Published in final edited form as:

Methods Mol Biol. 2016 ; 1364: 1–9. doi:10.1007/978-1-4939-3112-5_1.

Synthesis and Conjugation of Short Interfering RiboNucleic Neutral siRNNs

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Summary

Due to their high potency (EC₅₀ ~1 pM) and exquisite target selectivity for all expressed mRNAs, small interfering RNA (siRNA) induced RNAi responses hold significant promise as a therapeutic modality. However, the size and high negative charge of siRNAs renders them unable to enter cells without assistance from a delivery agent. Most current methods of siRNA delivery rely on encasing siRNA molecules in large nanoparticles or cationic liposomes. However, these approaches suffer from a number of problems, including a poor diffusion coefficient, cytotoxicity, and poor pharmacokinetics. To address the problem of siRNA *in vivo* delivery, we developed monomeric neutral RNAi prodrugs, termed siRiboNucleic Neutrals (siRNNs), that directly neutralize the phosphate backbone negative charge by synthesis with bioreversible phosphotriester groups that are enzymatically cleaved off in the cytoplasm of cells. Here we describe the synthesis and purification of siRNN conjugates that induce *in vivo* target gene knockdown following systemic delivery into mice.

Keywords

siRNN Prodrug; RNAi Delivery; siRNN Delivery; bioreversible phosphotriester; GalNAc

1. Introduction

siRNA-induced RNAi responses have become a standard methodology used in the study of gene function *in vitro*, but the biophysical properties of siRNAs have limited their use *in vivo*. siRNAs can be synthesized in a scalable manner, allowing for the rapid production of siRNAs directed against any target mRNA (1). However, for all the promise as a therapeutic, siRNAs have several significant negative attributes that limit their usefulness. The high negative charge of siRNA phosphodiester backbone and the 14,000 Dalton (Da) size prevent siRNA molecules from crossing the cellular membrane (2). These attributes also make siRNAs pharmacokinetically unfavorable, as naked siRNAs are removed from the bloodstream by the kidneys within minutes of injection into a mouse (3). Moreover, naked native charged siRNAs are rapidly degraded in blood by serum nucleases.

Here, we demonstrate the synthesis of monomeric neutral RNAi prodrugs, termed siRiboNucleic Neutrals (siRNNs). Due to neutralization of the negatively charged

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phosphodiester with bioreversible phosphotriester protecting groups, siRNNs show high stability against serum nucleases and can be used for *in vivo* delivery. Inside of cells, the S-acyl-2-thioethyl (SATE) phosphotriester group used in siRNNs is specifically converted into a charged native phosphodiester linkage by cytoplasmic thioesterases, resulting in a native wild type charged siRNA that is loaded into Ago2 to induce robust RNAi responses (4,5). RNN oligonucleotides can be synthesized on standard oligonucleotide synthesizers using commercially available phosphoramidites and RNN phosphoramidites that can be synthesized on site (6). Similar to RNA, RNN oligonucleotides are purified by reversed-phase high-performance liquid chromatography (RP-HPLC). Purified RNN oligonucleotides are then analyzed by mass spectrometry and urea denaturing gel electrophoresis. RNNs show both high coupling and purification yields on par with, if not better than RNA oligonucleotides. Finally, RNN oligonucleotides containing reactive phosphotriester groups are conjugated to Tris-GalNAc, a hepatocyte-specific targeting domain, and duplexed to complementary RNN oligonucleotides to form siRNN conjugates ready for animal injection (7).

2. Materials

Equipment Required

1. Oligonucleotide synthesizer.
2. Centrifugal evaporator.
3. RP-HPLC.
4. MALDI-TOF.
5. Lyophilizer.

2.1 RNN Oligonucleotide Synthesis + Purification Components

1. 1. Phosphotriester Phosphoramidites: Cannot contain a 2'-OH and must have ultramild exocyclic amino group protection (either phenoxyacetyl (PAC) or 4-isopropylphenoxyacetyl (iPr-PAC)) (see **Note** ¹).

Recommended RNA phosphoramidites:

- 2'-OMe-5'-O-DMTr-PAC-A-CE (Glen Research, Cat. # 10-3601-XX)
 - 2'-Fluoro-5'-O-DMTr-PAC-C-CE (R. I. Chemicals, Cat. # P1500-01)
 - 2'-OMe-5'-O-DMTr-iPr-PAC-G-CE (Glen Research, Cat. # 10-3621-XX)
 - 2'-Fluoro-5'-O-DMTr-U-CE (R. I. Chemicals, Cat. # F-1015)
2. RNN phosphoramidites (Synthesis methods described in Meade *et al.* [6]).
 - 2'-OMe-5'-O-DMTr-PAC-A-tBu-SATE

¹RNN oligonucleotide synthesis is incompatible with phosphoramidites that contain 2'-OH (following deprotection). Presence of 2'-OH groups will also lead to strand scission at locations of phosphotriester nucleotides.

- 2'-Fluoro-5'-O-DMTr-PAC-C-*t*Bu-SATE
- 2'-OMe-5'-O-DMTr-iPr-PAC-G-*t*Bu-SATE
- 2'-Fluoro-5'-O-DMTr-U-*t*Bu-SATE.
- Aldehyde A-SATE phosphoramidites of the above.
- dT-Q-CPG 500 (Glen Research, Cat. # 21-2230-XX) (*see Note* ²)

3. Oligonucleotide synthesis chemicals:

- Activator: 5-Benzylthio-1H-tetrazole (BTT) in Acetonitrile (Glen Research, Cat.# 30-3170-XX) (*see Note* ³)
- Deblocking Reagent: 3% TCA (w/v) (VWR, Cat. # EM-BI0830-0950)
- Cap A: Ultra Mild Cap A THF/Pyridine/Pac₂O (Glen Research, 40-4210-XX) (*see Note* ⁴)
- Cap B: 16% MeIm in THF (Glen Research, 40-4220-XX)
- Acetonitrile, Anhydrous (VWR, Cat. # AX0151-1)
- Oxidizing Reagent: 0.02M I₂O in THF/Pyridine/H₂O (70/20/10) (VWR, Cat. # EM-BI0420-4000)
- CPG cleavage solution: 10% diisopropylamine (DIA) (Sigma-Aldrich, Cat. # 386464), 90% anhydrous methanol (EMD Millipore, Cat. # EM-MX0472-7). Prepare immediately before use.

4. Purification components:

- HPLC Buffer A: 50 mM triethylamine acetate (TEAA) in ultrapure water
- HPLC Buffer B: 90% acetonitrile (ACN) in ultrapure water
- Stericup-GP, 0.22 μm, polyethersulfone, 1000 mL (EMD Millipore, Cat. # SCGPU11RE-1)
- Matrix for MALDI-TOF analysis of RNNs: 10 mg/mL 2',4',6'-Trihydroxyacetophenone (THAP) + 50 mg/mL diammonium citrate in 50% acetonitrile and deionized water (Sigma-Aldrich, Cat. # 91928) (*see Note* ⁵)
- DMT removal buffer: 80% glacial acetic acid, 10% ACN, 10% ultrapure water.

²Any solid support that is susceptible to cleavage by 10% diisopropylamine in methanol may be used for RNN oligonucleotide synthesis.

³BTT activator may form crystals on the ends of oligonucleotide synthesizer activator injection lines. If this occurs, increase activator priming time or number of primes to remove obstructing crystal and ensure proper activator injection.

⁴Ultra Mild Cap A must be used to avoid exchange of iPr-PAC or PAC protecting groups with acetyl groups.

⁵3-hydroxypicolinic acid (3-HPA) matrix is generally recommended for MALDI-TOF analysis of oligonucleotides >3,500 Da. However, THAP matrix is better suited for the analysis of fully 2'-OMe/F modified oligonucleotides.

2.2 RNN Oligonucleotide Analysis Components

1. Illustra NAP-10 Columns, Sephadex G-25 DNA Grade (GE Healthcare Life Sciences, Cat. # 17-0854-02)
2. 40% acrylamide/bis solution, 19:1.
3. 10× TBE (Tris-Borate-EDTA).
4. Ammonium persulfate: 10% solution in deionized water.
5. N,N,N',N'-Tetramethylethylenediamine (TEMED).
6. Gel running buffer: 1× TBE in deionized water.
7. 100× bromophenol blue (BPB) solution: 0.2 g Tris base, 0.2 g BPB dissolved in 9.9 mL of deionized water.
8. 2× Denaturing Gel Loading Buffer (10 mL): 9.8 mL of formamide, 100 µL of 0.5 M EDTA (pH 8.0), and 100 µL of 100× BPB solution.
9. Methylene blue stain: 250 µL saturated methylene blue solution (35 mg/L in water) and 19.75 mL of deionized water.

2.3 siRNN Conjugation Components

1. Aniline
2. GalNAc-HyNic (Synthesis methods described in Slidregt *et al.*, Rensen *et al.*, and Meade *et al.*) (6–8).
3. Ethanol (200 proof).

3. Methods

3.1 RNN Oligonucleotide Synthesis + Purification

1. Synthesize RNN oligonucleotide on standard solid-phase oligonucleotide synthesizer. Use oligonucleotide synthesis chemicals, dT-Q-CPG solid support, and phosphoramidites listed in section 2.1. Use 6 minute coupling time for all RNN phosphoramidites. Synthesize RNN oligonucleotides DMT-On for RP-HPLC purification (*see Note* ⁶).
2. After completion of RNN oligonucleotide synthesis, remove column from synthesizer and transfer resin into a 2 mL screw cap microcentrifuge tube. Add 1 mL of CPG cleavage solution to tube containing CPG-oligonucleotide. Incubate for 4 hours at room temperature on a rotisserie rotator.
3. After 4 hours, pellet CPG by brief centrifugation in a mini centrifuge (5 seconds at 2,000 × g). Transfer crude RNN oligonucleotide supernatant to a new 1.5 mL microcentrifuge tube. Wash CPG with 0.5 mL of anhydrous methanol and pool with crude RNN oligonucleotide solution.

⁶If synthesis of a phosphorothioate oligonucleotide is desired, RNN phosphoramidites are compatible with sulfurization during oligonucleotide synthesis (Sulfurizing Reagent II, Glen Research, Cat. # 40-4037-XX).

4. Dry crude RNN oligonucleotide by centrifugal evaporation (*see Note* ⁷).
5. Once dry, dissolve crude RNN oligonucleotide pellet in 50% ACN by briefly heating at 65°C and vortexing.
6. Inject into RP-HPLC column for purification with the following protocol:
 - Flow rate: 1 mL/min
 - Gradient:
 - 0 – 2 min (100% Buffer A/0% Buffer B)
 - 2 – 42 min (0% Buffer B to 60% Buffer B)
 (see **Notes** ⁸ and ⁹) (Fig. 1A).
7. Collect 500 µL fractions across DMT-On peak.
8. Analyze HPLC fractions for presence of full-length RNN oligonucleotide on MALDI-TOF using THAP matrix (Fig. 1B).
9. Freeze fractions containing full-length RNN oligonucleotide and lyophilize until dry (*see Note* ¹⁰).
10. Once dry, dissolve each fraction in 100 µL of 50% ACN in ultrapure water and pool with other fractions containing full-length RNN oligonucleotide. Freeze and lyophilize dry pooled RNN oligonucleotide.
11. To remove DMT group, add 200 µL of DMT-removal buffer to lyophilized RNN oligonucleotide pellet. Incubate solution for 1 hour at 65°C. After 1 hour, freeze and lyophilize solution.
12. For RNN oligonucleotide desalting, equilibrate NAP-10 column with 15 mL of 20% ACN in ultrapure water. Resuspend RNN oligonucleotide in 1 mL of 20% ACN in ultrapure water. Incubate RNN oligonucleotide solution at 65°C for several minutes and vortex periodically to bring oligonucleotide into solution. Once in solution, cool to room temperature and then add to equilibrated NAP-10 column. To elute RNN oligonucleotide add 1.4 mL of 20% ACN in ultrapure water to the NAP-10 column and capture eluent. Freeze and lyophilize eluent (*see Note* ¹¹).
13. Resuspend lyophilized RNN oligonucleotide pellet in 200 µL of 50% ACN in water. If necessary, briefly incubate at 65°C and vortex to bring oligonucleotide into solution. Measure absorbance of RNN oligonucleotide at 260 nm. Use the

⁷Do not heat during centrifugal evaporation as this may lead to SATE phosphotriester reversal.

⁸Buffer A should be filtered through a 0.22 µm filter prior to use.

⁹Buffer B can also be made with 50 mM TEAA. However, presence of TEAA in Buffer B does not generally confer a purification advantage and will necessitate more than 2 lyophilizations to fully remove all TEAA from the collected fractions.

¹⁰Do not pool fractions prior to the first lyophilization as this can lead to concentration of TEAA and reversal of SATE phosphotriesters.

¹¹RNN oligonucleotides that contain hydrophobic phosphotriesters (tBu-SATE) may be more difficult to solubilize. For these oligonucleotides it is advisable to add 400 µL of 50% ACN in ultrapure water to lyophilized oligonucleotide pellet, incubate solution at 65°C for several minutes with periodic vortexing, and then add 600 µL of ultrapure water to the solution to bring the final oligonucleotide solution to 1 mL of 20% ACN in ultrapure water.

calculated extinction coefficient for your oligonucleotide and the measured A260 to determine oligonucleotide concentration. Dilute oligonucleotide to desired concentration with 50% ACN in ultrapure water and store at -20°C .

3.2 RNN Oligonucleotide Analysis

1. Mix urea denaturing gel solution components in a 50 mL conical centrifugation tube. Add 10.5 g of urea, microwave for 9 seconds, and vortex until urea is fully dissolved. Add 200 μL of 10% ammonium persulfate, mix, add 20 μL of TEMED, and cast two gels within $7.25\text{ cm} \times 10\text{ cm} \times 1.5\text{ mm}$ gel cassettes. Immediately insert 10-well gel combs without introducing air bubbles. Allow gels to sit at room temperature for 10–15 minutes to ensure complete polymerization before use.
2. Aliquot 1 nmol of RNN oligonucleotide to be analyzed in a fresh microcentrifuge tube. Dry sample by centrifugal evaporation (without heat) or by lyophilization (*see Note* ¹²).
3. Once oligonucleotide samples are completely dry, dissolve pellets in 3–5 μL of $2\times$ denaturing gel loading buffer. Incubate samples in a 65°C heating block for 1 minute and cool on ice.
4. Place gels in running apparatus and fill with gel running buffer. Remove the comb gently and electrophorese for 10 minutes at 200 V constant to preheat the gel prior to gel loading.
5. Prior to gel loading, use a syringe and needle to clean out the wells of the gel. Load prepared oligonucleotide solution to each well and run at 200 V constant for 1 hour or until bromophenol blue dye front runs off of the gel (*see Note* ¹³).
6. Remove gel from running apparatus and glass plates. Place in dish containing 20 mL of methylene blue stain and rock gently at room temperature until desired level of staining is accomplished (*see Notes* ¹⁴ and ¹⁵).
7. Analyze purified RNN oligonucleotides on MALDI-TOF using THAP matrix to ensure that the final product has the expected mass (Fig. 1B).

3.3 GalNAc-siRNN conjugation

1. Set up the following conjugation reaction:
 - a. 120 μM RNN oligonucleotide (containing $1\times$ A-SATE phosphotriester)

¹²The acetonitrile present in RNN oligonucleotide stocks will cause gel loading errors if the sample is not fully dried.

¹³Urea will constantly diffuse out of the gel and into the wells that leads to poor resolution of samples. It is recommended that wells be flushed out with a syringe and needle, the flushed out wells loaded with sample, and then the process repeated until all samples have been loaded. The gel should be loaded and run promptly, as urea will continue to diffuse out of the gel and compress samples loaded into wells.

¹⁴Neutralizing phosphotriesters decrease methylene blue staining of oligonucleotides. This will cause lighter staining of RNN oligonucleotides relative to RNA oligonucleotides despite equimolar loading.

¹⁵5 – 10 minutes of incubation will generally be sufficient to stain 21-mer RNN oligonucleotides. Afterwards, destain gel in deionized water on a rocker at room temperature. Examine destained gel for the presence of fast-migrating truncation products that cannot be seen above background stain prior to destaining.

- b. 600 μ M GalNAc-HyNic
- c. 100 mM Aniline.

In 50% ACN and ultrapure water (*see Note* ¹⁶).

2. Incubate reaction for 1 hour at room temperature (*see Note* ¹⁷).
3. Freeze and lyophilize conjugation solution until dry.
4. Once dry, add 1 mL of ethanol to pellet. Incubate sample in a 65°C heating block for 5 minutes with intermittent vortexing to break up pellet.
5. Incubate solution at –80°C for 30 minutes.
6. Centrifuge sample for 5 minutes at 10,000 $\times$ g to pellet insoluble material.
7. Remove and discard supernatant (*see Note* ¹⁸).
8. Rinse pellet by adding 1 mL of –20°C ethanol to pellet and vortexing.
9. Centrifuge sample for 5 minutes at 10,000 $\times$ g. Remove and discard supernatant.
10. Dry GalNAc-RNN pellet by centrifugal evaporation.
11. Once dry, resuspend pellet in 50% ACN and measure absorbance of RNN oligonucleotide at 260 nm. Use the oligonucleotide extinction coefficient and the measured A260 to determine conjugate concentration (Fig. 1C).
12. To make GalNAc-siRNNs, aliquot an amount of GalNAc-RNN conjugate, add an equimolar amount of the complementary RNN oligonucleotide, heat at 65°C for 5 minutes, vortex, and incubate at room temperature for 15 minutes to allow for RNN oligonucleotide and conjugated RNN oligonucleotide to duplex.
13. Freeze GalNAc-siRNN sample and lyophilize dry (*see Note* ¹⁹).
14. Once dry, resuspended GalNAc-siRNN in water or saline. Sample is now ready for injection into animal models or to treat primary hepatocytes.

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¹⁶If conjugating to an RNN oligonucleotide containing more than 1 $\times$ A-SATE phosphotriester maintain a 1:5 molar ratio of A-SATE to GalNAc-HyNic. For example, if conjugating an RNN oligonucleotide containing 3 $\times$ A-SATEs the reaction should contain 120 μ M RNN oligonucleotide and 1.8 mM GalNAc-HyNic.

¹⁷For reaction volumes > 100 μ L, incubate on a rotisserie rotator.

¹⁸Supernatant contains unreacted GalNAc-HyNic and residual aniline. If desired, supernatant can be retained for re-purification of unreacted GalNAc-HyNic.

¹⁹To examine conjugation status of the RNN oligonucleotide at any time during this procedure, lyophilize 1 nmol of GalNAc-conjugated RNN oligonucleotide and 1 nmol of unconjugated RNN oligonucleotide until dry. Run unconjugated and GalNAc-conjugated RNN oligonucleotide side-by-side on a urea denaturing gel and stain with methylene blue as described in section 3.2 (Fig. 1C).

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Figure 1. Synthesis, purification, and conjugation of RNN oligonucleotides

(A) Representative RP-HPLC chromatogram of a crude RNN oligonucleotide. Gradient begins at 100% 50 mM TEAA in H₂O and ends at 90% ACN. Fractions are collected across the main peak and analyzed by MALDI-TOF mass spectrometry. Fractions containing full-length product are pooled, frozen, and lyophilized for further processing. (B) MALDI-TOF analysis of crude RNN oligonucleotide using THAP matrix. (C) Urea denaturing gel analysis of an RNN oligonucleotide containing one reactive A-SATE phosphotriester

conjugated to Tris-GalNAc. Left lane: unconjugated RNN oligonucleotide. Right lane: GalNAc-conjugated RNN oligonucleotide.