleared through the kidneys when administered *in vivo* [22,23]. For cellular uptake to occur, PNA oligomers must be conjugated, formulated, or contain some modifications in the backbone to facilitate membrane association/endocytosis. An early and effective solution for cellular delivery is to formulate the PNA as a duplex with sacrificial DNA and capitalize on DNA transfection technologies (Figure 1g) [24,25•]. Another general strategy is leveraged on cell-penetrating peptide (CPP) technologies. Conjugation of PNA oligomers to CPPs has been shown to enhance their cellular delivery (Figure 1h) [26]. The success of oligoarginines as a CPP tag inspired the development of guanidine-based peptide nucleic acids (GPNAs) wherein the glycine residue in the PNA backbone is replaced by a *D*-arginine residue (Figure 1i) [27]. GPNAs have been shown to be taken up both in cell culture [28,29] and *in vivo* [30•]. Interestingly, a head-to-head comparison of GPNA versus PNA conjugated to an oligoarginine tag demonstrated superior uptake for the GPNA [29]. However, endosomal trapping still limits the efficacy of delivery. More recently, a strategy that combines sacrificial complementary oligonucleotide sequences with CPP–PNA conjugates was reported [31]. A self-assembled oligonucleotide scaffold that includes a central complementary region for self-assembly and lateral regions complementing the PNA–CPP conjugates was shown to greatly enhance uptake (Figure 1j). This design enabled the incorporation of endosomolytic peptide-PNA conjugates that facilitate endosomal escape. Conversely, PNA–DNA hybridization has also been used to facilitate DNA cellular uptake without recourse to transfection agents. Specifically, a PNA beacon has been designed with a well-known CPP (TAT peptide) flanked by two short complementary PNAs in order to form a stable hairpin with a PNA overhang on one side. The overhang has been used for hybridization to cargo DNA and shown to confer cellular uptake [32]. Notably, uptake of the TAT–PNA hairpin is increased by 10-fold compared to the linear TAT analog. In order to use well-established cationic lipid transfection agents such as lipofectamine but without recourse to sacrificial DNA, two methods have been reported to synthesize PNA oligomers that are negatively charged. The first method involves the addition of 4–6 lysine residues derivatized with a bis-phosphonate (Figure 1k), [33•] and can be performed in an automated fashion following PNA synthesis. The conjugates were found to have antisense activity (splice correction) at low nM concentrations, which is significantly lower than the concentrations required with CPP–PNA conjugates or DNA–PNA hybrids. These results highlight the fact that endosomal trapping restricts the potency of most cellular uptake technologies. The second method to obtain negatively charged PNA oligomers suitable for direct transfection relies on a γ-modified backbone with a serine side chain functionalized with a sulfate (Figure 1k) [34].

While microscopy experiments have clearly shown that cationic PNA such as GPNA or PNA-$(Arg)_x$ conjugates (and other CPP–PNA) are quite efficiently taken up by cells through endocytosis, the PNAs remain trapped in the endosome to a large extent. It was recently demonstrated that conjugation of such PNAs to sensitizers of reactive oxygen species (ROS) greatly enhanced endosomal escape by compromising the endosomal membrane upon irradiation / ROS formation [35,36]. This endosomal escape was clearly visualized by relocation of a tetramethylrhodamine-PNA (TMR-PNA) after 10 min light irradiation. Similar enhancement can be achieved with chloroquine which promotes endosomal escape, albeit with some toxicity (Figure 1l).

The first example of a liposomal PNA formulation was recently reported to deliver unmodified PNA oligomers with unprecedented efficiency in cell culture [37]. Impressively, this formulation led to faster and higher cellular concentrations of PNA oligomers than PNA–Arg_8 conjugates. These results corroborate the fact that endosomal trapping limits cytosolic delivery of CPP–PNA conjugates. It is noteworthy that several liposomes are used in FDA approved drug formulations (Figure 1m).

An important technology that has been used in genome editing work is the use of nanoparticles (NPs) to simultaneously deliver PNA oligomers and donor DNA [38,39]. NPs prepared from a biodegradable polymer (poly(lactic-co-glycolic acid [PLGA]) and loaded with both oligomers have been shown to be effective in clinically relevant target cells such as hematopoietic stem and progenitor cells. This technology is also effective for *in vivo* delivery to hematopoietic cells using intravenous injection of NPs, [40,41••] and *in utero* delivery with intra-amniotic administration. NP formulations have also been used to deliver antisense PNA oligomers targeting microRNA (miR) [42••,43].

Graphene oxide (GO) binds single-strand nucleic acids, including PNA, and dissociates upon duplex formation. As such, it could be used as a delivery vehicle; however, its low dispersibility in cell culture media makes it unsuitable. Recently, it has been shown that nanosized GO modified with PEGs overcome this limitation and enables the delivery of antisense PNA (Figure 1n) [44].

Beyond CPPs, other conjugates for active transports have also been investigated. A recent addition to this arsenal is the conjugation to vitamin B12 which was shown to confer uptake in gram negative bacteria and could be used to deliver antisense PNA with higher efficacy than a control CPP–PNA conjugate [45]. The use of well-defined DNA nanostructures (DNA tetrahedron) has been investigated as a vector for drug delivery. Hybridization to one of the edges of the DNA tetrahedron was used to promote the uptake of antisense PNA into bacteria and shown to inhibit gene expression in methicillin-resistant *Staphylococcus aureus* [46].

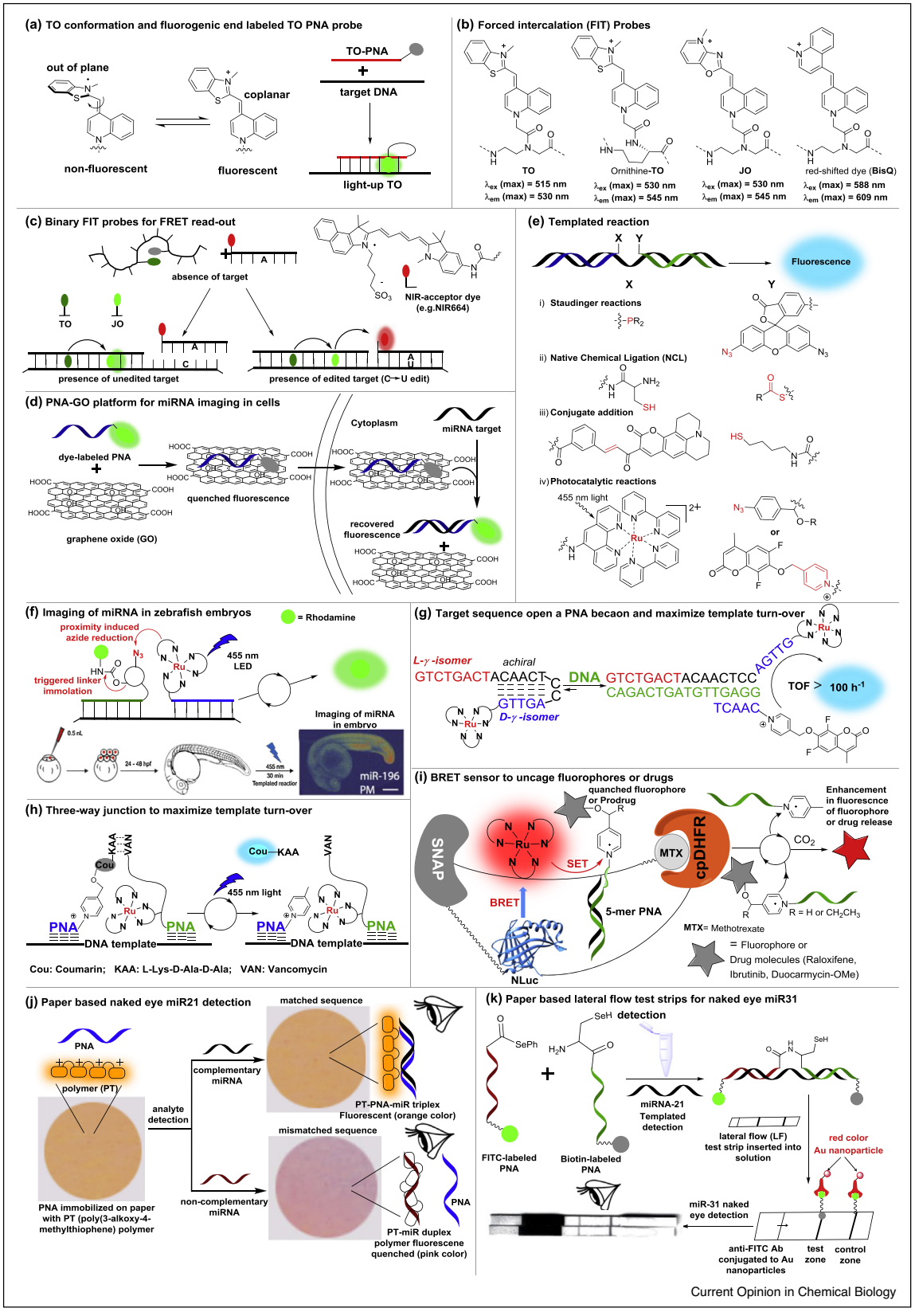
Nucleic acid sensing

Detection of specific nucleic acid sequences is critical in biomedical research and diagnosis. The unique hybridization properties and metabolic stability of PNA make it ideal for sensing in complex biological environments or even whole cells [47]. Two main strategies have been pursued for live cell imaging: fluorogenic probes that increase in fluorescence upon duplex formation and templated reactions in which a target sequence catalyzes the formation of a fluorescent product. For the fluorogenic probes, a very successful family of dyes is thiazole orange (TO) and its analogs (Figure 2a,b). An important property of these dyes is their fluorescence increase when planarized. Initial proof of principle was demonstrated with TO appended at the end of a PNA (Figure 2a) [48,49]. Subsequent studies have found heightened performance with TO inserted within the PNA sequence and acting as a base surrogate (Figure 2b) [50•]. Upon hybridization, a forced intercalation of the dye increases its planarity and fluorescence (FIT probes) [51]. FIT–PNA probes were found to be particularly sensitive to single base pair mismatch adjacent to the TO residue. Linking the TO through an ornithine backbone rather than through the aminoethylglycine of the PNA backbone yielded further improvements (Figure 2b) [52]. Notably, a comparison between a traditional DNA-molecular beacon and FIT–PNA for the detection of a KRAS oncogenic mutation (SNP) showed that the FIT probe performed better *in vitro* and *in cellulo* (PNA transfected with sacrificial complementary DNA) (Figure 2b) [53]. FIT–PNA probes with red-shifted dye (BisQ), optimal for detection in a cellular context, were effective in sensing SNP in live cells (Figure 2b) [54]. The utility of FIT–PNA probes has also been extended to the detection of triplex. As in the case of duplexes, TO functions as a universal base in a triplex and oligomers containing TO can report on triplex formation with single base pair discrimination [55,56]. A further refinement of FIT–PNA made use of binary probes for a FRET read-out (Figure 2c) [57•]. The use of binary probes allows the use of shorter oligomers with better mismatch discrimination while retaining unique targeting of longer oligonucleotide stretches. The combined use of TO and JO resulted in a FIT–PNA probe capable of FRET with a NIR-acceptor dye on the adjacent probe, thus enabling the discrimination of a C → U edit in an mRNA of live cells. The binary probe system also enables a colorimetric discrimination between the presence of a transcript and whether it is edited based on the emission wavelength.

An alternative technology to FIT probes is to capitalize on GO, which indiscriminately adsorbs ssPNAs and efficiently quenches a broad spectrum of fluorophores (Figure 2d) [58]. Upon hybridization, the PNA–RNA is desorbed and fluorescence is restored. As previously discussed, GO also facilitates cellular uptake of PNA. The FIT probes make use of a dye that acts as a universal base. Effort to design nucleobase-dyes hybrids that are environmentally sensitive have also been reported [59–61]. Probes that have leveraged this strategy have also been combined with a GO adsorption/quenching approach for a sensitive detection of human telomeric repeats [62].

As previously discussed, binary probes are attractive in light of the higher sequence resolution achieved by shorter oligomers while maintaining the benefit of unique targeting through the combined sequences of the binary probes. Fluorogenic probes, however, can only produce stoichiometric output of fluorescence relatively to a target hybridization. Alternatively, binary probes have been used to bring reagents in proximity following a hybridization in order to promote a reaction yielding fluorescent products (Figure 2e) [63–65]. Several chemistries have been reported with PNA, including Staudinger reactions, [66] native chemical ligation (NCL), [67] conjugate addition [68] and ruthenium photocatalyzed reactions [69] with amplifications reaching >1000 fold and detection thresholds of 5 pM. Because dynamic hybridization is sufficient to catalyze the reaction, antisense effects can be avoided. By virtue of its biorthogonality and leveraging aforementioned cellular delivery techniques, the Staudinger chemistry was used to detect mRNA [28] and miRNA in live cells [70]. The use of photocatalysis in templated reactions provides temporal control over the readout. This special feature has been harnessed to visualize microRNAs in developing zebra fish embryos by injecting the probe at the one-cell stage, and irradiating the organism at 24 hour-post-fertilization to localize microRNAs of interest through the development of fluorescence at specific tissues (Figure 2f) [71••]. It is noteworthy that this chemistry has been performed in live vertebrates without morphologic signs of toxicity.

Figure 2



Nucleic acid sensing: environment-sensitive nucleobases and templated reactions.

Templated reactions have also been adapted to detect specifically dsRNAs, making use of novel nucleobase (M, P, E) designed for triplex formation [72].

The rate of signal amplification in templated reactions is ultimately limited by the exchange of reagents on the template. Two approaches have recently been reported to maximize template turn-over. In the first, the target sequence opens a PNA beacon thus revealing a template stretch optimized for fast turnover (Figure 2g) [9•]. The second approach makes use of a three-way junction between the binary probe to yield a cleavage reaction and a product with lower affinity to the template (Figure 2h) [73].

Alternatively, it has been shown that performing templated reactions with PNA probes embedded within permeable agarose and alginate hydrogels reduces background reactions and enables more sensitive detection of a target sequence, affording a detection of DNA sequences down to 100 pM [74]. Another strategy for increasing signal amplification is using a templated reaction to generate an active catalyst from catalytically inactive precursors [75]. Once formed, high signal amplification (up to 15 000 turnovers) is achieved through the conversion of a pro-fluorescent substrate present at a high concentration (to maximize signal amplification) without compromising specificity of the templated reaction.

Templated reactions have also been used to synthesize or uncage bioactive molecules in response to a given nucleic acid input [76–78]. This chemistry was recently harnessed to convert a sensor into a responder; the bioluminescence resonance energy transfer (BRET) of the sensor was used to photoexcite a ruthenium photocatalyst which in turn uncaged diverse drugs (Figure 2i) [79]. A templated process was found to be essential in the latter step to ensure the effectiveness of uncaging, by virtue of the high effective concentrations of templated reactions. It was shown that the sensor operated with 64 turnovers enabling drug concentration $>0.5\ \mu M$ with 10 nM of the sensor.

While the aforementioned fluorescence readout for nucleic acid sensing is highly sensitive and effective, it is not practical for application outside of the laboratory, such as for point-of-care diagnostics. Several technologies using PNA probes have been shown to deliver naked eye detection of relevant oligonucleotide analytes. One method makes use of a light-harvesting conducting polymer to enhance the signal of a fluorescent PNA probe [80]. A paper-based device leveraging this concept has been shown to enable naked-eye detection of microRNA-21, a cancer biomarker, through color changes (Figure 2j) [81]. Another method makes use of a templated reaction to ligate two PNA probes labeled with analytes that are compatible with commercial paper-based lateral flow test strips (Figure 2k), [82] and has been used to detect microRNAs (miR-21, miR-31) from crude cell extracts with a detection threshold of 100 pM.

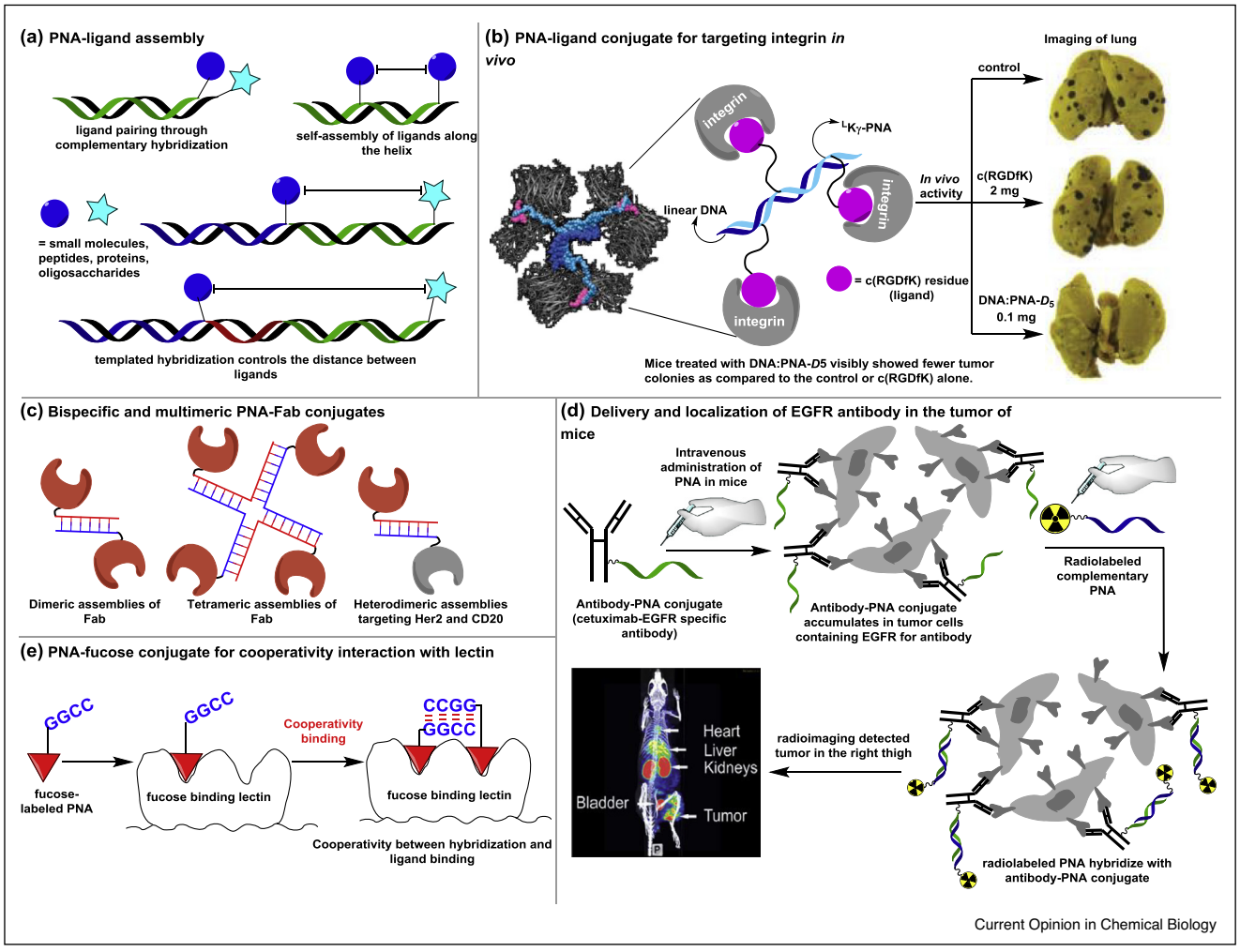
Supramolecular drugs

In the past decade, a number of studies have used PNA oligomer-ligand conjugates to program their self-assembly into larger structures through self-complementation or hybridization to a template (Figure 3). The geometry and distance between the ligands can be adjusted based on the hybridization instructions, providing a rapid assessment of the optimal solution for tight binding and efficacy (Figure 3a) [83–88]. Moreover, assemblies of PNA-ligand conjugates have been found to be functional *in vivo* (Figure 3b) [89••]. Specifically, a PNA-ligand conjugate that targets the $\alpha_v\beta_3$ integrin, a trimeric receptor overexpressed in many cancers, showed 100-fold enhanced binding when oligomerized, resulting in 50% reduction of tumor colonies in a mouse model. PNA-tagged macromolecules have been used to program the assembly of antibody fragments (Fab) in order to rapidly explore bispecific antibodies (Figure 3c) [90]. This methodology has been used to generate potent bispecific antibodies that recruit cytotoxic T lymphocytes to cells expressing a cancer marker (Her2 or CD20), as well as multimeric antibody fragments with enhanced activity. PNA-tagged antibodies have also been used to deliver SPECT tag. Cetuximab (EGFR-specific antibody) was tagged with a PNA and administered to mice; subsequently, the complementary strand with a radioactive tracer was injected, resulting in conjugation to the antibody through hybridization (Figure 3d) [91]. These previous studies were performed with PNA-based assemblies that form thermodynamically stable duplexes. Recently, it has been shown that transient hybridization of a short PNA oligomer can be sufficient to achieve cooperativity in dimeric ligand-protein interactions (Figure3e) [92]. Specifically, a fucose-binding lectin with adjacent binding sites was targeted by fucose conjugated to a short self-complementary PNA (4-mer). While the PNA oligomer was too short to form a stable duplex, its affinity for the lectin was enhanced by 100-fold relatively to the fucose ligand itself. This enhancement was comparable to a thermodynamically stable dimer and was also able to inhibit the lectin's interaction with epithelial cells. Beyond these applications, PNAs have also been used to tag small-molecule libraries, facilitating their preparation by traditional split-and-mix synthesis and assemblies in different combinations [93].

Antisense

The metabolic stability and strong binding affinity of PNA propelled this unnatural oligomer as a prime candidate for antisense and antigene therapy. Nearly two decades of research in the area has shown that, while PNA hybridization to an mRNA does not elicit an RNase H or dicer response as is the case with ribose-based antisense agents, [94] PNAs are steric blockers that

Figure 3

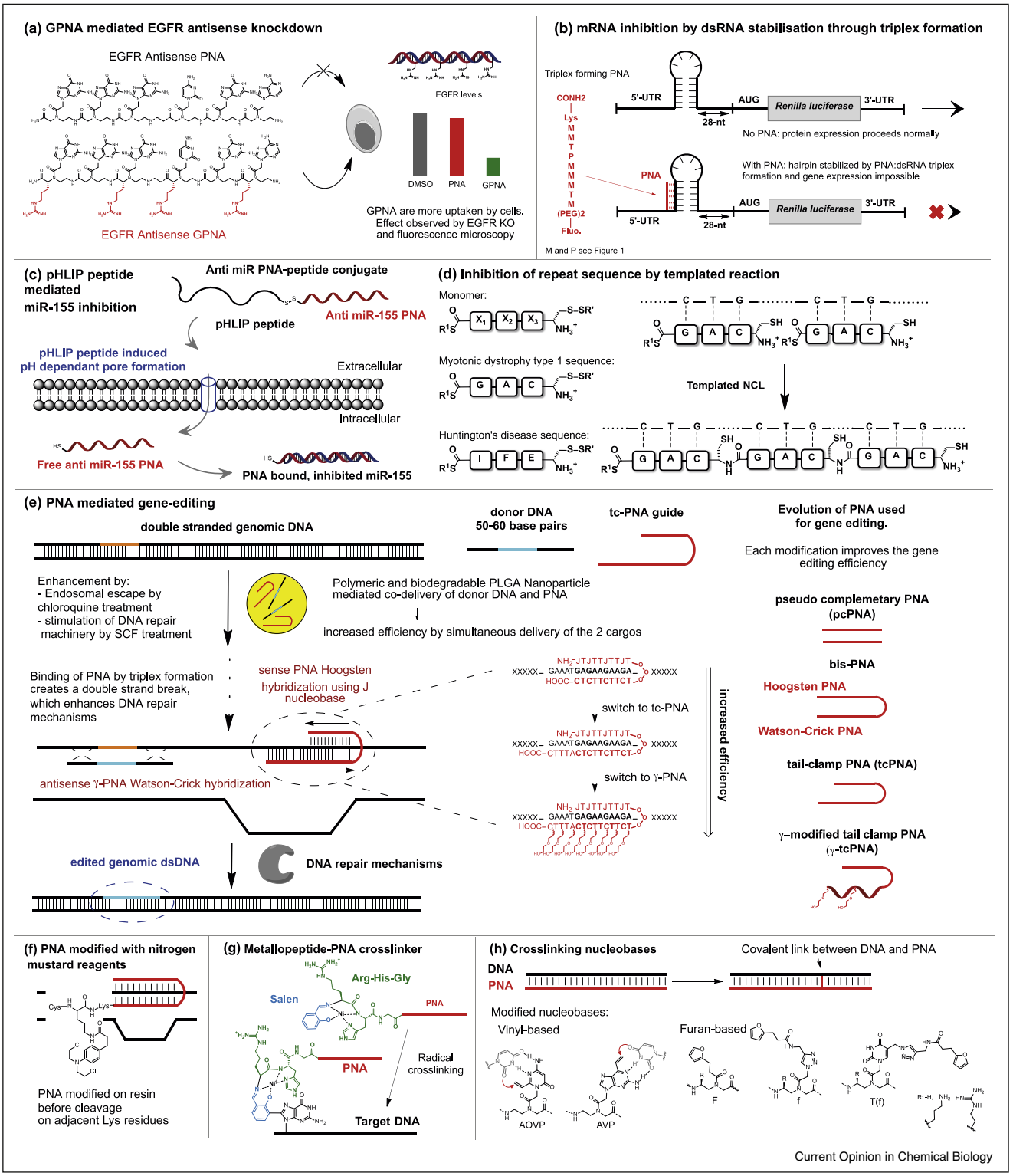


PNA-based assemblies as supramolecular drugs.

inhibit the splicing of a target mRNA or translation by binding to the initiation site [95]. While the aforementioned cellular permeability is an obstacle, particularly for systemic delivery, it was demonstrated 16 years ago that PNA functionalized with four lysines at the C-terminus were effective in correcting an aberrant splicing in a transgenic mouse, validating their potential as therapeutics [96]. In a more recent study, a GPNA was successfully used to suppress the expression of EGFR, an important driver in non-small-cell lung cancer, in a mouse model (Figure 4a) [30•]. While these examples are encouraging, the sparsity of follow-up work suggests that the full potential of PNAs for therapy has not been fully realized. In a recent innovation, it was shown that a PNA oligomer designed to form a triplex with dsRNA was able to suppress the translation of the targeted mRNA (Figure 4b) [19•]. Another recent advance demonstrated the reversible regulation of gene expression by PNA oligomers. In this case, an antisense PNA was designed to contain a non-complementary toehold. The antisense effect can be switched off by the addition of a second fully complementary PNA allowing translation to reoccur through toe-hold mediated strand displacement [97].

Beyond the regulation of protein-coding RNAs, the growing interest in non-coding RNAs and the fact that microRNA (miR) expression are correlated with many human diseases [98] begged the question: Can PNAs interfere with their function? PNA oligomers have been found to be efficient agents to inhibit the function of miRs [99,100]. A landmark study demonstrated that anti-miR PNA conjugated to a peptide with a low pH-induced transmembrane structure (pHLIP) selectively targeted the tumor microenvironment, transported the conjugate across plasma

Figure 4



PNA as antisense agents, gene editing tools and crosslinkers.

membranes under the acidic conditions found in solid tumors, and effectively inhibited the oncogenic miR-155 in a mouse model of lymphoma (Figure 4c) [101••]. Systemic delivery of PNA remains a major consideration and several technologies discussed above are applicable to antagonize the function of a miR (nanoparticle formulation, [43] cationic PNA [29], oligoarginine conjugate [102,103]).

A recent innovation for antisense agents is the use of template-directed ligation to target repeat sequences. Short PNA oligomers (3-mers) that only form weak and transient interactions with RNA are oligomerized in template-directed ligations to form concatenated oligomeric products that bind tightly to the RNA template. This strategy has been used to target the rCAG-repeat expansion associated with Huntington's disease and a number of other related neuromuscular and neurodegenerative disorders (Figure 4d) [21,104]. A supramolecular approach has also been reported to target repeat sequences. In this case, slightly longer PNA probes (6-mer) functionalized with pyrene moieties that can stabilize adjacent probe hybridization through stacking interactions were shown to be sufficient to disrupt a pathogenic interaction between the rCUG-expansion sequence and MBL1 complex [105]. These short PNA oligomers benefitted from the enhanced hybridization of γ-modifications.

Gene editing

A remarkable property of PNA oligomers is their ability to invade dsDNA and enable gene editing. The stability of the invasion complex can be further enhanced by forming a PNA_2/DNA_1 triplex (bis-PNA or tail-clamp-PNA, Figure 4e), in which antiparallel Watson–Crick and parallel Hoogsten base pairing contribute to the invasion complex's stability (incorporation of pseudoisocytosine is important for Hoogsten base pairing at neutral pH). Such invasion results in a modified helical structure that is recognized by the endogenous DNA repair machinery, and induces recombination with a donor DNA. In pioneering work, it was shown that this technology can be used to correct the mutation responsible for β-thalassemia in a model cell line [106]. Gene correction was achieved in 0.2% of the cells after electroporation and could be enhanced twofold by endosomal disruption using chloroquine. In subsequent work with optimized tcPNA oligomers, gene editing rate rose up to 2.8% in THP-1 and primary human CD34+ hematopoietic stem cells (HSC) [39]. This technology was used to introduce a stop codon in the sequence of the CCR5 receptor, thus resulting in a defective receptor that led to R5-tropic HIV-1 resistance. This modification could be detected up to four months after grafting the treated HSC cells on mice.

Genetic disorders not targeting the hematologic system cannot be treated *ex vivo* and transferred back into the patient; hence, an improved delivery method is needed. The next breakthrough came with the use of PLGA NPs loaded with both donor DNA and PNA allowing for the simultaneous delivery of both components after the uptake of NPs by cells. Cells transfected with these NPs showed a 1% site-specific gene modification with no loss in viability, a notable improvement compared to the poor cell viability observed with electroporation [38]. These NPs were used to modify CCR5 or β-globin genes in HSC engrafted mice. Analysis of gene modification *in vivo* by deep sequencing revealed a 0.43% efficiency in the whole spleen but with rates reaching 14–19% in specific cell types, [40] demonstrating the efficacy of PNA-mediated gene editing *in vivo*. Further gains in efficacy have been obtained with the use of γ-modified PNA (miniPEG) [107] and the stimulation of the DNA repair machinery, achieving gene correction yield of up to 15% in CD117+ bone marrow cells. The β-thalassemia mutation was corrected for 4% of total bone marrow cells by *in vivo* treatment of a thalassemic mouse model (and increased up to 6.9% in some specific cell subpopulation) [41••]. The technology has also been successfully applied for gene editing *in utero*. The β-globin mutation was corrected in the fetus of a β-thalassemia transgenic model mouse, after IV injection, at a frequency of about 6% and the editing rate rose up to 10% in isolated progenitor cells (sorted for specific presence or absence of proteins). This treatment led to immediate phenotypic response and improvement of the survival rate of the treated mice offspring and no toxicity of NP or PNA were observed [108]. In all these studies, the off-target rate remained extremely low, 0.00034 *in vivo* [41••] and 0.000002 *in utero* [108]. These remarkable breakthroughs in gene editing were aided by recent developments in PNA modifications and formulations. Further progress in DNA strand invasion and probe delivery will undoubtedly contribute to additional improvements in yield and efficacy.

Crosslinker

While PNAs form remarkably stable duplexes and triplexes with DNA or RNA, the thermodynamic stability is not sufficient to withstand displacement by the replication or translation machineries. To make the complexes irreversible, several chemistries leading to crosslinking in the hybridization complexes have been reported. For example, PNA-nitrogen mustard conjugates have been shown to form a covalent complex with the target genomic sequence. An oligomer targeting the HER-2/neu oncogene suppressed expression by up to 80%, significantly higher than the ssPNA (13%) or bis-PNA (55%) (Figure 4f) [109]. Covalent adducts have also been prepared with a nickel(II)-PNA conjugate, which upon oxidation ($KHSO_5$), promote a phenolic radical crosslinking (Figure 4g) [110]. More recently, modified nucleobases have been designed to serve as crosslinkers. PNA oligomers incorporating AOVP (4-amino-6-oxo-2-vinylpyrimidine) nucleobase at the N-terminus were found to form a covalent adduct at T with complementary DNA but not with RNA [111]. In accordance with the same design,

AVP (2-amino-6-vinylpurine) was found to react preferentially with U in RNA. The crosslinking reactions to pre-miR-122 with PNA containing AVP enhance the inhibition of Dicer processing; again illustrating the benefit of crosslinking (Figure 4h) [112]. Other PNA-crosslinking monomers include furan-derived nucleobases. Upon ROS activation, the furan is converted into an electrophile that can engage in crosslinking. Three nucleobases have been developed ranging from complete nucleobase replacement to thymine modification and have been included in γ-Lys-modified or γ-Arg-modified and non-modified PNA monomers. Sequence selective crosslinking was found to proceed best with γ-Lys-modified PNAs (Figure 4h) [113,114].

Conclusion

While PNAs are nearly 30 years old, the past decade still brought about important innovations in their chemistry and applications. Important developments include γ-substituents in the backbone that enhance hybridization while offering an array of physicochemical properties; delivery methods that target different microenvironments and function *in vivo*; enhanced dsDNA invasion that enable gene editing; fluorogenic probes and reactions that enable nucleic acid sensing; and the use of PNA as a supramolecular scaffold to organize drugs and proteins. While PNA chemistry is intrinsically simpler than that of ribosyl-based oligonucleotides, it is regrettably less accessible from commercial suppliers which undoubtedly curtailed broader use. We hope that the examples illustrated in this review will inspire the use of PNAs and promote further developments in the area.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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