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TLR9-Targeted SiRNA Delivery In Vivo

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Abstract

The SiRNA strategy is a potent and versatile method for modulating expression of any gene in various species for investigational or therapeutic purposes. Clinical translation of SiRNA-based approaches proved challenging, mainly due to the difficulty of targeted SiRNA delivery into cells of interest and the immunogenic side effects of oligonucleotide reagents. However, the intrinsic sensitivity of immune cells to nucleic acids can be utilized for the delivery of SiRNAs designed for the purpose of cancer immunotherapy. We have demonstrated that synthetic ligands for the intracellular receptor TLR9 can serve as targeting moiety for cell-specific delivery of SiRNAs. Chemically synthesized CpG-SiRNA conjugates are quickly internalized by TLR9-positive cells in the absence of transfection reagents, inducing target gene silencing. The CpG-SiRNA strategy allows for effective targeting of TLR9-positive cells in vivo after local or systemic administration of these oligonucleotides into mice.

Keywords

TLR9; SiRNA; CpG; Cancer; Myeloid cells; Leukemia; Oligonucleotides

1 Introduction

The discovery of RNA interference (RNAi) mediated by small-interfering RNA (SiRNA) created a unique opportunity for targeting almost any disease-related gene for therapeutic purposes [1 – 3]. The SiRNA-based therapies can overcome the challenges of non-enzymatic proteins, such as transcription factors. Preclinical studies in various disease models, including cancer, demonstrated in vivo efficacy of RNAi in rodents [2] and in nonhuman primates [4]. Recent, first-in-human clinical trial confirmed gene silencing after systemic

delivery of SiRNA to cancer patients [5]. Broad application of SiRNAs is still limited by the lack of cell-specific delivery, insufficient intracellular uptake, and cytoplasmic release. The effective target gene silencing usually requires encapsulating SiRNA into chemical formulations that may contribute to toxicities and side effects [1, 2]. Previously, several strategies have been implemented to enhance targeted cell delivery by conjugating SiRNAs with cholesterol, cationic peptides, cell-specific antibodies, RNA aptamers, or ligands for cell surface receptors [3, 6]. However, many of these strategies result in endosomal entrapment of SiRNAs with limited cytoplasmic release and thereby limited effect on target mRNAs [2].

We have recently developed an original method to deliver therapeutic SiRNAs into specific target cells by targeting endosomal receptor Toll-like receptor 9 (TLR9) [7]. TLR9-positive cells recognize and internalize single-stranded oligodeoxyribonucleotides containing an unmethylated CpG motif (CpG ODN) [8, 9]. The conjugates of CpG ODNs to Dicer substrate SiRNAs (CpG-SiRNAs) [10] are actively internalized by human and mouse TLR9-positive cells, without any transfection reagents [7, 11]. The CpG-SiRNA conjugates interact with Dicer after uptake into early endosomes (EE). Importantly, TLR9 plays essential role in facilitating the release of uncoupled SiRNA from EE and trafficking to RNA-induced silencing complexes (RISC) on the surface of endoplasmic reticulum (ER) [12, 13]. The CpG-SiRNA processing results in efficient target gene silencing in various human and mouse TLR9-positive cells in vivo. These include hematopoietic cells, such as dendritic cells (DCs), macrophages, and B cells, as well as in certain blood cancers, e.g., in acute myeloid leukemia (AML), multiple myeloma, or B cell lymphoma (BCL) [7, 11, 14]. The efficacy studies in mice demonstrated direct and/or immune-mediated antitumor activity of CpG-SiRNAs targeting various tumorigenic factors, including *STAT3*, *STAT5*, *RELA/p65*, *BCL2L1*, or *SIPR1* [11, 15, 16]. Overall, CpG-SiRNA strategy allows for a two-pronged targeting of both cancer cells and immune cells to augment therapeutic effects. In this chapter, we describe methods for the design and application of CpG-SiRNA conjugates to deliver SiRNA into specific target cells in mice, starting from the oligonucleotide synthesis to local/systemic delivery into TLR9-positive cells in vivo. Additionally, we discuss critical troubleshooting points for target cell selection and optimizing gene silencing efficiency of CpG-SiRNA conjugates.

2 Materials

2.1 CpG-siRNA Design and Synthesis

- The CpG-SiRNA conjugates consist of Dicer substrate SiRNA (25/27mer) [10] linked to the class A CpG/D19 (type A) or CpG1668 (type B) for human- or mouse-optimized versions of the reagent, respectively.
- The two strands of the CpG-SiRNA are synthesized separately using Akta OligoPilot100 (GE) as follows:
 - SiRNA(SS)—25mer sense strand with or without fluorochrome added to the 3' end (*see Note 1*).
 - 5'-NNNNNNNNNNNNNNNNNNNNNNNNNNNNdNdN-3'

- N = ribonucleotide; dN = deoxyribonucleotide.
 - CpG-SiRNA(AS)—27mer antisense strand.
 - *Human version* 5'-GGGGGGGG-linker-3'.
 - *Mouse version* 5'- TCCATGACGTTCTGATGCT -linker-NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3'.
 - The linker consists of 5 units of the C3 Spacer (Glen Research, Sterling, VA); underlined are phosphorothioated residues. The second nucleotide from the 3' end of CpG-SiRNA(AS) strand can be 2'O-methyl-modified to improve stability.
3. Both oligonucleotides are aliquoted and stored as lyophilized pellets of 50 nmol each below –20 °C until use.

2.2 Tissue Digestion

1. 10× Collagenase D stock solution: Determine enzyme activity in Wunsch units and prepare 4000 U Collagenase D per 1 mL of HBSS with calcium and magnesium; before use dilute in HBSS to 1× solution for tissue digestion.
2. 10× DNase I stock solution: Dissolve 10 mg DNase I in 1 mL of HBSS, and before use dilute in HBSS to 1× solution for tissue digestion.
3. 10× ACS buffer: For 1 L, dissolve 89.9 g NH_4Cl , 10.0 g KHCO_3 , and 370 mg EDTA in milli-Q water, then adjust to pH 7.3, and dilute to 1× buffer solution in water for erythrocyte lysis.
4. Histopaque®-1083 for isolating viable cells from mouse tissues.

2.3 7 M Urea/Polyacrylamide (PAGE) Gel

1. 10× TBE: For 500 mL, dissolve 54 g Tris base, 27.6 g boric acid, and 10 mL 0.5 M EDTA, pH 8.0 in water, adjust to pH 8.3, and dilute to 1× solution for gel running and staining.
2. 8 M Urea: For 200 mL, dissolve 96.1 g urea in water, and filter the solution.
3. 7 M Urea/20 % acrylamide solution: For 200 mL, dissolve 88.2 g urea in 100 mL of 40 % acrylamide/bis-acrylamide solution (19:1), adjust volume to 200 mL using milli-Q water and filter the solution, *see* Table 1 .
4. Ethidium bromide: Use 2 µL of ethidium bromide solution (10 mg/mL) in 25 mL of 1× TBE buffer for gel staining.
5. Double-stranded RNA marker.
6. Nuclease -free water: DNase/RNase-free water.

¹The choice of fluorochrome and its position in the conjugate depends on the application. For uptake and biodistribution studies, it is best to label 3' end of the siRNA(SS) to prevent interference with the intracellular processing of the conjugate. For in vivo applications, more intensive fluorochromes are preferred, e.g., Cy3 or Alexa 488 rather than fluorescein.

2.4 Flow Cytometry Reagents

1. Staining buffer: 1× PBS, 2 % FBS and 0.1 % NaN₃.
2. Antibodies: Fluorescently labeled antibodies specific to surface markers on immune and cancer cells.
3. Fc Block: Anti-CD16/CD32 antibody to block unspecific binding by Fcγ receptors.
4. Viability dye: 7AAD.
5. Flow cytometer : BD Accuri C6.

2.5 Time-Lapse Confocal Microscopy Reagents, Instruments and Software

1. Culture dishes: 35/14 mm #1.5 glass bottom tissue culture dishes.
2. Microscope: cLSM510-Meta inverted confocal microscope (Zeiss, Thornwood, NY).
3. Acquisition software: LSM software v.4.2 SP1 (Zeiss).
4. Post-acquisition analysis software: LSM Image Browser v.4.2.0.121 (Zeiss).

2.6 In Vivo CpG-siRNA Delivery

1. Syringes: 1 mL U-100 insulin syringe with 30G needle to inject CpG-SiRNA conjugates diluted in 1× PBS in vivo.
2. Anesthetic agents: Anesthesia induction chamber including isoflurane and oxygen supply.
3. Mice : For in vivo experiments C57BL/6 and NOD/SCID/IL-2RγKO (NSG) mice were purchased from National Cancer Institute (Frederick, MD) and the Jackson Laboratory (Bar Harbor, ME), respectively.

3 Methods

3.1 Hybridization of CpG-siRNA Conjugates

1. Add 250 μL of DNase/RNase-free water to each of the two 50 nmol aliquots of CpG-SiRNA(AS) and SiRNA(SS), mix well at 37 °C for 5 min, and make sure that both oligonucleotides are completely dissolved.
2. Combine both strands in a new 1.5 mL sterile tube to make 100 μM stock solution of CpG-SiRNA conjugate (use amber tubes for fluorescently labeled reagents).
3. Place the tube at 80 °C for 1 min; promptly transfer to the 37 °C water bath and incubate for 1 h.
4. Spin down and aliquot hybridized CpG-SiRNA conjugates into desired volumes for long-term storage at −80 °C.

3.2 Oligonucleotide Quality Control on the 7 M Urea/PAGE Gels

1. Assemble the gel plates in the gel casting chamber, prepare 7 M urea/15 % PAGE gel according to Table 1 and pour quickly between gel plates, insert comb, and allow polymerizing for 30 min.
2. Dismount the polymerized gel from casting chamber and assemble into gel apparatus according to manufacturer's instruction.
3. Fill the gel chamber with 1× TBE, carefully remove the comb, wash all wells and pre-run for 30 min.
4. Dilute oligonucleotides to 10 μ M, use 2 μ L of diluted samples to mix with 0.5 μ L of 0.5 M EDTA, pH 8.0 and 3 μ L of 2× RNA loading dye, load into the wells together with the RNA marker, and run the gel at 250 V for 1 h.
5. Remove the gel and carefully transfer into a dish containing 1× TBE plus ethidium bromide, stain for 15–20 min, and then examine the gel on the UV transilluminator (Fig. 1) (*see Note 2*).

3.3 The Cytofluorimetric Assessment of CpG-siRNA Internalization by Target Cells In Vitro

1. Culture the desired number of cells (usually 10^6 cells/mL) in the appropriate medium or, if using freshly isolated primary cells (*see Subheadings 3.7 – 3.9*), allow cells to adjust to the in vitro culture condition for 2–4 h (*see Note 3*).
2. Treat cells with various doses (10–500 nM) of fluorescently labeled CpG-SiRNA constructs, then collect cells at desired times to verify cellular uptake.
3. Collect cells, resuspend each sample in 100 μ L of the staining buffer, add 1 μ L of the Fc Block for 10 min, then stain cells with a combination of fluorescently labeled antibodies specific to immune cell markers for 20–30 min on ice, wash once with 2 ml of the staining buffer, and resuspend in the staining buffer plus 7AAD for exclusion of dead cells for the analysis using flow cytometry (Fig. 2a, b) (*see Note 4*).

3.4 Time-Lapse Confocal Microscopy to Verify Internalization of CpG-siRNA In Vitro

1. Plate and culture cells overnight at a desired density (usually 10^5 cells/well) in the appropriate medium using the 35/14 mm #1.5 glass-bottom tissue culture dishes.
2. Next day, wash cells twice in phenol red-free cell culture medium, then replace it with complete culture medium, and add fluorescently labeled CpG-SiRNA at 100–500 nM concentration.

²Differences in concentrations or formation of secondary structures between CpG part of the molecule and the SiRNA(AS) could potentially interfere with proper hybridization of both oligonucleotides. Therefore, it is important to confirm the quality and the proper formation of hybridized duplex using gel electrophoresis.

³For selection of optimal target cells, it is critical to confirm TLR9 expression. Cells lacking TLR9 internalize CpG-SiRNA but endosomal retention of the SiRNA is likely to prevent gene silencing effect [12].

⁴Majority of TLR9-positive immune cells and malignant cells, such as leukemia and lymphoma, internalize the conjugate within 1–4 h at concentrations from 100 to 500 nM. Higher concentrations of the CpG-SiRNA usually do not improve uptake any further.

3. Transfer culture dishes to the microscope chamber with environmental control (37 °C at 5 % CO₂), and incubate for desired times (0.25–48 h).
4. Acquire images at various time increments using cLSM510- Meta inverted confocal microscope, C-Apochromat 40×/1.2 water-immersed objective and LSM software v.4.2 SP1 (Zeiss).
5. Use LSM Image Browser v.4.2.0.121 for post-acquisition analysis (Zeiss) (Fig. 3) (*see Note 5*).

3.5 Local Peritumoral Delivery of CpG-siRNA to Target Cell Populations In Vivo

1. Prepare 7–10-week-old mice (C57BL/6 or NSG for syngeneic or xenotransplanted tumor models, respectively).
2. Inject tumor cells suspended in sterile PBS (usually $0.1\text{--}1 \times 10^6$ cells/100 µL) to generate subcutaneous (SC) tumor model.
3. After tumor size exceeds ~5 mm in diameter, prepare for injections of CpG-SiRNA.
4. Prepare solution of fluorescently-labeled CpG-SiRNA (e.g., CpG-SiRNA^{Cy3}) at 1–5 mg/kg (20–100 µg) in 100 µL of sterile PBS.
5. Use insulin syringe with 30G injection needle laid parallel to the skin and inserted flatly onto the tumor.
6. Slowly inject CpG-SiRNA solution in four different locations (~25 µL/location) without completely withdrawing the needle to reach maximum area without puncturing skin on the opposite side (*see Note 6*).
7. After the appropriate time (usually 1–24 h), euthanize mice, harvest tumors, tumor-draining, and peripheral lymph nodes, and prepare single-cell suspensions as described in Subheading 3.7 followed by the flow cytometric analysis of the oligonucleotide internalization as in Subheading 3.3 .

3.6 Systemic Delivery of CpG- siRNA to Target Cell Populations In Vivo

1. Prepare 7–10-week-old mice (C57BL/6 or NSG for syngeneic or xenotransplanted tumor models, respectively) and anesthetize using isoflurane inhalation.
2. Prepare solution of fluorescently labeled CpG-SiRNA (e.g., CpG-SiRNA^{Cy3}) at 1–5 mg/kg in 200 µL of sterile PBS.
3. Position the anesthetized mouse on its side, use index finger and thumb to draw back skin of above and below the eye then carefully insert the 30G needle at ~45° angle at the corner of the eye, lateral to the medial canthus.

⁵The purpose of testing conjugate uptake using confocal microscopy is to confirm that SiRNA is present intracellularly rather than associated with target cell surface. The exact localization of the construct cannot be distinguished by standard flow cytometry.

⁶For peritumoral injections, it is important not to withdraw needle completely and to avoid additional puncturing of the skin which can result in leakage of the reagent. It is also advisable to hold the needle in position for few seconds after injecting the total 100 µL to allow for penetration of the solution deeper into tissues to minimize potential reflux.

4. Slowly inject the CpG-SiRNA solution into the retrobulbar sinus then remove needle gently and apply ointment to reduce strain (*see Note 7*).
5. After the desired time (usually 1–24 h) euthanize mice, collect blood and harvest various organs (e.g., liver, spleen, kidney, bone marrow) to evaluate biodistribution of the fluorescently labeled oligonucleotides using as described in Subheadings 3.3 , 3.7 and 3.8 (Fig. 4) (*see Notes 8 and 9*).

3.7 Isolation of Cells from Soft Tissues and Tumors

1. Transfer harvested tissues into Petri dishes and wash twice with HBSS.
2. Add 5–10 mL of 1× Collagenase D (400 U/mL) and 1× DNase I (1 mg/mL) and mince tissues into small cubes (~1–2 mm in diameter) using surgical scissors, and incubate for 30 min/37 °C.
3. Add 10 mM EDTA (tissue culture grade) to stop enzymatic reaction and then pipette up and down to disperse tissues into single-cell suspension.
4. Filter cell suspensions through 70 µm cell strainers into 50 mL tubes, adjust volume to 20–30 mL with HBSS without calcium and magnesium and spin down at 500 × g/5 min/18 °C.
5. To lyse erythrocytes, add 5 mL of 1× ACS buffer to the cell pellet, resuspend, and incubate for 3 min/RT.
6. Add 25 mL HBSS without calcium and magnesium to stop the lysis, spin down, and resuspend cells into 10 mL HBSS without calcium and magnesium. To isolate viable cells, pipet 10 mL of cell suspension into 50 mL tube then carefully underlay with 5 mL of Histopaque[®]-1083 equilibrated to RT and centrifuge tubes at 400 × g/30 min/18 °C.
7. Collect viable cells from the interphase without touching the pellet, resuspend in HBSS without calcium and magnesium, spin down again, and then resuspend again for cell counting (Fig. 5) (*see Note 10*).

3.8 Isolation of Cells from the Bone Marrow

1. Euthanize mice, remove tibia and femur and place them in tubes with PBS.
2. Cleanly remove rests of muscles from bones, place them in a mortar and add 5 mL of PBS/3 % FBS, crush all bones using pestle for 1–2 min, and rinse with 1 mL of PBS/3 % FBS.

⁷In case of retroorbital injections, mice should not receive more than single injection per day. Additional injections are possible only when using alternate eyes with 1–2 days in between.

⁸Efficient *in vivo* delivery of the SiRNA to target cells depends on their ability to quickly internalize CpG-SiRNA (within 0.5–1 h). The best results are usually achieved targeting normal and malignant myeloid cells *in vitro* and *in vivo*.

⁹Conjugate degradation by serum nucleases as well as rapid kidney clearance limits CpG-SiRNA half-life in mouse circulation. Chemical modification of the oligonucleotide backbone and the conjugation of CpG-SiRNA to polyethylene glycol (PEG) can improve the pharmacokinetic profile of these conjugates.

¹⁰To induce target gene silencing *in vivo*, mice should be treated using CpG-SiRNA conjugates for at least 2 days using daily injections of the reagent.

3. Aspirate the cell suspension up and down using 5 mL pipette, and then filter the content of the mortar through a 70 μ m strainer into 50 mL tube and spin down at $500 \times g$ for 5 min.
4. To lyse erythrocytes, add 5 mL of $1 \times$ ACS buffer to the cell pellet, resuspend, and incubate for 3 min/RT.
5. Add 25 mL PBS then spin down and re-suspend cells into 10 mL PBS.
6. To isolate viable cells, pipet 10 mL of cell suspension into 50 mL tube, then carefully underlay with 5 mL of Histopaque®-1083 equilibrated to RT, and centrifuge tubes at $400 \times g/30$ min/ 18°C .
7. 7. Collect viable cells from the interphase without touching the pellet, resuspend in HBSS without calcium and magnesium, spin down, and resuspend the cell pellet again for cell counting (Fig. 5).

3.9 Processing of Blood Cells

1. Transfer collected blood into 15 mL tube containing 3–4 mL ACS buffer to lyse blood cells.
2. Incubate for 5–10 min at RT and spin down $500 \times g$ for 5 min.
3. Wash cell pellet twice with PBS.
4. Count cell number and proceed to next experiment (Fig. 5).

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Fig. 1. Quality control for the hybridized CpG-siRNA constructs. Mouse and human versions of siRNA(SS), CpG-siRNA(AS), and CpG-siRNA conjugate were loaded on the 7 M urea/ 15 % PAGE gel. After electrophoresis gel was stained using ethidium bromide to visualize oligonucleotides compared to a dsRNA marker

Fig. 2.

Human immune and leukemic cells efficiently internalize CpG-siRNA conjugates in vitro.

(a) Targeted delivery of CpG-*STAT3* siRNA into different primary human immune cells in vitro. Human PBMCs were incubated with fluorescently labeled CpG(A)-*STAT3* siRNA^{Cy3} or unconjugated *STAT3* siRNA^{Cy3} at 500 nM concentration for 1 h without any transfection reagents. Percentages of Cy3⁺ monocytes (CD14⁺), plasmacytoid dendritic cells (BDCA2⁺), B cells (CD19⁺), and T cells (CD3⁺) were assessed by flow cytometry. (b) CpG-siRNA internalization by human MM (KMS.11) and AML (MV4-11, KG1a) cells. Cells were incubated with 500 nM Cy3-labeled CpG-*STAT3* siRNA for 1 h. The percentage of Cy3⁺ leukemic cells was analyzed by flow cytometry

Fig. 3. Intracellular localization of CpG-siRNA after in vitro uptake. Representative images from time-lapse confocal microscopy of cultured primary bone-marrow derived macrophages incubated in the presence of 500 nM CpG-siRNA^{Cy3} for 0.5 h

Fig. 4.

CpG-*Stat3* siRNA is efficiently internalized by AML cells in vivo. C57BL/6 mice bearing *Cbfb-MYH11/Mpl*⁺ AML were injected with a single dose of CpG-*Stat3* siRNA^{Cy3} (5 mg/kg). Uptake of fluorescently labeled siRNA was assessed using flow cytometry in AML cells isolated from spleen and bone marrow 3 or 18 h after injection

Fig. 5.
Schematic of blood, bone, and soft tissue processing to assess organ and cellular biodistribution of CpG-siRNA conjugates

Table 1

Composition of 7 M urea/15 % PAGE

Reagent	For 10 mL
7 M Urea/20 % acrylamide solution	7.5 mL
8 M Urea	1.5 mL
10× TBE	1 mL
APS (10 %)	80 µL
TEMED	7 µL