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Lipophilic conjugates for carrier-free delivery of RNA importable into human mitochondria

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Running title: Synthesis of lipophilic conjugates

Lipophilic conjugates for carrier-free delivery of RNA importable into human mitochondria

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Summary

Defects in human mitochondrial genome can cause a wide range of clinical disorders which still do not have efficient therapies. The natural pathway of small non-coding RNA import can be exploited to address into mitochondria therapeutic RNAs. To create an approach of carrier-free targeting of RNA into living human cells, we designed conjugates containing a cholesterol residue and developed the protocols of chemical synthesis of oligoribonucleotides conjugated with cholesterol residue through cleavable pH-triggered hydrazone bond. The biodegradable conjugates of importable RNA with cholesterol can be internalized by cells in a carrier-free manner; RNA can then be released in the late endosomes due to a change in pH and partially targeted into mitochondria. Here we provide detailed protocols for solid-phase and “in solution” chemical synthesis of oligoribonucleotides conjugated to a cholesterol residue through a hydrazone bond. We describe the optimization of the carrier-free cell transfection with these conjugated RNA molecules and methods for evaluating the cellular and mitochondrial uptake of lipophilic conjugates.

Key Words: RNA therapeutics; carrier-free cell delivery; synthesis of lipophilic conjugates; mammalian cells transfection; fluorescent microscopy.

1. Introduction

Mitochondria harbour their own genetic system, but critically depend on the import of nuclear-encoded macromolecules. In all eukaryotes, selected non-coding RNAs (including tRNAs, miRNAs and lncRNAs) produced from the nuclear genome are partially redirected into the mitochondria, where they participate in gene expression (reviewed in (1)).

Defects in human mitochondrial genome can cause a wide range of clinical disorders, mainly neuromuscular diseases (2). Since the most of these diseases still do not have efficient therapies, gene therapy (or more precisely termed, “nucleic acid exploiting therapy”) represents an open possibility. The problem is that there are no methods of the directed transport of DNA or mRNA molecules into the mitochondria, since these organelles are surrounded by a double membrane bearing a negative charge.

The natural pathway of small non-coding RNA import can be exploited to address into mitochondria therapeutic RNAs. Our studies of the import of small non-coding RNAs into human mitochondria allowed the identification of short RNA structural domains serving as import determinants. RNA molecules containing these domains can penetrate into human mitochondria (3-5). This finding has been used to target to mitochondria various small non-coding RNAs, including antireplicative and guide RNAs. The antireplicative strategy aims to prevent the replication of mutant mtDNA by designed recombinant RNA that can be imported and annealed in the replication fork (6). We have demonstrated that small RNAs containing structural determinants for mitochondrial import and 20-nucleotide sequence corresponding to the mutated region of mtDNA, are able to anneal selectively to the mutated mitochondrial genomes. Capable to penetrate into mitochondria of cultured human cells, these RNAs induced a decrease of the proportion of mtDNA bearing pathogenic mutations (5,4,6). We also designed guide RNAs (a component of CRISPR/Cas9 system) targeting different regions of mtDNA and able to be targeted to human mitochondria (7).

A factor that significantly limits biomedical application of RNAs is their inefficient delivery to target cells and tissues (8) due to their large size, negative charge and low stability. Many various systems, including lipid nanoparticles or RNA modifications have been developed to overcome these obstacles (9-12). The successful system for nucleic acids delivery should meet a number of requirements: low cytotoxicity, resistance to nuclease degradation, possibility of endosomal escape and capability of entering the appropriate cellular compartment (13,14). One of the ways to create successful delivery systems is bioconjugation. Currently, for the synthesis of bioconjugates of oligonucleotides with ligands of different functionality, there are two main synthetic strategies - conjugation "in solution" and on solid-phase. Each of these approaches has its own advantages and disadvantages (e.g. see (15,16)). Both strategies allow the use of various types of stable or labile linkers for covalent bioconjugation (17,18).

To decrease the toxicity of the commonly used cell transfection procedure (RNA delivery in the complexes with commercial cationic lipids) and to create an approach of carrier-free targeting of various RNAs into living human cells, we designed conjugates containing a cholesterol residue. Because cholesterol could stall the mitochondrial import of therapeutic RNA due to attachment to the mitochondrial membranes, we developed the protocols of chemical synthesis of oligoribonucleotides conjugated with cholesterol residue through cleavable hydrazone bond (19,20). To obtain an optimal balance between this bond's lability under acidic conditions and stability under neutral conditions, we used the combination of an aromatic aldehyde with an aliphatic acyl hydrazide (21). We designed a linker containing 2 carbon atoms, hydrazone bridge and another 6 carbon atoms between 5'-nucleotide of RNA and cholesterol residue (Fig. 1). Conjugates containing pH-triggered hydrazone bond were characterized by efficient carrier-free cellular uptake and partial co-localization with mitochondrial network. Moreover, the imported oligoribonucleotide designed to target a pathogenic point mutation in mitochondrial DNA was able to induce a decrease in the proportion of mutant mitochondrial genomes (19).

The rationale of the methodology is to use the non-toxic cholesterol ligand to permit the conjugated RNA molecule to enter into the cell without additional formulation *via* endocytosis pathway, followed by a pH-dependent cleavage of the hydrazone bond in the late endosomes and release of the active cholesterol-free RNA, which then can be targeted into mitochondria and affect the replication of the mutant mtDNA (Fig.1).

Here we provide detailed protocols of the solid-phase and “in solution” chemical synthesis of oligoribonucleotides conjugated to a cholesterol residue through cleavable pH-triggered hydrazone bond, and the optimization of cultured cells transfection with these conjugated molecules.

2. Materials

2.1. Synthesis of hydrazide of 6-(cholesteryloxycarbonylamino)hexanoic acid

(*see Note 1*)

1. Pyridine (Py), water (H₂O) <0.01%.
2. 6-Aminohexanoic acid, ≥98.5%.
3. Chlorotrimethylsilane, ≥98.0%.
4. Cholesteryl chloroformate, 95%.
5. Dichloromethane (CH₂Cl₂), ≥99.8%, ~20 ppm amylene as stabilizer, water (H₂O) <0.02%.
6. Hydrochloric acid (HCl), 36.5-38.0%.
7. Sodium chloride (NaCl), ≥99.0%.
8. Sodium sulfate (Na₂SO₄), ≥99.0%, anhydrous, granular.
9. Ethanol, 94-96%.
10. Phosphorus trichloride (PCl₃), 99%.
11. Methanol (CH₃OH), ≥99.9%.
12. Sodium bicarbonate (NaHCO₃), ≥95.0%.
13. Hydrazine monohydrate (NH₂NH₂·H₂O).

14. Kieselgel F₂₅₄ thin-layer chromatography plate.
15. Silica gel, pore size 60 Å, 230-400 mesh.
16. Rotary evaporator system, Rotavapor R-200.
17. Standard organic laboratory glassware.
18. UV lamp (254 nm).
19. Bruker AVANCE III 400 NMR-spectrometer.

2.2. Synthesis of oligoribonucleotides and their aldehyde-modified analogs

1. Acetonitrile (AcN), water (H₂O) <0.02%.
2. Tetrahydrofuran (THF), ~300 ppm butylhydroxytoluene as inhibitor, water (H₂O) <0.02%.
3. Dichloromethane (CH₂Cl₂), ≥99.8%, ~20 ppm amylene as stabilizer, water (H₂O) <0.02%.
4. Pyridine (Py), water (H₂O) <0.01%.
5. 5'-Aldehyde-Modifier C2 phosphoramidite and 2'-*O*-TBDMS-5,*N*-protected ribophosphoramidites only with the UltraMILD base-protecting groups (e.g., acetyl, phenoxyacetyl, 4-isopropyl-phenoxyacetyl). Phosphoramidite solutions prepared in AcN at concentration 0.1M are generally stable for up to 2 weeks when stored under inert atmosphere (*see Notes 2, 3*).
6. Solid support: *N*-Protected 5'-*O*-DMTr-2'-*O*-acetyl-nucleoside -3'-succinoyl-long chain alkylamino-CPG, 500 Å pore size, ~24-35 μmol loading. For the synthesis of 3'-amino-containing oligonucleotide, use a special solid support as *N*-(6-(*O*-DMTr)-hexyl)-(2-carboxamide)-phthalimidyl-lcaa-CPG.
7. Activator for coupling: 0.25 M solution of 5-(ethylthio)-1H-tetrazole (ETT) in AcN (*see Note 2*).
8. Capping reagent A: THF/acetic anhydride/2,6-lutidine, 8/1/1, v/v/v.
9. Capping reagent B: THF/*N*-methylimidazole, 84/16, v/v (*see Note 2*).
10. Oxidizing reagent: 0.02 M solution of iodine in THF/H₂O/Py, 45/4.5/0.5, v/v/v.

11. Detritylating reagent: 3% Dichloroacetic acid (DCA) (v/v) in CH₂Cl₂.
12. DNA/RNA synthesizer, corresponding columns and filters. We use ASM-800 (Biosset).

2.3. Synthesis of oligoribonucleotides conjugated with cholesterol through hydrazone bond

1. Acetic acid, 99-100%.
2. Dioxane, 99.8%.
3. NaOAc buffer (0.1 M, pH 5.2).
4. HEPES-NaOH buffer (0.1 M, pH 7.5).
5. HEPES-NaOH buffer (0.1 M, pH 8.5).
6. Fluorescein isothiocyanate isomer I.
7. N-succinimidyl ester of ATTO-565 (ATTO-TEC).
8. Dimethyl sulfoxide (DMSO), ≥99.5%.
9. Ethanol, 94-96%.
10. Cleavage reagent 1 (AMA solution): 40% methylamine in water/concentrated ammonium hydroxide solution, 1/1, v/v, freshly prepared. Concentrated (28-30%) ammonium hydroxide solution store at -20 °C.
11. Cleavage reagent 2: 0.05M K₂CO₃ in methanol.
12. Washing reagent: AcN/H₂O/ethanol, 1/1/1, v/v/v.
13. Acetone, water (H₂O) <0.2%.
14. Solution for precipitation: 2% (w/v) NaClO₄ (≥98.0%) solution in acetone.
15. 2'-O-TBDMS deprotecting cocktail: N-methylpyrrolidinone/triethylamine/triethylamine-tris(hydrogen fluoride), 3/1.5/2, v/v/v, freshly prepared (*see Note 4*).
16. Ethoxytrimethylsilane, 98%;
17. Diethyl ether (Sigma-Aldrich).
18. Speedvac Concentrator Plus.
19. Thermomixer Comfort.

20. Centrifuge 5424 R.

2.4. Analysis and purification of lipophilic RNAconjugates

1. Ethanol, 94-96%.
2. Acetone, water (H₂O) <0.2%.
3. *N,N,N,N'*-Tetramethylethylenediamine (TEMED). Store at 4°C.
4. TBE×10 Buffer: 0.5 M Tris-HCl, pH 8.3, 0.5 M boric acid, 10 mM EDTA.
5. Ammonium persulfate (APS): 10 % (w/v) solution in water, freshly prepared (*see Note 5*).
6. Solution to prepare denaturing polyacrylamide gel for oligonucleotide isolation and analysis: 12% solution of acrylamide/*N,N'*-methylenebisacrylamide (30/0.5), 7 M urea, TBE×1 Buffer (*see Note 6*).
7. Loading Buffer: 7 M urea, 0.025% bromphenol blue, 0.025% xylene cyanol FF.
8. Stains-All: 0.05% (w/v) solution in formamide/water, 1/1, v/v.
9. Ethidium Bromide solution (1 µg/mL) in water; UV-transilluminator.
10. Elution buffer: 0.3 M NaOAc solution in water, pH 7.0. Store at 4 °C.
11. Standard equipment for polyacrylamide gel electrophoresis.
12. Kieselgel F254 thin-layer chromatography plate covered with polyethylene or polypropylene film for visualizing oligonucleotides and their conjugates on gels by UV shadowing.
13. Thermomixer Comfort.
14. Speedvac Concentrator.
15. Spectrophotometer NanoDrop.

2.5. Transfection of human cultured cells with lipophilic conjugates

1. Cultured human cells: HepG2, HEK293T, 143B or human skin fibroblasts.
2. EMEM (Essential Modified Eagle's Medium) or MEM (Minimum Essential Medium Eagle) containing 1 mg/mL glucose.
3. OptiMEM, Reduced Serum medium for transfection.

4. PBS, Dulbecco's phosphate buffered saline without CaCl₂.
5. Streptomycin, penicillin, Fungizone, 100x solutions.
6. FBS, fetal bovine serum.
7. MitoTracker Deep Red (Molecular Probes) dissolved in dimethyl sulfoxide (DMSO) at 100 μM concentration, aliquoted and stored at -20°C.

3. Methods

Here we describe in details the chemical synthesis of the oligoribonucleotides conjugated with cholesterol residue through hydrazone bond.

For this, hydrazide of 6-(cholesteryloxycarbonylamino)hexanoic acid should be synthesized (Fig. 2, Subheading 3.1) and then conjugated with 5'-O-Aldehyde-modified oligoribonucleotide. This reaction can be performed by conjugation "in solution" or on solid-phase (Figs. 3, 5).

3.1. Synthesis of hydrazide of 6-(cholesteryloxycarbonylamino)hexanoic acid

3.1.1. 6-(Cholesteryloxycarbonylamino)-hexanoic acid (compound **1** Fig. 2, step i) (22)

Suspend 6-aminohexanoic acid (0.9 g, 6.6 mmol) in dry pyridine (15 ml), then add chlorotrimethylsilane (3.3 ml, 26.4 mmol) dropwise at 0°C. Stir the mixture until the solution became clear, then add cholesteryl chloroformate (1 g, 2.2 mmol) and stir the reaction mixture for 3h at r.t. Evaporate pyridine under reduced pressure, dissolve the residue in CH₂Cl₂ (100 ml), wash with 0.7 M HCl (50 ml) followed by saturated aqueous NaCl (50 ml). Dry the organic phase under anhydrous Na₂SO₄ and evaporate under reduced pressure. Purify the residue by Silica gel column chromatography (CH₂Cl₂/EtOH, 0-30%) to obtain compound **1** with a yield 0.95 g (80%). ¹H-NMR (400 MHz, CDCl₃, δ, ppm): 0.69 (s, 3H, H-18/19 cholesterol); 0.87 (d, 3H, H-26/27 cholesterol); 0.89 (d, 3H, H-26/27 cholesterol); 0.93 (d, 3H, H-21 cholesterol); 1.025 (s, 3H, H-18/19 cholesterol); 2.33 (t, 2H, -CH₂-COOH); 3.35 (dd, 2H, -CH₂-NH-); 4.51 (m, 1H, H-3 cholesterol); 4.88 (t, 1H, -NH-); 5.4 (d, 1H, H-6 cholesterol).

3.1.2. Methyl 6-(cholesteryloxy carbonylamino)hexanoate (compound **2** Fig. 2, **step ii**)

Dissolve compound **1** (0.2 g, 0.37 mmol) in dry CH₂Cl₂ (5 ml), add phosphorus trichloride (13 μ l, 0.149 mmol) and stir the reaction mixture for 3 h under argon at 50 °C. Add absolute methanol (1 ml), dilute by CH₂Cl₂ (45 ml), wash with saturated aqueous NaHCO₃ (50 ml) and twice with water (50 ml). Dry the organic phase under anhydrous Na₂SO₄ and evaporate to oily residue. Purify the product **2** by Silica gel column chromatography (CH₂Cl₂/EtOH, 0-2.5%) (yield 0.165 g, 80%). ¹H-NMR (400 MHz, CDCl₃, δ , ppm): 0.69 (s, 3H, H-18/19 cholesterol); 0.87 (d, 3H, H-26/27 cholesterol); 0.89 (d, 3H, H-26/27 cholesterol); 0.93 (d, 3H, H-21 cholesterol); 1.025 (s, 3H, H-18/19 cholesterol); 2.29 (t, 2H, -CH₂-C(O)OCH₃); 3.35 (dd, 2H, -CH₂-NH-); 3.65 (s, 3H, -CH₂-C(O)OCH₃); 4.51 (m, 1H, H-3 cholesterol); 4.88 (t, 1H, -NH-); 5.4 (d, 1H, H-6 cholesterol).

3.1.3. Hydrazone of 6-(cholesteryloxy carbonylamino)hexanoic acid (compound **3** Fig. 2, **step iii**)

Dissolve compound **2** (0.1 g, 0.18 mmol) in methanol (5 ml) (**23**). Add hydrazine monohydrate (1.25 mL, 49 mmol) dropwise, and incubate the reaction mixture for 8h at r. t. Precipitate compound **3** in cold water (100 ml), filter it off, wash with water and dry (yield 0.089 g, 89%). ¹H-NMR (400 MHz, CDCl₃, δ , ppm): 0.69 (s, 3H, H-18/19 cholesterol); 0.87 (d, 3H, H-26/27 cholesterol); 0.89 (d, 3H, H-26/27 cholesterol); 0.93 (d, 3H, H-21 cholesterol); 1.025 (s, 3H, H-18/19 cholesterol); 2.13 (t, 2H, -CH₂-C(O)NH-NH₂); 3.35 (dd, 2H, -CH₂-NH-); 3.85 (s, 2H, -CH₂-C(O)NH-NH₂); 4.51 (m, 1H, H-3 cholesterol); 4.88 (t, 1H, -NH-); 5.4 (d, 1H, H-6 cholesterol); 6.75 (s, H, -CH₂-C(O)NH-NH₂).

3.2. Automated phosphoramidite synthesis of oligoribonucleotides and their aldehyde-modified analogs

Here we provide detailed protocol for the oligonucleotide synthesis on an automatic DNA/RNA synthesizer ASM-800 at 0.4 μ mol scale using the solid phase phosphoramidite technique (*see*

Notes 2, 3, 7). Synthesis of any oligonucleotide is carried out according to the synthesizer's program. The steps of each cycle: detritylation, coupling, capping and oxidation with appropriate washings (**steps 2-10**).

1. Pack 10-15 mg of required solid support in the appropriate column, place on the automated ASM-800 synthesizer and wash with AcN (280 μ l) during 15 s.
2. Wash with CH_2Cl_2 (190 μ l) 12 s.
3. Detritylation: remove 5'-*O*-dimethoxytrityl group from the first support-bound nucleoside during 120 s with detritylating reagent (350 μ l).
4. Wash with AcN (300 μ l) during 15 s.
5. Coupling: add a mixture (1/1, v/v) of phosphoramidite monomer solution and 0.25 M ETT (140 μ l) for 10 min (for 2'-*O*-TBDMS-protected RNA phosphoramidites).
6. Wash with AcN (100 μ l) during 5 s.
7. Capping: add a mixture of capping reagents A and B (1/1, v/v) (240 μ l) during 30 s for capping of unreacted 5'-hydroxyl groups of oligonucleotides.
8. Wash with AcN (100 μ l) during 10 s.
9. Oxidation: oxidize the phosphite-triester to phosphotriester with oxidizing reagent (160 μ l) during 65 s.
10. Wash with AcN (400 μ l) during 30 s.
11. Carry as many cycles as necessary for the synthesis of oligoribonucleotide.
12. Repeat **steps 2-10** using 5'-Aldehyde-Modifier C2 phosphoramidite to introduce protected aldehyde function at the 5'-end of oligonucleotide chain (*see Note 8*).
13. Wash the column with AcN and dry thoroughly.

3.3. Solid-phase synthesis of oligoribonucleotides conjugated with cholesterol through hydrazone bond

3.3.1. Conjugation of 5'-aldehyde-modified polymer-bound protected RNAs with hydrazide of 6-(cholesteryloxycarbonylamino)hexanoic acid (Fig. 3)

1. Transfer fully protected 5'-aldehyde-modified CPG-bound RNA (10-15 mg, 0.24-0.35 μmol , Subheading 3.2) from the synthesis column to a 0.6 mL tube and incubate with 80% acetic acid (400 μl) 1 h at r.t. to remove the acetal protecting group.
2. Wash polymer-bound RNA with water (3x200 μl), AcN (2x200 μl) and acetone (2x200 μl) and dry thoroughly.
3. Dissolve hydrazide of 6-(cholesteryloxycarbonylamino)hexanoic acid (**3**) (1.9 mg, 3.5 μmol) in dioxane (50 μl), mix with the same volume of 0.1 M NaOAc pH 5.2 and add to the 5'-aldehyde-containing CPG-bound protected RNA.
4. Carry out the reaction 16 h at r.t. with stirring.
5. Wash the support-bound conjugate with dioxane (3x200 μl), AcN (2x200 μl), acetone (2x200 μl) to remove an uncoupled lipophilic compound, and then dry.

3.3.2. Cleavage of lipophilic conjugates of RNAs from the support and removal of base-labile and 2'-O-TBDMS protecting groups

1. Transfer solid support with lipophilic conjugates of RNAs to a new 0.6 mL tube.
2. Add 0.05M K_2CO_3 in methanol (400 μL) and incubate at r.t. for 16 h with shaking (*see Note 9*). Use the microtube cap locks (Thomas Scientific) to avoid the loss of material.
3. Transfer the soluble material to 1.5-mL tube, wash the solid support with AcN/ H_2O /ethanol (1/1/1 v/v/v) (150 μL) twice, combine all the solutions, neutralize with acetic acid (2 μL) (*see Note 10*) and evaporate in a Speedvac up to final volume 100 μL (*see Note 11*). Precipitate by addition of ten volumes 2% NaClO_4 in acetone (1mL) to obtain the dry pellet.
4. Add the freshly prepared and preheated to 65 $^\circ\text{C}$ 2'-O-TBDMS deprotecting cocktail (200 μL) to the pellet and incubate the reaction mixture at 65 $^\circ\text{C}$ for 90 min with shaking (*see Note 4*).
5. To quench the reaction, add ethoxytrimethylsilane (300 μL) and mix well. Be careful, it is important to hold the tube cap and open it slightly to avoid loss of material because during this step abundant gas evolution occurs. Incubate the crude reaction mixture for 10 min at r.t., add

diethyl ether (1mL) to precipitate oligonucleotide, and then incubate the tube for 10 min at -20 °C and centrifuge for 3 min at 14000g.

6. Remove the supernatant, rinse the pellet with acetone and air dry until the smell of organic solvents disappears (do not dry off the oligoribonucleotide pellet), dissolve the dry pellet in 100 μ L of 50 mM HEPES-NaOH buffer pH 7.5 (*see Note 12*).

3.3.3. Analysis and purification of crude lipophilic conjugates of RNAs

1. Measure the amount of crude lipophilic conjugates by NanoDrop spectrophotometer.

2. Check crude modified lipophilic conjugates of RNAs by analytical electrophoresis in 12% denaturing polyacrylamide gel. About ~0.05-0.1 OD₂₆₀ (~1- 2 μ g) of oligonucleotide can be loaded per 0.5 cm width well, visualize the bands by Stains-all or Ethidium Bromide staining (Fig. 4, *see Notes 6, 13*).

3. For gel-purification, load 10-15 OD₂₆₀ of the synthesized lipophilic conjugates mixed with an equal volume of the Loading buffer per the 5 cm width well.

4. After migration at 5-10 V/cm, detect the band of the lipophilic conjugates of RNA by a UV-shadowing method (254 nm irradiation), cut from gel and elute by shaking in 600-1000 μ L of 0.3M NaOAc (pH 7.0) for 16 h at 25 °C.

5. Take the supernatant, add four volumes of ethanol, and incubate for 4-16 h at -20 °C.

6. After centrifugation, wash the pellet twice with chilled 80% ethanol (until no acetic acid smell), and dry in a SpeedVac for 1 minute. Dissolve the pellet in 100 μ L of 50 mM HEPES-NaOH buffer pH 7.5 (*see Note 12*), measure the amount and check the purity of lipophilic conjugates of RNAs by analytical electrophoresis as detailed above (**steps 1, 2**) (*see Note 13*).

7. Presence of cleavable hydrazone bond can be assayed by incubation of an aliquot of the conjugate in acidic conditions (*see Note 10*). For this, mix 10 μ L (1 μ g) of purified conjugate (**step 6**) and 40 μ L acetic acid, incubate at 37 °C for 10 min, precipitate with ten volumes of 2%

NaClO₄ in acetone, dry the pellet on air until the smell of acetone disappears, dissolve in 50 mM HEPES-NaOH buffer pH 7.5 and load on 12% polyacrylamide-urea gel (Fig. 4, lane 5).

3.4. Synthesis “in solution” of oligoribonucleotides and their fluorophore labelled derivatives conjugated with cholesterol through hydrazone bond

3.4.1. Cleavage of aldehyde-modified RNAs from the support and removal of base-labile and 2'-O-TBDMS protecting groups (Fig. 5)

1. Transfer fully protected 5'-aldehyde-modified CPG-bound RNA from the synthesis column to a 0.6 mL tube.
2. Add freshly prepared AMA solution (400 µL, Subheading 2.3) and incubate at 65 °C for 15 min with shaking. Use the microtube cap locks (e.g. Thomas Scientific) or the tubes with screw caps to prevent the opening the tube.
3. Cool the tube at 4 °C for 15 min before opening.
4. Transfer the soluble material to 1.5-mL tube, wash the solid support with AcN/H₂O/ethanol (150 µL) twice, combine all the solutions and evaporate in a Speedvac to obtain the dry pellet.
5. Remove of 2'-O-TBDMS protecting groups (Subheading 3.3.2, **steps 4-6**).
6. Analyze the quality of the oligoribonucleotide by electrophoresis in a 12% polyacrylamide-urea gel and purify if necessary (Subheading 3.3.3).

3.4.2. Conjugation of 5'-aldehyde-containing RNAs with hydrazide of 6-(cholesteryloxycarbonylamino)hexanoic acid “in solution”

1. Incubate RNA containing protected aldehyde function (5 units A₂₆₀) with 80% acetic acid 1 h at r.t. to remove the acetal protecting group.
2. Add ten volumes of 2% NaClO₄ in acetone; mix well and centrifuge 3 min at 14000g.
3. Remove supernatant, rinse the pellet with acetone and dry the pellet on air until the smell of acetone disappears.

4. Re-dissolve the pellet of 5'-aldehyde-containing RNA in 0.1 M NaOAc pH 5.2 (50 μ L) (*see Note 12*).
5. Dissolve hydrazide of 6-(cholesteryloxycarbonylamino)hexanoic acid (**3**) (3 mg, 5.2 mmol) in dioxane (50 μ l) and mix with the solution of 5'-aldehyde-containing RNA.
6. Carry out the reaction 12 h at r.t. with permanent stirring. Precipitate the oligonucleotide material by adding ten volumes of 2% NaClO₄ in acetone/diethyl ether (1/1 v/v), keep 15 min at -20°C and centrifuge 10 min at 16000g, 4°C. Remove supernatant, rinse the pellet with acetone and dry the pellet on air until the smell of organic solvents disappears.
7. Analyze the quality of the lipophilic conjugate by electrophoresis in a 12% polyacrylamide-urea gel (Subheading 3.3.3).

3.4.3. Synthesis of fluorophore labelled conjugates

For labelling of lipophilic conjugates with fluorophores, the conjugate should contain 3'-terminal aminolinker (Fig. 6). For the synthesis of 3'-amino-containing oligonucleotide, use special amino-containing solid support as *N*-(6-(*O*-DMTr)-hexyl)-(2-carboxamide)-phthalimidyl-lcaa-CPG.

1. Add ten volumes of 2% NaClO₄ in acetone to the solution of 3'-amino-containing lipophilic conjugate (1 unit A₂₆₀, Subheading 3.4.2), mix well, incubate at -20°C for 15 min and centrifuge 10 min at 16000g.
2. Remove the supernatant; wash the pellet with acetone and dry.
3. For labelling with FITC (*see Note 14*), dissolve the pellet in 30 μ L of 0.1 M HEPES-NaOH (pH 8.5) (*see Note 12*). Add FITC solution (0.3 mg in 30 μ l DMSO) and mix well. Use black tubes, avoid the bright light during all the manipulations with the fluorescent dyes and labelled RNA.

4. For labelling with ATTO-565 (*see Note 14*), dissolve the pellet (**step 2**) in 85 μL of 0.1 M HEPES-NaOH (pH 8.5) and mix with 15 μL of *N*-succinimidyl ester of ATTO-565 in dry DMSO (5 mg/ml). Use black tubes.
5. Carry out the reaction with FITC or ATTO-565 overnight at 25° with permanent stirring (800 rpm).
6. Precipitate the conjugate by adding four volumes of ethanol; incubate 4 h at -20 °C.
7. After centrifugation at 16000g for 10 min, remove the supernatant; wash the pellet with chilled ethanol and air dry (in the dark) until the smell of ethanol disappears. Dissolve the pellet in 100 μL of 50 mM HEPES-NaOH buffer pH 7.5.
8. Remove the non-incorporated fluorescent dye by Bio-Spin columns P6 or P30 (Bio-Rad).
9. To check the efficiency of the labelling, compare the fluorescent dye absorbance and the oligonucleotide nitrous bases absorbance (260 nm) using NanoDrop Microarray Program (**24**).

3.5. Transfection of cultured human cells with lipophilic conjugates

To detect and quantify the cellular uptake of RNA conjugated with cholesterol, cultured human cells incubated with fluorescently labelled conjugates can be analyzed by flow cytometry and/or fluorescent confocal microscopy.

1. Grow the cells in 2 cm^2 wells in EMEM or MEM medium supplemented with 10% FBS, penicillin, streptomycin, and fungizone in a CO₂ incubator (at 37 °C, 5% CO₂) to the confluence of 70%.
2. Dilute purified fluorescently labelled conjugates in OptiMEM to final concentration 70-200 nM (*see Note 15*).
3. Wash the cells with phosphate-buffered saline (PBS), add 500 μl /well of OptiMEM containing conjugates. Incubate cells overnight (15 h) at 37 °C, 5% CO₂, keeping them in the dark.
4. Remove the transfection mixture and add 0.5 ml of EMEM medium for 1 h to eliminate non-internalized oligonucleotides from the cells surface.

5. Wash the cells twice with PBS, detach, centrifuge at 600g for 10 min, resuspend the pellet of cells in ice-cold PBS. Keep the cells protected from direct light.
6. Perform flow cytometry analysis to estimate the percentage of fluo-positive cells in the population (normally ~ 90%, *see Note 16*). We use CyFlow[®] Space (*Partec*), equipped with a 488 nm laser and a 592 nm bandpass filter for detecting the cells internalized ATTO-565 labelled conjugates (Fig. 7A).
7. For fluorescent confocal microscopy, grow the cells in 0.5 mL of EMEM on chambered coverglass for microscopy with 4 wells (2 cm²) (Lab-Tek) to the confluence of 60-80%.
8. Transfect cells with fluorescently labelled conjugates as described above (**steps 2-3**).
9. Replace the medium with 0.5 mL of DMEM containing MitoTracker Deep Red (or other MitoTracker dye) at final concentration 100 nM, incubate for 15 min in a CO₂ incubator at 37°C.
10. Aspirate medium and wash cells gently 3 times with PBS, add 1 mL of DMEM without red phenol. Perform cell imaging using a confocal laser scanning microscope (Fig. 7B). We use the LSM 700 confocal scanning microscope (Zeiss LSM 700) in conjunction with Zen imaging software and a Zeiss 40x/1.30 oil immersion objective.

4. Notes

1. Organic solvents and chemicals should be of high quality (Sigma-Aldrich, Acros, Merck), and stored in a dark place. For personal protection, use a lab coat, safety glasses, and gloves. Work with all chemicals in a well-ventilated chemical hood.
2. You may use phosphoramidites, reagents and solvents from different manufacturers (Glen Research, ChemGen, Sigma-Aldrich, TriLink, etc.). To improve the quality and yield of the oligonucleotide synthesis, remove the trace amounts of moisture by adding molecular sieves (3 Å, 8 to 12 mesh) to phosphoramidite solutions, activator solution, capping reagent B, and acetonitrile.

3. All steps of chemical synthesis should be handled under the inert atmosphere. We use helium gas for operating ASM-800 DNA/RNA synthesizer, for the other synthesizers use gas recommended by the manufacturer.
4. Use the 2'-*O*-TBDMS deprotecting cocktail only freshly prepared and hot (65 °C).
5. The APS solution can be stored at 4°C for no more than one week. Longer storage can affect the acrylamide polymerization and the quality of oligonucleotide separation.
6. To separate the oligoribonucleotides and their lipophilic conjugates (Fig. 4), we use polyacrylamide gels with a small proportion of the *N,N'*-methylenebisacrylamide linker (30/0.5). The stock solution must be prepared from the individual components supplied in powder form (toxic by inhalation, please use a mask!). Commercial stock solution acrylamide/*N,N'*-methylenebisacrylamide (37.5/1) can also be used. Solution of 12% acrylamide with 7M urea can be stored at 4°C for no more than three weeks.
7. You may synthesize oligonucleotides using other automatic DNA/RNA synthesizers (for example, from Applied Biosystems or Mermade) and protocols optimized for these instruments. To prevent the nuclease degradation of oligoribonucleotides and their analogs and conjugates, use only fresh ultrapure water (for example, by Simplicity® Water Purification System, Millipore, 18 MΩ-cm at 25°C) and sterile or autoclaved plastic tubes and tips; wear gloves when handling RNA samples; store all oligonucleotides and their conjugates at -20 °C.
8. Normally, the program of automatic DNA/RNA synthesizer includes the function of the final detritylation. This step must be canceled; otherwise, the acetal protection of the aldehyde group can be partially removed, which will lead to undesirable modification at the step of AMA (cleavage reagent 1, Subheading 3.4.1) treatment.
9. Cleavage reagent 2 (0.05 M K₂CO₃ in methanol) is not suitable for the cleavage of oligonucleotides synthesized on the amino-containing solid support.
10. To avoid cleavage of the hydrazone bond, it is necessary to carefully control the pH of all solutions. We tested the lability of the hydrazone link and demonstrated that it was stable at pH

>6.6, while at pH <6.0, the cholesterol residue was quickly split from the oligoribonucleotide (19). Since RNA molecules degrade at pH>8.8, there is an interval of 6.8<pH<8.5 which must be respected to avoid the cleavage of conjugated molecules.

11. At this step, avoid complete evaporation of the solution as this may lead to the degradation of the conjugated oligoribonucleotide.
12. Be careful when handling the tube with a dry pellet of oligonucleotide conjugate. Centrifuge the tube before opening and open it very carefully; otherwise, the pellet can fly out of the tube upon opening.
13. Samples of crude lipophilic conjugates obtained after cleavage from the support and removal of protecting groups (Subheading 3.3.2) contain, besides lipophilic conjugates (Fig. 4, lane 2, upper band), also non-conjugated oligoribonucleotides (lower band) and the products of incomplete synthesis visible as a smear. Therefore, before labeling the lipophilic conjugates with fluorophores and/or transfection of human cells, the conjugated molecules should be gel-purified (Subheading 3.3.3). Unfortunately, the efficiency of elution of the conjugates from the gel is rather low. We have tested various elution techniques, and the best one (Subheading 3.3.3) offers only a yield of around 50%.
14. For the choice of a fluorescent dye, we should take into account two characteristics: the charge of the fluorescent dye and its possible interaction with lipid bilayers (membrane interaction factor, MIF). Positively charged fluorophores can induce a nonspecific accumulation of the oligoribonucleotide on the mitochondrial surface (25), providing the false positive results of mitochondrial targeting. The interaction of fluorescent dye with lipid bilayer (26) can lead to an important decrease of the labelled RNA import into mitochondria. Here we provide protocols for two dyes, Fluorescein isothiocyanate (FITC, green fluorescence) and ATTO-565 (red fluorescence). Fluorophore labelling can affect the cellular accumulation and biological activity of RNA conjugates, as it was recently demonstrated for siRNA (27). Noteworthy, our blot hybridization experiments also demonstrated that unlabelled RNA molecules were more

efficiently imported into mammalian mitochondria compared to fluorescently labelled RNAs (not shown).

15. Oligoribonucleotide components of conjugated molecules can be rapidly degraded by nucleases, which are present in FBS (fetal bovine serum). The RNA molecules incubated in culture media supplemented with FBS are quickly degraded (28). Therefore, the carrier-free cell transfection with lipophilic oligoribonucleotide conjugates should be performed in a medium with reduced serum content (such as OptiMem). Cells grown in complete medium should be washed before transfection (Subheading 3.5) to remove traces of FBS.
16. The efficiency of cell transfection was evaluated with a gel-purified fluorescently labelled conjugate (19). When using the crude reaction mixture, it is important to estimate the proportion of lipophilic conjugate compared to unconjugated oligoribonucleotide (Fig. 4, see also Note 13).

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Figure Captions

Figure 1. The strategy of the carrier-free cell delivery (adapted from (11)). **A**, Biodegradable conjugate of the importable RNA (shown by a helix) with cholesterol (in pink) can be internalized by endocytosis in a non-toxic and formulation-free manner; then RNA is released in late endosomes due to the change of pH and can be partially targeted into mitochondria. **B**,

Structure of the lipophilic conjugate of RNA. In blue, anti-replicative RNA; in pink, cholesterol residue; in green, pH-dependent hydrazone bond.

Figure 2. Scheme of the chemical synthesis of hydrazide of 6-(cholesteryloxycarbonyl amino)hexanoic acid. Reagents: *i*) $(\text{CH}_3)_3\text{SiCl}$, Py, 4 °C; CholOC(O)Cl, Py, r.t.; H_3O^+ ; *ii*) PCl_3 , CH_2Cl_2 , argon, 50 °C; MeOH_{abs} , r.t.; *iii*) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, MeOH, r.t. (from (**19**) with modifications).

Figure 3. Scheme of the solid-phase synthesis of oligoribonucleotides (OLIGO) conjugated with cholesterol (R-) through covalent hydrazone bond.

Figure 4. PAGE separation of the following compounds: lane 1, oligoribonucleotide containing 5'-aldehyde group (ON); lane 2, an aliquot of reaction mixture for synthesis of lipophilic conjugate (Subheading 3.3.2); lanes 3-4, lipophilic conjugates purified by PAGE (Chol~ON); lane 5, gel-purified lipophilic conjugate incubated under acidic conditions to partially cleave the hydrazone bond.

Figure 5. Scheme of the synthesis “in solution” of lipophilic conjugates containing hydrazone bond.

Figure 6. Labelling of lipophilic conjugates with fluorophore dyes.

Figure 7. Evaluation of the cellular uptake of lipophilic conjugates. **A**, An example of flow cytometry analysis, cells were incubated 15 h with 70 nM ATTO-565 labelled conjugate. In gray, signal corresponding to auto-fluorescence of non-transfected cells. **B**, Confocal microscopy images of human skin fibroblasts transfected with 70 nM ATTO-565 labelled conjugate (upper panel, yellow signal), 26 h post-transfection; lower panel represents control non-transfected cells. Mitochondrial network was visualized by MitoTracker DeepRed staining. The excitation/emission laser wavelengths were 555/585 nm (yellow channel) and 639/660 nm (red magenta channel).

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