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Polymer nanoparticle-mediated delivery of microRNA inhibition and alternative splicing

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Abstract

The crux of current RNA-based therapeutics relies on association of synthetic nucleic acids with cellular RNA targets. Antisense oligonucleotide binding to mature microRNA and splicing junctions on pre-mRNA represent methods of gene therapy that respectively inhibit microRNA-mediated gene regulation and induce alternative splicing. We have developed biodegradable polymer nanoparticles--which are coated with cell-penetrating peptides--that can effectively deliver chemically-modified oligonucleotide analogs to achieve these forms of gene regulation. We found that this nanoparticle system could block the activity of the oncogenic microRNA, miR-155, as well as modulate splicing to attenuate the expression of the proto-oncogene, MCL-1. Regulation of these genes in human cancer cells reduced cell viability and produced pro-apoptotic effects. These findings establish polymer nanoparticles as delivery vectors for non-conventional forms of gene therapy activated by cellular delivery of RNA-targeted molecules, which have strong therapeutic implications.

Keywords

gene therapy; microRNA (miRNA) inhibition; alternative splicing; poly(lactic-co-glycolic acid) (PLGA) polymer nanoparticles; peptide nucleic acids (PNA); phosphorodiamidate morpholinos (PMO)

Introduction

Antisense oligonucleotides can be designed to inhibit microRNA (miRNA) and alter splicing, and their efficacy can be increased through conjugation to cell-penetrating peptides (CPP) as well as various other chemical modifications.^{1–4} Oligonucleotide delivery can also be improved by encapsulation into nanoscale vehicles, which offer various benefits including: enhanced stability, high loading densities, and favorable pharmacokinetics. We have previously developed a poly(lactic-co-glycolic acid) (PLGA) polymer nanoparticle (NP) system that effectively transports small RNAs into cells and tissues.^{5,6} As with oligonucleotide-CPP conjugates, CPPs can be attached to these NPs to further improve delivery.⁵ Here we show that NPs modified with the CPP, nona-arginine (ARG),⁷ can

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Conflict of Interest

The authors declare no conflict of interest.

effectively deliver chemically-modified oligonucleotide analogs to cancer cells to achieve therapeutic miRNA inhibition or alternative splicing.

Phosphorodiamidate morpholino oligomers (PMO) and peptide nucleic acids (PNA) are nucleic acid analogs that exhibit excellent stability and enhanced binding affinity for target nucleic acids compared with conventional DNA- or RNA-based antisense oligonucleotides. Over the past decade, microRNAs have emerged as potent therapeutic targets due to their regulation of numerous biological processes, particularly those related to diseases such as cancer.⁸ Through binding to complementary microRNA (miRNA), anti-miR oligonucleotide analogs, such as PMOs and PNAs, can suppress miRNA activity and thus de-suppress miRNA-based downregulation of gene expression (Figure 1).⁹ Additionally, PMOs and PNAs can modulate splicing of pre-mRNA. Through binding to the 3' acceptor and 5' donor splice sites flanking an exon, splice-shifting oligonucleotides can mask exons from the splicing machinery, which can yield variant isoforms (Figure 1). As with miRNAs, many diseases, including cancer, can be linked to aberrant splicing or the presence of unwanted gene isoforms.^{10,11} In this study, we investigate the inhibition of a specific miRNA, miR-155, which is one of the first identified miRNA oncogenes, as well as the splice-shifting of Mcl-1, which is a member of the Bcl-2 family that has both anti-apoptotic and pro-apoptotic isoforms.^{12–14}

Only a few prior studies describe the encapsulation of either PMOs or PNAs into nanoscale delivery systems. Previously, PMOs have been conjugated to protein- and dendrimer-based nanovehicles.^{15,16} These systems have shown success in the delivery of antisense agents *in vivo*; however, we hypothesized that loading a high density of freely distributed PMOs into NPs may improve delivery efficacy. We have previously shown that NPs can deliver PNAs in association with DNA in order to direct site-specific genomic recombination; however, the DNA component required the addition of a polycation counter-ion, spermidine, for efficient loading.¹⁷ Like most nucleic acid delivery vehicles, NPs can comprise a cationic component that facilitates association with anionic DNA or RNA. However, such polycations may have deleterious returns, for example: once inside a cell, the electrostatic complexes must dissociate to release bioactive nucleic acids; cationic agents may have non-specific interactions with off-target cellular components; and molecules that can become heavily protonated may induce cellular toxicity through osmotic swelling of intracellular vesicles.¹⁸ A cationic component is not essential for NP formation, which can be based on an emulsion-driven process. Therefore, we hypothesized that NPs devoid of cationic additives could load high densities of charge-neutral oligonucleotide analogs. Unlike other nucleic acids, the backbones of PMOs and PNAs have a neutral charge density; therefore, loading of PMOs and PNAs into NPs presents a platform for the delivery of antisense-based gene therapy that does not rely on electrostatic interactions.

Materials and Methods

Nanoparticle Synthesis

NPs were fabricated using a double emulsion solvent evaporation method. PLGA (Durect Corporation, Pelham, AL, USA) with terminal ester group (50:50 monomer ratio and 0.55–0.75 dL/g inherent viscosity) was dissolved in methylene chloride. Nucleic acids (PMO, PNA, and ssDNA) suspended in water were added to the polymer mixture (at a ratio of 1 nmole of nucleic acid per mg of polymer; note that splice-shifting nucleic acids were co-loaded into NPs each at a ratio of 1 nmole/mg) and sonicated. This primary emulsion was then added to a secondary aqueous solution of 5% (w/v) polyvinyl alcohol (PVA) and sonicated. This secondary emulsion was added to a solution of 0.3% (w/v) PVA and incubated for 3–4 hours to allow the methylene chloride to evaporate and NPs to harden. NPs were then washed and lyophilized with the cryoprotectant, trehalose, at an equal mass

ratio of polymer to carbohydrate. For NPs loaded with ssDNA and cationic counter-ion, prior to NP synthesis spermidine (Sigma-Aldrich, St. Louis, MO, USA) was complexed with ssDNA at an N-to-P ratio of 8:1. NPs were surface modified as previously described,⁵ except ARG (Keck Peptide Synthesis Facility at Yale University, New Haven, CT, USA) was used as the CPP with the sequence: RRRRRRRRRGGC.

Nanoparticle Characterization

PMOs (Gene Tools LLC., Philomath, OR, USA), PNAs (Bio-Synthesis Inc., Lewisville, TX, USA), and ssDNA (Keck Oligonucleotide Synthesis Facility at Yale University, New Haven, CT, USA) were synthesized using the following sequences:

anti-LUC: 5'-TCTTAATGTTTTTGGCATCTTCCAT-3'

anti-CRL: 5'-ACCCAATCGTCAAATTCCATATA-3'.

anti-miR-155: 5'-CCCCTATCACGATTAGCATTAA-3',

Mcl-1 3' acceptor: 5'-CGAAGCATGCCTGAGAAAGAAAAGC-3'

Mcl-1 5' donor: 5'-TTAAGGCAAACCTACCCAGCCTCTT-3'

For quantification studies, fluorescein or rhodamine was conjugated to the 3' end of the nucleic acids. Loading was measured by spectrofluorescence of NPs hydrolyzed in 1N NaOH or dissolved in DMSO. Controlled release was measured by quantifying the release of fluorescein-labeled PNA, PMO, and ssDNA at various time points from NPs suspended in 1 ml of phosphate buffered saline (PBS, pH 7.4) and incubated at 37°C with gentle shaking. At each time point (~ 2, 7, 34, 57, 102, and 171 hrs), NPs were pelleted by centrifugation (16,100 rcf for 5 min) and 0.9 ml of the supernatant was collected and analyzed by spectrofluorescence (excitation: 485 nm; emission: 515 nm) using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). NPs were resuspended with the addition of 0.9 ml of fresh PBS and used for serial controlled release analysis. NP size and surface charge was measured using a Zetasizer Nano ZS (Malvern Instruments Inc, Westborough, MA, USA); analysis was conducted using NPs (0.5 mg/ml) suspended in distilled water (pH 6) at room temperature. Internalization of CPP-modified NP was monitored using flow cytometry. Cells were incubated with Coumarin 6-labeled NP at a NP to cell ratio of 8×10^4 . After incubating for 30 min at 37°C, cells were washed with PBS and harvested using cell dissociation buffer (Invitrogen, Carlsbad, CA, USA). Internalized NP were enriched by incubating cells with 0.1% (w/v) Trypan Blue for 5 min to quench any extracellular fluorescence of live cells (i.e. surface-associated NP). Cells were washed with 1% BSA in PBS and then analyzed on a FACScan (BD Biosciences, San Jose, CA, USA).

Microscopy

For all cell culture experiments, KB cells were (ATCC, Manassas, VA, USA) maintained in folate-free RPMI-1640 medium from (Invitrogen) containing 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA).

For SEM, NPs were sputter-coated with a thin layer of gold and visualized using a XL-30 ESEM-FEG SEM (FEI, Hillsboro, OR, USA). Size distribution was measured by micrograph analysis of at least 1,000 NPs using image analysis software (ImageJ, National Institutes of Health). For TEM, NPs were loaded with 5% (w/w) OsO₄ during synthesis and incubated with cells overnight. Cells were washed, fixed with 2.5% glutaraldehyde, dehydrated and embedded in resin. Sections (50 nm-thick) were cut, mounted onto a glow-discharged carbon-coated formvar grid, and then stained with uranyl acetate and lead citrate by standard methods. Samples were viewed using a Tecnai Biotwin TEM (FEI). For confocal imaging, ARG-NPs loaded with FITC-labeled PMOs were incubated overnight

with cells grown on chambered coverglass (Lab-Tek™, Rochester, NY, USA) at a NP to cell ratio of 6×10^5 . Cells were washed with 10% FBS (v/v) in PBS, fixed in 4% paraformaldehyde, and permeabilized using 0.1% Triton X-100 in PBS. Per the manufacturer's instructions, actin was stained with Texas Red®-X phalloidin (Invitrogen) and nuclei were stained with Hoescht 33342 (Invitrogen). Cells were mounted in Slow Fade Gold (Invitrogen) and visualized using a TCS SP5 Spectral Confocal Microscope (Leica, Bannockburn, IL, USA).

Luciferase Assays

For antisense luciferase experiments, NPs containing anti-LUC or anti-CRL oligonucleotide analogs were incubated in 1 ml PBS (4 mg/ml) at the indicated times, NPs were pelleted by centrifugation (16,100 rcf for 5 min) and the entire volume of PBS was removed and replaced with fresh buffer. Samples containing released oligonucleotide analogs were concentrated under vacuum and resuspended in 5 μ l H₂O. These aliquots were added to TnT® Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA); luciferase activity was measured using the manufacturer's protocol. For dual-luciferase reporter experiments, the miRNA sensor was generated by inserting the target sequence for miR-155 into the 3'UTR of Renilla luciferase on a psiCHECK™-2 vector (Promega). Cells were transfected with the reporter using Lipofectamine 2000 (Invitrogen) using the manufacturer's protocol. After 24 hours, cells were treated with ARG-NPs containing anti-miR or relevant controls at a concentration of 1 μ M. Cell lysates were measured for luciferase activity 48 hours after initial transfection using the Dual-Luciferase Reporter Assay System (Promega).

qPCR Analysis of miRNA levels

Cells were incubated with ARG-NPs containing anti-miR-155 or relevant controls at a concentration of 1 μ M. After 24 hours, total RNA was extracted using Trizol (Invitrogen) according to standard procedures. DNA was digested (Qiagen, Valencia, CA, USA) and RNA was further isolated by repeating Trizol extraction. RT and qPCR were performed using Taqman® MicroRNA System for hsa-miR-155 (Applied Biosystems, Foster City, CA, USA) and MyiQ Real-Time Thermal Cycler (Bio-Rad, Hercules, CA, USA); miR-155 levels were normalized to U6 internal control (Ambion, Austin, TX, USA).

RT-PCR Analysis of Pre-mRNA Splicing

Cells were incubated with ARG-NPs containing each splice-shifting PMO (binding the 3' acceptor and 5' donor splice sites that flank exon 2 of Mcl-1) or relevant controls at a total PMO concentration of 2 μ M. For Endo-Porter group, cells were incubated with 1 μ M of each splice-shifting PMO as well as 6 μ M of Endo-Porter. After 24 hours, total RNA was extracted as earlier described. RT was performed using iScript cDNA Synthesis Kit (Bio-Rad). PCR was performed using Titanium Taq DNA Polymerase (Clontech, Mountain View, CA, USA). PCR products were analyzed by 2% agarose gel electrophoresis and visualized with SYBR® Safe (Invitrogen). The following primers were used: Mcl-1 FWD: 5'-GTTGGTCGGGGAATCTGGTA-3', Mcl-1 REV: 5'-AAATTAATGAATTCGGCGGG-3', β -actin FWD: 5'-ATTGCCGACAGGATGCAGAA-3', β -actin REV: 5'-GCTGATCCACATCTGCTGGAA-3'

Cell Viability and Apoptosis

For dose response experiments, cells were incubated with various ARG-NP formulations at the indicated PMO concentrations for 96 hours. We note that due to the co-loading of splice-shifting PMOs for Mcl-1 the total nucleic acid concentration was 2-fold the indicated amount

for the dose response and apoptosis studies. CellTiter-Blue® (Promega) was used to assay for cell viability. For apoptotic DNA fragmentation analysis, cells were incubated with 1 μ M of the indicated PMOs in ARG-NPs. After 24 hours, Cell Death ELISA Kit (Roche Applied Science) was used to determine the amount of apoptotic DNA fragmentation. The DNA fragmentation index was determined by quantifying the cytosolic histone-associated mono- and oligonucleosomes.

Statistical Analysis

Where applicable, either Student's t-test or 2-way ANOVA was used to determine statistical significance. Unless otherwise noted, data are reported as an average and standard deviation, representative of n=3 independent experiments.

Results

Nanoparticle Design and Characterization

NPs with a size range of 100–200 nm (Table 1) were coated with the arginine-rich CPP, ARG (Figure 1); BCA assay (Thermo Fisher Scientific Inc., Rockford, IL, USA) measured the surface density as 15.1 ± 0.9 μ g of peptide/mg of NP, which corresponds to ~12,000 ARG molecules per NP—the number of NP for a given mass was calculated using the PLGA NP density of 1.23 g/cm³ and assuming a spherical volume for each NP.¹⁹ Flow cytometry of fluorescently labeled NPs revealed the impact of the surface modification: NPs that were coated with ARG (ARG-NPs) demonstrated greater association with human KB oral carcinoma cells than unmodified NPs and NPs coated with other CPPs, TAT and ANTP (Figure 2). Transmission electron microscopy (TEM) confirmed that ARG-NP localized to the cytosol (Figure 1).

NPs were characterized for their ability to encapsulate charge-neutral PNAs and PMOs. Loading densities of 410 ± 55 pmoles of PNA/mg of NP and 410 ± 34 pmoles of PMO/mg of NP were achieved (Table 1), and this degree of encapsulation was substantially greater than what was observed for NPs loaded with either single-stranded DNA (ssDNA, 25 ± 5 pmoles of DNA/mg of NP) or ssDNA in complex with a cationic counter-ion, spermidine, (ssDNA:cation, 49 ± 11 pmoles of DNA/mg of NP). Coating NPs with ARG adds a cationic component to the surface of the nanoparticles; however, the charged surface ligand did not appear to impact loading of nucleic acids. During ARG-NP synthesis, ARG is added to the nanoparticle surface after the nucleic acids have been emulsified into the nascent nanoparticle phase. Note that for all cargos, surface-modified ARG-NPs had similar loading values compared to uncoated NPs: ARG-NPs encapsulating PNA, PMO, ssDNA, and ssDNA:cation loaded 419 ± 51 , 416 ± 23 , 32 ± 6 , and 56 ± 13 pmoles/mg of NP, respectively. NP surface charge was dependent on the charge of the encapsulated agents (Table 1). NPs loaded with charge-neutral PMOs and PNAs had ζ -potential values of -19.5 ± 0.3 and -22.6 ± 0.2 , respectively; these values were similar to that of PLGA NPs with no encapsulated agents. NPs loaded with ssDNA and ssDNA:cation had ζ -potential values of -30.7 ± 1.0 and -6.6 ± 0.1 , respectively; these values were influenced by the presence of charged components.

Release of Charge-Neutral Oligonucleotide Analogs

Controlled release of PNA and PMO occurred by an initial burst over the first few hours followed by sustained release for days to weeks; after one week, ~50% of either encapsulated PNA or PMO was released from NPs (Figure 3A). Both free DNA and DNA in complex with a cationic counter ion also have a biphasic sustained release profile, but due to the lower loading densities, the net amount of DNA released per NP was much less than the charge-neutral oligonucleotide analogs (Figure 3A). We note that coating NPs with surface

ligands that have a high cationic charge density (i.e. most CPPs) may alter release dynamics of encapsulated anionic nucleic acids; however, charge-neutral oligonucleotide analogs can avoid these electrostatic interactions and more freely diffuse out of nanoparticles.^{5,20} However, for all encapsulated nucleic acid cargos, the release profiles of uncoated NPs and ARG-NPs (data not shown) were not significantly different. Based off of the ARG coating density and NP surface area, less than 10% of the NP surface is covered with ligand. Therefore, at this density, even for charged molecules, release of nucleic acid cargo was not significantly occluded by electrostatic interactions.”

To demonstrate the utility of controlled release systems in delivering bioactive charge-neutral nucleic acids, NPs were loaded with antisense PMOs that can bind to luciferase mRNA to inhibit translation. Over the span of one week, the total amount of released PMO was collected at various time intervals. These samples were then added to a cell-free coupled *in vitro* transcription/translation mixture (Promega, Madison, WI, USA) to inhibit the translation of luciferase (Figure 3B). Samples from the first 2 hours and subsequent 10 hours produced over 80% inhibition of luciferase activity and expression. Samples from the next 12 and then 24 hours achieved less inhibition than the aforementioned burst release phase samples, probably due to the slower PMO release rate (and resulting fewer PMO molecules collected in the sample supernatant) during the collection period. Incubating the NPs for an additional five days resulted in the sustained release of enough PMO to again achieve over 80% inhibition.

Nanoparticle-Mediated Inhibition of MicroRNA

On the basis of their enhanced cellular uptake, cytosolic localization, high loading density, and favorable release rates we postulated that ARG-NPs loaded with charge-neutral anti-miRs could effectively inhibit miRNA, specifically miR-155. To monitor the inhibition of miR-155 we used a dual luciferase reporter system in which the target binding sequence for miR-155 was inserted into the 3'UTR of Renilla luciferase; Firefly luciferase was used for normalization. Endogenous miR-155 represses Renilla luciferase expression, but this can be de-repressed in the presence of anti-miR-155. KB cells transfected with this sensor exhibited more than a 4-fold increase in normalized Renilla luciferase activity after being exposed to ARG-NPs delivering anti-miR-155 (Figure 4A). In accordance with attenuation of miR-155 function, detectable levels of miR-155 in KB cells decreased in response to anti-miR-155 delivered by ARG-NPs (Figure 4B). Importantly, ARG-NPs decreased miR-155 levels to ~60% while NPs with no surface modification only decreased levels to ~80% relative to untreated cells. Cells treated with ARG-NPs loaded with a scrambled control PMO anti-miR (CRL) showed no miR-155 loss, which further emphasizes the specificity of this miRNA-based therapeutic strategy. Interestingly, despite the greater RNA binding affinity of PNAs compared to PMOs, both PNAs and PMOs functioned comparably as anti-miRs. Therefore, for the rest of this study, we focused on the nanoparticulate-based delivery of PMOs.

Nanoparticle-Mediated Alternative Splicing

Gene therapy typically involves either increasing (e.g. DNA delivery) or down-regulating (e.g. RNA interference) the levels of specific genes. Modulation of splicing patterns presents a multimodal form of gene regulation that can accomplish both of these processes. Oligonucleotides that mask exons simultaneously reduce the production of a specific gene isoform and induce the splicing and subsequent transcription of another isoform. A unique property of charge-neutral oligonucleotide analogs is their ability to passively diffuse into the nucleus, the site-of-action for alternative splicing.²¹ Therefore, we postulated that ARG-NPs could effectively transport charge-neutral PMOs into cells and to the cytosol (Figure 1) where subsequently released PMOs could translocate to the nucleus and induce alternative splicing. We observed that FITC-labeled PMOs delivered by ARG-NPs were able to enter

the nucleus of KB cells (Figure 5A). We do not exclude the possibility that ARG-NPs have a direct role in facilitating nuclear transport of PMOs; however, it is more likely that sustained release of PMOs from ARG-NPs generated a diffusion gradient into the nucleus.

Once in the nucleus, splice-blocking oligonucleotides are free to bind to pre-mRNA and alter splicing. The *MCL1* gene is an interesting alternative splicing model because it has two isoforms of different size and function (Figure 1): Mcl-1L is anti-apoptotic (1005 bp) and Mcl-1S is pro-apoptotic (757 bp).^{13,14,22} In KB cells, ARG-NPs that deliver PMOs that mask exon 2 of Mcl-1 pre-mRNA manipulate the splicing machinery to predominantly produce the Mcl-1S isoform as opposed the Mcl-1L isoform (Figure 5B). Densitometry of the RT-PCR results was used to measure the Mcl-1S/Mcl-1L ratio as ~ 2. The splicing pattern of ARG-NPs delivering scrambled control PMOs (Mcl-1S/Mcl-1L ~ 0.03) was unchanged from that of untreated cells (Mcl-1S/Mcl-1L ~ 0.03). Delivery of PMOs by ARG-NPs was observed to be as effective as the positive control, Endo-Porter (Gene Tools LLC., Philomath, OR, USA), in altering splicing (Mcl-1S/Mcl-1L ~ 2; Figure 5B). Note that both PMOs and PNAs require a delivery vector (e.g. nanoparticles or Endo-Porter) in order to efficiently enter cells.²³ Endo-Porter functions by promoting non-specific uptake of the extracellular milieu, so a key difference is that ARG-NPs have applicability as a therapeutic delivery system.²⁴

Gene Therapy Using Nanoparticles that Deliver Charge-Neutral Oligonucleotide Analogs

We chose to investigate miR-155 and Mcl-1 as gene therapy targets due to their functional relevance in cell maintenance.^{12,25} We observed that KB cells were more sensitive to ARG-NPs that delivered anti-miR-155 (IC₅₀ ~ 80 nM) than Mcl-1 splice-switching PMOs (IC₅₀ ~ 360 nM) (Figure 6A). The delivery of scramble control PMOs (anti-CRL) using ARG-NPs, as well as empty ARG-NPs with no encapsulated agents showed no significant cytotoxicity (Figure 6A). Mechanistically, both therapeutic strategies can promote apoptotic signaling. DNA fragmentation and enrichment in the cytoplasm is a hallmark of apoptosis. In accordance with the varied cytotoxicity response, the extent of cytosolic DNA fragments in cells treated with anti-miR-155 and Mcl-1 splice switching PMOs was ~2-fold and ~1.3-fold greater than untreated cells, respectively (Figure 6B).

Discussion

Effective gene therapy delivery vehicles must be able to traverse cell membranes and transport their payloads to the cytosol. We have previously demonstrated that NPs can capitalize on the membrane transduction properties of surface-attached CPPs.⁵ Whether through direct membrane translocation, endocytosis, a combination of both, or an entirely different mechanism, ARG enters cells and ultimately reaches the cytoplasm.^{1,26,27} These processes are likely confounded by the tethering of polymer nanoparticles to the peptide; however, ARG-NPs were observed to be freely distributed in the cytosol (Figure 1). We note that the seemingly greater degree of ARG-NP uptake by cells when visualized by confocal microscopy (Figure 5A) compared to TEM (Figure 1) is a result of both the larger field of view and the thicker imaged section for the confocal imaging—the TEM slice thickness was 50 nm while the confocal imaging plane z-height was 0.9 μ m. Based on these images, we estimate that the intracellular density of ARG-NPs is between 0.1 NP/ μ m³ (which we estimate from Fig 5A) and 1 NP/ μ m³ (which we estimate from Fig 1).” Escaping endolysosomal degradation and vesicular sequestration is paramount for nucleic acids that are intended to bind miRNA in the cytosol or pre-mRNA in the nucleus.

These forms of gene therapy can be induced by PMOs and PNAs, but due their charge-neutral backbone, these nucleic acid analogs cannot effectively utilize conventional oligonucleotide delivery systems that rely on electrostatic interactions. Fabrication of PLGA

NPs relies on the hydrophobic effect instead of charged interactions. Due to their charge-neutral backbone, PMOs and PNAs demonstrated higher loading into NPs compared to anionic nucleic acids (Table 1). Additionally, PNAs and PMOs had comparably high encapsulation densities which can be attributed to their similar chemical compositions and physical properties. NPs were formed using a double emulsion solvent evaporation process; therefore, denser loading of charge-neutral oligonucleotide analogs was likely due to the lack of intermolecular electrostatic repulsion that promotes outward diffusion of nucleic acids during NP fabrication. Furthermore, PLGA NPs have widespread applicability as a delivery platform because they can function as a reservoir for controlled release of encapsulated drugs. Agents entrapped in PLGA NPs are gradually released through diffusion and hydrolysis of the polymer matrix. Although the release of PMOs, PNAs, and ssDNA followed similar kinetics (Figure 3A), the greater loading density of PMOs and PNAs makes them potentially effective agents for NP-mediated gene therapy.

In recent years, inhibiting miRNA with anti-miR molecules and altering splicing with splice-shifting oligonucleotides have emerged as promising therapeutic strategies.^{21,28,29} Nonetheless, currently there are only a few strategies for delivery of agents to induce these forms of gene therapy that have shown promise *in vivo*.^{30–34} The most effective delivery strategies involve the administration of free chemically-modified antisense molecules, so encapsulation of a high density of these agents into PLGA NPs may provide a dosing advantage using a delivery system that has a history of *in vivo* success.³⁵ The observed loading amounts of PNAs and PMOs into NPs were therapeutically relevant, particularly for an anti-miR-based approach. We calculated that a single NP contains ~500 anti-miR molecules. Cancers that are connected to miRNA oncogenes typically have between 100–10,000 copies of the aberrantly expressed miRNA per cell.^{36–38} Therefore, delivery of between 1 and 20 nanoparticles per cancer cell would be sufficient to match the anti-miR dose with target miRNA levels. Similar calculations are less predictive when targeting pre-mRNA, since a single alternatively spliced mRNA can be translated to produce numerous copies of a protein.

ARG-NPs effectively delivered PMOs to exploit both miR-155 inhibition and altered Mcl-1 splicing for therapeutic purposes, but anti-miR-155 therapy was more efficacious. This disparity was likely due to relative sites of action: ARG-NPs are more efficient at delivering PMOs to the site-of-action for miRNA blocking (cytosol) than alternative splicing (cytosol and then nucleus). Additionally, inhibition of miR-155 and splice-shifting of Mcl-1 induces apoptosis via different biological pathways. miR-155 downregulates a multitude of cellular targets that directly promote apoptosis including TP53INP1, CASP3, SHIP1, and FOXO3a, as well as ancillary anti-survival targets such as SOCS1 and SMAD5.^{39–44} In contrast, the apoptotic role of Mcl-1 is defined by its involvement with Bcl-2 apoptosis regulator proteins.¹³ Therefore, inhibition of miR-155 may induce a variety of cellular responses; while splice-shifting of Mcl-1 to the pro-apoptotic isoform (Mcl-1S) has a more defined—yet possibly less potent—outcome.

This study presents a delivery technology that can inhibit miRNA and alter splicing. As gene therapy delivery vehicles, NPs have several key advantages: (1) NPs provide a safe, biodegradable matrix to protect encapsulated nucleic acids; (2) NPs can be coated with surface ligands (e.g. ARG) to enhance cellular uptake and impart other functionalities; (3) NPs can deliver high doses of charge-neutral oligonucleotide analogs over a sustained period of time; (4) and NPs are also effective at *in vivo* delivery, in a related study we have developed NPs that inhibit oncogenic miRNA in an aggressive lymphoma tumor model (Babar and Cheng et al., unpublished results). Regulating miRNA activity and altering splicing are relatively nascent therapeutic strategies; we envision that our nanoparticulate delivery system will advance the widespread use of these forms of gene therapy.

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Figure 1.

Workflow schematic of alternative splicing and miRNA inhibition induced by nona-arginine-coated nanoparticles (ARG-NPs) that deliver charge-neutral oligonucleotide analogs. ARG-NPs comprise a spherical PLGA core coated with PEGylated ARG. Dehydrated ARG-NPs were visualized by scanning electron microscopy (SEM), intracellular uptake of osmium tetroxide-loaded ARG-NPs was visualized by transmission electron microscopy (TEM); scale bars for SEM and TEM micrographs represent 1 μ m.

Figure 2.

Comparison of Coumarin 6-labeled NPs coated with ANTP, ARG, and TAT; unmodified NPs and PEGylated NPs were used as controls. CPP density (~3, 8, and 15 ug of peptide per mg of NP) on the surface of NPs increases from left-to-right for individual groups. Cellular association and internalization was monitored using flow cytometry and Trypan Blue quenching of extracellular fluorescence. Shown is the mean channel (FL-1) fluorescence, $n=3$; statistical analysis was performed with respect to unmodified nanoparticles for either the associated or internalized groups, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 3.

(A) Controlled release profiles of nucleic acids from uncoated NPs. The inset depicts initial burst release; axes are the same as the parental figure. Cation refers to the positively-charged counter-ion, spermidine, which was used to enhance loading of anionic ssDNA. (B) Antisense translation-based inhibition of *in vitro* expression of luciferase. Each group comprised the total amount of PMOs released from NPs over the indicated time interval. *** $p < 0.0005$; * $p < 0.05$.

Figure 4.

(A) miR-155 dual luciferase sensor demonstrating the inhibition of miR-155 activity in KB cells by anti-miRs (PMOs or PNAs as indicated) that were delivered by ARG-NPs. Renilla luciferase was normalized to Firefly luciferase signal. *** $p < 0.0001$. (B) Relative levels of miR-155 in KB cells treated with PMO anti-miRs loaded in NPs. *** $p < 0.0001$.

Figure 5.

(A) Confocal microscopy of cellular localization of ARG-NPs delivering FITC-labeled PMOs. Green = PMO; red = actin; blue = nucleus; scale bar represents 25 μ m (B) Electrophoretic fractionation of Mcl-1 splicing isoforms, Mcl-1L and Mcl-1S. Both isoforms were amplified from the same set of primers; Mcl-S (757 bp) is a truncation of Mcl-1L (1005 bp). β -actin was used as a loading control.

Figure 6.

(A) Dose response of KB cells to treatment with anti-mir-155 or Mcl-1 splice-shifting PMOs. Data presented as percent survival relative to untreated cells. 2-way ANOVA was used for statistical analysis relative to ARG-NP anti-CRL group. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. (B) Extent of apoptotic DNA fragmentation measured by quantifying cytosolic mono- and oligonucleosomes in cells treated with anti-mir-155 or Mcl-1 splice-shifting PMOs. Data presented as fold-change relative to untreated cells. ** $p < 0.005$.

Table 1

Surface charge and loading properties of NPs encapsulating nucleic acids.

Formulation	ζ -potential (mV)	Diameter (nm)	Loading (pmoles/mg)	Encapsulation Efficiency (%)	Molecules per NP
PMO	-19.5 ± 0.3	166 ± 34	400 ± 34	41 ± 3	520 ± 40
PNA	-22.6 ± 0.2	172 ± 47	410 ± 55	41 ± 6	530 ± 70
ssDNA	-30.7 ± 1.0	159 ± 38	25 ± 5	3 ± 1	30 ± 6
ssDNA:cation	-6.6 ± 0.1	168 ± 44	49 ± 11	5 ± 1	60 ± 10