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Interfering nanoparticles (iNOPs) for silencing microRNAs

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Abstract

MicroRNAs (miRNAs) are single-stranded non-coding RNAs ~21 nt in length and regulate gene expression at posttranscriptional level. miRNAs are involved in almost every area of biology, including developmental processes, disease pathogenesis, and host-pathogen interactions. Dysregulation of miRNAs in various disease states makes them potential targets for therapeutic intervention. Specific miRNAs can be silenced by anti-microRNAs (anti-miRs) which are chemically modified antisense oligonucleotides complimentary to mature miRNA sequences. *In vivo* delivery of anti-miRs is the main barrier in achieving efficient silencing of target miRNAs. A new systemic delivery agent, interfering nanoparticles (iNOPs), was designed and prepared from lipid-functionalized poly-L-lysine dendrimer. iNOPs can efficiently deliver small RNAs including short interfering RNAs (siRNAs), miRNA mimics and anti-miRs. Systemic delivery of a chemically stabilized anti-miR-122 by iNOPs effectively silences miR-122 in mouse liver. Intravenous administration of 2mg/kg-1 anti-miR-122 complexed with iNOP-7 results in 83% specific silencing of target miRNA. The specific silencing of miR-122 by iNOP-7 is long lasting and does not induce an immune response.

1. INTRODUCTION

MicroRNAs (miRNAs) are single-stranded RNAs ~21 nt in length that are involved in developmental processes, disease pathogenesis, and host-pathogen interactions (Ambros, 2011; Kim et al., 2009; Krol et al., 2010). The biogenesis of mature miRNAs depends on cleavage of the precursor RNA hairpin structure by two members of the RNase III family, Drosha and Dicer, while other miRNAs can be generated through splicing of miR-coding introns (Carthew and Sontheimer, 2009; Kim et al., 2009). For functional assemblies, miRNAs are loaded into a ribonucleoprotein assembly called the RNA-induced silencing complex (RISC), which serves as the catalytic engine for miRNA-mediated post-transcriptional regulation. Although some studies have suggested a potential role for miRNAs in translational activation (Henke et al., 2008; Orom et al., 2008; Vasudevan et al., 2007), the more common mechanism of miRNA-mediated gene regulation involves repression. In general, miRNAs bind imperfectly to the 3' UTR of target mRNA and block their expression by directly inhibiting the translational steps and/or by enhancing mRNA destabilization (Bagga et al., 2005; Fabian et al., 2010; Guo et al., 2010). Recent studies have identified the role of GW182 protein in the molecular mechanism of miRNA-mediated mRNA deadenylation (Behm-Ansmant et al., 2006; Eulalio et al., 2007; Iwasaki and Tomari, 2009). GW182 directly interacts with all members of the Ago protein family and is localized within P-bodies in the cytoplasm of mammalian cells (Fabian et al., 2010). Another P-body protein, RCK/p54, a DEAD box helicase, has been shown to interact with the argonaute proteins, Ago1 and Ago2, and modulate miRNA function (Chu and Rana,

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2006). RCK/p54 facilitates formation of P-bodies and is a general repressor of translation, suggesting that miRNAs are transferred to P-bodies for further decay or storage (Chu and Rana, 2006).

Many disease-affected tissues have characteristic miRNA expression patterns (Chang et al., 2008; Munker and Calin, 2011; Suzuki et al., 2011). Selective elimination of upregulated miRNAs in disease-affected tissue could provide a potential therapeutic approach (Krutzfeldt et al., 2005; Montgomery and van Rooij, 2011). Specific disease-related endogenous miRNAs can be silenced by their complementary antisense sequence, anti-miRs. However, development of anti-miR-based therapies faces many challenges including stabilization and optimization of anti-miR sequences and efficient delivery of these sequences to the tissues. While many anti-miR sequences and chemical modifications have been successfully designed to target miRNAs (Lennox and Behlke, 2010), developing efficient in vivo delivery agents is essential for successful therapeutic development. An efficient delivery agent should protect anti-miR from degradation by endonuclease in circulation and in tissue, facilitate cellular uptake and release the cargo in a tissue specific manner (Baigude et al., 2007; Baigude and Rana, 2009; Su et al., 2011).

2. DELIVERY OF SHORT THERAPEUTIC RNAS

2.1 NANOPARTICLES FOR SHORT INTERFERING RNA DELIVERY

Nanoparticles have attracted much attention as nonviral carriers for in vivo delivery of short synthetic RNAs. Traditional drug delivery method involving liposome formulation has been readily adopted and optimized for siRNA delivery. Highly efficient in vivo siRNA delivery based on liposome has been reported. By optimizing structure and components of lipid molecules together with formulation methods, Love et al was able to knock down 80% a clinically relevant gene transthyretin at a dose as low as 0.03 mg/kg-1 (Love et al., 2010; Semple et al., 2010). Although several approaches have been reported to attenuate liposome for tissue specific siRNA delivery (Peer et al., 2008), main target tissue of lipid-based siRNA delivery has been limited to liver. Cationic polymer-based delivery is another highly attractive approach for systemic delivery of short therapeutic RNAs. Both synthetic polymer such as polyethylenimine (PEI) (Nimesh and Chandra, 2009; Urban-Klein et al., 2005) and naturally occurring polycation such as Chitosan (Howard et al., 2006; Liu et al., 2007; Pille et al., 2006) have been reported to be useful for in vitro and in vivo siRNA delivery. SiRNA can also be conjugated to cationic polymers for more efficient systemic delivery. A polyconjugate was prepared by covalently attaching siRNA to an amphipathic poly(vinyl ether) and was functionalized with hepatocyte targeting ligand *N*-acetylgalactosamine and polyethylene glycol (PEG) (Rozema et al., 2007). The 10nm polyconjugate nanoparticle successfully delivered siRNA to mouse liver, significantly knocking down the target gene. Cationic peptides have also been used for nanoparticle formulation and siRNA delivery. Apart from popular ones such as Tat peptide (Chiu et al., 2004) and poly-arginine (Kim et al., 2006), peptide transduction domains (PTDs) have been reported for targeted siRNA delivery (Eguchi and Dowdy, 2010; Palm-Apergi et al., 2011). Remarkably, a poly-arginine functionalized 29-amino acid peptide derived from rabies virus glycoprotein (RVG) enables the transvascular delivery of small interfering RNA (siRNA) to mouse brain by specifically binding to the acetylcholine receptor expressed by neuronal cells (Kumar et al., 2007). Nanoparticles or nanomaterials have also been reported to deliver anti-miRNA in vitro (Dong et al., 2011; Kim et al., 2011).

2.2 DELIVERY OF MIRNA INHIBITORS

Mature miRNAs are single-stranded ~21 nucleotides long RNA molecules. An anti-miR is a single-stranded oligonucleotides containing a complementary sequence to a mature miRNA.

Single-stranded 2'-O-methyl modified sequences that are complementary to endogenous microRNA can inhibit miRNA function by interacting with the miRNA-RISC nucleoprotein complex (Hutvagner et al., 2004; Meister et al., 2004). Transfection reagents that are used for siRNA delivery can also be used for anti-miRs delivery. Commercially available transfection reagents such as lipofectamine 2000 can deliver anti-miRs to the cells in vitro. Chemically stabilized cholesterol-conjugated anti-miRs (antagomirs) have been used for in vivo silencing of endogenous miRNA (Krutzfeldt et al., 2005). However, a relatively higher dose (80 mg kg⁻¹) had to be administered to achieve significant knockdown of miRNA. Chemically stabilized anti-miR sequences with 2'-fluoro and/or methoxyethyl groups have increased inhibitory activities (Rayner et al., 2011). The anti-miR efficiency of a complementary sequence of miRNA can be further enhanced by incorporating locked nucleic acids (LNA) nucleotides in the sequence (Chan et al., 2005; Elmen et al., 2008; Lecellier et al., 2005; Wang et al., 2011). Incorporation of double-stranded flanking regions significantly increases inhibitor function (Vermeulen et al., 2007).

We designed and created a series of iNOPs for short RNA delivery in vitro and in vivo. iNOP-7 is polymeric nanoparticles chemically synthesized by functionalizing a generation 4 (G4) poly-l-lysine dendrimer (LDG4) with lipids (such as oleic acid) (Baigude et al., 2007). Chemically synthesized LDG4 is highly branched poly-l-lysine bearing 32 amino groups on the surface. The amino groups, which are positively charged at physiological condition, provide excellent binding affinity for any negatively charged macromolecules, such as oligonucleotides, siRNA, microRNA and plasmid DNA. The amino groups are also viable to various chemical modifications for further functionalization of the nanoparticles such as tissue specific delivery of nucleic acids. For example, to increase hydrophobicity of the dendrimer to facilitate cellular uptake of the nanoparticles, we chemically conjugated lipid chain with different degree of saturation and different length (C12~C18) and found that oleic acid is one of the best lipids for this kind of purpose, although linking other lipids to LDG4 can also increase the hydrophobicity of dendrimer/nucleic acid complex and cellular uptake. By carefully adjusting the number of oleic acid (6 to 13 oleoyl groups) coupled to LDG4, we were able to find the optimal number which make the resulting dendrimer least toxic and most efficient in siRNA or microRNA delivery in vitro and in vivo. iNOP-7, which is composed of LDG with 7 oleic acids moiety, is non-toxic at a concentration of up to 1.5 μ M for in vitro delivery of siRNA. More importantly, iNOP-7 can deliver siRNA (against ApoB) in mice and obtained at least 50% of decrease in ApoB mRNA level (Figure 1 and 2).

The soluble, 15 nm nanoparticles readily bind to short nucleic acids (siRNA and/or microRNA) at neutral pH and form nanoparticles with diameter less than 200 nm (Fig 3). Since iNOP-7 is made from a naturally occurring amino acid, it is less toxic and more biocompatible in contrast to other non-degradable polymeric nanoparticles and inorganic nanoparticles. Besides, iNOP-7 is more flexible in terms of surface functionalization due to the more accessible surface groups. One of the iNOPs, iNOP-7 was able to deliver siRNA against apoB in mouse liver (Baigude et al., 2007). Moreover, iNOP-7 was able to deliver chemically stabilized anti-miR-122 to mouse liver and significantly silenced the target miRNA-122 (Su et al., 2011).

3. PROTOCOLS

3.1 Determining the silencing efficiency of anti-miRs

There are two main methods to detect the endogenous miRNA level after transfecting anti-miRs to the cells or tissues: (1) Northern blotting and (2) quantitative PCR. Although there has been no direct proof so far, a duplex of miRNA-anti-miR could potentially degraded by nuclease present in the cell. Northern blotting is routinely used methods for detection of

miRNA levels. The silencing effect of anti-miR can also be evaluated by using commercially available quantitative PCR kits.

3.1.1 Determining the silencing efficiency by northern blotting—RNA from cell culture or tissue can be isolated by homogenizing in TRIZOL according to the manufacturer's instructions. Total RNA can be separated on a 14% acrylamide containing 20% formamide and 8M urea gel, then electroblotted onto Hybond-XL nylon membrane (GE Healthcare, Piscataway, NJ, USA). The probe with g-32P-labeled oligonucleotides for miRNA or rRNA is hybridized to the membrane at 42 °C. The blots are visualized by scanning in a FLA-5000 scanner (Fujifilm, Stamford, CT, USA).

3.1.2 Determining the silencing efficiency by quantitative PCR—Silencing efficiency of anti-miR delivered to the cells can be determined by RT-qPCR. The total RNA is used to quantify miR expression level using Mir-X™ miRNA First-Strand Synthesis and SYBR qRT-PCR kit according to the manufacturer's instruction.

3.1.3 Dual luciferase assay—Anti-miR transfection efficiency can be determined by in vitro dual luciferase assay. Following is a protocol that we used for preliminary determination of anti-miR transfection efficiency by iNOP-7.

The miR-122 luciferase constructs were engineered by inserting the full 23-bp sequence complementary to the mature miR-122 into the 3'-UTR of pGL3-Control as described previously (Chu and Rana, 2006) (Promega, Madison WI, USA). Huh-7 cells were seeded in 24-well culture plates and transfected with 0.1 mg miR-122 pGL3-control plasmid and 0.015 mg pRL-TK plasmid (Promega, Madison WI, USA) for normalization using iNOP-7. After 4 h of transfection, cells were treated with complete media. Cells were lysed 48 h later, unless otherwise indicated, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison WI, USA). These quantitative assays were conducted in multiple replicates for each concentration.

3.2 Oligonucleotides

The anti-miR efficiency of iNOP-7 can further be increased by using chemically modified RNAs (Chiu et al., 2004; Chiu and Rana, 2002, 2003). For example, to knockdown endogenous microRNA-122 we designed a chemically stabilized 23–24 nucleotides length anti-miR-122 as following (Dharmacon, Lafayette, CO, USA):

anti-miR-122 (CM): 5'-AC^FAAAC^FAC^FC^FAUUGUCACACUCCA-3';

mismatch anti-miR-122 (MM): 5'-UCAC^FAAAC^FCUCC^FUAGAAAGAGUAGA-3'.

The superscript letter F represents 2'-O-F modified nucleotides. Chemical modifications were designed based on our previously published rules established in our laboratory (Rana, 2007).

3.3 In vitro silencing of microRNA by iNOP-7

3.3.1 Preparation of materials for in vitro transfection—iNOP-7 readily dissolves in water. A stock solution of iNOP-7 (0.2 mM) is prepared by dissolving 1.16 mg iNOP-7 in 1.0 ml RNase free water. The stock solution is sterilized by filtering through a 0.22 um spin filter. No prepare a buffered solution if iNOP-7, first dissolve it in water at 2X concentration and then dilute with equal volume of buffer of choice. The aqueous solution of iNOP-7 can be preserved at 4 °C for at least 6 months without losing any apparent transfection efficiency.

A 50 μ M stock solution of anti-miRs is prepared by suspending the powders provided by the manufacturer in 1X siRNA buffer.

3.3.2 Preparation of cells for in vitro transfection—To achieve the highest transfection efficiency with iNOP-7, seed actively growing cells in six-well plate 15 h to 20 h before transfection. The confluency of the cells at the time of transfection should be about 70%. The state of the cells before transfection can impact anti-miR transfection efficiency. Use cells with lower passage number and monitor cellular morphology. Use cells with similar passage number and appropriate cell density to achieve reproducible transfection results.

For example, Huh-7 cells were maintained at 37 °C with 5% CO₂ in DMEM with High Glucose culture medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml-1 penicillin and 100 mg ml⁻¹ streptomycin. Cells were regularly passaged and plated in 6-well culture plates for 16 h before transfection at 70% confluency. The anti-miRs were transfected in Opti-MEM serum free culture medium for 4 h at 37 °C, and then changed to normal medium with 10% FBS.

3.3.3 Transfection of adherent cells in six-well plate using iNOP-7

3.3.3.1 Serum free transfection

1. Check the cells under microscope for optimal confluency.
2. Add 500ul of serum-free medium (such as Opti-MEM) to a sterile plastic tube and add 5 ul of iNOP-7. Vortex gently to mix.
3. Incubate at room temperature for 5 min.
4. Add anti-miR (1.0 ul of a 50 μ M siRNA stock) to another sterile plastic tube containing 500 ul of Opti-MEM.
5. To allow nanoparticle formation incubate at room temperature for 20 min.
6. Remove cell culture medium and wash the cells twice with PBS pH7.4.
7. Add iNOP-7/anti-miR complex dropwise to the cells, and gently rock the plate to disperse the complexes.
8. Incubate at 37 °C for 2 h to 3 h.
9. Remove transfection medium and add 2.0 ml complete medium.
10. Incubate for 24 h

3.3.3.2 Transfection in complete medium

1. Check the cells under microscope for optimal confluency.
2. Add 250ul of serum-free medium (such as Opti-MEM) to a sterile plastic tube and add 5 ul of iNOP-7. Vortex gently to mix.
3. Incubate at room temperature for 5 min.
4. Add anti-miR (1.0 ul of a 50 μ M siRNA stock) to another sterile plastic tube containing 250 ul of Opti-MEM.
5. To allow nanoparticle formation incubate at room temperature for 20 min.
6. Remove 500 ul of complete medium from each wells of six-well plate and add iNOP-7/anti-miR complex.

7. Gently rock the plate to disperse the complex.

Different cell lines have different sensitivity to iNOP-7. Therefore optimal amount of iNOP-7 used for a certain cell line must be determined. We recommend to test a few different concentrations of iNOP-7 as a preliminary experiment to optimize the conditions. At least three serial concentrations should be tested: 2.5 μ l, 5.0 μ l and 7.5 μ l for each six-well plate, which give 0.5 μ M, 1.0 μ M and 1.5 μ M concentration, respectively.

3.4 In vivo silencing of microRNA by iNOP-7

3.4.1 Formulation of iNOP-7/anti-miR-122

1. Prepare a stock solution of anti-miRs by resuspending RNAs in 1X siRNA buffer. The concentration of stock RNA solution is 1.0 mM, which can be stored at -80°C . To prevent frequent freeze-thawing, the stock solution should be frozen in small aliquots.
2. Prepare a stock solution of nanoparticle-7 in RNase free water at 3.0 to 5.0 mg/ml concentration. Sterilize by filtering. The stock solution of nanoparticles-7 can be preserved at $+4^{\circ}\text{C}$ for at least 6 months without losing any apparent activity.
3. Assemble iNOP-7 in a sterile plastic tube. Take anti-miR and add to 1X HEPES buffer. Amount of RNA varies depending on the dosage. For example, 50 μ g anti-miR is added to 100 μ l 1X HEPES buffer for 2.0 mg/kg-1 dose for a 25 g mouse. Mix well and keep on ice.
4. Dilute 250 μ g stock nanoparticle-7 in 1X HEPES buffer to make 100 μ l solution. Mix well.
5. Carefully add 100 μ l anti-miR solution to 100 μ l nanoparticles-7. Mix by pipetting up and down for 5 to 10 times.
6. Incubate at room temperature for 20 min to form iNOP-7.

3.4.2 In vivo delivery of anti-miR-122

1. Animal: six- to eight-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) are used for in vivo anti-miR (or siRNA) delivery experiment. Mice should be maintained under a 12 h dark cycle in a pathogen-free animal facility.
2. Inject iNOP-7 via the lateral tail vein on three consecutive days with iNOP-7 complexes of anti-miR or its mm anti-miR. Daily dosages of 0.20–5 mg kg $^{-1}$ RNA can be delivered in a final volume of 0.2 mL.

3.4.3 Determining the delivery efficiency—The delivery efficiency of iNOP-7 can be determined twenty-four hours after the final injection. In case of siRNA delivery, tissue levels of target mRNA can be determined by RT-qPCR, and the level of protein can be determined by Western blotting, providing that an antibody against target protein is available. In case anti-miR delivery, endogenous level of microRNA can be determined by either RT-qPCR or Northern blotting.

To determine mRNA levels in tissues, small uniform samples are collected from three regions of the tissue. Total RNA is extracted with Trizol and treated with DNase I before quantification. mRNA level is determined by qPCR as described above. After anti-miR treatment, the mRNA levels of genes regulated by microRNA can be determined by RT-qPCR.

4. CONCLUDING REMARKS

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that regulate post-transcriptional gene expression and are important in many biological processes. Disease-associated miRNAs have been shown to become potential targets for therapeutic intervention. The newly designed nanoparticles (iNOP-7) can be used to deliver single-stranded anti-miR oligonucleotides to silence disease-related endogenous miRNA. iNOP-7 containing chemically modified anti-miRs specifically silence miRNAs in liver. iNOP-7 treatment is non-toxic and do not induce an immune response. Furthermore, iNOP-7 can be modified to target specific tissues and develop tissue specific RNAi-based therapies.

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Figure 1.

(A) Schematic illustration of nanoparticles-siRNA complex (iNOP-7) initiating cellular entry. (B) Chemical structure of the nanoparticles used to construct iNOP-7. Particle size of iNOP-7 before (C) and after (D) complexed with short RNA (in this case, a 21-mer duplex RNA) measured by dynamic light scattering

Figure 2.
Schematic illustration of iNOP-7 bringing siRNA into cytoplasm and knocking down endogenous mRNAs.

Figure 3.

Localization of siRNA. (A) Cells were transfected with iNOP-7 containing Cy3-labeled siRNA. Localization of the duplex siRNA after 24 h was monitored by confocal microscopy. Overlay images of siRNA and nuclear DNA (4',6-diamidino-2-phenylindole [DAPI] stained) are shown. (B) Cells transfected with iNOP-7/Cy3 siRNA complex were stained fluorescein labeled Phalloidin to show cell outlines. (C) Fluorescence microscopy analysis of siRNA distribution in vivo. Histology slide of liver shows the distribution of siRNA in liver 4 hours after intravenous administration of iNOP-7 was injected. Scale bar: 100 micron. (D) Bright field microscopic image of (C).

Figure 4.

(A) In vitro delivery of ApoB siRNA to FL83B cells by iNOP-7. More than 90% decrease in ApoB mRNA was achieved by using 1.0 uM concentration of iNOP-7 complexed to ApoB siRNA (50 nM). (B) In vivo delivery of ApoB siRNA to mouse. A 1.5 mg kg⁻¹ dose of iNOP-7 complexed to ApoB siRNA was injected to C57BL/6 mice via tail vein. After 48 h, liver mRNA level of ApoB was analyzed by quantitative PCR.

Figure 5.

(A) Silencing of miR-122 by iNOP-7 in vitro. Huh-7 cells were transfected by iNOP-7 containing anti-miR-122 at varying concentrations as indicated for 4 h. Total RNA was isolated from cells 24 h after transfection and separated on 14% polyacrylamide gels. Membrane was probed for miR-122 and miR-22, respectively. 5S rRNA is shown as a loading control. (B) Specific silencing of miR-122 in mice treated with iNOP-7 assembled with chemically modified anti-miR-122. Mice were intravenously injected with 2mg kg⁻¹ of iNOP-7 containing chemically modified anti-miR-122, mismatched chemically modified anti-miR-122, or PBS at 0, 12 and 36 h. Tissues were harvested at 24 h after the last injection to measure miRNA or mRNA levels. Total RNA was isolated from mouse liver 24 h after last injection. Samples were separated in 14% polyacrylamide gel and membrane was probed for miR-122, miR-22 and 5S rRNA. 5S rRNA is shown as a loading control.