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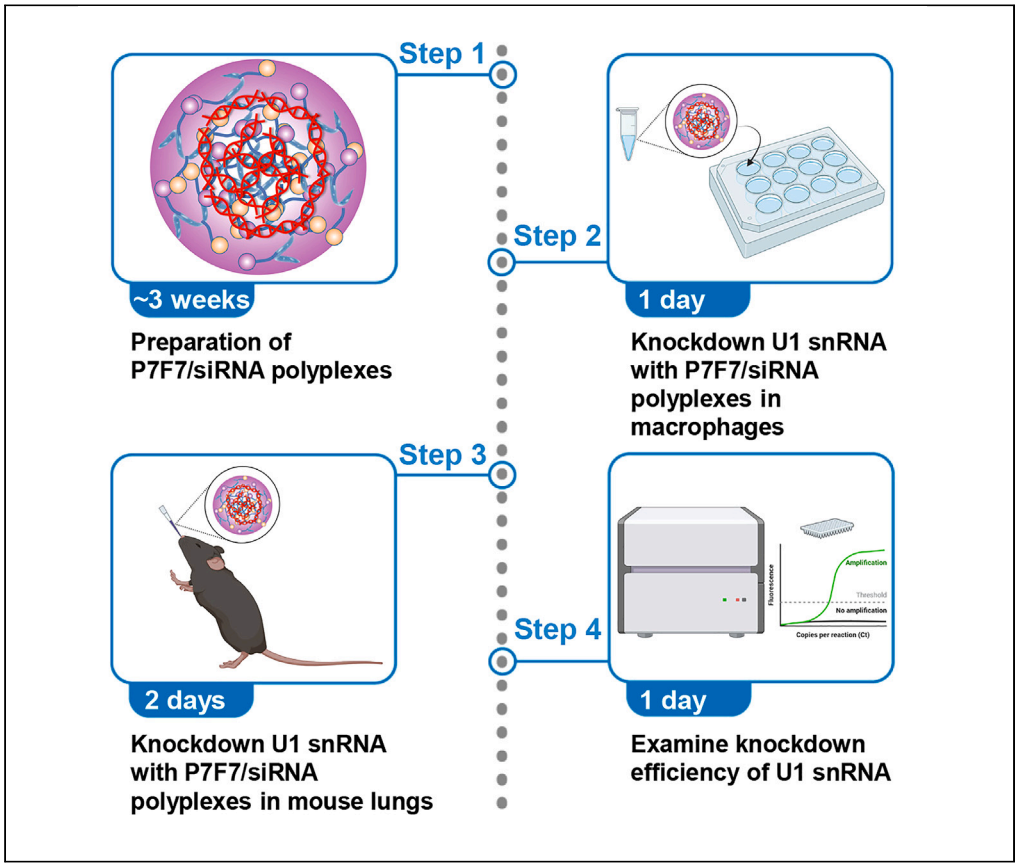
Protocol

Protocol for siRNA-mediated U1 snRNA knockdown using fluorinated α-helical polypeptide *in vitro* and *in vivo*



Here, we present a protocol for small interfering RNA (siRNA)-mediated U1 small nuclear RNA (snRNA) knockdown using fluorinated α-helical polypeptide in macrophages and mouse lungs, providing a dependable approach to silence U1 snRNA *in vitro* and *in vivo*. We describe steps for preparing P7F7/siRNA polyplexes and silencing U1 snRNA with P7F7/siRNA polyplexes in macrophages and mouse lungs. Knockdown efficiency is validated through reverse-transcription quantitative real-time PCR analysis. This protocol is applicable for studying the physiological or pathophysiological function of U1 snRNA.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Using fluorinated α-helical polypeptide P7F7 for efficient siRNA delivery

Silencing U1 snRNA with P7F7/siRNA polyplexes *in vitro* and *in vivo*

Approach to study the physiological or pathophysiological function of U1 snRNA

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Protocol for siRNA-mediated U1 snRNA knockdown using fluorinated α-helical polypeptide *in vitro* and *in vivo*

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SUMMARY

Here, we present a protocol for small interfering RNA (siRNA)-mediated U1 small nuclear RNA (snRNA) knockdown using fluorinated α-helical polypeptide in macrophages and mouse lungs, providing a dependable approach to silence U1 snRNA *in vitro* and *in vivo*. We describe steps for preparing P7F7/siRNA polyplexes and silencing U1 snRNA with P7F7/siRNA polyplexes in macrophages and mouse lungs. Knockdown efficiency is validated through reverse-transcription quantitative real-time PCR analysis. This protocol is applicable for studying the physiological or pathophysiological function of U1 snRNA.

For complete details on the use and execution of this protocol, please refer to Zhang et al.[1]

BEFORE YOU BEGIN

U1 small nuclear RNA (snRNA) is essential for pre-mRNA splicing, encoded by numerous U1 genes and expressed widely with high transcript levels.[2,3] Its multi-locus expression and essential physiological functions pose challenges in generating U1 snRNA knockout mice for *in vivo* studies. Instead, small interfering RNA (siRNA)-induced RNA interference (RNAi) technology is a simpler and more efficient approach for investigating genes with multiple copies at distinct loci, like U1 snRNA. Additionally, generating knockout mice for essential genes is not feasible due to the potential embryonic lethality. Therefore, using siRNA for gene knockdown is a better choice to study essential genes and the genes with multiple copies.

In our prior publication, we achieved the knockdown of U1 snRNA in mouse lungs via the fluorinated α-helical polypeptide P7F7-mediated siRNA delivery system.[1] Fluorocarbon modification of polycations enhances mucus permeation by stabilizing the polyplexes via steric hindrance against the adsorption of mucin glycoproteins. Therefore, fluorinated P7F7 has mucus permeation capability.[4] Cationic helical polypeptides have an efficient internalization efficacy through the "pore formation" mechanism. Moreover, cationic polypeptides with α-helical conformation and abundant positively charged guanidine groups can impart strong membrane penetrating activity to facilitate the transmembrane delivery of siRNA. With the synergistic effect of guanidine and hydrophobic domains, guanidine-containing polypeptides outperformed the commercial reagent, Lipofectamine 2000,

by 3–6 folds regarding the *in vitro* transfection efficiency.[5] Therefore, guanidinated α-helical P7F7 has stronger cell membrane penetration ability.[4–6] Overall, compared to other siRNA transfection methods, fluorinated and guanidinated bifunctional α-helical P7F7 shows better mucus and cell membrane penetration capabilities, thereby enhancing the efficiency of siRNA delivery *in vitro* and *in vivo*. The present article provides a detailed description of siRNA-mediated U1 snRNA knockdown using fluorinated α-helical polypeptide *in vitro* and *in vivo*.

Institutional permissions

All animal experiments were conducted according to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Service Center of Suzhou Institute of Systems Medicine (ISM-IACUC-0011-R). It is important to note that ethical guidelines and standards vary across countries and institutions. Before starting your experiments, ensure you have the required approval from your institution's relevant committee.

Preparation of siRNAs

Timing: ~3 days

1. Order siRNAs and the siRNA sequences are listed in the key resources table.

   Note: We recommend choosing pre-designed siRNAs from commercial sources. If pre-designed siRNAs are unavailable, design custom siRNA sequences using online tools, such as siDirect (http://sidirect2.rnai.jp/).[7–9] Due to the potential off-target effects, we recommend designing two or three distinct siRNA sequences for each target gene.

   Note: Methoxy modification can improve the stability of siRNA, and locked nucleic acid modification can improve the specificity of siRNA.

2. Dissolve powdered siRNA in DEPC-treated water to make a 1 mg/mL stock solution, and store at −80°C.

Preparation of fluorinated α-helical polypeptide P7F7

Timing: ~2 weeks

Fluorinated α-helical polypeptide P7F7 can efficiently deliver siRNA to mouse lungs by facilitating both mucus permeation and cell penetration.[4] P7F7-mediated siRNA delivery offers a dependable approach to silence any gene. P7F7 is synthesized via ring-opening polymerization (ROP) of *N*-carboxyanhydride (NCA) and click chemistry.[10–12] First, the polypeptide PPOBLG is synthesized via *n*-butylamine-initiated ROP of γ-(4-propargyloxybenzyl)-$_L$-glutamic acid-based *N*-carboxyanhydride (POBLG-NCA) at the molar ratio of monomer and initiator ([M]/[I]) of 100. Then, azido fluorocarbon 7F-N_3 is synthesized via esterification and azide reactions. Finally, the side chain of PPOBLG is modified with azido guanidine (6-azidohexyl guanidine) and 7F-N_3 at preselected molar ratios of 93/7 via click chemistry, thus yielding water-soluble cationic polypeptide with both guanidine and fluorocarbon groups on the side chain.

3. Synthesis of POBLG-NCA (Figure 1A) according to reported procedures.[5]
   a. Mix γ-(4-propargyloxybenzyl)-$_L$-glutamic acid (1 g, 3.43 mmol) and triphosgene (0.41 g, 1.37 mmol) in anhydrous tetrahydrofuran (THF) (40 mL) in a round-bottom flask.
   b. Stir the mixture at 74°C for 3.5 h.
   c. Remove the solvent under vacuum using a rotary evaporator at 20°C–25°C (Caution: POBLG-NCA is sensitive to heat, therefore the temperature cannot exceed 40°C).

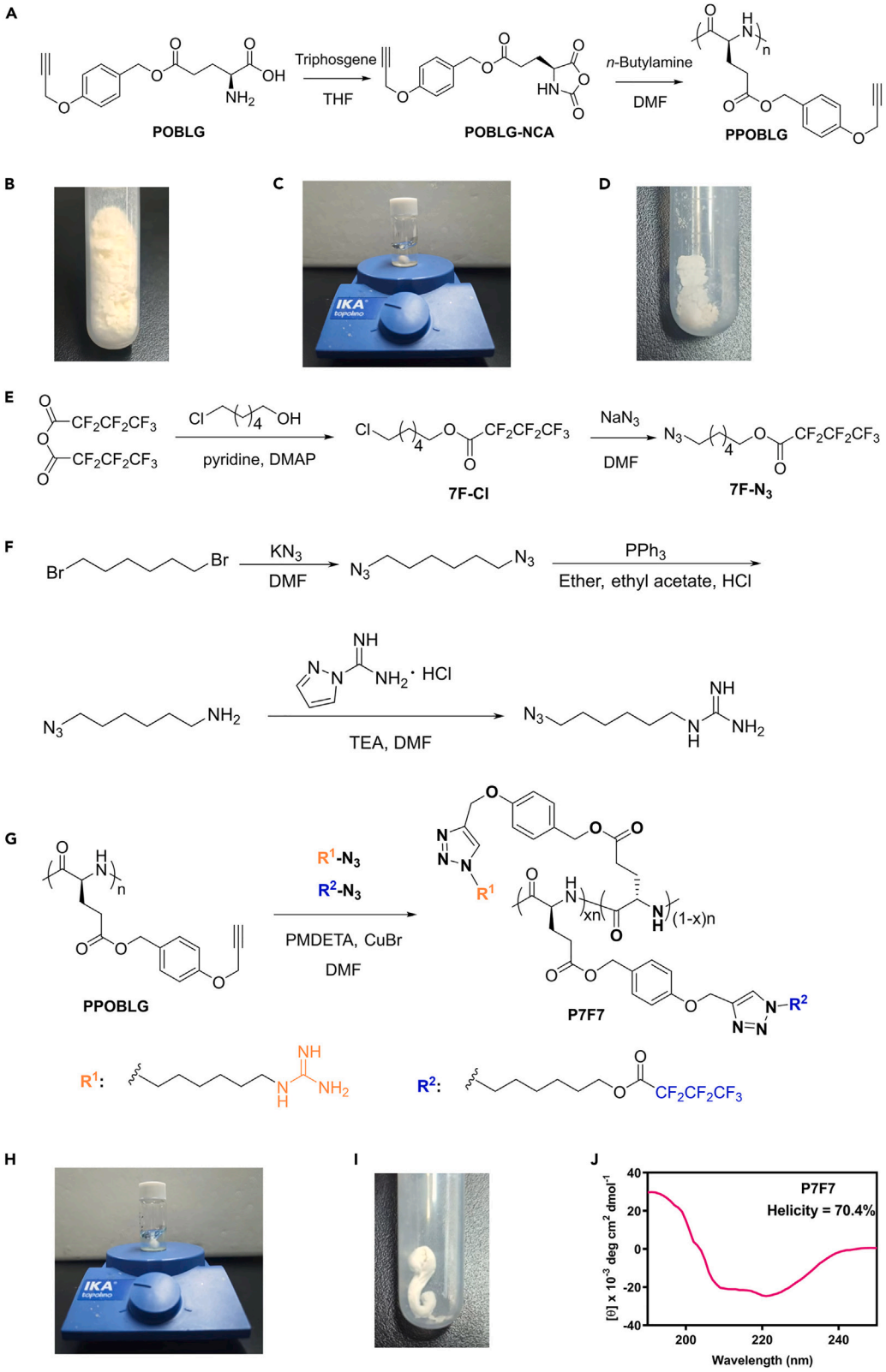
A
POBLG
Triphosgene
THF
POBLG-NCA
n-Butylamine
DMF
PPOBLG
B
C
D
E
pyridine, DMAP
7F-Cl
NaN3
DMF
7F-N3
F
KN3
DMF
PPh3
Ether, ethyl acetate, HCl
TEA, DMF
G
PPOBLG
PMDETA, CuBr
DMF
P7F7
R1:
R2:
H
I
J
P7F7
Helicity = 70.4%
[θ] x 10-3 deg cm2 dmol-1
Wavelength (nm)

Figure 1. Synthesis and characterization of P7F7
(A) Synthetic route of PPOBLG.
(B) Photograph showing of POBLG-NCA.
(C) Photograph showing of ring-opening polymerization of POBLG-NCA.
(D) Photograph showing of PPOBLG.
(E) Synthetic route of 7F−N_3.
(F) Synthetic routes of 6-azidohexyl guanidine.
(G) Synthetic route of P7F7 (n = 130, x = 0.07).
(H) Photograph showing of click chemistry of PPOBLG.
(I) Photograph showing of P7F7.
(J) Circular dichroism (CD) spectrum of P7F7 (0.2 mg/mL) in distilled water.

d. Dissolve the crude product in ethyl acetate (5 mL) and recrystallize the mixture using *n*-hexane (50 mL) at −20°C three times.
e. Remove the solvent through filtration and collect POBLG-NCA as white solid after vacuum drying (yield 76%, which is calculated by the ratio of the obtained mass to the theoretical mass).

Note: The POBLG-NCA (Figure 1B) should be stored at −20°C (the structure remains stable for at least 6 months), and its structure can be characterized by ^{1}H nuclear magnetic resonance (^{1}H-NMR). The pure NCA monomers are soluble in ethyl acetate. If the product is insoluble or partially insoluble, it indicates the oligomerization of NCA monomers, which is unusable.

4. Synthesis of PPOBLG (Figure 1A).
   a. Dissolve the POBLG-NCA (0.86 g, 2.71 mmol) in anhydrous dimethylformamide (DMF) (5 mL) in a 10 mL vial under a nitrogen atmosphere.

   Note: DMF should be dried by a solvent purification system (EX-SPS8-800) quipped in a column packed with alumina before use.

   b. Add a solution of *n*-butylamine in DMF (0.1 mol/L, 271 μL, 0.027 mmol) using a pipette.
   c. Stir the mixture (Figure 1C) at 20°C–25°C for 72 h.
   d. Precipitate the mixture with distilled water (60 mL) to afford crude product, wash the crude product with distilled water for three times, and collect the product PPOBLG as white solid (yield 94%, which is calculated by the ratio of the obtained mass to the theoretical mass).

   Note: The PPOBLG (Figure 1D) should be stored at −20°C (the structure remains stable for at least 3 years), and its structure can be characterized by ^{1}H-NMR.

5. Synthesis of 7F-Cl (Figure 1E).
   a. Mix the 6-chlorohexanol (0.97 mL, 7.32 mmol), heptafluorobutyric anhydride (2.18 mL, 8.78 mmol), pyridine (1.77 mL, 21.96 mmol), and 4-dimethylaminopyridine (DMAP) (60 mg, 0.488 mmol) in a 10 mL vial in an ice-water bath.
   b. Stir the solution at 20°C–25°C for 84 h.

   Note: The solution should turn yellow, and precipitate should appear during this process.

   c. Dissolve the mixture in dichloromethane (50 mL) and wash it once with 50 mL of saturated sodium chloride solution, four times with 1 M HCl (50 mL each time), and twice with saturated sodium chloride solution (50 mL each time) using a separating funnel.
   d. Remove the solvent under vacuum using a rotary evaporator at 20°C–25°C.
   e. Obtain the crude product as light-yellow oil.
   f. Add the distilled water (20 mL) to the crude product and stir at 60°C for 1 h.
   g. Dissolve the mixture in dichloromethane (50 mL) and wash it twice with saturated $NaHCO_3$ solution (50 mL each time) and twice with saturated sodium chloride solution (50 mL each time).

h. Dry the organic phase with anhydrous sodium sulfate at 4°C for 2 h, and filter to afford 7F-Cl as light-yellow liquid after removal of the solvent under vacuum using a rotary evaporator at 20°C–25°C (yield 79%, which is calculated by the ratio of the obtained mass to the theoretical mass).

Note: The 7F-Cl should be stored at −20°C (the structure remains stable for at least 1 year), and its structure can be characterized by ^{1}H-NMR.

6. Synthesis of 7F-N_3 (Figure 1E).
   a. Mix 7F-Cl (1.30 g, 3.91 mmol) and NaN_3 (1.27 g, 19.54 mmol) in DMF (3 mL), and stir at 60°C for 48 h.
   b. Dissolve the mixture in hexane (50 mL).

   Note: Hexane should be dried by a column packed with alumina before use.

   c. Wash four times with saturated sodium chloride solution (50 mL each time) and dry over anhydrous sodium sulfate at 4°C for 2 h.
   d. Obtain 7F-N_3 as transparent liquid after filtration and removal of the solvent under vacuum using a rotary evaporator at 20°C–25°C (yield 81%, which is calculated by the ratio of the obtained mass to the theoretical mass).

   Note: The 7F-N_3 should be stored at −20°C (the structure remains stable for at least 1 year), and its structure can be characterized by ^{1}H-NMR.

7. Synthesis of 6-azidohexyl guanidine (Figure 1F) according to reported procedures.[5]
   a. Mix 1,6-dibromohexane (3.85 mL, 25 mmol) and KN_3 (6.07 g, 75 mmol) in DMF and stir at 60°C for 24 h.
   b. Add distilled water (150 mL) and extract the product from distilled water four times with diethyl ether (50 mL each time).
   c. Collect the organic phase and dry over anhydrous sodium sulfate at 4°C for 2 h.
   d. Obtain 1,6-diazohexane as transparent liquid after filtration using a funnel and removal of the solvent under vacuum using a rotary evaporator at 20°C–25°C.
   e. Dissolve 1,6-diazohexane (3.88 g, 23.1 mmol) in the mixture of diethyl ether (15 mL) and ethyl acetate (15 mL).
   f. Add 5% HCl (30 mL) to the mixture.
   g. In an ice-water bath, add triphenylphosphine (6.32 g, 24.1 mmol) to the mixture slowly.
   h. Stir the mixture at 20°C–25°C for 24 h.
   i. Extract the crude product from the mixture with 30 mL of 1 M HCl and wash the aqueous phase three times with dichloromethane (20 mL each time).
   j. Adjust the pH of aqueous phase to 12 using 0.1 M NaOH.
   k. Extract the product from the mixture five times with dichloromethane (30 mL each time) and dry over anhydrous sodium sulfate at 4°C for 2 h.
   l. Obtain 6-azihexamine as transparent liquid after filtration using a funnel and removal of the solvent under vacuum using a rotary evaporator at 20°C–25°C.
   m. Dissolve 6-azihexamine (1.35 g, 9.53 mmol), 1H-pyrazole-1-carboxamidine hydrochloride (1.40 g, 9.53 mmol), and triethylamine (1.06 g, 10.48 mmol) in DMF (5 mL).
   n. Stir the mixture at 20°C–25°C for 24 h.
   o. Precipitate the product 6-azidohexyl guanidine with diethyl ether (50 mL) and collect it as white solid through filtration (yield 72%, which is calculated by the ratio of the obtained mass to the theoretical mass).

   Note: The 6-azidohexyl guanidine should be stored at −20°C (the structure remains stable for at least 1 year).

8. Synthesis of P7F7 (Figure 1G).
   a. Dissolve PPOBLG (30 mg, 0.11 mmol), 6-azidohexyl guanidine (20.7 mg, 0.11 mmol), and 7F-N_3 (2.9 mg, 0.01 mmol) in DMF (3 mL) in a glass vial (10 mL) in the glovebox.
   b. Add *N,N,N′,N″,N″*-pentamethyl diethylene-triamine (PMDETA, 34 μL, 0.125 mmol) and copper bromide (CuBr) (18 mg, 0.125 mmol).

   Note: In this process, CuBr serves as a catalyst and PMDETA serves as a complexant to increase the solubility of cuprous ions in DMF.

   c. Stir the mixture (Figure 1H) at 20°C–25°C for 24 h and quench by exposure to air.
   d. Add HCl (1 M, 3–4 mL) until the solution becomes colorless.
   e. Dialyze the product against distilled water (MWCO = 3500 Da) for three days and lyophilize to afford P7F7 as white solid (yield 92%, which is calculated by the ratio of the obtained mass to the theoretical mass).
   f. Dissolve P7F7 in DEPC-treated water to make a 10 mg/mL stock solution, and store it at 4°C.

   Note: The solid P7F7 (Figure 1I) can be stored at 20°C–25°C (the structure remains stable for at least 1 year), and the structure of P7F7 can be characterized by ^{1}H-NMR. If the yield of P7F7 is low (<70%), refer to troubleshooting 1. If P7F7 dissolves incompletely, refer to troubleshooting 2.

9. Characterization of P7F7.

   Note: Circular dichroism (CD) measurement is recommended to determine the characterization of P7F7.

   a. Dissolve P7F7 in distilled water to a concentration of 0.2 mg/mL.
   b. Place the solution in a quartz cell with a path length of 1 mm.
   c. Calculate the mean residue molar ellipticity of P7F7 based on the measured apparent ellipticity according to reported formulas[13]: Ellipticity ([θ] in deg·cm^2·dmol^{-1}) = (millidegrees × mean residue weight)/(path length in millimeters × concentration of polypeptide in mg/mL) (Figure 1J).
   d. Calculate the helicity of P7F7 according to the following equation: Helicity = $(-[\theta_{222}] + 3{,}000)/39{,}000$ (Figure 1J).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Copper bromide (CuBr, 99%)	Sigma-Aldrich	CAS# 7787-70-4
N,N,N′,N″,N″-pentamethyl diethylene-triamine (PMDETA, 99%)	Sigma-Aldrich	CAS# 3030-47-5
n-Butylamine	Energy Chemical	CAS# 109-73-9
Heptafluorobutyric anhydride	Energy Chemical	CAS# 336-59-4
4-Dimethylaminopyridine (DMAP)	Energy Chemical	CAS# 1122-58-3
6-Chlorohexanol	Energy Chemical	CAS# 2009-83-8
Dimethylformamide (DMF)	Sigma-Aldrich	CAS# 68-12-2
Hexane	Sigma-Aldrich	CAS# 110-54-3
Pyridine	Sigma-Aldrich	CAS# 110-86-1
Dichloromethane	Sigma-Aldrich	CAS# 75-09-2
HCl	Sigma-Aldrich	CAS# 7647-01-0
$NaHCO_3$	Sigma-Aldrich	CAS# 144-55-8
NaN_3	Sigma-Aldrich	CAS# 26628-22-8

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anhydrous sodium sulfate	Sigma-Aldrich	CAS# 7757-82-6
Heparin	Sigma-Aldrich	CAS# 9041-08-1
Triphosgene	Sigma-Aldrich	CAS# 32315-10-9
Tetrahydrofuran	Sigma-Aldrich	CAS# 109-99-9
Ethyl acetate	Sigma-Aldrich	CAS# 141-78-6
1,6-Dibromohexane	Sigma-Aldrich	CAS# 629-03-8
KN_3	Sigma-Aldrich	CAS# 20762-60-1
NaOH	Sigma-Aldrich	CAS# 1310-73-2
Diethyl ether	Sigma-Aldrich	CAS# 60-29-7
Triphenylphosphine	Sigma-Aldrich	CAS# 603-35-0
Triethylamine	Sigma-Aldrich	CAS# 121-44-8
1H-Pyrazole-1-carboxamidine hydrochloride	Sigma-Aldrich	CAS# 4023-02-3
M-CSF	Sigma-Aldrich	CAS# 81627-83-0
Chloroform	Sigma-Aldrich	CAS# 67-66-3
Isopropyl alcohol	Sigma-Aldrich	CAS# 67-63-0
Ethanol	Sigma-Aldrich	CAS# 64-17-5
Sodium chloride solution	Sigma-Aldrich	Cat# 71392
PBS	Gibco	Cat# C1001050013T
DEPC-treated water	Solarbio	Cat# R1600
RIPA lysis buffer	Beyotime	Cat# P0013B
Dulbecco's modified Eagle's medium (DMEM)	Gibco	Cat# C11995500BT
Fetal bovine serum	Bovogen	Cat# SFBS-NZ
Penicillin-Streptomycin	Gibco	Cat# 15140122
TRIzol	Invitrogen	Cat# 15596018
Critical commercial assays		
MTT assay kit	Invitrogen	Cat# V13154
BCA protein assay kit	Takara	Cat# T9300A
PrimeScript RT master mix (perfect real time)	Takara	Cat# RR036B
TB Green Premix Ex Taq (Tli RNaseH Plus)	Takara	Cat# RR420B
Experimental models: Cell lines		
RAW264.7	ATCC	Cat# TIB-71
Experimental models: Organisms/strains		
C57BL/6J mice (male, 6–8 weeks)	Vital River Laboratory Animal Technology	N/A
Oligonucleotides		
siU1-1: 5′-GAGAUACCAUGAUCACGAA-3′	This paper	N/A
siU1-2: 5′-GGGAAACUCGACUGCAUAA-3′	This paper	N/A
Cy3-siRNA	Ge et al.[4]	N/A
qPCR primer for U1-forward: 5′-CTTACCTGGCAGGGGAGATA-3′	This paper	N/A
qPCR primer for U1-reverse: 5′-GCAGTCGAGTTTCCCACATT-3′	This paper	N/A
qPCR primer for L32-forward: 5′-TTAAGCGAAACTGGCGGAAAC-3′	This paper	N/A
qPCR primer for L32-reverse: 5′-TTGTTGCTCCCATAACCGATG-3′	This paper	N/A
Software and algorithms		
GraphPad Prism 8	GraphPad software, Inc.	http://www.graphpad.com
Other		
CD spectrometer	JASCO	J-700
^{1}H nuclear magnetic resonance (^{1}H-NMR)	Bruker	ARX400 MHz
Round-bottom flask	Glassco	N/A
10 mL vial	Synthware	N/A
Magnetic stirrer	IKA	topolino
Glovebox	SUPER	MIKROUNA
Dialysis bag (MWCO = 3500 Da)	G-CLONE	N/A
Quartz cell	Shimadzu	N/A
Separatory funnel	Synthware	N/A
Vortex oscillator	Thermo Fisher Scientific	Maxi Mix II

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell culture incubator	Shanghai Muni	CHW-80L-GW
Water bath	Lichen	HH-2
Electronic balance	Taiheng	FA200
Rotary evaporator	IKA	RV10
Vacuum pump	Edwards	RV8
Ultrapure water system	Honbo	HBII-C
C1000 Touch Thermal Cycler	Bio-Rad	1851148
qTOWER^3	Analytik Jena	N/A
Multi-Mode microplate reader	Molecular Devices	SpectraMax i3
NanoDrop 2000	Thermo Fisher Scientific	ND-2000
Tissue homogenizer	MP Biomedicals	FastPrep-24
Tissue grinding beads	Beyotime	F6653
Gel permeation chromatography	Agilent	1260 Infinity II

STEP-BY-STEP METHOD DETAILS

Preparation of P7F7/siRNA polyplexes

Timing: 3 days

In this step, we describe how to configure P7F7/siRNA polyplexes before silencing genes *in vitro* and *in vivo*. We also assess the cytotoxicity and cellular internalization level of P7F7/siRNA polyplexes to evaluate the effectiveness of P7F7.

1. Configuration of P7F7/siRNA polyplexes.
   a. Add the P7F7 solution into the siRNA solution at weight ratio of 15.
   b. Vortex for 10 s.
   c. Incubate at 37°C for 30 min.

   Note: P7F7/siRNA polyplexes should be used immediately after preparation.

2. Cytotoxicity analysis of P7F7/siRNA polyplexes.
   a. Seed RAW264.7 cells (1×10^4 cells/well, 3.13×10^4 cells/cm^2) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in the 96-well plate and culture 24 h.
   b. Replace the medium of RAW264.7 cells with serum-free DMEM medium.
   c. Treat RAW264.7 cells with P7F7/siRNA polyplexes at the polymer concentration of 15 μg/mL for 4 h.
   d. Replace the medium with DMEM containing 10% FBS and 1% P/S.
   e. Incubated cells for 20 h.
   f. Assess cell viability using a MTT assay kit available from vendors, such as Sigma-Aldrich and Thermo Fisher Scientific.
   g. Result is presented as percentage viability of control cells that receive no treatment with polyplexes (Figure 2A).
3. Cell uptake analysis of P7F7/siRNA polyplexes.
   a. Seed RAW264.7 cells (1×10^4 cells/well, 3.13×10^4 cells/cm^2) in DMEM supplemented with 10% FBS and 1% P/S in the 96-well plate and culture 24 h.
   b. Replace the medium of RAW264.7 cells with serum-free DMEM medium.
   c. Treat RAW264.7 cells with P7F7/Cy3-siRNA polyplexes at a concentration of 1 μg Cy3-siRNA/mL for 4 h.
   d. Wash cells with cold PBS containing heparin (20 U/mL) for three times.

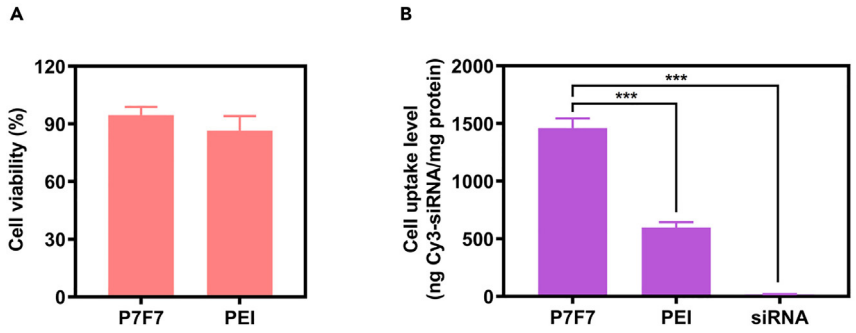

Figure 2. Cytotoxicity and cellular internalization level of P7F7/siRNA polyplexes

(A) Cytotoxicity of P7F7/siNC polyplexes (w/w = 15) and PEI/siNC polyplexes (w/w = 5) at the polymer concentration of 15 μg/mL in RAW264.7 cells (n = 3).

(B) Cellular uptake levels of P7F7/Cy3-siRNA polyplexes following 4 h incubation in RAW264.7 cells (n = 3). Naked siRNA and PEI/siRNA polyplexes (w/w = 5) served as controls.

Data are presented as mean ± SD (***p < 0.001; one-way ANOVA).

e. Lyse cells with the RIPA lysis buffer (100 μL/well).
f. Monitor Cy3-siRNA content in the lysate by spectrofluorimetry (λ_{ex} = 550 nm, λ_{em} = 565 nm).
g. Quantify protein level using a BCA protein assay kit available from vendors, such as Takara and Thermo Fisher Scientific.
h. The uptake level is determined as ng of Cy3-siRNA per mg of cellular protein (Figure 2B). If the cell uptake level of P7F7/siRNA polyplexes is low (<500 ng of siRNA per mg of cellular protein), refer to troubleshooting 3.

Silencing U1 with P7F7/siRNA polyplexes *in vitro*

Timing: 1 day

In this step, we describe the procedure to knockdown U1 snRNA with P7F7/siRNA polyplexes in bone marrow-derived macrophages (BMDMs). This method can also be applied to other cell types.

4. Preparation of BMDMs according to the reported protocol.[14]

The detailed steps mainly include collection of bone marrow cells, differentiation into BMDMs, and preparation for *in vitro* stimulation using BMDMs.

Note: Mature BMDMs which are already differentiated can be observed on day 7 of BMDMs differentiation and can be used for subsequent experiments.

5. Knockdown of U1 snRNA in BMDMs.
   a. Prepare P7F7/siRNA polyplexes according to step 1.
   b. Replace the medium of BMDMs with serum-free DMEM medium.
   c. Treat BMDMs with P7F7/siRNA polyplexes at 0.5 μg/mL siRNA for 4 h.
   d. Replace the medium with DMEM supplemented with 10% heat-inactivated FBS, 1% P/S, and 1% M-CSF-conditioned medium.
   e. Incubate cells for additional 20 h.
   f. Discard the medium and wash the cells with PBS buffer.
   g. Add TRIzol (0.5 mL/well for 12 well plate) to each cell sample and pipette vigorously several times to lyse the cells.
   h. Perform RNA extraction[15] immediately or store at −80°C.

Silencing U1 with P7F7/siRNA polyplexes *in vivo*

Timing: 2 days

In this step, we describe the procedure for knocking down U1 in mouse lungs using intranasal administration of P7F7/siRNA polyplexes.

6. Knockdown of U1 in mouse lungs.
   a. Prepare P7F7/siRNA polyplexes (5 μg siRNA/mouse) according to step 1.
   b. Anesthetize mice based on body weight using the approved reagents, such as via an intraperitoneal injection of pentobarbital sodium (50–90 mg/kg).[16]
   c. Place mice on a clean drape facing up with a heating pad underneath to maintain body temperature.
   d. Pick up the mouse and ensure that its neck and chin are flat and parallel to the floor.

   Note: The chin should be as close to a 180-degree angle as possible with the neck to assist with intranasal delivery.

   e. With a micropipette take 10 μL of the P7F7/siRNA polyplexes. Place the tip of the filled micropipette near the mouse's left nostril, usually at a 45-degree angle.
   f. Slowly drop 2–3 μL of solution into left nostril each time until the whole volume has been deposited, and leave time between drops to allow the mouse to breathe the solution in.

   Note: Mice can suffer from respiratory distress during this procedure so they should be left to assimilate each drop prior to administrate the next one to prevent it as much as possible.

   g. Re-fill the micropipette with another 10 μL and repeat the operation for the right nostril (Figure 3A). The total inoculum volume is 20 μL per mouse.
   h. After 24 h, euthanize mice with CO_2.
   i. Place the mice in supine position fixed on a flat and disinfected surface.
   j. Make an incision and remove the skin from chest region to expose the thoracic area.
   k. Extract the lungs using sterile scissors and forceps and remove trachea and connective tissue.

   Note: For lung collection, readers can also refer to Fuentes et al.[17] Additionally, we recommend that readers refer to an excellent video that details the lung collection procedure (https://app.jove.com/v/10298/sterile-tissue-harvest).

   l. Transfer the lungs of each mouse into a tube containing 1 mL of TRIzol and 2-3 tissue grinding beads (maintain on ice until processing).
   m. Thoroughly homogenize the tissue with a tissue homogenizer.
   n. Perform RNA extraction immediately or store the samples at −80°C.

Assess knockdown efficiency of U1

Timing: 1 day

In this step, we introduce in detail procedures for examine the knockdown efficiency of U1, including RNA extraction, synthesis of cDNA, and qPCR experiments.

7. RNA extraction.
   a. Perform RNA extraction following manufacture's instruction (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Ftrizol_reagent.pdf).

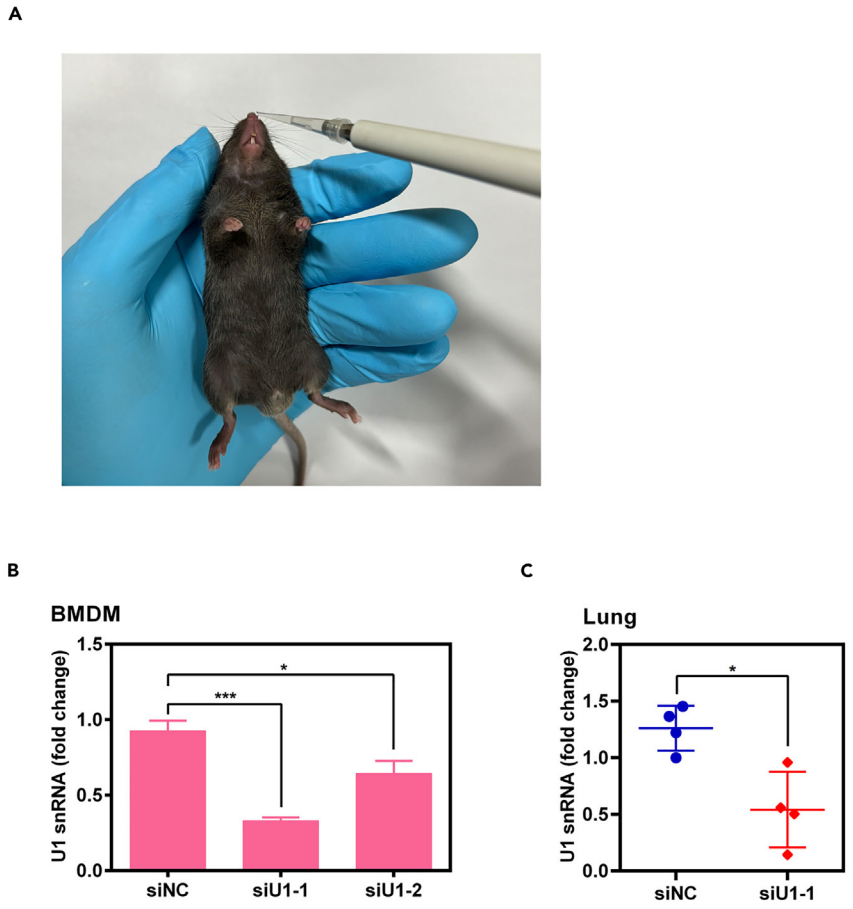

Figure 3. siRNA-mediated U1 snRNA knockdown using P7F7 *in vitro* and *in vivo*

(A) Photograph showing the intranasal administration using a pipette for an anesthetized mouse.

(B) RT-qPCR detection of the U1 knockdown efficiency in BMDMs treated with P7F7/siNC, P7F7/siU1-1, or P7F7/siU1-2 polyplexes (n = 3).

(C) RT-qPCR detection of the U1 knockdown efficiency in mouse lungs from the littermates intranasally administrated with P7F7/siNC or P7F7/siU1-1 polyplexes (n = 4).

Data of (B) and (C) are presented as mean ± SD (*p < 0.05, ***p < 0.001; one-way ANOVA or Student's t-test).

b. Determination of RNA concentration using NanoDrop 2000.

Note: The RNA should be stored at −80°C.

8. Synthesis of cDNA.
   a. Prepare the reverse-transcription reaction solution on ice, following manufacture's instruction (https://www.takarabio.com/documents/User%20Manual/RR036A_e.v2008Da.pdf).

   Note: During the preparation of the reaction solution, the RNA should be kept on ice.

   b. Mix the reaction solution gently.
   c. Perform the reverse-transcription reaction, following manufacture's instruction (https://www.takarabio.com/documents/User%20Manual/RR036A_e.v2008Da.pdf).
9. Examine knockdown efficiency of U1 through qPCR.
   a. Prepare the qPCR mixture on ice and perform qPCR program, following manufacture's instruction (https://www.takarabio.com/documents/User%20Manual/RR420A/RR420A_UM.pdf).

Note: Before preparation of the qPCR mixture, dilute the cDNA to a concentration of 1/10 for use.

b. Calculate relative expression of U1 in relation to the reference gene using the $2^{-\Delta\Delta Ct}$ cycle threshold method (Figures 3B and 3C).[18] If the qPCR results present large variations among biological replicates, refer to troubleshooting 4. If the knockdown efficiency is low, refer to troubleshooting 5.

EXPECTED OUTCOMES

This experiment aims to efficiently knockdown U1 snRNA *in vitro* and *in vivo* by delivering siRNA with fluorinated α-helical polypeptide. We describe the synthesis and characterization of fluorinated α-helical polypeptide P7F7 (Figure 1). Following this protocol, the anticipated yield of P7F7 is approximately 90% or more. The characterization of P7F7 is based on circular dichroism measurement, which includes the mean residue molar ellipticity and the helicity, as depicted in Figure 1. After P7F7 is prepared, the cytotoxicity and cellular internalization level of P7F7/siRNA polyplexes should be tested to determine the efficacy of P7F7, as depicted in Figure 2. When delivering with siRNA against U1 snRNA using P7F7 in BMDMs and mouse lungs, we typically observe that the expression level of U1 snRNA is substantially reduced, as determined by RT-qPCR shown in Figure 3. The approach of siRNA-mediated U1 snRNA knockdown using P7F7 can be applied in other scenarios where transient or conditional U1 snRNA silencing is required. Moreover, this protocol is suitable for achieving efficient knockdown of long non-coding RNA (lncRNA), circular RNA (circRNA), or coding genes *in vitro* and *in vivo* via P7F7/siRNA delivery system.

LIMITATIONS

While siRNA delivery systems are effective for achieving gene knockdown in mice, generating gene knockout mice enables a more complete loss of gene function and is more effective for *in vivo* studies. Therefore, this protocol is more suitable for genes that cannot generate knockout mice, such as essential genes and genes with multiple copies. In addition, the relative short half-lives of siRNAs make them unsuitable for *in vivo* experiments with longer durations. Moreover, the off-target effect of siRNA has a significant impact on gene knockdown.[19]

The serum stability of P7F7/siRNA polyplexes is poor, with siRNA easily leaking out of the polyplexes. Therefore, achieving systemic knockdown with the same formulation, particularly intravenous injection, is not quite feasible. Instead, it is more suitable for local administration, especially in the respiratory tract or gastrointestinal tract, due to the excellent mucus permeation capability of P7F7. Furthermore, the synthesis steps of P7F7 are relatively complicated, with many raw materials and synthetic intermediates, and it is not easy to prepare in large quantities.

TROUBLESHOOTING

Problem 1

Insufficient yield of P7F7 (related to step: Synthesis of P7F7).

Potential solution

Extend the reaction time of click chemistry to ensure a conversion rate of 100% (related to synthesis of P7F7: step 8c).

Problem 2

P7F7 is not completely dissolved (related to step: Synthesis of P7F7).

Potential solution

Heating and ultrasonic dissolution allow P7F7 to dissolve completely.

Problem 3
The cell uptake level of P7F7/siRNA polyplexes is low (related to step 3).

Potential solution
Adjust the pH of P7F7 solution to 7.

Problem 4
qPCR results present large variations among biological replicates (related to step 9).

Potential solution

- Prevent RNA degradation and improve RNA quality.
- Measure RNA concentration accurately.
- Prepare the qPCR mixture on ice and avoid exposure to light.
- For *in vivo* experiments, littermates should be used to reduce individual differences.

Problem 5
Low knockdown efficiency (related to step 9).

Potential solution

- Ensure the validity of P7F7 by assessing the cytotoxicity and cellular internalization level of P7F7/siRNA polyplexes according to steps 2 and 3.
- Multiple freeze-thaw cycles of the siRNA affect its efficacy. Prepare and use a fresh siRNA stock.
- Try alternative siRNA sequences.
- For cell experiments, it is necessary to ensure the healthy status of cells. For example, perform mycoplasma detection before treating cells, and change the medium in time after treatment.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Feng Ma (maf@ism.pumc.edu.cn).

Technical contact
For specific questions and details regarding the technical aspects of the protocol, please address correspondence to the technical contact, Fan Zhang (zhangfan_msj@163.com).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate unique datasets or code.

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AUTHOR CONTRIBUTIONS
F.M. and L.Y. conceived and designed the project. F.Z., C.G., Z.Q., and Y.H. performed the experiments. F.Z., C.G., and F.M. wrote the manuscript.

DECLARATION OF INTERESTS

C.G. and L.Y. report a patent application related to this study.

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