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REVIEW

Applications of PNA-laden nanoparticles for hematological disorders

Shipra Malik[1] · Stanley Oyaghire[2] · Raman Bahal[1]

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Abstract
Safe and efficient genome editing has been an unmitigated goal for biomedical researchers since its inception. The most prevalent strategy for gene editing is the use of engineered nucleases that induce DNA damage and take advantage of cellular DNA repair machinery. This includes meganucleases, zinc-finger nucleases, transcription activator-like effector nucleases, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) systems. However, the clinical viability of these nucleases is marred by their off-target cleavage activity (≥ 50% in RNA-guided endonucleases). In addition, in vivo applications of CRISPR require systemic administration of Cas9 protein, mRNA, or DNA, which presents a significant delivery challenge. The development of nucleic acid probes that can recognize specific double-stranded DNA (dsDNA) regions and activate endogenous DNA repair machinery holds great promise for gene editing applications. Triplex-forming oligonucleotides (TFOs), which were introduced more than 25 years ago, are among the most extensively studied oligomeric dsDNA-targeting agents. TFOs bind duplex DNA to create a distorted helical structure, which can stimulate DNA repair and the exchange of a nearby mutated region—otherwise leading to an undesired phenotype—for a short single-stranded donor DNA that contains the corrective nucleotide sequence. Recombination can be induced within several hundred base-pairs of the TFO binding site and has been shown to depend on triplex-induced initiation of the nucleotide excision repair pathway and engagement of the homology-dependent repair pathway. Since TFOs do not possess any direct nuclease activity, their off-target effects are minimal when compared to engineered nucleases. This review comprehensively covers the advances made in peptide nucleic acid-based TFOs for site-specific gene editing and their therapeutic applications.

Keywords PNA · PLGA nanoparticles · Gamma PNA · Gene editing · Anemia

Introduction

Genome engineering offers the promise of remediating disease phenotypes by manipulating their underlying genotypes [1]. This prospect is most alluring in hematological disorders wherein, inherited genetic variations result in abnormal expression of genes crucial for the viability of hematopoietic lineages or exogenous pathogenic agents threaten the survival of hematopoietic subpopulations vital to immune function. Indeed, these disorders possess many of the features required to reap the potential benefits of this burgeoning technology, particularly: monogenicity, implying that palliative effects could be imparted by appropriate modifications to a single gene; compartmentalization, as genetic manipulation of cells in a single tissue is usually sufficient to remediate disease; and growth selectivity of even a small fraction of correctly modified cells in the population of interest—a useful feature for a field populated by reagents with substantial diversity in efficacy and safety profiles.

The predominant reagents currently available to genetic engineers are the exogenous nucleases that are engineered to bind target genomic loci and introduce double-strand breaks (DSB) [2]. DSBs are structural aberrations that trigger endogenous repair mechanisms which strive to restore the structural integrity of the DNA duplex. In the context of hematology, activation of DNA repair can facilitate various outcomes. It can correct the pathological mutation in the disease-associated gene, direct the targeted insertion of coding sequences for the deficient factors, disrupt genes

Shipra Malik and Stanley Oyaghire contributed equally to this work.

✉ Raman Bahal
Raman.Bahal@uconn.edu

[1] Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT, USA

[2] Department of Therapeutic Radiology, Yale University, New Haven, CT, USA

encoding repressors of surrogates for the defective gene product, or perturb coding sequences for surface epitopes that render some hematopoietic lineages labile to destruction by invading pathogens [3]. While nuclease-based strategies have been effective in principle, important challenges remain in the prevailing methods for targeted reagent delivery and, perhaps more concerning, the avidity and activity of these reagents for/at off-target loci [4–6].

In most applications, nuclease reagents (or their precursory plasmids) are effectively delivered in vitro by electroporation [7]. Though successful for the introduction of diverse cargo to a variety of clinically relevant primary cell types, it remains unfeasible in vivo, and adversely affects survival of treated cells when used ex vivo. The later limitation is especially important for some hematological disorders where as a consequence of depletion in relevant hematopoietic lineages, there exist low basal levels of relevant primary cells available for harvesting and modification ex vivo.

Even when efficient intracellular transfer of the requisite nucleases is achieved, the destruction of the DNA duplex stimulates repair pathways that produce a wide spectrum of molecular outcomes. A majority off-repair events are mediated by non-homologous end joining (NHEJ), a pathway that prioritizes DNA structure over the DNA sequence; while a minority of the modifications are mediated by homology-directed repair (HDR) that preserves the sequence of the DNA template. Although both NHEJ and HDR are required for genome engineering in hematological disorders, the stochastic distribution of molecular outcomes resulting from combination of both these homologous regions of duplex DNA targets, with resultant local helical distortion—as reagents for provoking gene modification [8, 9]. However, the utility of these oligomers is hindered by their lability to cellular nuclease.

Peptide nucleic acids (PNAs) are synthetic nucleic acid analogs with pseudo-peptide backbone which imparts resistance to nuclease enzymes. PNAs consist of *N*-(2-aminoethyl)-glycine units-based backbone. Further nucleobases [Adenine (A), Guanine (G), Cytosine (C) and Thymine (T)] are attached to the backbone by a methylene carbonyl linkage (Fig. 1). PNAs charge-neutral property enables strong binding with DNA and RNA targets via Watson–Crick (WC) base pairing. This binding could be further increased by introducing cationic functionalities to PNA oligomer [10, 11]. It has been demonstrated that different designs of PNA can invade double-stranded DNA (dsDNA) and activate DNA repair and recombination events in the mammalian cells to induce site specific gene editing. In addition, PNAs circumvent many of the aforementioned limitations associated with nuclease and other class of oligonucleotide-based gene editing reagents. A variety of PNA designs and combinations have been developed for gene editing applications, including bis-PNAs [12, 13], tail clamp PNAs (tcPNAs) [14], pseudo-complementary PNAs (pcPNAs) [15] and new generation gamma PNAs (γPNAs) [16] (Fig. 2).

Poor cellular uptake properties of PNAs limit their broader clinical application. In past, several attempts have been made for increasing cellular uptake of PNAs. These strategies include conjugation with cell-penetrating peptides (CPP) [17] like penetratin, nuclear localization signal (NLS) and conjugation with pH low insertion peptide (pHLIP) targeting tumor microenvironment [18]. Few promising strategies include inclusion of cell transduction domain (guanidinium) onto PNAs to increase its uptake [19]. Recently, nanoconstruct-based approach has garnered great attention to deliver therapeutically active PNAs both ex vivo as well

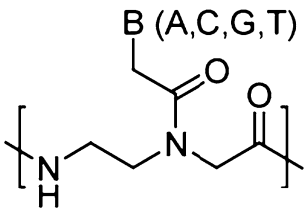

Fig. 1 Chemical structure of regular PNA. B denotes nucleobases (A, C, G and T)

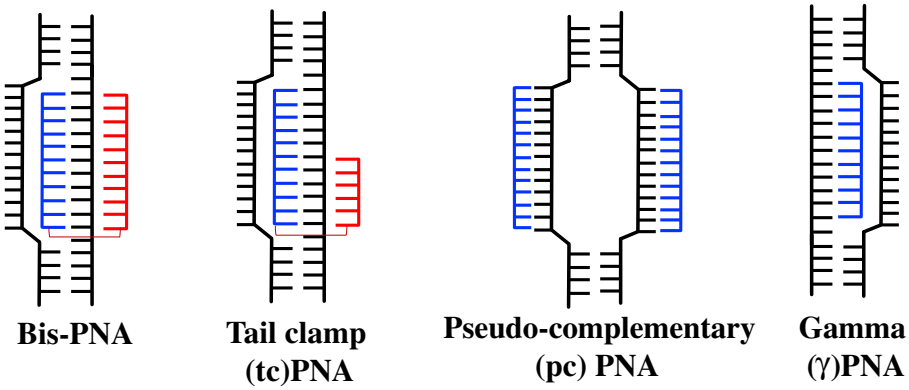

Fig. 2 DNA binding modes of different PNAs

as in vivo. Several polymeric nanoparticles (NPs) have been used for PNAs delivery as enlisted in Table 1.

Bis-PNA

Bis-PNAs consist of two PNA strands linked via a flexible poly diethylene glycol linker and targets only homopurine region of genomic DNA. One PNA strand binds to the target DNA via WC base pairing in antiparallel orientation, whereas another PNA strand binds to the homopurine region of DNA via Hoogsteen base pairing forming a PNA/DNA/PNA triplex clamp (Fig. 3). In bis-PNA, pseudoisocytosine (also called J nucleobase) is used instead of cytosine for pH-independent base pairing to G in Hoogsteen binding domain to form a stable triplex structure at physiological pH [20]. Triplex clamp formed by bis-PNAs are highly stable and exhibit thermal denaturation temperature (Tm) > 70 °C. Triplex clamp results in displacement of homologous DNA strand forming D-loop (Fig. 2). Glazer and co-worker have demonstrated that triplex helix created by bis-PNA activates the nucleotide excision repair (NER) mechanism in cells and induce homologous recombination of donor DNA strand (containing the correct sequence/base) at the mutated site [12, 13]. The potential of bis-PNAs to induce recombination was studied in plasmid vector pSupFG1/G144C in vitro. Recombination frequencies were compared between bis-PNA–donor DNA conjugate, a mixture of bis-PNA with donor DNA, and bifunctional TFO–donor DNA (A-AG30). Bis-PNA–donor DNA conjugate induced higher recombination frequency (62×10^{-5}) in comparison to TFO–donor DNA conjugate (38×10^{-5}). However, maximum recombination frequency (81×10^{-5}) was observed in mixture of bis-PNA and donor DNA that was fivefold higher than the donor DNA alone. These results demonstrated that a triplex clamp created by bis-PNA stimulates recombination of donor DNA at the target site.

Further the role of nucleotide exclusion factor, Xeroderma Pigmentosum (XPA) in NER pathway was assessed by comparing recombination frequency in XPA-depleted (−XPA) cell extracts and extracts treated with recombinant XPA protein supplement (+XPA) [21]. The extracts were depleted of XPA by rabbit polyclonal antibody against recombinant human XPA protein followed by immunoprecipitation. UV-exposed samples acted as a control because DNA repair due to UV damage is mediated by XPA. XPA depletion resulted in reduced DNA repair both in bis-PNA and UV-treated extracts and addition of XPA protein restored the DNA repair indicating that XPA plays an important role in DNA repair activity induced by bis-PNAs. Further, XPA depletion resulted in 14% decrease in recombination activity for mixture of bis-PNA and donor DNA, while 39% decrease

Table 1 Nanoparticle-based strategies for enhanced delivery of PNAs

Polymeric nanoparticles	Application	References
N,*N*,*N*-trimethyl-*O*-alkyl chitosans (TMACs) NPs	Drug delivery	[38]
PEGylated nanosized graphene oxide(PEG-nGO) constructs	Antisense (Cancer)	[39]
Mesoporous Silica NPs	Antisense (Cancer)	[40, 41]
Membrane penetrating oxidized carbon (MPOCs) NPs	Gene therapy	[42]
Porous-silicon (PSi) films	Drug delivery and biosensing	[43, 44]
Zeolite-L-nanocrystals	Drug delivery	[45]
Poly-lactic-co-glycolic acid (PLGA) NPs	Antisense, Antigene	[8, 46–48]
Poly-beta-amino-esters (PBAE) NPs	Antisense, Antigene	[33]
Surface modified PLGA/PBAE NPs	Antisense, Antigene	[49]
Peptide coated PLGA NPs	Antigene	[50]
Cationic shell-cross-linked knedel-like (cSCK) NPs	Drug Delivery	[51]
Avidin-labeled protein nanoparticles	Antisense (HIV infections)	[52]

Fig. 3 Schematic of the PNA–DNA system. The 50–60-mer donor DNA is homologous to the gene target of choice except for a several base-pair mutation. The bis-PNA binds near the target and catalyzes homologous recombination of the donor strand into the target. Allele-specific PCR (AS-PCR) can distinguish between modified (mutant) and unmodified (wild-type) genomic DNA

in activity was reported for bis-PNA–donor DNA conjugate establishing that DNA repair is mediated by NER pathway [13].

Prior work has demonstrated that triplex-forming bis-PNAs effectively bind the beta (β)-globin gene and stimulate modification at a β-thalassemia-associated site in human CD34+ hematopoietic stem cells (HSCs) without loss of pluripotency [13]. Glazer and co-worker designed several triplex-forming bis-PNAs that mediate recombination at the first position of intron 2 (IVS2-1) of the β-globin gene and achieved recombination frequencies of 0.1–0.5% in a CHO cell GFP/β-globin gene fusion model with gene editing verified at the protein, mRNA (by qRT-PCR), and genomic DNA (by direct sequencing) levels. Primary CD34+ HSCs transfected with bis-PNAs and donor DNAs showed the gene editing at the β-thalassemia locus, with the presence of the mutation detected in HSC-derived cells grown in erythroid and neutrophil differentiating conditions. Transfection was accomplished via the Amaxa nucleofector [22], which although useful for proof-of-principle studies, is toxic to hematopoietic cells and is applicable only for ex vivo research applications.

In another study, it was demonstrated that Poly (lactic co-glycolic acids) (PLGA) NP-based delivery of bis-PNAs/donor DNA combination can lead to site-specific gene editing of CD34+ HSCs [14]. PLGA is a commonly used biodegradable polymer for drug delivery systems and medical devices. It has been approved by both the US Food and Drug Administration (USFDA) and European Medical Agency in a variety of clinical applications. An appealing feature of PLGA is that it degrades by hydrolysis into endogenous non-toxic metabolites (lactic acid and glycolic acid), which enhances its biocompatibility for in vivo delivery. Dye-loaded PLGA NPs showed surprisingly efficient uptake in CD34+ HSCs. Bis-PNAs/donor DNA were formulated into 150 nm spherical PLGA NPs, with ample loading of nucleic acids (250–450 pmol/mg NPs). Further, the PLGA NPs loaded with bis-PNAs/donor DNA combinations stimulated genomic recombination to modify the IVS2-1 splice site within the β-globin gene. Allele-specific PCR confirmed that NPs-delivered bis-PNA/donor DNA mediate site-specific modification in CD34+ HSCs. Importantly, the PLGA NPs with bis-PNA/donor DNA are not toxic to the progenitor cells. Progenitor cells that were genetically modified with NPs were differentiated into both erythroid and neutrophil populations, without loss of the gene modification.

Pseudo-complementary PNAs (pcPNAs)

Unlike bis-PNAs, pcPNAs consists of two PNA strands where each PNA binds to the complementary DNA strand in a sequence unrestricted manner via double duplex invasion-based mechanism (Fig. 2). Since, PNA–PNA duplex binding is stronger than PNA–DNA duplex binding, to prevent the self-quenching between two strands of pcPNAs, 2,6 diaminopurine (D) and 2-thiouracil (U) modified nucleobase are used instead of regular A and T nucleobases [20]. Due to the presence of modified nucleobases, the two PNA strands do not form a stable PNA–PNA duplex [23]. It was indicated that pcPNAs can bind to the dsDNA containing mixed purine and pyrimidine sequences (~40% AT rich sequences). In gene editing-based experiments, pcPNAs induced higher gene editing frequency (0.65%) in *sup*F reporter gene in comparison to TFO (0.14%) and bis-PNA (0.21%). However, in CHO-GFP/IVS2-1 reporter cell, pcPNAs/donor DNA combinations could stimulate the recombination at β-thalassemic mutation site (IVS2-1) only at frequency of 0.012%. Further pre-treatment with histone deacetylase (HDAC) inhibitor (SAHA) improved the gene editing frequency to 0.17% that was threefold higher than only donor DNA [15].

Tail Clamp PNAs (tcPNAs)

Another promising PNA design has been used for gene editing-based application called tail clamp PNAs (tcPNAs). In tcPNAs, the WC binding domain is extended so that it can bind beyond the homopurine region and bind to a longer target site and thereby enhance the binding specificity. This creates an even larger helical distortion by increasing the length of the strand invasion and P-loop complex (Fig. 2). In binding studies, tcPNAs show greater affinity and specificity compared to bis-PNAs. Importantly, high binding affinity to DNA by tcPNAs does not require a long homopurine run; in fact, homopurine stretches as short as 5 bp are sufficient.

In a collaborative effort, it has been demonstrated that tcPNAs/donor DNA combination, delivered via modified PLGA NPs, demonstrated significant gene editing in F508del mutation in cystic fibrosis transmembrane conductance regulator (CFTR) gene in cystic fibrosis (CF) [9]. CF is a multi-system genetic disease affecting the respiratory, gastrointestinal and reproductive tracts [24]. Although the average life expectancy is 37 years, 50% of individuals with CF die in childhood from respiratory failure making it a very serious pediatric health problem. CF is most commonly caused by a three base-pair deletion (F508del) mutation in CFTR, an ion channel that mediates chloride transport [25]. Although CF is one of the most rigorously characterized genetic diseases, current treatment of patients with CF focuses on symptomatic management rather than primary correction of the genetic defect. CFTR is considered not readily amenable to gene therapy because of challenges including in vivo gene delivery, inflammatory reactions, and transient gene expression.

NPs formulated from a blend of PLGA and PBAE and further surface modified with the nuclear localization sequence-containing cell-penetrating peptide MPG (modified PLGA/PBAE/MPG NPs) demonstrated superior gene editing efficiency as compared to PLGA NPs alone. Intranasal administration of modified PLGA NPs encapsulating tcPNA/donor DNA in CF mice showed nasal potential difference (NPD) similar to wild-type mice. Gene editing frequencies were reported to be > 5% in nasal epithelium and > 1% in lungs. Further 10% editing was noticed in human CFBE cells treated in vitro with modified PLGA NPs.

Gamma PNAs (γPNAs)

In addition, Ly and co-workers have developed another novel class of PNAs called gamma PNAs (γPNAs) [26, 27]. Compared to classical PNAs, γPNAs are highly water soluble; they neither aggregate nor adhere to surfaces or other macromolecules in a nonspecific manner (Fig. 4) [28, 29]. As individual strands, γPNAs adopt a right-handed helical motif—as confirmed by circular dichroism (CD), NMR, and X-ray crystallography—and hybridize to DNA or RNA strands with unusually higher affinity and sequence specificity [30]. In addition, prior studies also revealed that on average each γ-backbone modification stabilizes a PNA–DNA duplex by 4 °C [31]. They are the only class of oligonucleotide molecules developed to date that has been shown to be capable of invading any sequence of double helical genomic DNA at physiological conditions, with recognition occurring through WC base-pairing. γPNAs have been exploited in a number of biological and biomedical applications, from electronic barcoding of single gene [32] to gene correction (achieving clinically acceptable correction frequencies with extremely low off-target effects, as compared to that of zinc-finger nucleases or CRISPR/Cas system) [16, 33, 34]. In this section, we discussed the recent gene editing results of γPNAs-based probes.

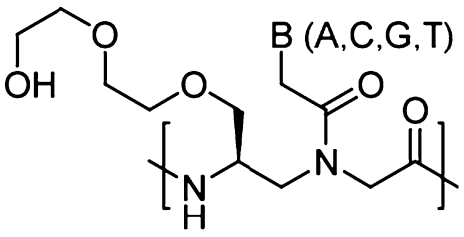

Fig. 4 Chemical structure of gamma PNA (γPNA) containing an ethylene glycol (MP) at the γ-position. B denotes nucleobases (A, C, G and T)

PLGA NPs containing ss γPNAs for site-specific gene editing

Effectiveness of ss γPNAs/donor DNA combination encapsulated in PLGA NPs for successful gene editing have been assessed in green fluorescent protein (GFP) transgenic mouse model. This transgenic mouse model has a β-globin/GFP fusion transgene consisting of human β-globin intron 2 carrying a thalassemia-associated IVS2-654 (C → T) mutation embedded within the GFP coding sequence. The presence of IVS2-654 (C → T) mutation results in improper splicing of β-globin/GFP mRNA and lack of GFP expression [35]. This model allows for robust quantification of gene editing frequencies [33]. To test whether ss γPNAs can induce gene editing, series of γPNA oligomers were designed and synthesized. Further, γPNA/donor DNA was encapsulated in PLGA NPs using double emulsion solvent evaporation technique. Due to the presence of ethylene glycol units in γPNAs, γPNA/donor DNA complex formed a clear solution at room temperature and higher total nucleic acid loading was observed in γPNA/donor DNA in comparison to regular PNA/donor DNA samples. NPs containing γPNA/donor DNA resulted in gene editing frequency of 0.1% in ex vivo studies using bone marrow cells of GFP transgenic mice treated with 2 mg/mL of NPs, which was higher than the levels observed with regular PNA/donor DNA NPs (0.02%). PBAE/PLGA NPs with 15% PBAE (poly (beta-amino) ester) and 85% PLGA led to improved loading of γPNA/donor DNA combination and sustained release of nucleic acids. Significantly higher gene editing frequency (0.8%) was observed in PBAE/PLGA NPs containing γPNA/donor DNA combination. Further, these PNAs were able to induce gene editing up to a distance of ~ 100 bp (0.43%) and ~ 250 bp (0.23%) away from the donor DNA binding site. SS γPNA/donor DNA combination was found to be non-toxic without any impact on differentiation potential of hematopoietic progenitor cells. Further in vivo studies were conducted by administering four retro-orbital injections of 2 mg PBAE/PLGA NPs containing regular PNA or γPNA/donor DNA combinations. Deep sequencing analysis resulted in gene editing frequency of 0.077% in bone marrow cells of mice treated with PBAE/PLGA NPs containing γPNA/donor DNA with minimum off-target effects (≤ 0.01%). To study the inflammatory response, RT-PCR analysis was performed on bone marrow cells to determine levels of inflammatory markers like interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) and no significant difference was observed in their levels between PBAE/PLGA NPs-treated cells and untreated cells. Overall, it was successfully demonstrated that ss γPNA encapsulated in PBAE/PLGA NPs exhibit higher loading, binding affinity and gene editing abilities without any sequence restriction as established via both ex vivo and in vivo studies in transgenic mice model.

Enhanced gene editing by gamma-modified tcPNAs (γtcPNAs)

In our published work, we showed that tcPNAs with poly diethylene glycol substitution at γ position results in higher gene editing frequencies in transgenic GFP mouse model. PNA-mediated triplex formation induces DNA repair and recombination of the genomic site with a 60-nucleotide ss donor DNA that is homologous to a portion of the β-globin intron 2 sequences except for providing a wild-type nucleotide at the IVS2-654 position in transgenic GFP mouse model. Via recombination, the splice-site mutation is corrected resulting in expression of functional GFP. Hence, GFP expression provides a direct phenotypic assessment of genome editing frequencies that can be quantified by flow cytometry.

In a recent study, we designed a series of tcPNAs to bind to the β-globin intron 2 near the IVS2-654 (C → T) mutation [16]. These PNAs were combined with the donor DNA and formulated into PLGA NPs. These NPs were added to the culture medium of bone marrow (BM) cells from GFP mice, and 2 days later, the cells were scored for gene editing by flow cytometry to quantify GFP expression. We identified the most active tcPNA oligomer. Further, we performed chemical modifications onto regular tcPNA with every other residue in the WC domain substituted with γPNA. A scrambled sequence control was also made with the same base composition as that of test γtcPNA.

We treated mouse BM cells with PLGA NPs containing regular tcPNA and γtcPNA, as well as its scrambled control. We also sorted the BM cells based on cell surface markers, so that we could interrogate the extent of gene editing in individual stem and progenitor cell populations. We made two key findings: (1) The gene editing occurred primarily in CD117+ cells. CD117 is the product of the c-Kit gene and is a receptor tyrosine kinase that marks stem cell populations. This was encouraging because results demonstrated that HSCs may be particularly susceptible to PNA-mediated gene editing, and HSCs are the most desirable cell population to edit. (2) The NPs containing γtcPNA gave substantially higher levels of gene editing (up to 9% in a single treatment) compared to regular tcPNA (about 2.5%) indicating the superior activity of the γPNAs. There was no effect with scrambled control. We attribute this increased efficacy of γtcPNA to the enhanced DNA binding properties of γPNAs, which take on a pre-organized helical conformation enforced by the γ substitution.

Gene editing in mice with thalassemia and amelioration of the disease phenotype

Prompted by these results in reporter mice, we tested gene editing in a β-thalassemia mouse model [36]. These mice carry the human β-globin gene replacing the mouse β-globin gene locus and containing the same β-thalassemia splicing mutation at IVS2-654 as in the GFP reporter mice. We treated these mice via simple IV injection with γtcPNA/donor DNA NPs given four times at two-day intervals. Because we had determined that activation of the c-Kit pathway with stem cell factor (SCF) boosts gene editing (an effect associated with increased DNA repair in the c-Kit + cells), we also treated the mice with SCF. This regimen yielded gene editing at a frequency of 7% in Lin-Sca1 + cKit + CD150 + CD135 cells, a population that is highly enriched for long-term HSCs [16]. We also observed gene editing in other progenitor population cells. This treatment produced amelioration of the disease phenotype in the thalassemic mice with sustained reversal of the anemia, normalization of hemoglobin concentrations, and decrease in reticulocytosis. In parallel, there was also reduced extramedullary hematopoiesis and marked reduction in splenomegaly with improvement in splenic architecture on histologic examination.

Gene editing ability was further studied in human CD34+ cells, after introducing the mutation at IVS2-654 of normal human CD34+ cells. These cells were then treated with blank PLGA NPs, SCF plus the γtcPNA/donor DNA containing PLGA NPs. Deep sequencing analysis showed gene editing frequency of 5% at IVS2-654 position in case of SCF plus the γtcPNA/donor DNA and low off-target effects; 400,000-fold lower when compared to the 5% frequency at the targeted site. Bone marrow transplantation studies in NOD-*scid IL2rg*^{null} mice with γtcPNA/donor DNA nanoparticles and SCF treated cells showed 3.4% gene editing frequency.

Conclusion

Overall, this review present various novel approaches for site-specific gene editing based on nanoparticle-delivered PNA-based strategies. It has been well documented that PNAs can invade double duplex DNA creating a loop in double- stranded DNA which instigates the NER pathway allowing the homologous donor DNA to provide the correct genetic sequence at target site. Each PNA invades the dsDNA via a specific mechanism (Fig. 2 and Table 2) resulting in gene editing of mutated region. Further chemical modifications of PNAs at γ position led to more efficient γPNAs which can bind specifically to the target site without any sequence restriction.

The critical difference between PNAs and nuclease-based gene editing is important since off-target strand breaks caused by nucleases could lead to leukemias and other malignancies. This technology is known as "minimally invasive" gene repair, as gene editing occurs in situ

Table 2 Advantages and gene editing efficiencies of PNAs

PNA	Advantages	Gene editing efficiency
Bis-PNA	PNA/DNA/PNA clamps are stable and can target only homopurine regions Exhibit high binding affinity and specificity	60-fold higher gene correction than the background in SupFG1 reporter gene in vitro [12] 0.2% of gene editing reported in CHO/IVS2-1 reporter cell assay using bisPNA targeting IVS2-194 in combination with donor DNA in S-Phase of cell cycle [13] 0.2% of gene editing observed in CD34+ hematopoietic progenitor cells treated with PLGA NPs encapsulating bis-PNA (targeting IVS2-194) and donor DNA [14]
pcPNA	Targets mixed sequence containing ≥ 40% A:T bp High binding affinity and specificity Modified 2,6-diaminopurine and 6-thiouracil bases prevent formation of PNA:PNA duplex Binds to target DNA strand only via WC base pairing	0.65% of gene editing in an episomal target site using supF reporter gene [15] 0.012% gene editing in CHO/IVS2-1 reporter cell assay after single transfection [15] Increased gene editing frequency observed in S-Phase (0.19%) and combination of S-Phase with chloroquine (0.25%) and histone deacetylase inhibitor, SAHA (0.78%) [15]
tcPNA	Targets homopurine regions of dsDNA Extended WC domain increases the specificity Binds to target site via both WC and Hoogsteen base pairing	2.8% CCR5 gene editing in primary human CD34+ cells with transfection of tcPNA and donor DNA together in order to impart HIV-1 resistance [9] 5.7% CFTR gene editing in nasal epithelium using PBAE/PLGA/MPG NPs in CF mouse model [9]
γPNA	Targets mixed DNA sequence without any sequence restriction Capable of targeting 0–100% GC rich DNA sequence Higher aqueous solubility Improved loading in NPs	0.8% of gene editing frequency in bone marrow cells of GFP mouse model treated with PBAE/PLGA NPs [33] Up to 4.0% gene editing in β-thalassemic mice with marked improvement in the phenotypic features [16]

via recruitment of the cells' own DNA repair machinery and without the need for viral vectors. Because this approach seeks to edit the mutated gene at the specific mutation site, it avoids the risk of deleterious ectopic integration in the genome that has been seen with virus-mediated gene therapy [37].

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