Chapter 17

Poly(Lactic-*co*-Glycolic Acid) Nanoparticle Delivery of Peptide Nucleic Acids In Vivo

Stanley N. Oyaghire, Elias Quijano, Alexandra S. Piotrowski-Daspit, W. Mark Saltzman, and Peter M. Glazer

Abstract

Many important biological applications of peptide nucleic acids (PNAs) target nucleic acid binding in eukaryotic cells, which requires PNA translocation across at least one membrane barrier. The delivery challenge is further exacerbated for applications in whole organisms, where clearance mechanisms rapidly deplete and/or deactivate exogenous agents. We have demonstrated that nanoparticles (NPs) composed of biodegradable polymers can encapsulate and release PNAs (alone or with co-reagents) in amounts sufficient to mediate desired effects in vitro and in vivo without deleterious reactions in the recipient cell or organism. For example, poly(lactic-*co*-glycolic acid) (PLGA) NPs can encapsulate and deliver PNAs and accompanying reagents to mediate gene editing outcomes in cells and animals, or PNAs alone to target oncogenic drivers in cells and correct cancer phenotypes in animal models. In this chapter, we provide a primer on PNA-induced gene editing and microRNA targeting—the two PNA-based biotechnological applications where NPs have enhanced and/or enabled in vivo demonstrations—as well as an introduction to the PLGA material and detailed protocols for formulation and robust characterization of PNA/DNA-laden PLGA NPs.

Key words Peptide nucleic acid (PNA), Poly(lactic-co-glycolic acid) (PLGA), Nanoparticles (NP), Gene editing, Anti-microRNA (antimiR)

1 Introduction

1.1 Primer on PNA-Induced Gene Editing

Appropriately designed PNAs trigger repair activity after binding to their target sequences in genomic or episomal DNA, on the scale of those elicited by more overt, direct forms of DNA damage, presumably due to tight DNA binding and consequent helical distortion [1]. The repair-associated DNA syntheses, in turn, lead to DNA modification within [2, 3] or proximal [3] to the PNA binding site. Further, we have demonstrated that this exogenously induced but endogenously controlled DNA metabolism can result in gene disruption [2–5] due to stochastic repair events or precise gene modification [1, 6–17] when templated by a donor DNA

Peter E. Nielsen (ed.), *Peptide Nucleic Acids: Methods and Protocols*, Methods in Molecular Biology, vol. 2105,
https://doi.org/10.1007/978-1-0716-0243-0_17,

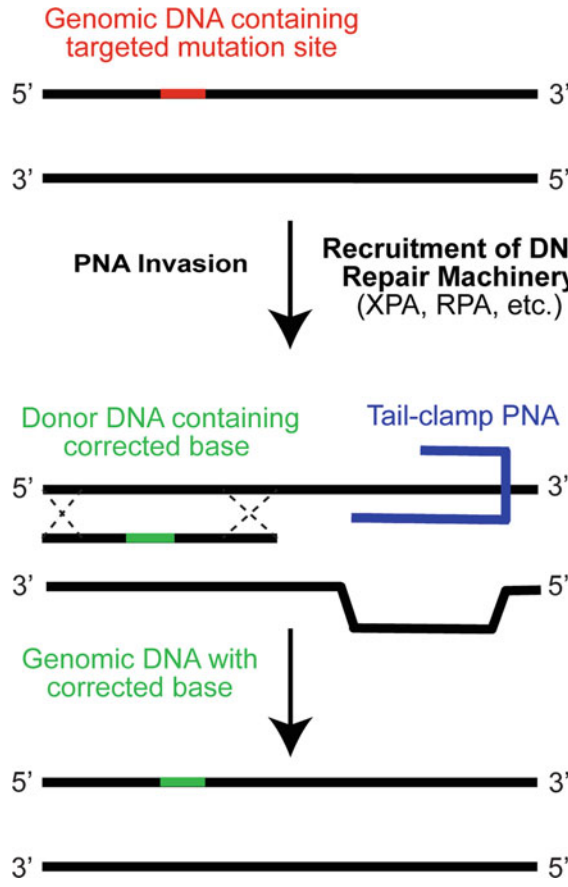

Fig. 1 Gene editing by triplex-forming oligonucleotides. PNAs stimulate recombination of short (60 bp) DNA fragments into genomic DNA. Binding of the PNA subsequently produces a structural change within the dsDNA that activates cellular repair mechanisms, which are initiated by nucleotide excision repair (NER)

oligomer introduced with the PNA (Fig. 1). The precision of this latter application has been leveraged by our group to correct pathologic mutations in disease-related genes [6, 7, 9, 15–17], and introduce nonnatural but benign (and in some contexts, beneficial) genomic modifications [8, 10, 12] in normal genetic backgrounds (Fig. 1).

A survey of the range of PNA designs deployed for gene modification has been covered elsewhere in recent reviews [18, 19]. Here, we summarize (Fig. 2) the PNA structural space explored for gene *correction* in our lab, in part because PLGA NP-assisted delivery of PNA has been demonstrated for this application in several reports [9, 10, 13–17]. As proof of principle for PNA-induced gene correction, we first reported that a **bisPNA** oligomer—a PNA structural variant [20, 21] possessing unique tethered domains designed to recognize the Watson-Crick and

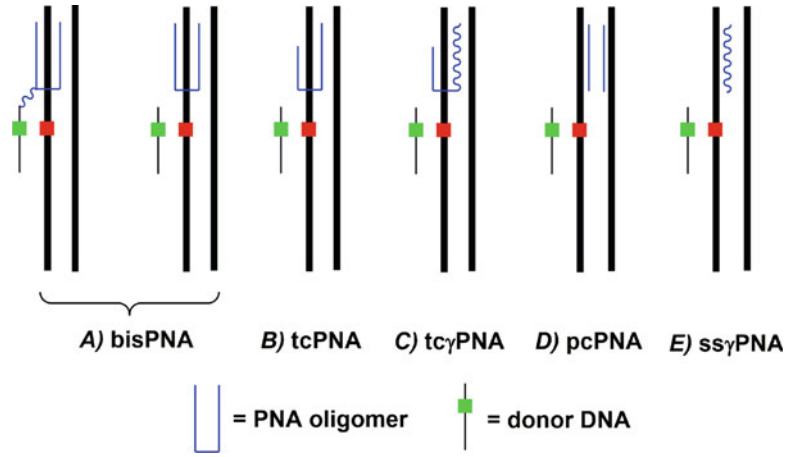

Fig. 2 PNA design variations applied for gene correction. (**a**) bisPNA, (**b**) tail-clamp (tc) PNA, (**c**) tail-clamp gamma (γ) PNA, (**d**) pseudo-complementary (pc) PNA, (**e**) single-stranded (ss) γPNA

Hoogsteen faces of purine-rich DNA sequences—can stimulate recombination reactions between an episomal DNA target and a donor DNA oligomer [1] (Fig. 2a). In this demonstration, the bisPNA was directed toward a purine-rich binding site within the coding sequence for a reporter gene harboring an inactivating mutation and was tethered to or uncoupled from a donor DNA (Fig. 2a) designed to restore reporter activity by recombination-induced transversion of the mutation [1]. Although of limited therapeutic utility, this work has had profound implications on subsequent applications of this gene editing technology by establishing foundational parameters for PNA/DNA design. Namely, the finding that a PNA/donor DNA reagent pair was more effective for gene correction when both oligomers were untethered from (instead of conjugated to) each other has simplified PNA/DNA preparation and design while also extending this technology to applications where the PNA binding and donor DNA target sites are relatively distal [1]. However, the requirement for simultaneous introduction of separated reagents reinforces the delivery challenge inherent in this strategy, since optimal results will require co-delivery of two components with significantly different chemical properties.

The same bisPNA targeting strategy has been used to stimulate recombination reactions in the β-globin gene (*HBB*) [6, 9], in which pathologic mutations underlie the primary pathophysiology of β-thalassemia and sickle cell disease. Several bisPNA oligomers, directed to different purine stretches in intron 2 (IVS2) of *HBB*, were shown to be useful for stimulating recombination between the gene and a donor DNA designed to correct a thalassemia-associated mutation at position 1 (hence IVS2-1) of the target

intron [6]. In addition to demonstrating the feasibility of this correction paradigm in a genomic, endogenous, disease-relevant target, we also reported that gene correction frequencies were enhanced by chloroquine treatment subsequent to nucleofection—presumably due to lysosomal disruption [6] and ostensibly improved PNA/DNA bioavailability. This result again suggested that reagents capable of delivering PNA/DNA oligomers to specific intracellular compartments where they are active, or at least diverting them away from ineffective compartments, will be useful additions to this platform.

Tail-clamp PNA (tcPNA) oligomers [22] have also been utilized to stimulate recombination reactions within *HBB* IVS2 [16] (Fig. 2b). In contrast to bisPNA oligomers, which feature Watson-Crick and Hoogsteen binding domains of equal length, this targeting modality incorporates into the PNA an extended Watson-Crick binding domain that enhances target duplex invasion [22]. Already demonstrated to significantly enhance donor recombination events in endogenous chromosomal targets significantly above those obtained with bisPNAs [8] (presumably due to enhanced duplex invasion and consequent helical distortion), we reported that tcPNA ligands directed proximal (~70–200 bp) to the location of another thalassemia-associated mutation in IVS2 were able to stimulate recombination-induced correction by apposite donor DNAs [16]. This targeting and correction modality was enhanced even further by the introduction of gamma (γ) PNA residues [23, 24] into the Watson-Crick binding domain of the tcPNAs, thus creating **tcγPNAs** [16] (Fig. 2c). When incorporated intermittently or completely into PNA oligomers, γPNA monomers—which feature chemical substituents in the γ position of the monomer backbone—impose conformational selection in the composite oligomers, the nature of which is determined by the stereochemistry at the γ position [23, 24]. Several reports from Ly and coworkers establish that γPNA monomers of appropriate stereochemistry can preorganize composite PNA oligomers into right-handed helices [23, 24] that are more effective for duplex DNA strand invasion [25], in addition to other benefits (such as solubility [24]) determined by the chemical nature of the γ substituents themselves. The judicious introduction of γPNA monomers into an already active tcPNA (in addition to other important improvements) led to an important demonstration of the therapeutic utility of our gene correction paradigm (*see* Subheading 1.3).

Although less extensively explored, we have also demonstrated that pseudo-complementary (pc) PNA oligomers [26] are useful reagents for mediating PNA-induced gene correction [7] (Fig. 2d). By engaging both strands of target DNA duplexes [26], **pcPNAs** can overcome the targeting restriction imposed by bis/tcPNAs: the requirement for pronounced asymmetry in the strand distribution of purines and pyrimidines for effective invasion. (In the context of

DNA repair induction, it is possible that dual-strand engagement yields stronger helical distortion and higher repair/recombination than single-strand targeting, although this theory is yet to be systematically evaluated.) We have reported that pcPNAs stimulate recombination of a donor DNA into *HBB* IVS2-1 at modest frequencies [7]. Here, as with bisPNA-induced editing, the observation that correction frequencies were marginally improved by chloroquine treatment [7] suggests that simultaneous delivery of all requisite co-reagents (2 pcPNAs + 1 donor DNA) will be an important challenge to address. Appropriately designed single-stranded (ss) PNAs—defined here as PNA oligomers for which target hybridization is mediated by only a single domain and engaging only one strand of the duplex—are also effective reagents for gene correction [13]. In this context, we have reported that ssPNAs can stimulate donor recombination into position 654 of *HBB* IVS2. Although requiring, in this example, γ-modifications [hence **ssγPNA** (Fig. 2e)] to achieve even modest levels of correction [13], this targeting modality offers the potential to overcome the sequence limitations of other targeting modalities, while significantly simplifying reagent design.

1.2 The Imperative of Nanoparticle-Mediated PNA/DNA Delivery for Gene Correction

While exploration of PNA variants useful for inducing gene correction should continue, even greater gains in editing efficacy—defined here as the yield of modified cells posttreatment—have been obtained by nanoparticle-assisted delivery of existing reagents. One demonstration was provided almost 10 years ago, in work by McNeer et al. [9], wherein bisPNA and donor DNA oligomers targeting *HBB* IVS2-1 were delivered by PLGA NPs or optimized nucleofections to primary human $CD34^+$ cells—a population of interest in many gene-targeting therapeutic programs. Our results indicated that the nucleofection protocol itself decreased cell viability ~40%, 24 h after treatment—with toxicity increasing to 60% at 72 h. Introduction of the requisite PNA/DNA oligomers into the nucleofection cocktail was even more deleterious and increased nucleofection-associated toxicity to ~80% and 90% at 24 h and 72 h, respectively [9]. In contrast, essentially no toxicity was observed when the cells were exposed to PLGA NPs possessing no cargo (blanks) or PNA/DNA oligomers, at either time point. Further, while cells in all treatment groups, for either delivery method, showed a time-dependent attrition in CD34 expression—presumably due to spontaneous differentiation in culture—marker depletion was more rapid for nucleofected samples [9]. While the mechanisms of PLGA NP-mediated delivery of PNA/DNA oligomers are still being delineated, it is clear from our data that this transfection method is less inimical to cell viability than the transient membrane distortions created during nucleofection [27].

Further, the intended modifications were detected in genomic DNA isolated from cells exposed to reagent-laden PLGA NPs at 3, 8, and 30 days posttreatment, demonstrating that these reagents, although benign, remain effective for delivering active PNA/DNA oligomers to the nucleus. Importantly, the induced modification frequencies detected in NP-treated cells were ~seven- fold higher than those in nucleofected cells. Conflated with the survival advantage (on day 3), this superiority translates to ~63-fold enhancement in editing efficacy mediated by improved delivery of PNA/DNA reagents [9].

While even more seminal demonstrations of gene correction/modification by PNA/DNA-loaded PLGA NPs are described in the next section, it is worth examining some of the important considerations arising from the data summarized above: (1) even with its associated toxicity, nucleofection remains a transfection protocol of choice in many applications of gene editing, including those involving the nuclease-based reagents that produce higher modification frequencies than those induced by PNA [28]. (2) Consequently, for many applications, the realistic goals are to modify stem cells ex vivo, select for the modified population, and transplant into patients [29, 30]. Achieving any/all of these goals will require that treated cells survive long enough and in large enough quantities for additional manipulation posttreatment [29, 30]. (3) Accruing evidence that cells have potent mechanisms [31, 32] to detect and destroy exogenous nucleic acids entails that the transfection protocols for gene editing reagents must conceal them from such surveillance mechanisms long enough for the intended DNA metabolism to occur. The evidence suggests that reagent-laden PLGA NPs circumvent these challenges, as their introduction to cells in culture perturb neither cell survival, proliferation, differentiation capacity, nor lineage commitment [9]. Moreover, the reagents remain immunologically inert [11, 15–17], evading innate and/or humoral immune mechanisms, possibly because they physically (if transiently) conceal their cargo from surveillance pathways and release them in controlled amounts beneath detection thresholds.

1.3 In Vivo Demonstrations of PNA/DNA-Induced Gene Editing Enabled by PLGA NPs

The biologically benign properties of PLGA NPs and editing precision achievable with PNA/DNA oligomers in vitro have incentivized the extension of this gene modification paradigm to proof-of-principle studies in humanized mouse models and therapeutic demonstrations in genetically engineered animal models that conservatively recapitulate human disease pathology. In early work, we demonstrated that PLGA NPs containing tcPNA and donor DNA oligomers previously designed and demonstrated to target and introduce stop codon mutations in human *CCR5* [8]—mimicking a naturally occurring genotype [33] associated with R5-tropic HIV1 resistance—were able to mediate *CCR5* modification in

1.3.1 Modification of CCR5

engrafted human cells in mice [11]. Although occurring at relatively low frequencies (0.4% in spleen; 0.05% in bone marrow), the targeted modifications were also detected in bone marrow cells from secondary recipient mice previously engrafted themselves with cells from NP-treated donor mice [11]. These results demonstrated that the initial NP treatments (in donor mice) were able to achieve targeted modifications in hematopoietic compartments populated by primitive stem cells that can persist, proliferate, and populate the hematopoietic system of nominally untreated recipients. Such demonstrations are crucial in the context of therapeutic gene editing, since, as alluded to above, the realistic goals of ex vivo manipulation and transplantation of autologous stem cells will be effective only if modifications occur in primitive cells without compromising their viability, proliferation, pluripotency, and engraftment—already a keen challenge for otherwise highly effective reagents [34, 35].

Expectedly, the genomic modification mediated by the reagent-laden NPs resulted in expression of an altered mRNA transcript in lung samples from treated humanized mice [11]. The functional relevance of this genomic modification and associated mRNA alteration was further demonstrated [11] by NP treatment of mice engrafted with peripheral blood mononuclear cells isolated from individuals heterozygous for the Δ32 mutation—the naturally occurring *CCR5* deletion mutation conferring resistance to HIV-1. Following HIV-1 infection, mice receiving reagent-laden NPs showed T-cell retention at levels significantly higher than those receiving blank NPs, validating that PNA/DNA-mediated editing enabled by PLGA NP delivery led to increased resistance to HIV1-mediated T-cell cytotoxicity [11]. While the therapeutic implications of such findings for AIDS prevention/treatment are clear, the mechanistic implications are especially salient, as they suggest that NPs can deliver the active reagents to potentiate editing outcomes in circulating definitive $CD4^+$ T-cells and/or primitive $CD34^+$ cells in bone marrow (that can themselves differentiate into T-cells).

1.3.2 Gene Correction in Cystic Fibrosis Models

In vivo correction of F508del, the trinucleotide deletion mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that causes protein instability and is a predominant genotype responsible for cystic fibrosis (CF) lung disease [36] has been achieved by intranasal administration of NPs conveying apposite tcPNA and donor DNA oligomers [15]. The polymeric material deployed in this example was based primarily but not entirely on PLGA, and the resulting particles were further modified with a cell-penetrating peptide harboring a nuclear-localization sequence—adjustments which were shown to enhance delivery of plasmid DNA to primary human lung cells in vitro and improve PNA/DNA-mediated correction in a reporter model in vivo [14].

Intranasal administration of reagent-laden NPs to CF mouse models resulted in correction frequencies of ~6% in the nasal epithelium and ~1% in the lung [15], modification frequencies at least an order of magnitude higher than those reported by us for previous demonstrations in vitro. Moreover, the increased efficacies, mediated in part by iterative NP treatments, did not coincide with any increases in inflammatory cytokines or histological changes in modified tissues [15], suggesting that in vivo editing efficacies can be enhanced for these reagents in a manner that remains innocuous to recipients. Importantly, the elevated genotypic correction was functionally relevant, as nasal potential difference readings (an indication of chloride efflux, the primary function of CFTR) for CF mice homozygous for the F508del mutation receiving loaded NPs approached the wild-type range, in contrast to mice receiving blank NPs [15].

1.3.3 Gene Correction in β-Thalassemia Models

Correction of a β-thalassemia-associated polymorphism in a mouse model of the disease represents our clearest demonstration of the therapeutic utility of PNA-mediated gene editing. Delivered in PLGA NPs, appropriately designed tcγPNA and donor DNA oligomers were shown to mediate reversion of mutant *HBB* alleles in vivo without genotoxic or deleterious immunological outcomes [16]. NP-mediated delivery, as extrapolated from the detection of allele correction potentiated by the nucleic acid encapsulants, was achieved in total bone marrow cells, including those possessing markers characteristic of primitive progenitors [16], which are difficult to transfect [37] but remain imperative for any modality of gene therapy or engineering in the context of β-thalassemia [29]. Importantly, successful transfection of and allele correction in hematopoietic components populated by primitive stem cells led to remediation of various hematological and anatomical disease phenotypes [16], demonstrating that modest correction frequencies (~4%), if directed toward the right cell populations by the delivery vehicle, can be therapeutically effective.

This latter point has been emphasized in our most recent demonstration of therapeutic PNA-mediated correction in utero, wherein PNA/DNA-loaded PLGA NPs delivered intravenously to mouse fetuses mediated genotypic and phenotypic correction of β-thalassemia in resulting pups, with the therapeutic effects persisting into adulthood [17]. We showed that this delivery route, at the appropriate gestational age in mice, could direct NPs to the fetal liver [17], a prominent site of hematopoietic stem cell (HSC) expansion during murine hematopoiesis. Coupled with other factors, such as high basal expression levels of repair factors relevant to PNA-induced editing in HSCs [16], this targeting of NPs to cell populations amenable to correction and relevant for the disease led to allele modification frequencies (~6%) higher than those (~4%) obtained by iterative administration in adult models [16], despite requiring a single, smaller NP dose [17].

1.4 In Vivo Applications of AntimiR PNAs Enabled by PLGA NPs

1.4.1 Primer on AntimiR PNAs

MicroRNAs (miRNAs) are short (22 nt long) RNAs that are endogenously expressed and regulate mRNA expression [38]. Following transcription, as well as nuclear and cytoplasmic processing, a mature miRNA binds to an RNA-induced silencing complex (RISC). The miRNA in turn acts as a molecular guide, targeting complementary mRNA for degradation or translational repression [39]. Though once poorly understood, miRNAs have now been implicated in diverse processes including embryological development, cellular differentiation, and cancer. These broad roles have led to a boom in miRNA therapeutics, including ones based on PNA technology [40]. Yet rather than replenish therapeutic miRNAs (miRNA mimic), PNAs have been primarily used to suppress the effects of aberrantly expressed, oncogenic miRNAs.

The first reported use of a PNA as an antimiR was by Fabani and Gait, who synthesized PNAs complementary to miR-122. Using in vitro models of hepatocellular carcinoma, they demonstrated that PNAs with four terminal lysine residues could sufficiently inhibit miR-122 activity in cells [41]. Using a terminal cysteine to conjugate the PNA to R_6-penetratin, they further showed that cell-penetrating peptides (CPPs) could be used to deliver antimiR PNAs into cells, resulting in lower levels of endogenous miR-122 and increased expression of target genes [41]. The use of CPPs to deliver antimiR PNAs was further expanded by Oh et al., who systematically evaluated CPPs to deliver PNAs targeting miR-21 [42]. Using reporter plasmids transfected into cells, the group found that TAT-modified peptides were more effective than R_6-penetratin in delivering antimiR-21 PNA [42]. While both of these examples demonstrated the effectiveness of antimiR PNAs, their clinical translatability was limited by the need for high doses in vitro (1 μM) [41, 42]. To overcome these limitations, our group has recently focused on encapsulating antimiR PNAs into PLGA NPs, which have a history of being effective in vitro and in vivo at significantly lower doses [43].

1.4.2 NP-Mediated Delivery of AntimiR-155 PNA

miR-155 is a critically important oncogenic miRNA, which has been shown to be upregulated in solid tumors of the lung, liver, kidney, gliomas, and pancreas, as well as B cell lymphoma and lymphoid leukemia [40]. Molecularly, miR-155 targets the SH2 domain-containing inositol 5′-phosphatase 1 (SHIP1) protein, reducing its expression through translational inhibition [44]. The resulting reductions in SHIP1 have now been implicated in the onset of acute myeloid leukemia (AML), as well as large B cell lymphoma [44]. Another group has also shown that miR-155 expression is essential for the survival of malignant lymphocytes in a mouse model of lymphoma [45].

Using PNAs targeting mature miR-155, we developed a method to encapsulate antimiR PNAs into PLGA NPs [43]. Although traditional oligonucleotide encapsulation is

improved by the use of counterions to condense RNA- or DNA-based molecules, the use of charge-neutral PNAs, with slightly hydrophobic characteristics, enabled efficient loading into PLGA without complexation [43]. The use of polymeric NPs, rather than CPPs, provided further advantages by creating a reservoir of antisense molecules that are released over time after administration, extending inhibitory effects beyond the initial dose. Using a mouse model of miR-155-dependent lymphoma, antimir-155 PNA NPs inhibited miR-155 in vitro and in vivo [45]. NP-mediated depletion of miR-155 also significantly reduced tumor volumes when administered locally or systemically [45]. Importantly, the use of PLGA NPs densely loaded with PNA provided a critical step toward clinical translatability, reducing the in vivo dose from two doses at 50 mg/kg [46] to one dose at 1.5 mg/kg [45]. As interest in antimiR PNAs continues to expand, we are investigating novel peptides as well as peptide-modified NPs to enhance in vivo delivery [45, 47].

1.4.3 NP-Mediated Delivery of PNA AntimiR-210

Although miRNA expression is regulated by several factors, miR-210 is partly expressed in response to hypoxia, a hallmark of the tumor microenvironment [48]. Molecularly, miR-210 targets the succinate dehydrogenase complex subunit D (SDHD), resulting in mitochondrial dysfunction and aberrant sensing of cellular O_2. Consequently, reductions in SDHD lead to increased expression of hypoxia-inducible factor 1α (HIF-1α), which leads to further upregulation of miR-210 [49]. Our group has shown that miR-210 also directly targets the 3′UTR of RAD52 leading to reduced homology-dependent repair (HDR) activity and greater genomic instability in hypoxic cells [50].

Given its central role in cancer progression, we developed a strategy to target miR-210 using PNAs encapsulated in PLGA NPs [51]. Unlike our previous work targeting miR-155, which made use of standard PNAs [45], we developed chemically modified versions with diethylene glycol substitutions at the γ position of each PNA monomer ($^{mp}\gamma$PNA) [51]. Using these modified monomers, we synthesized $^{mp}\gamma$-modified antimiR-210 PNA ($^{mp}\gamma P_{210}$). As expected, $^{mp}\gamma P_{210}$ was preorganized into a right-handed helical structure, with superior target hybridization relative to a chemically unmodified PNA (P_{210}). Local administration of $^{mp}\gamma P_{210}$-loaded NPs in a xenograft model effectively reduced miR-210 in vivo and increased miR-210 target protein (ISCU) expression. Importantly, in vivo administration of $^{mp}\gamma P_{210}$ NPs significantly reduced tumor volumes, demonstrating for the first time that γPNAs have superior in vivo antimiR effects relative to unmodified controls. As before, the use of densely loaded PLGA NPs, with modifications in PNA chemistry, allowed for further reductions in antimiR dose to 0.8 mg/kg [51]. We are currently exploring several alternative

NPs to enhance the in vivo effects of PNA, including polymer blends of PLGA with cationic poly(β-amino esters) (PBAE) [14, 15, 52] and a novel block copolymer of poly(lactic acid) and hyperbranched polyglycerol (PLA-HPG) [53].

1.5 PLGA NPs as Vehicles for Delivery of Bioactive PNA/DNA Reagents

Major benefits of polymeric carrier systems include their potential biodegradable, biocompatible, and controlled release properties in addition to well-defined chemistries and physical characteristics. Many synthetic polymers have versatile chemistries that are controllable through synthesis. Polymer physicochemical properties can be designed and modified (e.g., composition, molecular weight, polydispersity) according to desired specifications or applications. Examples of widely used biodegradable synthetic polymers are poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and the copolymer PLGA. The latter is an aliphatic polyester composed of lactic acid and glycolic acid in fixed ratios [54]. These materials degrade slowly via bulk hydrolysis in aqueous environments, providing the benefit of sustained release of cargo. PLGA degradation products are lactic and glycolic acid, which are eliminated via natural mechanisms such as the citric acid cycle [55]. We have primarily used PLGA-based formulations composed of a 50:50 lactic acid/glycolic acid ratio to encapsulate PNA-based therapeutics. PLGA-based delivery systems have been used for a wide variety of therapeutic agents [56], including nucleic acids such as siRNA, miRNA, and PNA alone [45, 57, 58]. Therapeutics encapsulated in PLGA NPs have demonstrated enhanced activity in several disease applications due in part to the protection from cargo degradation, increased biological half-life, and reduced side effects offered by NP encapsulation [59]. Notably, PLGA is a major component in drug delivery devices that have been approved by the FDA; its safety after introduction by a variety of routes of administration is well known, increasing the potential for clinical translation of new therapeutics [60]. In the context of PNA-based gene editing (introduced in Subheading 1.1), PLGA NPs serve as nontoxic and efficient delivery vehicles for PNA oligomers and/or co-reagents (e.g., donor DNA) [9].

PLGA PNA/donor DNA NPs for gene editing applications can be formulated using a water-oil-water double emulsion solvent evaporation technique (Fig. 3). Using this method, PNA and DNA oligomers are usually encapsulated in a 2:1 molar ratio, although this can be adjusted as desired. A detailed protocol is provided in Subheading 3. Briefly, nucleic acids in an aqueous phase are emulsified with polymer dissolved in organic solvent (i.e., the oil phase). Energy in the form of sonication is added to the system to promote the formation of polymer droplets. Next, the first emulsion is emulsified with a surfactant (e.g., poly(vinyl alcohol)) in water and sonicated again. The second emulsion is then diluted into a larger aqueous volume and stirred over several hours

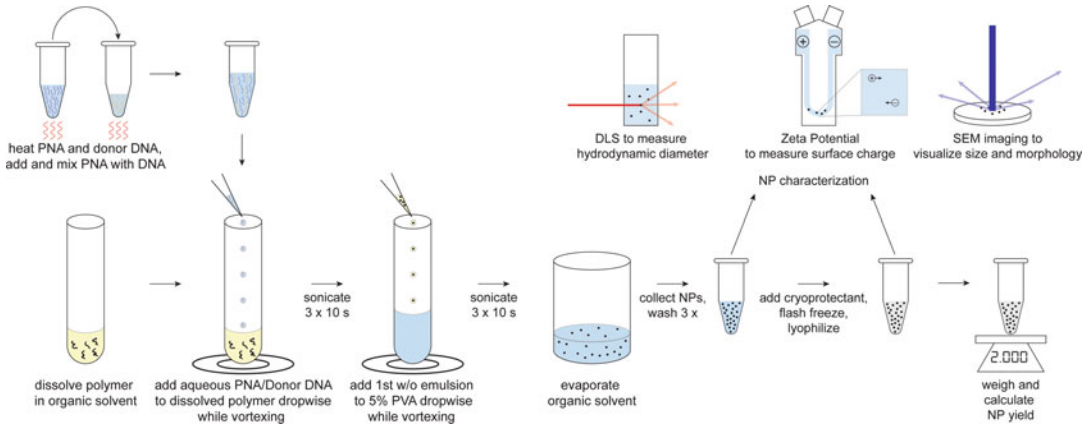

Fig. 3 Schematic of PLGA PNA/DNA NP formulation protocol and NP characterization

to evaporate the organic solvent. After the hardening phase, NPs are collected and washed by centrifugation at high speeds prior to lyophilization and storage. Following formulation, PLGA NPs are characterized; NP hydrodynamic diameter and surface charge are determined by dynamic light scattering (DLS) and zeta potential measurements, respectively, and NP size and surface morphology are determined using scanning electron microscopy (SEM). PNA/DNA NPs are spherical in morphology with diameters ranging from ~150 to 300 nm and exhibit a negative surface charge (illustrated in Subheading 3.3, Fig. 4). These formulations are biocompatible in vitro and ex vivo and are well-tolerated following in vivo administration. While PLGA-based formulations have many desirable properties for drug delivery, the reproducible formulation of NPs can be challenging, with variety being introduced by equipment, batch variation in reagents, and subtle differences in the method of emulsification. However, the detailed annotated protocol we provide here is designed to support a high degree of reproducibility in PLGA NP formulation.

2 Materials

2.1 Instruments and General Laboratory Equipment

1. 750 W ultrasonic processors with temperature controller (Cole-Parmer®) or comparable model.
2. Sterile 150 mL flat-bottom beaker with stir bar.
3. Sterile 18 × 150 mm disposable test tubes.
4. Stir plate (Corning) or comparable model.
5. Standard pipettes (P1000, P200, and pipette controller for large volumes).
6. Water bath sonicator (Branson Ultrasonics).

7. Zetasizer Nano ZS (Malvern Instruments) or comparable model.
8. Disposable square polystyrene cuvettes (Malvern Instruments).
9. Disposable folded capillary cell (Malvern Instruments).
10. Scanning electron microscopy pin stub (Agar Scientific).
11. Carbon conductive double-sided adhesive tape (Agar Scientific).
12. XL-30 scanning electron microscope (FEI, Hillsboro, Oregon) or similar instrument.

2.2 Chemicals

1. 50:50 poly(DL-lactide-*co*-glycolide), ester terminated, inherent viscosity 0.55–0.75 (dL/g) (LACTEL absorbable polymers, Birmingham, AL).
2. Poly(vinyl alcohol) (PVA), average molecular weight 30,000–70,000 (Sigma-Aldrich, St. Louis, MO).
3. Dichloromethane (Sigma-Aldrich, St. Louis, MO).
4. Trehalose (Sigma-Aldrich, St. Louis, MO).
5. 1× TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4).
6. Quant-iT™ OliGreen™ ssDNA Assay Kit (Invitrogen).

2.3 Surfactant Solutions

1. 5% PVA (w/v): dissolve 5 g of PVA in 100 mL of diH_2O. Stir overnight or until PVA is fully dissolved (*see* **Note 1**).
2. 0.3% PVA (w/v): dissolve 300 mg of PVA in 100 mL diH_2O. Stir overnight or until PVA is fully dissolved (*see* **Note 1**).

2.4 Nucleic Acids

1. PNAs can be synthesized using standard solid-phase techniques and purified as previously described [61]. Purified PNAs should be diluted to a 1 mM stock concentration prior to nanoparticle formulation.
2. Donor DNA can be purchased from Midland Certified Reagent Company Inc. (Midland, Texas, U.S.A.) or similar vendor. Donor DNA should be diluted to a 1 mM stock concentration prior to nanoparticle formulation (*see* **Note 2**).

3 Methods

A detailed graphical representation of the methods below has been described in Fig. 3 to be used as a quick reference when formulating PNA/donor DNA NPs. When formulating NPs containing either PNA or donor DNA alone, follow the protocol below, adding the PNA or donor DNA alone dropwise to the polymer solution.

3.1 Nanoparticle Formulation

3.1.1 Polymer Preparation (Day 1)

1. Dissolve 50 mg of PLGA in 1 mL of DCM in a 18 × 150 mm test tube in a chemical fume hood.
2. Cover the top of the tube with aluminum foil and parafilm, tightly securing the foil around the edges.
3. Mark the level of the solvent on the test tube. Allow the polymer to dissolve overnight. If evaporation occurs, add DCM the next day to the previously marked level.

3.1.2 PNA and Donor DNA Encapsulation (Day 2)

1. Heat 100 nmole of PNA (100 μL of 1 mM stock) and 50 nmole of donor DNA (50 μL of 1 mM stock) separately to 65 °C for 10 min using a heating block. In this case, the final ratio of PNA/DNA/PLGA starting material will be 100 nmole:50 nmole:50 mg or 2 nmole:1 nmole:1 mg (*see* **Notes 3** and **4**).
2. Prepare a work area with a vortex, P1000 pipette, P200 pipette, as well as solvent compatible tips in a chemical fume hood.
3. Pipette 2 mL of 5% PVA into a disposable 18 × 150 mm test tube.
4. Pipette 25 mL of 0.3% PVA into a 150 mL flat-bottom beaker with a stir bar. Place the beaker on a stir plate and set the stir speed to 360 rpm.
5. Remove the parafilm and aluminum foil covering the PLGA solution. Add DCM to the previously marked level if any has evaporated.
6. While mixing the PLGA solution using a vortex, quickly add and mix the PNA with the donor DNA solution. Add the resulting mixture dropwise to the PLGA. This will form the first water-in-oil emulsion (w/o).
7. Quickly sonicate the solution using an ultrasonic processor set for 10 s with an amplitude of 38%. Pause after each sonication step, and allow the solution to cool on ice for approximately 5 s. Repeat each step two more times for a total of three sonication steps.
8. Mix the 5% PVA solution by vortexing. While vortexing, add the first w/o emulsion to the 5% PVA solution dropwise.
9. Quickly sonicate the resulting w/o/w emulsion as described above (**step 7**).
10. Directly transfer the final w/o/w emulsion to the 0.3% PVA solution. 1–5 mL of the stirring 0.3% PVA solution may be used to dilute the w/o/w emulsion prior to transfer.
11. Allow the solution to stir for 3 h as the DCM evaporates and NPs harden.

3.1.3 Nanoparticle Collection

1. After 3 h, transfer the hardened NPs to a sterile 50 mL Falcon tube.
2. Centrifuge the NPs for 15 min at 16,100 × *g*.
3. Discard the supernatant, and resuspend the NP pellet in 5 mL of water using a water bath sonicator, and vortex until the pellet is fully resuspended.
4. Add 15 mL of diH_2O to the resuspended pellet, and centrifuge for 15 min at 16,100 × *g*.
5. Repeat **steps 3** and **4** for a total of three centrifugation steps.
6. Following the final centrifugation, discard the supernatant, and resuspend the pellet in 4–5 mL of diH_2O. A weight ratio of 1:1 trehalose/PLGA may be added as a cryoprotectant (*see* **Note 5**).
7. Transfer the resuspended NPs to 1.7 mL pre-weighed Eppendorf tubes, equally dividing the final volume among 10–15 tubes, depending on NP yield.
8. Flash freeze the NP aliquots with liquid nitrogen for 5 min.
9. Lyophilize the samples for 72 h.

3.2 Nanoparticle Characterization

1. Prepare a 0.05 mg/mL solution of NPs in diH_2O, taking care to rigorously resuspend the nanoparticles through water bath sonication and vortex (*see* **Note 6**).

3.2.1 Nanoparticle Diameter

2. Load 1 mL of the sample into the square polystyrene cuvette with a pipette, taking extra care to avoid introducing air bubbles.
3. Insert sample and allow temperature to equilibrate for 3 min.
4. Perform three independent size (hydrodynamic diameter) measurements, taking note of the correlation data to ensure measurement stability.

3.2.2 Nanoparticle Zeta Potential

1. Prepare a 0.05 mg/mL solution of NPs in diH_2O as above (*see* **Note 6**).
2. Load 1 mL of the sample into a disposable folded capillary cell, taking care to avoid introducing bubbles.
3. Insert sample and allow temperature to equilibrate for 2 min.
4. Perform three independent zeta potential measurements.

3.2.3 Nanoparticle Surface Morphology

1. Place double-sided carbon tape on an SEM pin stub (*see* **Note 7**).
2. Using a metal or disposable spatula, spread a thin layer of lyophilized NPs across the tape.
3. Sputter coat the sample with gold for 30 s.
4. Image gold-coated NPs using an XL-30 scanning electron microscope (FEI) or similar instrument.

3.2.4 Nanoparticle Loading

1. Dissolve 2 mg of NPs in 0.5 mL of DCM overnight.
2. Add 0.5 mL of TE buffer to the dissolved NPs.
3. Mix vigorously by vortex and spin the sample at 12,000 rpm at 4 °C.
4. Repeat **steps 2** and **3** for a total of two volumes of 1× TE buffer.
5. Measure the OD at 260 nm of the combined 1 mL fraction. For quantification of donor DNA alone, use the Quant-iT™ OliGreen™ ssDNA Assay Kit (Invitrogen) according to the manufacturer's protocol (*see* **Note 8**).

3.3 Representative Results

Using the methods above, one can reasonably expect to obtain PLGA NPs loaded with PNA and donor DNA molecules. While diameter measurements may vary depending on the methods used to quantify this parameter, we have found that the diameter of our PLGA PNA/DNA NPs are typically between 250 and 290 nm as measured by **DLS**. As DLS is a diffusion-based measurement, it is particularly sensitive to the effects of particle concentration, as well as salt concentration used in the buffer [62]. Therefore, while particle concentrations and buffers can be changed to explore parameters, such as size stability over time, it is critical to accurately report nanoparticle concentration and buffer conditions to ensure reproducibility. Figure 4a highlights a typical distribution of nanoparticle sizes as measured by DLS. In this example, NPs were resuspended in diH_2O at a concentration of 0.05 mg/mL. Here, the average NP diameter is approximately 280 nm.

The **zeta potential**, or surface charge of NPs, is likewise sensitive to buffer conditions and in particular pH [63]. Therefore, as above, it is critical to carefully report the buffer used to measure zeta potential to ensure reproducibility. Figure 4b highlights a typical distribution of zeta potential values for PLGA PNA/DNA NPs as measured in diH_2O at a concentration of 0.05 mg/mL. In this example, the zeta potential of our NPs is approximately −23 mV, which is typical of such preparations.

The use of **SEM** to study NP morphology, though seemingly straightforward, may be complicated by differences in sputter coating methods and in the materials used to coat NPs. In particular, the length of time during which NPs are coated will lead to variations in coating thickness, which can create imaging artifacts or result in poor resolution. Though several options are available for coating NPs, PLGA PNA/DNA NPs have been primarily imaged by SEM following gold-palladium coating. Figure 4c is a typical SEM image of NPs. As seen in the figure, NPs are spherical and homogeneous, with little to no observable surface defects.

Loading of PNA and donor DNA is typically measured using absorbance readings at 260 nm, which are normalized to NP mass

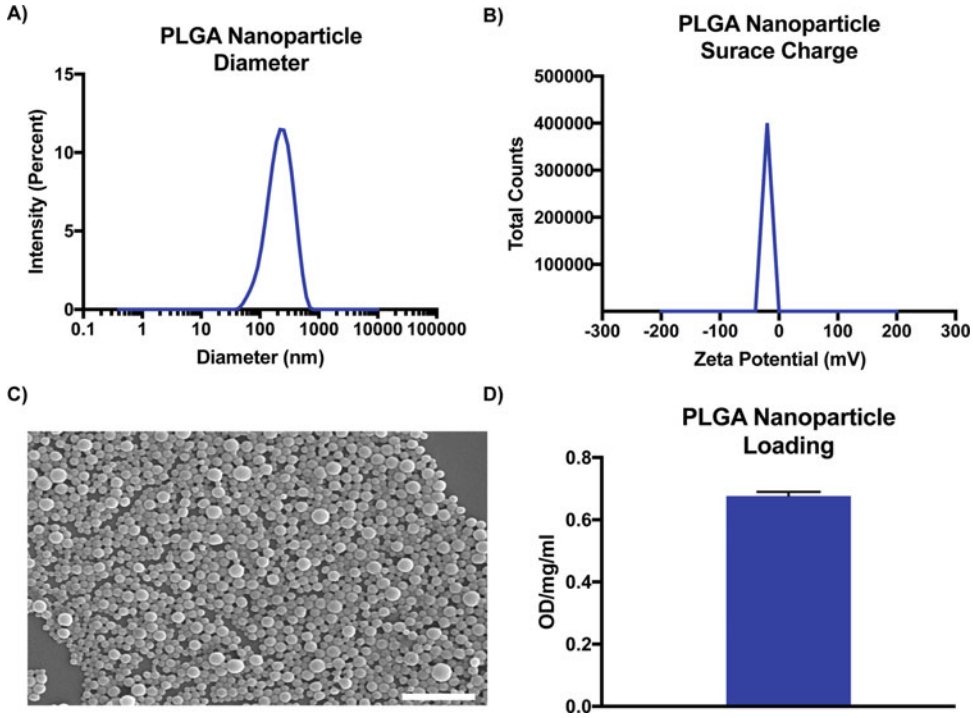

Fig. 4 Typical characteristics of PLGA NPs encapsulating donor DNA and PNA. (**a**) Dynamic light scattering measurement of NP diameter. (**b**) NP surface charge as measured by zeta potential. (**c**) SEM image of NPs. Scale bar is equal to 2 μm. (**d**) Total nucleic acid loading (PNA and donor DNA molecules) as measured by absorbance at 260 nm

and volume used for extraction (**OD/mg/mL**) [15]. In recent work, we have also used the Quant-iT™ OliGreen™ ssDNA Assay Kit (Invitrogen) to quantify loading of donor DNA alone. By using this kit, as well as fluorescently labeled PNAs, it is possible to precisely quantify loading of donor DNA and PNA independently, though use of fluorescently tagged PNA may alter loading of the PNA or donor DNA molecules [15]. As in Fig. 4d, typical OD/mg/mL values range between 0.4 and 0.6. So far, we have found that NPs with loading below 0.4 OD/mg/mL do not successfully edit genes in vitro.

4 Notes

1. Dissolving PVA requires vigorous stirring. Use of a stir bar and stir plate set to maximum speed is highly recommended. It is preferable to dissolve PVA slowly overtime, rather than use high heat to accelerate the process.
2. The typical length of donor DNA is 60 nucleotides. 5′ and 3′ ends can contain three phosphorothioate internucleotide

linkages to prevent degradation. Donor DNA is purified by reversed-phase high-performance liquid chromatography (HPLC).

3. Typically, PNA is added to donor DNA seconds prior to formation of the first water in oil emulsion. Mixing nucleic acids several minutes ahead of time is not recommended, as PNA/DNA complexes may precipitate out of solution.
4. Typically, the ratio of PNA to donor DNA to starting material of PLGA is kept at 2 nmole PNA:1 nmole DNA:1 mg PLGA. These ratios may be adjusted to further improve PNA and donor DNA loading.
5. The mass of trehalose added to the final NP suspension is based on NP yield, not starting mass of PLGA. Prepare empty NPs to determine typical yields prior to formulating NPs with the addition of cryoprotectant.
6. When characterizing NPs by DLS and zeta potential, it is critical to report NP concentration and buffer selection, as these factors greatly influence the values obtained. While DLS and zeta values are typically reported for NPs after lyophilization, it is generally recommended to perform these measurements prior to drying, as a quality control step.
7. The addition of cryoprotectant may introduce artifacts when imaging by SEM. To avoid this, a small sample of nanoparticles (100 μL) can be separately frozen and lyophilized prior to the third centrifugation step. Alternatively, cryoprotectant can be removed from the final product following three washes with diH_2O. The washed NPs can then be air-dried on a glass-covered SEM stub and processed and coated as described.
8. Loading of PNA and donor DNA may vary based on sequence, with typical loadings between 0.4 and 0.6 OD/mg/mL observed in a majority of PLGA NPs.

Acknowledgments

This work was supported by the NIGMS Medical Scientist Training Program T32GM07205 (to E.Q.); National Institutes of Health grants R01HL125892, R01AI112443, and UG3HL147352 (to W.M.S. and P.M.G.); and institutional training grant 5T32GM007223-43 (to E.Q.). A.S.P. was supported by NIH National Research Service Awards (NRSAs): T32 (GM86287) training grant and F32 (HL142144) individual postdoctoral fellowship. S.N.O was supported by UG3HL147352 (to P.M.G. and W.M.S.).

Disclosures/Competing interests: E.Q., A.S.P., W.M.S., and P.M.G. are consultants for Trucode Gene Repair, Inc. W.M.S. and P.M.G also have equity interests in Trucode Gene Repair, Inc.

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