Methods in Molecular Biology 1364

Springer Protocols

Kato Shum
John Rossi *Editors*

# SiRNA Delivery Methods

## Methods and Protocols

Humana Press

*Editors*
Kato Shum
Beckman Research Institute of City of Hope
Duarte, CA, USA

John Rossi
Beckman Research Institute of City of Hope
Duarte, CA, USA

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-3111-8 ISBN 978-1-4939-3112-5 (eBook)
DOI 10.1007/978-1-4939-3112-5

Library of Congress Control Number: 2015947262

Springer New York Heidelberg Dordrecht London


Printed on acid-free paper

Humana Press is a brand of Springer
Springer Science+Business Media LLC New York is part of Springer Science+Business Media (www.springer.com)

# Chapter 4

# Highly Efficient SiRNA Delivery Mediated by Cationic Helical Polypeptides and Polypeptide-Based Nanosystems

**Lichen Yin, Nan Zheng, and Jianjun Cheng**

## Abstract

RNA interference (RNAi) mediated by small interfering RNA (SiRNA) has recently emerged as a potent machinery in regulating gene expression at the post-translation step. Despite its efficiency and specificity, the biggest hurdle against its wide application is the safe and effective delivery of the SiRNA cargo into target cells. Here, we describe the highly effective SiRNA delivery mediated by the cationic helical polypeptides and polypeptide-based nanosystems both in vitro and in vivo via oral administration.

**Key words** Helical polypeptide, SiRNA delivery, Gene silencing, Oral delivery, Tumor necrosis factor α (TNF-α), Acute hepatic injury

## 1 Introduction

RNA interference (RNAi) mediated by small interfering RNA (SiRNA) is an important mechanism that regulates gene expression in eukaryotic cells via site-specific mRNA cleavage and degradation. Because of its efficiency and sequence specificity, the RNAi machinery affords an exciting modality for the treatment of various human diseases [1–4]. To realize the efficiency of the RNAi, an effective and safe delivery vector/method is required to deliver the SiRNA into target cells.

Nonviral delivery vectors, exemplified by cationic lipids and polymers, possess desired biocompatibility and minimal mutagenesis, and thus serve as desired candidates for SiRNA delivery. However, in comparison with their viral counterparts, nonviral vectors often suffer from low transfection efficiencies, mainly due to the various systemic barriers. For instance, SiRNA is liable to nuclease-assisted degradation; it can hardly traverse the cell membranes and the internalized SiRNA molecules tend to be entrapped by endosomes/lysosomes and ultimately get degraded.

Kato Shum and John Rossi (eds.), *SiRNA Delivery Methods: Methods and Protocols*, Methods in Molecular Biology, vol. 1364, DOI 10.1007/978-1-4939-3112-5_4, 

Here, we report a newly developed nonviral gene delivery material, the cationic helical polypeptide [5, 6] and polypeptide-based supramolecular self-assembled nanoparticles (SSNPs) [7], which can effectively overcome the aforementioned barriers to mediate effective SiRNA delivery both in vitro and in vivo. Particularly, the stabilized helical structure allows the polypeptide to create pores on cell membranes and endosomal membranes, thereby facilitating the cellular internalization as well as endosomal escape of the SiRNA cargo. When the polypeptide was assembled with other rationally designed materials, the obtained SSNPs can target intestinal M cells and gut-associated macrophages (GAMs) after oral administration to greatly enhance the intestinal absorption of SiRNA against tumor necrosis factor-α (TNF-α) and mediate effective gene knockdown in macrophages, thus triggering systemic TNF-α silencing towards the treatment of acute inflammation. We herein describe the protocol for in vitro and in vivo SiRNA delivery mediated by the helical polypeptide (PVBLG-8) and PVBLG-8-containing SSNPs. We also demonstrate that the helical PVBLG-8, but not the random coiled analogue PVBDLG-8, can trigger effective knockdown of the luciferase gene in HeLa-Luc cells. Compared with the commercial reagent Lipofectamine 2000, the SSNPs mediated an approximately tenfold higher TNF-α knockdown efficiency in macrophages in vitro in terms of reduced SiRNA dose, and after oral administration, they prevented the lipopolysaccharide (LPS)-induced systemic TNF-α production to protect mice from inflammatory hepatic injury at the low dose of 200 μg SiRNA/kg. This finding also represents a 1–2 orders of magnitude improvement over existing delivery vehicles, which typically require dosing from 500 μg/kg to 50 mg/kg in mice via i.v. injection [8–10].

## 2 Materials

1. Oleyl-trimethyl chitosan chloride (OTMC, MW = 200 kDa, quaternization degree of 28.7 %, oleyl conjugation ratio of 20.3 %, structure shown in Fig 1), oleyl-$PEG_{3400}$-mannose (structure shown in Fig. 1.), and oleyl-$PEG_{3400}$-cysteamine (structure shown in Fig. 1.) were synthesized according to previously published protocols [7]. Poly(γ-4-((2-(piperidin-1-yl) ethyl)aminomethyl)-benzyl-L-glutamate) (PVBLG-8, degree of polymerization 200, structure shown in Fig. 1), a cationic helical polypeptide, was synthesized according to our published protocols [5, 6]. Poly(γ-4-((2-(piperidin-1-yl)ethyl) aminomethyl)-benzyl-D,L-glutamate) (PVBDLG-8), a random coiled analogue of PVBLG-8 was also synthesized according to our published protocols.

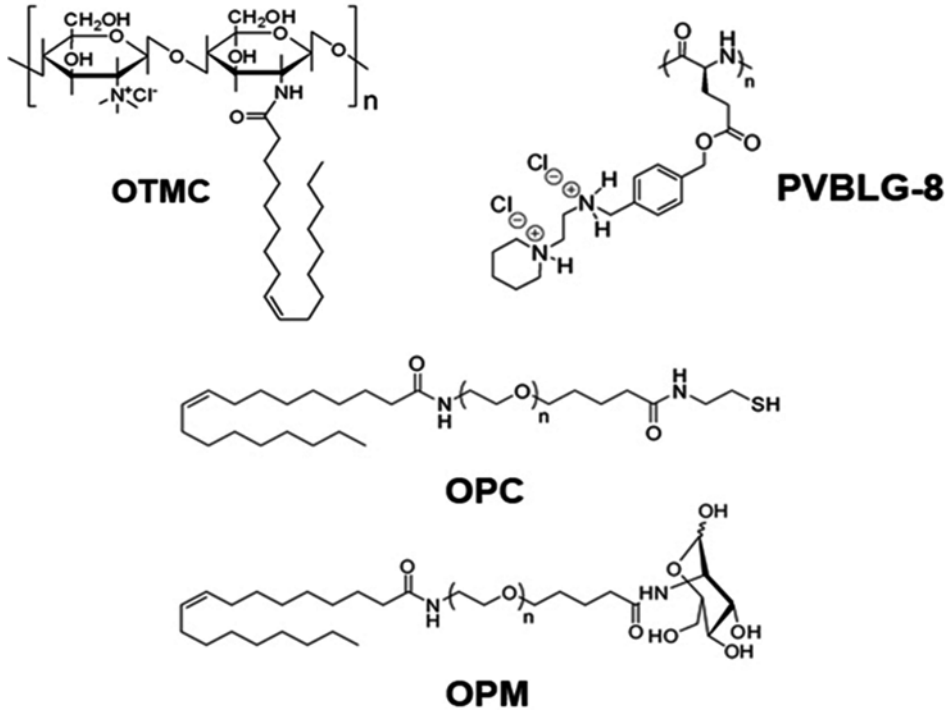


**Fig. 1** Chemical structures of OTMC, OPC, OPM, and PVBLG-8

2. SiRNA duplex against mouse tumor necrosis factor-α (TNF-α) has the sequence of sense strand: 5′-GUCUCAGCCUCUUC UCAUUCCUGct-3′ and antisense strand: 5′-AGCAGGA AmUGmAGmAAmGAmGGmCUmGAmGAmCmAmU-3′, wherein the (m) pattern mN represents a 2′-*O*-methyl base. Sequences of the primers used were as follows. TNF-α F:CCACCACGCTCTTTCTGTCTACTG, TNF-α R: GGG CTACAGGCTTGTCACTCG.
3. SiRNA duplex against luciferase (Luc SiRNA) has the sequence of sense strand: 5′-CUUACGCUGAGUACUUCGAtt-3′ and antisense strand: 5′-UCGAAGUACUCAGCGUAAGtt-3′.
4. Weigh 2 mg lipopolysaccharide (LPS, from *E. coli* 0111:B4) in a biosafety cabinet, transfer to the 25-mL glass vial, and dissolve by 20 mL sterilized PBS to obtain a stock solution of 100 μg/mL. Aliquot to 1 mL/vial in 1.5-mL centrifuge tubes, and store at −20 °C. Thaw the solution at room temperature before use, take out 10 μL, transfer into a 25-mL glass vial, dilute with 9990 μL cell culture medium to obtain a solution of 100 ng/mL (for use in RAW 264.7 cells). Alternatively, take out 100 μL of the 100 μg/mL solution, transfer into a 25-mL glass vial, dilute with 9900 μL PBS to obtain a solution of 1 μg/mL (for use in mouse). Freshly prepare the solution directly before use.

5. Weigh 1 g D-galactosamine (D-GalN), transfer to the 25-mL glass vial, and dissolve by 10 mL sterilized PBS to obtain a solution of 100 mg/mL. Freshly prepare the solution directly before use.
6. Prepare all solutions using diethylpyrocarbonate (DEPC)-treated water and analytical grade reagents.
7. Pre-warm the cell culture media to 37 °C before use.
8. Sterilize all glass vials and centrifuge tubes before use.
9. Diligently follow all waste disposal regulations when disposing waste materials.

## 3 Methods

### 3.1 In Vitro siRNA Delivery by PVBLG-8

1. Weigh 10 mg PVBLG-8 and transfer to the 25-mL glass vial. Add 10 mL DEPC-treated water to make the 1 mg/mL solution. Add 1 M hydrogen chloride to adjust the pH to 6.5 (*see* **Note 1**). Aliquot to 1 mL/vial in 1.5-mL centrifuge tubes, and store at −20 °C (*see* **Note 2**). Thaw the solution at room temperature before use.
2. Take out 200 μL PVBLG-8 solution, transfer into a 1.5-mL centrifuge tube, add 800 μL NaCl solution (150 mM, containing 20 mM HEPES) to obtain a final concentration of 0.2 mg/mL.
3. Dissolve Luc SiRNA with DEPC water at 0.2 mg/mL. Aliquot to 50 μL/tube in 0.5-mL centrifuge tubes, and store at −80 °C. Thaw the solution in ice bath before use (*see* **Note 3**).
4. Transfer 20 μL SiRNA solution into a 1.5-mL centrifuge tube. Add PVBLG-8 solution into SiRNA at determined PVBLG-8/SiRNA weight ratios (e.g., 20, 100, and 200 μL PVBLG-8 equals to PVBLG-8/SiRNA weight ratios of 1, 5, and 10, respectively), and mix by gentle pipetting (*see* **Note 4**).
5. Vortex the mixture for 30 s at 1500 rpm/min and incubate the mixture at RT for 15 min to allow formation of complexes (*see* **Note 5**).
6. Culture HeLa-Luc cells (purchased from the American Type Culture Collection) in DMEM supplemented with 10 % horse serum, 1 % penicillin–streptomycin, 1 % L-glutamine according to the manufacturer's protocol (ATCC website). Passage the cells for more than 3 times while less than 20 times before transfection studies (*see* **Note 6**).
7. Harvest the cells and resuspend in the cell culture media (*see* **Note 7**) at $1 \times 10^5$ cells/mL. Add 0.1 mL of the cell suspension into each well of 96-well plates at the seeding density of $1 \times 10^4$ cells/well (*see* **Note 8**). Slightly shake (left and right for 10 times, then up and down for 10 times), and incubate at 37 °C for 24 h (*see* **Note 9**).

8. Aspirate the media, wash each well once with 500 μL PBS, then add 500 μL serum-free DMEM (or Opti-MEM) to each well.
9. Add different volume of freshly prepared PVBLG-8/SiRNA complexes to the media at the final SiRNA concentrations of 0.4, 0.8, 1.2, and 1.6 μg/mL, respectively (*see* **Note 10**).
10. Shake the plate slightly (left and right for 5 times, then up and down for 5 times), and incubate the cells at 37 °C for 4 h. As a control, the complexes formed between SiRNA and PVBDLG-8, a random coiled analogue of PVBLG-8 with diminished membrane activities, can be prepared and used to transfect HeLa-Luc cells with the same method as described for PVBLG-8.
11. Aspirate all the media in each well, wash the cells once with 500 μL PBS, and add 500 μL serum-containing DMEM/well. Culture the cells at 37 °C for another 20 h.
12. Remove the cell culture medium and immediately add 50 μL of the Bright-Glo luciferase reagent (Promega) to each well. Measure the luminescence intensity using a microplate reader according to the manufacturer's protocols.
13. Calculate the gene silencing efficiency of PVBLG-8 which was denoted as the percentage luminescence intensity of control cells that did not receive PVBLG-8/SiRNA complex treatment (*see* Fig. 2.).

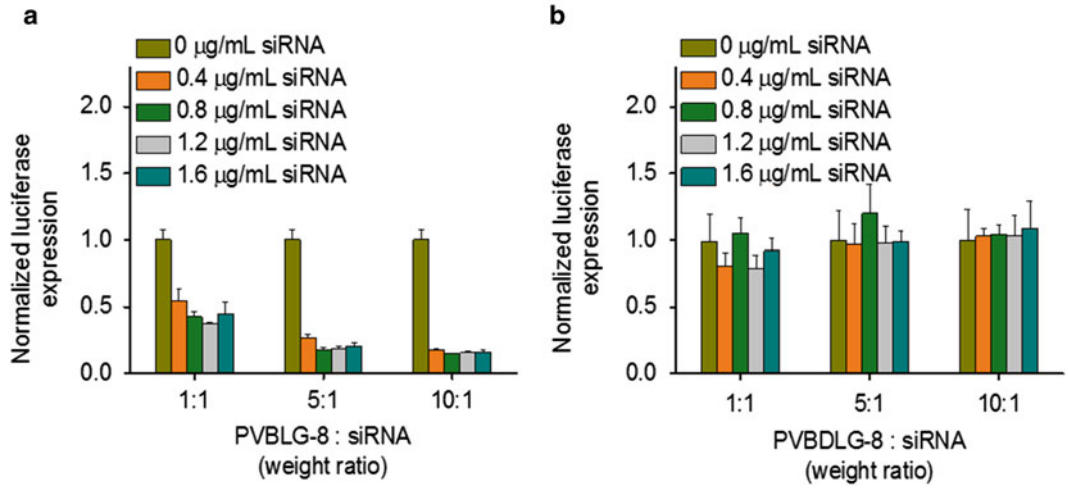


**Fig. 2** (**a**) In vitro transfection of HeLa-Luc cells with luciferase siRNA at various PVBLG-8:siRNA weight ratios and siRNA concentrations. (**b**) In vitro transfection of HeLa-Luc cells with luciferase siRNA at various PVBDLG-8:siRNA weight ratios and siRNA concentrations (reproduced from ref. [6] with permission from Nature Publishing Group)

### 3.2 In Vitro TNF-α siRNA Delivery to Macrophages by Supramolecular Self-Assembled Nanoparticles (SSNPs)

#### 3.2.1 Preparation of SSNPs

1. Weigh 20 mg OTMC and transfer to the 25-mL glass vial. Add 10 mL DEPC-treated water to make the 2 mg/mL solution.
2. Add 1 M hydrogen chloride to adjust the pH to 6.5 (*see* **Note 1**). Aliquot to 2 mL/vial in 7-mL vials, and store at −20 °C. Thaw the solution in 37 °C water bath before use (*see* **Note 11**).
3. Weigh 10 mg PVBLG-8 and transfer to the 25-mL glass vial. Add 10 mL DEPC-treated water to make the 1 mg/mL solution.
4. Add 1 M hydrogen chloride to adjust the pH to 6.5. Aliquot to 1 mL/vial in 1.5-mL centrifuge tubes, and store at −20 °C. Thaw the solution at room temperature before use (*see* **Note 2**).
5. Weigh 100 mg OPM or OPC and transfer to the 25-mL glass vial. Add 10 mL DEPC-treated water to make the 10 mg/mL solution. Aliquot to 1 mL/vial in 1.5-mL centrifuge tubes, and store at −20 °C. Thaw the solution in 37 °C water bath before use.
6. Weigh 10 mg sodium tripolyphosphate (TPP) and transfer to the 25-mL glass vial. Add 10 mL DEPC-treated water to make the 1 mg/mL solution. Aliquot to 1 mL/tube in 1.5-mL centrifuge tubes, and store at −20 °C. Thaw the solution at room temperature before use.
7. Dissolve SiRNA with DEPC water at 0.2 mg/mL. Aliquot to 50 μL/tube in 0.5-mL centrifuge tubes, and store at −80 °C. Thaw the solution in ice bath before use (*see* **Note 3**).
8. Add 20 μL SiRNA solution and 50 μL TPP solution into a 1.5-mL centrifuge tube and mix by gentle pipetting.
9. Add 200 μL OTMC, 80 μL PVBLG-8, 40 μL OPM, and 40 μL OPC into a 1.5-mL centrifuge tube and mix by gentle pipetting.
10. Transfer the OTMC/PVBLG-8/OPM/OPC mixture into the SiRNA/TPP mixture (*see* **Note 12**) and pipette for 10 times. Vortex for 30 s at 1500 rpm/min and incubate the mixture in 37 °C water bath for 30 min to allow formation of SSNPs (*see* Fig. 3a).

#### 3.2.2 Transfection of siRNA to Mouse Macrophages

1. Culture RAW 264.7 cells (mouse monocyte macrophage, purchased from the American Type Culture Collection) in DMEM supplemented with 10 % fetal bovine serum (FBS) according to the manufacturer's protocol (ATCC website). Passage the cells for more than 3 times while less than 20 times before transfection studies (*see* **Note 13**).
2. Harvest the cells and resuspend in the cell culture media at $1 \times 10^5$ cells/mL. Add 0.5 mL of the cell suspension into each well of 24-well plates at the seeding density of $5 \times 10^4$ cells/well.

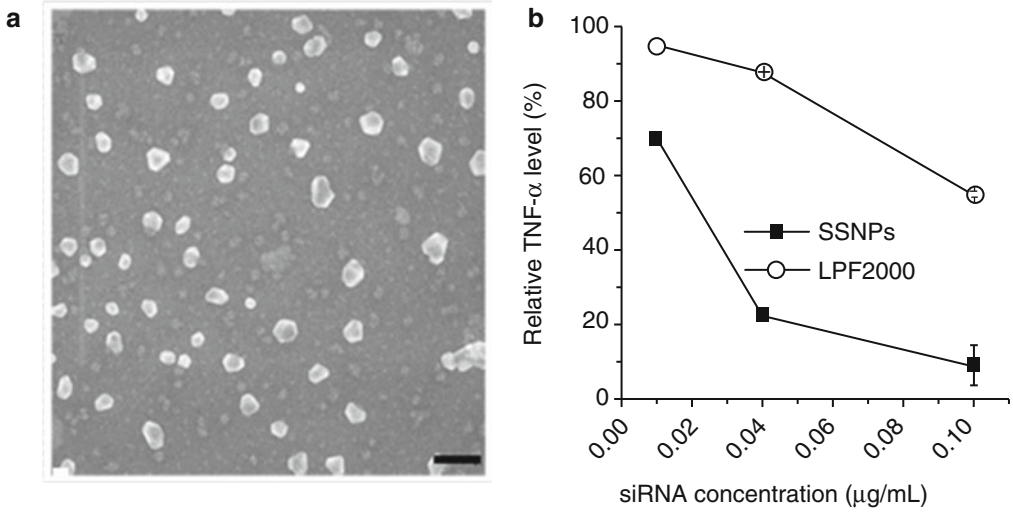


**Fig. 3** (**a**) SEM image of SSNPs (bar = 200 nm). (**b**) TNF-α knockdown efficiencies of SSNPs and LPF2000/siRNA complexes at various siRNA doses ($n = 3$) (reproduced from ref. [7] with permission from Wiley)

Slightly shake (left and right for 10 times, then up and down for 10 times), and incubate at 37 °C for 24 h (*see* **Note 7**).

3. Aspirate the media (*see* **Note 14**), wash each well once with 500 μL PBS, then add 500 μL serum-free DMEM (or Opti-MEM) to each well. Add the freshly prepared SSNPs (5.4, 2.1, and 0.5 μL) to the media at the final concentrations of 0.1, 0.04 and 0.01 μg/mL, respectively (*see* **Note 10**).
4. Shake the plate slightly (left and right for 5 times, then up and down for 5 times), and incubate the cells at 37 °C for 4 h. Commercial reagent Lipofectamine 2000 (LPF2000) as an internal control was also used to transfect the RAW 264.7 cells according to the manufacturer's protocol.
5. Aspirate all the media in each well, wash the cells once with 500 μL PBS, and add 500 μL serum-containing DMEM/well. Culture the cells at 37 °C for another 20 h.
6. Aspirate the culture media, add 500 μL 100 ng/mL LPS solution, and incubate the cells at 37 °C for 3 h.
7. Take out 2 μL of the cell culture media and measure the extracellular TNF-α level by the mouse TNF-α ELISA kit according to the manufacturer's protocol (*see* **Note 15**).
8. Aspirate the cell culture media, wash the cells with 3× 500 μL PBS, and extract the total RNA using TRIzol according to the manufacturer's protocol (*see* **Note 16**).
9. Synthesize cDNA from 500-ng total RNA using the high capacity cDNA reverse transcription kit according to the manufacturer's protocol.

10. Measure the TNF-α mRNA level using an ABI PRISM 7900HT Real-Time PCR system according to the manufacturer's suggested protocols.
11. Measure the extracellular TNF-α level or the mRNA level of control cells that did not receive SSNPs treatment but were treated by the same method as described above.
12. Calculate the gene silencing efficiency of SSNPs which was denoted as the percentage of TNF-α or TNF-α mRNA levels of the control cells (*see* Fig. 3b).

### 3.3 Oral Delivery of TNF-α siRNA by Supramolecular Self-Assembled Nanoparticles (SSNPs) Against Hepatic Injury

1. Orally deliver SSNPs (430 μL/mouse, 200 μg SiRNA/kg) to male C57/BL-6 mice (20–22 g) via gavage.
2. Take 2 mL LPS solution (1 μg/mL) and 2 mL D-GalN solution (100 mg/mL), transfer to a 7-mL vial, and mix by gentle pipetting (*see* **Note 17**).
3. Inject the freshly prepared LPS/D-GalN solution (500 μL/mouse, equals to 12.5 μg LPS/kg and 1250 mg D-GalN/kg) intraperitoneally 24 h after oral gavage of SSNPs.
4. Anesthetize the mice with isoflurane 1.5 h after i.p. injection (*see* **Note 18**). Collect 0.5 mL by retro-orbital bleeding, put the blood in 1.5-mL centrifuge tube at RT for 10 min to let it coagulate, centrifuge at 10,500 × *g* for 5 min, and collect the serum.
5. Measure the serum TNF-α level using a mouse TNF-α ELISA kit according to the manufacturer's protocol.
6. Sacrifice the mice 5 h after i.p. injection of LPS/D-GalN. Harvest liver, spleen, and lung, wash with PBS, and homogenize with TRIzol reagent in ice (use the whole spleen and lung, while cut a small piece (0.2 g) of liver). Extract the RNA according to the manufacturer's protocol and measure the TNF-α mRNA level using real-time PCR as described for RAW 264.7 cells.
7. Measure the serum TNF-α level or the mRNA levels in each specific organ of control mice that received oral gavage of PBS (430 μL/animal) instead of SSNPs and were treated by the same method as described above.
8. Calculate the systemic TNF-α silencing efficiency of SSNPs which was represented as the percentage of TNF-αor TNF-α mRNA levels of the control animals (*see* Fig. 4).
9. In another experiment, gavage the SSNPs and i.p. inject the LPS/D-GalN in the same manner as **steps 1–8**.
10. Anesthetize the mice with isoflurane 5 h after i.p. injection. Collect 1 mL blood by retro-orbital bleeding, put the blood in 1.5-mL centrifuge tube at RT for 10 min to let it coagulate, centrifuge at 10,500 × *g* for 5 min, and collect the serum.

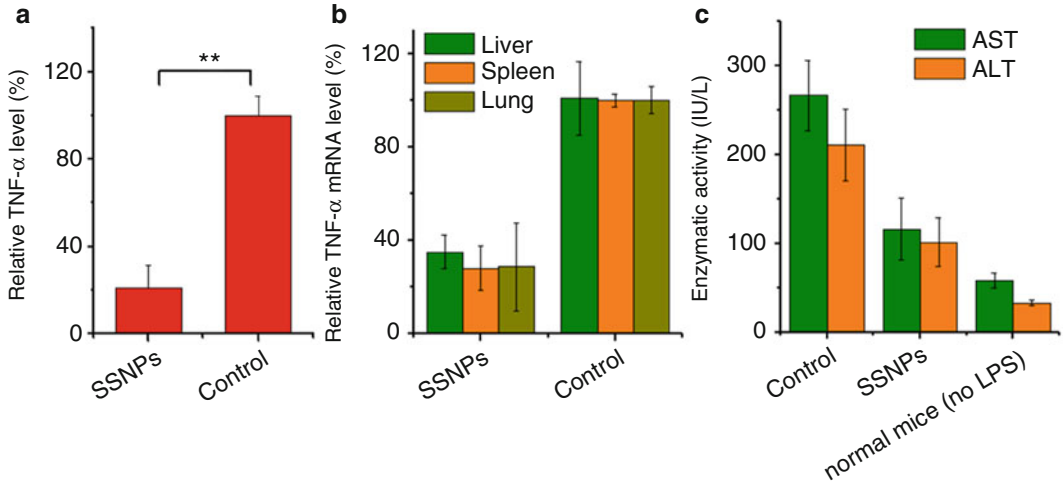


**Fig. 4** (**a**) Serum TNF-α level of mice gavaged with SSNPs at 200 μg siRNA/kg ($n = 6$). (**b**) Relative TNF-α mRNA levels in mouse liver, spleen, and lung 24 h after oral gavage of SSNPs ($n = 3$). (**c**) Serum ALT and AST levels of mice 5 h after LPS/D-GalN stimulation ($n = 4$) (reproduced from ref. [7] with permission from Wiley)

11. Measure the serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels using commercial kits according to the manufacturer's protocols. As a control, measure the serum AST/ALT levels of mice that received oral gavage of PBS (430 μL/animal) rather than SSNPs (*see* Fig. 4).

## 4 Notes

1. Using a pH paper to indicate the pH. pH values within the range of 6.2–6.8 are allowed.
2. Avoid repeated ice-thawing processes of PVBLG-8. Recommend less than five ice-thawing cycles for each preserved tube.
3. Avoid repeated ice-thawing processes of SiRNA. Recommend less than three ice-thawing cycles for each preserved tube.
4. Quickly add the PVBLG-8 solution into SiRNA at once and immediately mix for several times.
5. Incubation time can be longer than 15 min but less than 30 min.
6. Cell condition is critical to the success of transfection. Passage the cells at the confluence of 70–80 % and never let cells grow over-confluence. For each passage, keep the passage ratio higher than 1:3.
7. Culture the cells using antibiotic-containing media. However, use antibiotic-free media to seed cells. Also use antibiotic-free

media during and after transfection. Because PVBLG-8 mediates effective SiRNA delivery mainly via the pore-formation mechanism, it will also allow excessive diffusion of antibiotics into cells to hamper the cell viability.

8. For the convenient measurement of luciferase activity, seed the cells on white plates instead of transparent plates.
9. Usually do the transfection at the cell density of 70 %. Because we cannot visualize the cells grown on white plates, we usually seed some cells on another transparent plate at the same time for the purpose of monitoring cell density.
10. We recommend slowly add the complexes onto the surface of the culture media in small droplets. Do not touch the inner surface of each well. It will lead to inconsistence of the transfection results.
11. Fully thaw the solution at 37 °C until it becomes completely transparent. Thawing at room temperature sometimes cannot lead to incomplete dissolution because the amphiphilic polymer has the tendency to self-assemble.
12. Quickly add the OTMC/PVBLG-8/OPM/OPC mixture into the SiRNA/TPP at once and immediately mix for several times. Vortex vigorously to promote nanoparticle formation.
13. RAW 264.7 cells tend to be activated by the treatment of trypsin. It is therefore required to passage the cells using scrappers, which is also suggested by the ATCC. Scrape 3–5 times for each passage, and pipette 10–15 times to dissociate the cells. Avoid excessive scraping or pipetting that will also activate or damage the cells.
14. Do not let the cells stay in the dried condition for too long (usually less than 1 min). RAW 264.7 cells tend to detach from the plate when get dried.
15. The supernatant can be preserved at −20 °C if not assayed directly. However, avoid repeated ice-thawing process that would otherwise damage the TNF-α inside. It may need to dilute the sample with cell culture media if the TNF-α concentration exceeds the detection limit of the ELISA kit.
16. The homogenate can be preserved at −20 °C if not assayed directly and also avoid repeated ice-thawing processes.
17. i.p. injection of LPS/D-GalN establishes the acute hepatic injury model that is 100 % lethal to animals. This mixed solution needs to be freshly prepared and used within 20 min otherwise it would hamper its efficiency in inducing hepatic injury.
18. We recommend anesthetizing the animal before blood sampling because excessive struggling of the animal body may lead to inconsistent serum TNF-α levels.

## References


1. Davis ME, Zuckerman JE, Choi CHJ, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A (2010) Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature 464:1067–1070
2. Lee H, Lytton-Jean AKR, Chen Y, Love KT, Park AI, Karagiannis ED, Sehgal A, Querbes W, Zurenko CS, Jayaraman M, Peng CG, Charisse K, Borodovsky A, Manoharan M, Donahoe JS, Truelove J, Nahrendorf M, Langer R, Anderson DG (2012) Molecularly self-assembled nucleic acid nanoparticles for targeted in vivo siRNA delivery. Nat Nanotechnol 7:389–393
3. Woodrow KA, Cu Y, Booth CJ, Saucier-Sawyer JK, Wood MJ, Saltzman WM (2009) Intravaginal gene silencing using biodegradable polymer nanoparticles densely loaded with small-interfering RNA. Nat Mater 8:526–533
4. Dassie JP, Liu XY, Thomas GS, Whitaker RM, Thiel KW, Stockdale KR, Meyerholz DK, McCaffrey AP, McNamara JO, Giangrande PH (2009) Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. Nat Biotechnol 27:839–849
5. Gabrielson NP, Lu H, Yin LC, Li D, Wang F, Cheng JJ (2012) Reactive and bioactive cationic α-helical polypeptide template for nonviral gene delivery. Angew Chem Int Ed Engl 51:1143–1147
6. Gabrielson NP, Lu H, Yin LC, Kim KH, Cheng JJ (2012) A cell-penetrating helical polymer for siRNA delivery to mammalian cells. Mol Ther 20:1599–1609
7. Yin LC, Song ZY, Qu QH, Kim KH, Zheng N, Yao C, Chaudhury I, Tang HY, Gabrielson NP, Uckun FM, Cheng JJ (2013) Supramolecular self-assembled nanoparticles mediate oral delivery of therapeutic TNF-alpha siRNA against systemic inflammation. Angew Chem Int Ed Engl 52:5757–5761
8. Filleur S, Courtin A, Ait-Si-Ali S, Guglielmi J, Merle C, Harel-Bellan A, Clezardin P, Cabon F (2003) SiRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor resistance to antiangiogenic thrombospondin-1 and slows tumor vascularization and growth. Cancer Res 63:3919–3922
9. McCaffrey AP, Meuse L, Pham TTT, Conklin DS, Hannon GJ, Kay MA (2002) Gene expression—RNA interference in adult mice. Nature 418:38–39
10. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, Vornlocher HP (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432:173–178