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Nanotechnology for Delivery of Peptide Nucleic Acids (PNA)

Anisha Gupta^{1,†}, Raman Bahal^{1,†}, Meera Gupta^{2,3}, Peter M Glazer^{1,4,*}, and W. Mark Saltzman^{2,*}

¹Department of Therapeutic Radiology, Yale University, New Haven, CT, USA

²Department of Biomedical Engineering, Yale University, New Haven, CT, USA

³Department of Chemical Engineering, Indian Institute of Technology-Delhi, New Delhi, India

⁴Department of Genetics, Yale University, New Haven, CT, USA

Abstract

Over the past three decades, peptide nucleic acids have been employed in numerous chemical and biological applications. Peptide nucleic acids possess enormous potential because of their superior biophysical properties, compared to other oligonucleotide chemistries. However, for therapeutic applications, intracellular delivery of peptide nucleic acids remains a challenge. In this review, we summarize the progress that has been made in delivering peptide nucleic acids to intracellular targets. In addition, we emphasize recent nanoparticle-based strategies for efficient delivery of conventional and chemically-modified peptides nucleic acids. Keywords: nanoparticles; peptide nucleic acids; gene editing; antisense therapy

Graphical Abstract

This review highlight the novel nanoparticle based delivery strategies employed for delivery of regular as well as chemically modified PNAs both *ex vivo* as well as *in vivo*

*Correspondence to: Dr. W. Mark Saltzman, mark.saltzman@yale.edu and Dr. Peter M. Glazer, peter.glazer@yale.edu.

†The first two authors contributed equally to this work

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Keywords

nanoparticles; peptide nucleic acids; gene editing; antisense therapy

1. Introduction

Nucleic acid structures play an important role in directing transcriptional as well as post-transcriptional events during protein synthesis and their subsequent effects on gene expression. Point mutations in DNA and RNA sequences lead to myriads of diseases, such as various forms of cancer and rare genetic disorders. Hence, small molecule based drugs targeting nucleic acids have attained significant attention as anticancer therapeutics [1–4]. Though promising results have been obtained, off target toxicities produced by small molecules often limit their clinical use. An alternate approach is to target diseases at the genetic level, using the specificity of complementary binding by nucleic acids to minimize off-target effects. Gene therapy approaches rely heavily on targeted reagents, which can recognize DNA and RNA sequences selectively.

Bio-organic reagents have been developed to mimic the chemical features of DNA and RNA but exhibit improved properties. Examples include phosphorothioates [5, 6], morpholinos [7, 8], and locked nucleic acids (LNA) [9, 10]. Rigorous chemical structure optimization has been done on these nucleic acids analogues to provide improved features such as reduced off-target binding, resistance to enzymatic degradation, and improved aqueous solubility. One promising class of reagents is peptide nucleic acids (PNAs) [11, 12]. PNAs, which were introduced by Nielsen and coworkers in 1991, are synthetic DNA analogues in which the phosphodiester backbone is replaced with unchanged 2-N-aminoethylglycine units (Fig 1). Importantly, PNAs are resistant to enzymatic degradation by nucleases and proteases [13]. This feature makes PNAs attractive candidates for numerous therapeutic and biomedical based applications [14]. Additionally, unlike other reagents, the neutral PNA backbone confers strong hybridization properties with its complementary DNA/RNA targets in a sequence specific manner [15, 16]. Over the last two decades, PNAs have been extensively studied as antisense (targeting mRNA or microRNAs) as well as antigene agents (targeting genomic DNA) to control the gene expression and regulation [17–19].

Intracellular delivery of PNAs is a challenge [20]. Therefore, a number of strategies have been investigated for enhancing PNA delivery into cells [21–23]. These strategies include electroporation, transfection-based methods, inclusion of cationic peptides, use of chemically modified PNAs, and entrapment of PNA in polymer nanoparticles [24, 25]. Though all of these strategies have yielded some interesting results, issues such as selectivity, endosomal entrapment, and unwieldy synthetic procedures limit their broader applications. Here, we review current PNA delivery strategies and their limitations, focusing on the use of nanoparticle-based delivery strategies. Table 1 summarizes recent reports on nanoparticle-based approaches for PNA delivery.

2. Peptide Nucleic Acids and their delivery methods

PNA-based agents have shown promise *in vitro* due to their favorable biophysical properties, but broader applications of PNAs have been more difficult to achieve due to their limited cellular uptake. To increase uptake, various methods have been tested for increasing PNA transport across the cell membrane (Fig. 2).

Hanvey *et al.* first demonstrated the antisense and antigene properties of PNAs by microinjecting the PNAs into fibroblast cells [19]. PNAs were delivered inside cells via electroporation for modulating alternative splicing [42] and for induction of human gamma globin gene [43]. Corey et al. found that co-transfection of PNA/DNA complexes can lead to significant uptake in cells [44–47]. Direct permeabilization of cells using streptolysin-O enhances permeability to PNAs, which leads to PNA based mutations in supFG1 reporter gene [48]. These direct delivery methods have been useful in showing the biological activity of PNAs when introduced into via mechanical or electrical transduction (microinjection, electroporation and transduction), but they appear to be restricted to small-scale experiments, and likely are not translatable into clinical applications.

Another promising strategy for PNA delivery involves conjugation to cell penetrating peptides (Table 2). However, PNAs delivered via conjugated peptides appear to be largely entrapped in endocytic vesicles, where they are degraded or recycled back to the cell surface: escape into the cytoplasm and transport to the nucleus appears limited [49]. On the other hand, one particular approach, using a peptide called pHLIP (pH low insertion peptide) [50, 51], appears to be particularly promising for selective delivery to cancer cells. pHLIP undergoes uptake only under acidic conditions through a pH-dependent cellular translocation mechanism. This technology is promising, but is limited to delivery to cells in acidic microenvironments [18].

Further, PNAs have also been conjugated with lipophilic moieties such as adamantyl acetic acid to facilitate their cellular uptake [52]. In addition to adamantyl acetic acid, triphenyl phosphonium based cations have also been used for PNA delivery in the human fibroblast cells [53].

In a different strategy, chemical modifications are introduced in the PNA backbone to impart positive charge characteristics, which facilitates cellular uptake properties [59–61]. These positively-charged groups include guanidinium, which can be placed at the gamma or alpha position on the PNA backbone (Fig. 3).

Studies have shown that PNA-peptide conjugates are more toxic than GPNAs. This is likely due to the amphipathic nature of the PNA peptide conjugates, which result in disruption or permeabilization of cell membranes. Guanidinium containing PNAs are less toxic because they are less amphipathic than PNA peptide conjugates [60].

These strategies have shown promise in intracellular uptake of PNA and in targeting beta-catenin [62] as well as E-cadherin genes [60] which play an important role in Wnt pathway. However, PNA backbone modification requires complex chemical synthetic procedures, and will require further optimization for more widespread use.

3. PLGA based nanoparticles for PNA delivery

Poly (lactic co-glycolic acids) (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 [63] in a variety of clinical applications [64]. 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 [65]. Microparticles of PLGA have been used clinically for prostate cancer treatment in the form of Lupron ® and Trelstar ®. Another advantage of PLGA is that the properties of the material can be adjusted, to some extent, by variation of the copolymerization ratio. For example, PLGA 80:20 identifies a copolymer consisting of 80% lactic acid and 20% glycolic acid, which exhibits different physical properties and degradation rates, than homopolymers of poly(lactic acid) (PLA) or poly(glycolic acid) (PGA). Interestingly, PLGA nanoparticles have been shown to facilitate uptake of various macromolecules ranging from proteins, lipopeptides, peptides, viruses, cell lysates and the plasmid DNA into the cytoplasm [64]. PLGA nanoparticles can enhance the activity of vaccines, by providing controlled and targeted delivery of antigens [66]. In addition to delivering purified antigen, PLGA nanoparticles containing tumor lysates have been tested as anticancer vaccines based on tumor-associated antigens (TAAs) [67]. Numerous reports suggest that PLGA nanoparticles can be used to encapsulate antibiotics for more effective antibacterial applications [68].

PLGA-nanoparticles appear to permeate the cell membrane by a combination of fluid phase pinocytosis and clathrin-mediated endocytosis. Cells of the reticuloendothelial system (RES) can phagocytose PLGA nanoparticles and eliminate them from the blood stream, predominantly by uptake in the liver. To prevent this unwanted uptake, PLGA nanoparticles have been decorated with chemical functionalities such as PEG and certain peptides, to avoid elimination by the RES and prolong the half-life for nanoparticle circulation in the blood [69, 70]. In this regard, another important characteristic of PLGA nanoparticles is their surface charge density, which can be manipulated by chemical conjugation or fabrication in the presence of surfactants. It was well established that PLGA nanoparticles that possess positive charge undergo more cellular uptake, as well as more efficient endosomal release [71–73]. Recent work has explored the utility of PLGA nanoparticles to deliver PNAs for their antisense and antigene applications [29, 31].

3a. Targeting microRNAs

MicroRNAs are members of a class of small non-coding RNAs (22–23nt), which originate from the precursor pre-microRNA, and are known to regulate the translation and stability of mRNA during post-transcriptional events in the cytoplasm. miRNA-profiling experiments have revealed that miRNAs are widely over-expressed in several types of cancers. Hence, development of novel therapeutic molecules to inhibit miRs (miR therapeutics) is an intriguing approach to cancer therapy [74, 75].

Engineered antisense molecules, such as locked nucleic acids (LNA) and phosphorothioates, have been developed to target specific miRs, but they require extensive optimization and/or elaborate screening in terms of cellular delivery, off target toxicity, tumor selectivity and

enzymatic resistance properties. As an alternate, PNAs have been shown to target miRs and exhibit antisense properties. The chemical properties of PNA make it an attractive agent for targeting miRs, but delivery of PNAs across the cell membrane is a challenge. In early work, Fabani, Gait and co-workers demonstrated that PNAs exhibit inhibitory activity against miR-122 in human and rat liver cells when delivered by electroporation, or when modified by appending cell-penetrating peptide (R6-Penetratin), or by adding four lysines to the PNA oligomer [76]. Other studies found that polyarginine–PNAs can be employed to target miR-210, which is associated with hypoxia and erythroid differentiation. Gait and co-workers have shown that lysine-derivatized PNA and cysteine-containing PNA can target miR-122, which is involved in lipid metabolism and hepatitis C virus replication [77].

We have shown that PNA-based anti-miRs encapsulated in PLGA nanoparticles can inhibit miR properties [30, 78]. In these studies, nanoparticles of PLGA, composed of a 50:50 monomer ratio with terminal ester groups, were loaded with anti-miR-155 PNAs using a double emulsion solvent evaporation method [30, 79]. In addition to enzymatic resistance, we discovered that the neutral PNA backbone also facilitates loading of anti-miR PNAs into PLGA nanoparticles, when compared to oligonucleotides with a charged phosphodiester backbone. In order to enhance accumulation of anti-miR-155 in target cells, CPP was also attached to the surface of nanoparticles. Loading was significantly higher for PNA (410 ± 55 pmoles of PNA/mg of nanoparticles) than single-stranded DNA (ssDNA, 25 ± 5 pmoles of DNA/mg of nanoparticles) [30]. It has been noticed that coating of nanoparticles with CPP adds a cationic component to the surface of the nanoparticles; however, the charged surface ligand did not appear to impact loading of nucleic acids. A dual luciferase reporter assay was employed to measure the miR-155 inhibition. In this assay, a target binding sequence for miR-155 was inserted into the 3'UTR of renilla luciferase and firefly luciferase was used for normalization. Cells treated with CPP-coated PLGA nanoparticles containing anti-miR-155 PNA decreased miR-155 activity to ~60% of control values, whereas nanoparticles with no surface modification only reduced miR155 activity to ~80% of control.

The *in vivo* effect of nanoparticle delivered anti-miR-155 PNAs was tested in an oncomir-addicted murine model NesCre8; mir-155LSL^{TA} [30]. miR-155 expression in this mouse leads to disseminated lymphoma characterized by a clonal, transplantable pre-B-cell population of neoplastic lymphocytes. Withdrawal of miR-155 results in rapid regression of lymphadenopathy, which shows that these tumors are dependent on miR-155 expression. Injection of PLGA nanoparticles loaded with anti-miR-155 PNAs—either into the tumor or intravenously—provided significant delays in tumor growth. To facilitate nanoparticle uptake in tumor cells, the nanoparticles were surface modified with the peptide penetratin, a cell-penetrating peptide. The effectiveness of the nanoparticle carrier is further supported by histopathological analysis (Fig. 4). Enhanced permeability and retention (EPR) is an important feature of formulations such as nanoparticles, allowing preferential accumulation in tumor tissues as compared to normal tissues because of lack of normal architecture of tumor blood vessels. Presumably due to the EPR effect, penetratin-coated nanoparticles showed preferential targeting to tumor tissue as compared to other organs i.e lungs, liver, spleen, stomach, brain and heart. The presence of penetratin on their surface facilitated the uptake of nanoparticles and intracellular delivery of the anti-miR PNA. We note that

dependence on the EPR effect may limit the generalizability of these results, and have reviewed this issue in a recent commentary [80].

3b. Targeting mRNAs

Improved generations of PNAs have been developed where different functional groups (guanidinium, alanine, etc) are inserted at the gamma position in certain monomers, providing enhanced properties. PLGA-based nanoparticles can be used to deliver chemically-modified gamma PNAs (γ PNAs) possessing antisense properties. γ PNAs are modified PNAs where a chiral center is introduced at gamma position (Fig. 5) [81]. The presence of chirality at the gamma position provides right-handed helical characteristics to the PNAs. Biophysical and crystallographic studies have been shown that γ PNAs possess high binding affinity with complementary DNA and RNA strands because of their helicity [82, 83]. Incorporation of a single gamma monomer stabilized a PNA-DNA and PNA-RNA duplex by 3–5°C. [82] The extent of stabilization gradually increased with additional gamma monomers. In addition to conferring backbone preorganization, making it possible for PNAs to invade mixed-sequence B-DNA at physiological temperature, introduction of a chiral mini-PEG substituent at the γ -position significantly improves the water solubility and specificity of the system [84, 85].

PLGA-based nanoparticle delivery systems were used to deliver antisense molecules based on γ PNAs targeting CCR5 mRNA [29]. CCR5 is a membrane protein receptor, which is required by R5-tropic HIV-1 virus to gain entry into CD4+ T-cells [86]. When the CCR5 receptor is unavailable, HIV virus cannot engage with CD4+ T-cells to infect the cell. Using *in vitro* assays, it was demonstrated that PLGA nanoparticles loaded with γ PNAs exhibit an antisense effect that reduces CCR5 expression. PLGA nanoparticles containing γ PNAs possess uniform size distribution as evident from scanning electron microscopy images. In studies to date, PLGA nanoparticles were shown capable of delivering different γ PNAs including miniPEG as well as guanidinium containing γ PNAs. Using RT-PCR and western blot analysis, it was shown that antisense γ PNA, delivered into cells by PLGA nanoparticles, decrease CCR5 mRNA levels by about 60%.

3c. Targeting genomic DNA for gene editing

Hematopoietic stem cells are capable of self-renewal and differentiation into diverse components of blood throughout an individual's lifetime [87–89]. Genetic manipulation of hematopoietic stem and progenitor cells (HSPCs) could provide curative treatments for single-gene disorders of the blood such as beta (β) thalassemia. If successful, gene therapy could provide a cure for genetic diseases such as sickle cell anemia and thalassemia by generating blood stem cells carrying expression constructs for wild-type adult β -globin gene. However, gene modification of HSPCs remains a challenge due to their quiescent nature and relative resistance to both viral and non-viral methods to introduce foreign genetic material [90, 91]. In addition, nuclease-based technologies such as CRISPR/Cas9, ZFNs, and TALENs—which offer alternate methods for gene editing—present many challenges, particularly the risk of making unwanted changes in other parts of the genome (off-target effects) [92–94], which are not commonly found using PNAs.

Triplex-forming PNAs (TFPs) have been used to stimulate the recombination of short 50–60 bp —donor DNA fragments into genomic DNA to site specifically correct a thalassemia-causing splice site mutation at the chromosomal DNA level [95–98]. TFPs can form PNA/DNA/PNA triplexes with displacement of one strand of the duplex DNA; by binding, the TFPs produce structures that are highly provocative of DNA repair via a mechanism initiated by the nucleotide excision repair (NER) pathway (Fig. 6) [99, 100].

Most early PNAs for gene editing were designed based on using dimeric PNAs or —bis-PNAs, in which the two PNA strands (one strand for Watson-Crick pairing and the other strand for Hoogsteen pairing) are joined by a linker [101, 102]. Further, another promising design has employed tail-clamp PNAs (tcPNAs) [103–105]. In tcPNAs, the Watson-Crick binding segment in a bis-PNA is extended so that it can bind beyond the homopurine run in order to bind 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. In binding studies, tcPNAs show greater affinity and specificity compared to bis-PNAs [26, 98, 106]. Importantly, high-affinity binding to DNA by tcPNAs does not require a long homopurine run; in fact, homopurine stretches as short as 5 bp are sufficient [86]. This technology is known as —minimally invasivell gene repair, as gene modification occurs *in situ* via recruitment of the cells' own DNA repair machinery and without the need for viral vectors. We have shown that TFPs can be used to induce modification of the β -globin gene in HSPCs by stimulating recombination at the desired position in the β -globin locus with co-transfected donor DNAs that are homologous to the β -globin gene except for the base pairs to be changed [98].

PLGA nanoparticle-based delivery of TFPs and donor DNA can lead to site-specific gene editing of CD34+ HSPCs [26, 107]. Dye-loaded nanoparticles showed surprisingly efficient uptake in CD34+ HPSCs. TFPs and donor DNA were formulated into 150 nm spherical PLGA nanoparticles, with ample loading of nucleic acids (250–450 pmole/mg nanoparticles). Further, the PLGA nanoparticles loaded with TFP/donor DNA combinations stimulated genomic recombination to modify the IVS2-1 splice site within the β -globin gene. Allele-specific PCR confirmed that nanoparticle-delivered TFPs and donor DNA mediate site-specific modification in CD34+ cells. Importantly the PLGA nanoparticles with TFPs/DNA are not toxic to the progenitor cells. Progenitor cells that were genetically modified with nanoparticles were differentiated into both erythroid and neutrophil populations, without loss of the gene modification.

In another approach, PLGA nanoparticles were loaded with TFPs/donor DNA for site-specific gene editing in CCR5 gene [28]. As discussed above, CCR5 is required for HIV-1 entry into T cells. It has been reported that an HIV-1-positive individual treated by transplant of hematopoietic stem and progenitor cells from a CCR5-32 homozygous donor was cured of HIV-AIDS, with no detectable HIV-1 even after discontinuation of antiretroviral therapy for more than 5 years. In addition, individuals containing heterozygous CCR5 alleles (with only one containing the protective mutation) also have a substantially reduced disease progression rates. PLGA nanoparticles loaded with TFPs/donor DNAs for recombination-mediated editing of the CCR5 gene were formulated for delivery into human peripheral blood mononuclear cells (PBMCs). The nanoparticles efficiently entered PBMCs

without toxicity. Further, deep-sequencing revealed that a single treatment produced a ~1% gene editing frequency in the CCR5 gene and a low off-target frequency (0.004%) in the CCR2 gene. The nanoparticle treated PBMCs were efficiently engrafted into immunodeficient NOD-scid IL-2ry (–/–) mice, and the targeted CCR5 modification was detected in splenic lymphocytes 4 weeks post transplantation. After infection with an R5-tropic strain of HIV-1, humanized mice containing CCR5-NP-treated PBMCs displayed significantly higher levels of CD4+ T cells and reduced plasma viral RNA loads compared with control mice engrafted with mock-treated PBMCs, which show drastic depletion of CD4+ T cells and high levels of viremia [107].

4. PBAE: PLGA based nanoparticles for PNA delivery

PLGA is a popular component for nanoparticles because of its biocompatibility and long history of use in medicine. But PLGA formulations have limitations; for example, it is a challenge to load high levels of plasmid DNA [108]. To improve loading of oligonucleotides, cationic polymers, such as poly(beta-amino-esters) (PBAE), have been explored (Fig. 7). PBAE is synthesized by Michael-like addition of bifunctional amines to diacrylate esters, producing a cationic polymer that is also biodegradable [109]. The cationic nature of PBAE polymers enables it to condense negatively-charged oligonucleotides more efficiently than PLGA. Additionally, the cationic surface density of PBAE materials also acts as a buffer in the low pH environment of endosomes, which further leads to its disruption, and release of the encapsulated molecules. However, because of its cationic character, PBAE is toxic to many cells. Blends of PLGA and PBAE can be used to produce nanoparticles that are less toxic than those with PBAE alone. For example, microparticle delivery systems composed of 15% to 25% PBAE with PLGA result in controlled release of plasmid DNA to antigen presenting cells (APC) *in vivo*.

PLGA/PBAE based nanoparticles have been used to deliver small molecules like paclitaxel to MCF7 tumor bearing mice in a sustained manner [110]. In addition, it has been noticed that PLGA/PBAE nanoparticles increase the retention time of drug at tumor sites by reducing its clearance rate. PLGA/PBAE blends can also be used to administer two small molecules, paclitaxel and c6 ceramide, in a single formulation in a very effective manner.

4a. Targeting genomic DNA for gene editing

PBAE:PLGA nanoparticles have been employed for delivery of gamma PNAs for site specific gene editing. A transgenic murine model containing a β -globin/GFP fusion gene, consisting of intron 2 of human β -globin inserted within the GFP coding regions, was used to test these materials [27, 111]. The intron contains the IVS2–654 (C->T) mutation, which is a common cause of thalassemia in individuals of Southeast Asian heritage. The β -globin/GFP fusion gene in the mice contains a cryptic splice site that causes incorrect splicing of the intron and prevents GFP expression [8, 9, 112]. For *ex vivo* and *in vivo* delivery to mouse bone marrow cells, mini-PEG modified gamma PNAs and 60 nt donor DNAs were loaded into PLGA/PBAE nanoparticles. In comparison to the standard regular PNAs/donor DNA mixtures, mini-PEG modified gamma PNA/donor DNA mixtures showed substantial improvements in the encapsulation and subsequent release of nucleic acids. In

gene targeting assays, miniPEG modified gamma PNA/donor DNA containing PBAE:PLGA nanoparticles showed site-specific genome editing of the IVS2–654 (C->T) mutation site *in vivo* in mouse bone marrow at a frequency of 0.1%, with an off target frequency at a partially homologous site more than 10,000 fold lower. In addition, no toxicity was observed with multiple injections of PBAE:PLGA nanoparticles. Moreover, *ex vivo* treatment of mouse bone marrow cells revealed a gene correction frequency of 0.8% with a single treatment of PBAE:PLGA nanoparticles.

When PBAE/PLGA and PLGA nanoparticles were compared for DNA delivery, it was found that 15% PBAE:PLGA system are the most effective [113]. To reduce the toxicity and increase uptake into target cells, PBAE:PLGA particles were surface-modified with various CPPs via a PEGylated phospholipid linker (DSPE-PEG2000). Out of a number of CPPs tested, MPG decorated nanoparticles provided the most improved loading and transfection efficiency in CFBE cells.

MPG coated PBAE/PLGA nanoparticles are also effective in the *in vivo* delivery of C6 coumarin molecules in the lungs of mice [111]. Using the transgenic reporter mouse described above, it was demonstrated that MPG coated PBAE:PLGA nanoparticles effectively deliver triplex PNAs/ donor DNA to airway cells in the lung: MPG-coated PBAE:PLGA nanoparticles delivered PNAs induce gene editing efficiency of up to 0.6 % in the alveolar epithelial cells and 0.3 % in the lung macrophages.

PBAE:PLGA nanoparticles were used to deliver tcPNA and donor DNA to the lung to correct a mutation associated with cystic fibrosis (CF) [31]. CF is a lethal genetic disorder most commonly caused by the F508del mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. PNAs provide the opportunity for a different, safer strategy: tcPNAs and donor DNA molecules loaded into MPG-coated PBAE/PLGA based nanoparticles corrected the F508del CFTR mutation *in vitro* in human bronchial epithelial cells and *in vivo* in a CF mouse [31]. *In vitro*, after a single treatment, approximately 7% of CFBE cells treated with nanoparticles demonstrated Cl⁻ efflux similar to control animals (without a CF gene defect). When these CFBE cells were treated repeatedly with nanoparticles, 25% of the cells demonstrated Cl⁻ efflux equivalent to positive controls. *In vivo*, after intranasal delivery of tcPNAs and donor DNA loaded PBAE:PLGA nanoparticles, CF mice exhibited changes in the electrochemical potential of the nasal epithelium that indicated successful restoration of CFTR function.

5. Other Polymer based delivery of PNAs

In addition to PLGA and PBAE-based delivery systems, other nanoparticle formulation methods have been also tested for PNA delivery. Promising results have been shown in formulating and encapsulating PNAs, as well as delivery *in vitro*. *In vivo* delivery of nanoparticles remains to be tested, however.

5a. Avidin-labeled protein nanoparticles for PNA delivery

Kreuter and coworkers have shown that protein based nanoparticles can be engineered using sulfhydryl-based chemistry for the delivery of 15mer biotinylated antisense PNA targeting

the pol gene of HIV type 1 and 2 [32]. The protein nanoparticles were produced by a double desolvation procedure, and then sulphhydryl residues were introduced on the nanoparticle surface by reacting the free amines with Traut's reagent (2-imino thiolate). Activated NeutrAvidin was coupled to the nanoparticles via a thioether linkage between the sulfhydryl groups and the sulphhydryl reactive maleinimide group of the activated NeutrAvidin. Further biotinylated PNAs were loaded by incubation with the avidin nanoparticles followed by ultracentrifugation. Hence, protein nanoparticles can be engineered using biotin-avidin interactions to generate stable and effective carriers for biotinylated PNAs.

5b. Zeolite –L-nanocrystal mediated PNA delivery

Cola and coworkers have explored inorganic materials to simultaneously deliver PNA and organic molecules into the cells. Porous zeolite-L-nanoparticles were used to deliver a model drug and a PNA to living cells [33]. The PNA used in the nanoparticles was complementary to a DNA sequence bearing a single-point mutation (W1282X) implicated in human CF. Briefly, the zeolite-L-crystals were filled with fluorescent guest molecules and the outer particle surface was functionalized with (3-aminopropyl)triethoxysilane (APTES). The amine groups were converted into carboxylic acid groups which were further converted into NHS esters, so that the PNA probes H-(AEEA) 2 -CTTTCCTTCACTGTT-NH 2 (AEEA = 2-(2-aminoethoxy) ethoxyacetyl spacer) could be covalently attached via acyl coupling reactions. Finally a thin coating with poly-L-lysine (PLL), a cationic polymer, was added, in order to favor cell uptake. The PNA retained its DNA/RNA-binding activity when attached to the zeolites. By imaging after exposure to HeLa cells, it was observed that the PNA-functionalized zeolites exhibited rapid uptake in the cells. Further it was evaluated that these PNA-zeolite particles did not exert any toxic effect on the cells. Hence, zeolite nanosystems can be used to deliver specific PNA sequences with a combined drug in living cells opening new possibilities for bioimaging and drug delivery

5c. Fluorescent Mesoporous Silica nanoparticles deliver antisense PNAs

Another interesting approach to deliver antisense PNAs is based on mesoporous silica nanoparticles (MSNP) [34]. In an attempt to silence the Bcl-2 protein, which decreases cell apoptotic cell death and causes resistance in chemotherapy, antisense PNA sequences complementary to the first six codons of Bcl-2 mRNA were designed. MSNP were formulated for intracellular delivery of this PNA. The PNA was covalently conjugated with the MSNP via amidation between the amino group of PNAs and carboxylic group on MSNP surface (Fig. 8). The release of PNA from the MSNP depended on the cleavage of disulphide bond embedded on the MSNP. Due to the presence of an intracellular reducing environment—provided by intracellular glutathione (GSH)—the disulfide bond was cleaved facilitating the release of PNA inside cancer cells. Using this system for redox-triggered PNA release, it was shown that intracellular uptake effectively silenced Bcl-2 expression *in vitro*.

5d. Psi nanoparticles for PNA delivery

Duvall et al. reported an interesting strategy for intracellular delivery of an anti-miR PNA using porous silicon (Psi) [114]. Proof of concept was illustrated using a well-characterized anti-miR-122 PNA [76]; the 23-mer anti-miR-122 PNA targets a liver-specific miRNA, the suppression of which leads to decrease in hepatitis C viremia [115]. The PNA was

synthesized *in situ* from Psi substrate leading to the formation of anti-miR-122 PNA loaded multilayer Psi films. For delivery into the cytoplasm, these films were ultrasonically fractured to produce PNA functionalized Psi nanoparticles (PSNP). The activity of this complex was tested using Huh 7 human hepatic carcinoma cells expressing increased levels of miR-122. Cells treated with PNA-PSNP exhibited higher intracellular delivery of PNA as compared to those treated with free PNA, with no reduction in cell viability. Using a luciferase based reporter assay [116], PSNP delivery of 2 μ M PNA resulted in significant inhibition of miR 122 in Huh7 cells as evident from 44 % increase in luciferase activity.

5e. Cationic shell-cross-linked knedel-like (cSCK) nanoparticles for PNA delivery

Another PNA delivery approach uses cationic shell cross-linked knedel-like nanoparticles (cSCK) [35–38]. cSCK nanoparticles consist of a hydrophobic core and a positively charged highly functionalizable cross-linked shell. SCKs belong to a large family of cross-linked block copolymer micelles in which shape, composition, and functionality can be tailored to their particular utility for applications [117]. To formulate cSCK, amphiphilic block copolymers are synthesized: for example, polystyrene blocks linked to a poly(acrylic acid) segment were used in one study. Coupling the carboxylic acids to monoprotected diamine, followed by deprotection, generates primary amines. These amines are largely protonated at neutral pH 7, leading to the formation of a micelle which consists of a hydrophobic polystyrene core and a hydrophilic, positively charged shell. The micelle is stabilized by covalently cross-linking of the shell by amide formation between chains with an activated diester. cSCKs were complexed by electrostatic interactions with negatively charged PNA-ODN (DNA oligonucleotide) hybrid, and via covalent attachment of a PNA through a bio-reductively cleavable disulfide bond. These PNA-conjugated cSCKs facilitated endocytosis and endosomal release of the PNAs into Hela cells. To test the activity of these constructs, a luciferase splice correction assay developed by Kole and coworkers [118] was used. In this assay, a luciferase gene (pLuc705) results in a longer, mis-spliced mRNA, which encodes a defective luciferase. The designed PNA was complementary to the aberrant splice site and upon binding of the PNA, splicing is restored leading to shorter mRNA and an active luciferase. In Hela cells, the cSCK-PNA.ODN conjugates resulted in three times higher bioactivity and two-fold lower toxicity than commercially available Lipofectamine 2000. Likewise, the bio-reductively cleavable cSCK-SS-PNA₂ conjugates also showed superior properties demonstrating the utility of these nanoparticles.

The potential *in vitro* and *in vivo* applications of cSCK nanoparticles for PNA delivery were demonstrated using inducible nitric oxide synthase (iNOS) mRNA targeting for improved diagnostic and treatment strategies for acute lung injury (ALI) [35]. ALI is a life threatening medical condition characterized by widespread inflammation in the lungs [119]. iNOS, an enzyme abundant in activated lung macrophages, is one of the mediators commonly upregulated in ALI. Increased expression of iNOS in macrophages produces nitric oxide (NO), which results in tissue damage [120, 121]. Inhibition of iNOS reduces the accumulation of NO leading to improved outcome from ALI in experimental models [122]. An iNOS imaging probe was prepared by electrostatic complexation between a radiolabelled antisense PNA-YR₉. oligonucleotide (ODN) hybrid and cSCK nanoparticle [35]. The PNA was labeled with ¹²³I and an R₉ (arginine₉) peptide was used to facilitate the exit of

untargeted PNA from the cell. The radiolabelled, antisense PNA showed significant sequence-specific retention in both iNOS expressing cells *in vitro* and in mouse lung as compared with control mismatch PNA.

In another study, cSCK nanoparticles were used to deliver PNA-based strand displacement probes for live-cell imaging of iNOS mRNA [39]. Subsequently, this cSCK nanoparticle was used for delivery of antisense PNA. DNA binary FRET probes for both *in vitro* and *in vivo* imaging [38].

6. Conclusions

Since its discovery in 1991, PNA technology has been employed in diverse biomedical applications. Although PNA has shown promise in nucleic acid based therapeutics, intracellular delivery of PNA is a major issue that needs to be resolved for clinical applications. Numerous peptide based methods have been deployed for delivery of PNAs in cells, but this work remains hampered by toxicity and lack of specificity. Because of this, recent attention has focused on nanoparticle-based delivery methods, which offer the potential for targeted and sustained delivery of PNA-based therapeutics. PLGA and PBAE:PLGA nanoparticles have been shown to deliver PNAs into the cytoplasm and nucleus of cells, which enables antisense and gene editing by PNAs. In addition, PLGA and PBAE:PLGA nanoparticles are non-toxic after multiple injections systemically in mice. Recently, some promising results have been shown for other delivery materials such as zeolite and mesoporous silica for encapsulation of PNAs. Still comprehensive *in vivo* testing need be performed to evaluate the toxicity profile of these new materials. Hence, we believe that engineered nanocarriers, perhaps decorated with targeting ligands, will open new avenues for diagnostic and therapeutic applications of PNAs.

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Fig. 1.
Chemical structures of DNA (RNA) and PNA

Fig. 2.
Different delivery methods of PNA

α GPNA γ GPNA

Fig. 3.
Chemical structures of α GPNA and γ GPNA

Fig. 4.
 (A) Change in tumor volume after single local injection of ANTP coated nanoparticles delivering anti-miR-155 PNA molecules. (B) H & E analysis of locally treated subcutaneous lymphoid tumors. Scale bar represents 100 μ m for both low and high magnification images [30]

Fig. 5.
New generation gamma PNAs

Genomic DNA containing mutation site

“Donor DNA” and Triplex forming oligos targeting the genome

Genomic DNA containing corrected base pair site

Fig. 6.
Gene editing strategy using triplex forming oligonucleotides.

Poly (lactic-co-glycolic) acid (PLGA)

Poly (beta amino) ester (PBAE)

Fig. 7.
Chemical structures of PLGA and PBAE units.

Fig. 8.
Description of conjugation of PNA with FITC labeled MSNP and the release of Cy5 labeled PNA by redox-triggered mechanism inside the cells [34].

Table 1

Nanoparticles for delivery of PNA

Nanoparticles	Applications	Study design	Ref
PLGA	Gene editing	Cell culture (CD34+ and THP1) Preclinical (in mice)	[26–28]
	Antisense	Cell culture (THP1 and HeLa)	[29]
Peptide coated PLGA	Antisense	Preclinical (in mice)	[30]
PBAE/PLGA	Gene editing	Preclinical (in mice)	[27]
MPG coated PBAE/PLGA	Gene editing	Preclinical (in mice)	[31]
Avidin-labeled nanoparticles	Delivery vehicle	Preliminary characterization	[32]
Zeolites-L-Nanocrystals	Delivery vehicle	Cell culture (HeLa)	[33]
Mesoporous silica nanoparticles	Antisense	Cell culture (HeLa)	[34]
Cationic shell cross-linked knedel-like nanoparticles	Antisense	Preclinical (in mice)	[35, 36]
	Splice correction	Cell culture (HeLa)	[37]
	Imaging mRNAs	Cell culture (RAW 264.7)	[38, 39]
Gold nanoparticles	RNA detection	Cell culture (Vero cells)	[40]
Cobalt ferrite core/metallic shell	PNA/DNA based biosensors	Solution based <i>in vitro</i> assay	[41]

Table 2

Peptides used for delivery of PNA

Name of the peptide	Sequence of the peptide	Ref
Penetratin	RQIKIWFQNRRMKWKK	[54, 55]
Transportan	GWTLSAGYLLGKINLAALAKKIL	[56]
Retro-inverso penetratin	(D)-KKWKMRNRFWIKIQR-(D)	[57]
Phenyl leucine	FLFLFL	[23]
Nuclear localization peptide	PKKKRKV	[57]
Cell wall/Membrane active Peptide	KFFKFFKFFK	[60]
Tat peptide	GRKKRRQRRPPQ	[58]
Polylysine	KKKK	[23]
pH low insertion peptide (pHLIP)	AAEQNPYWARYADWLFTTPLLLL DLALLVDADEGTCG	[18]