In Vivo Delivery of RNAi by Reducible Interfering Nanoparticles (iNOPs)

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1. Materials and Measurements

All chemicals were purchase from Aldrich. Dimethylformamide (DMF) was distilled after drying. Dialysis tube (cut-off molecular weight of 3000; Spectrum Laboratories, Inc.) was used for the purification of iNOP-7 derivatives. ^{1}H-NMR was recorded on an Oxford 400 NMR spectrometer. Chemical shifts (δ = 0 ppm) were referred to TMS with the residual proton of the deuterated solvent. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra were recorded on a Waters Micromass MALDI-LR instrument. 2, 5-Dihydroxybenzoic acid (15 mg/ml in 50% acetanitrile solution containing 0.1% trifluoroacetic acid) was used as matrix. A mixture of the sample solution (1mL, 1.0 mg/ml in 50% acetanitrile solution containing 0.1% trifluoroacetic acid) and the matrix solution was applied to the MALDI probe. The sample was allowed to dry by air evaporation and was subjected to MS analysis.

2. Synthesis

2.1. 3-(2-tert-butoxycarbonylamino-ethyldisulfanyl)-propanoic acid (spacer I)

Ethanol solution (4 ml) of 3-mercaptopropionic acid (0.18 g, 1.7 mmol) was added dropwise to 2,2'-dithiodipyridine (0.75 g, 3.4mmol in 6ml ethanol) over 20 min by stirring vigorously. Glacial acetic acid (80 ul) was added and the reaction mixture was stirred at room temperature for 2 hours. Then the solvent was removed, and product was purified by basic aluminum oxide chromatography. The columnm was first washed using dichloromethane and ethanol (3:2) to flow byproducts and impurities, then the product was eluted using dichloromethane and ethanol (3:2) containing 4% acidic acid to give 3-(2-Pyridyldithio)propionic acid as a white foam (249 mg, yield: 68%) (1) . 3-(2-Pyridyldithio)propionic acid (405 mg, 1.9 mmol) was reacted with 2-(Boc-amino)ethanethiol (330 mg, 1.9 mmol) in ethanol (4 ml) to give 3-(2-tert-butoxycarbonylamino-ethyldisulfanyl)-propanoic acid (400 mg, yield: 75%). 1H NMR: (CDCl3) δ 1.44 (9H, 3xCH3), 2.52 (2H, CH2), 2.78 and 2.92 (4H, CH2), 3.43 (2H, CH2). MS m/z: calculated for $C_{10}H_{19}NNaO_4S_2$ $(M+Na)^+$ 304.40, found 304.46.

2.2. Synthesis of iNOP-7DS

A mixture of iNOP-7 (20mg, 3.4μmol), spacer **I** (72mg, 256μmol) and DIEA (0.49 ml, 2.8 mmol) was suspended in 0.6 ml DMF under nitrogen atmosphere. After the suspension was cooled to 0 ºC, BOP (221mg) was added. The reaction was performed at 0 ºC for 30 min and then at room temperature for 24 h. Then the solvent was removed under reduced pressure, and the obtained syrup was washed repeatedly with ethyl acetate. The product was further purified by recrystalization from methanol -ethyl acetate (1 mL:15 mL) system to give a white powder. After TFA deprotection, 34mg of white powdery iNOP-7DS was obtained. MALDI-TOF mass spectrum shows two broad peaks. The major peak ce ntered at m/z 9432.0 corresponds to a highly substituted moelcule, and the one at m/z 4730.9 corresponds to $[M+2H^+]$.

3. Biological Experiment Methods

3.1. siRNAs

All siRNAs used in in vivo studies were chemically synthesized using silyl ethers to protect 5′-hydroxyls and acid-labile orthoesters to protect 2′-hydroxyls (2′-ACE) (Dharmacon, Lafayette, CO). After deprotection and purification, siRNA strands were annealed as described previously (2). ApoB siRNA (ORF position 10049-10071): CM sense 5′-G*U^{F}C^{F}AU^{F}C^{F}ACACUGAAUAC^F*C^{F}AA*U^F-3′, CM antisense 5′-AU^{F}U^{F}GGUAUUCAGUGUGAU^{F}GAC^F*A*C-3'; CM-mm sense 5′-G*U^{F}GAU^{F}C^{F}AGACUCAAUAC^{F}GAA*U^F- 3′, CM-mm antisense 5′-AU^{F}U^{F}CGUAUUGAGUCUGAU^{F}CAC^F*A*C-3'. The superscript letter F represents 2′-O-F modified nucleotides; asterisk represents phosphorothioate linkage. iNOP-7 derivatives were prepared by mixing siRNA and modified poly-L-lysine dendrimers in 150 mM sucrose or Opti-MEM culture medium (Invitrogen, Carlsbad, CA) and incubating at room temperature for 20 min to complex siRNA with nanoparticles. For in vitro experiment, siGENOME Non-Targeting siRNA (from Dharmacon) was used as negative control.

DDB1 siRNA: antisense 5′-UAACAUGAGAACUCUUGUC-3′, sense 5‘-GACAAGAGUUCUCAUGUUA

3.2. *In Vitro* RNAi Activity of iNOP-7DS

For in vitro siRNA transfection, iNOP-7DS (1.0 uM ~ 1.5 uM final concentration) was complexed to siRNA (50 nM final concentration) and incubated at r.t. for 20 min before placed on cells. FL83B (mouse hepatocytes) cells were maintained at 37°C with 5% CO_2 in F-12K culture medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cells were regularly passaged and plated in 6-well culture plates for 16 h before transfection at 70% confluency. Cells were transfected with 1 mL per well of complex (siRNA-nanoparticles) for 4 h at 37°C. Efficiency of RNAi was determined as described previously (3, 4).

HCT116 cells and HepG2 cells were maintained at 37 oC with 5% Co2 in DMEM medium supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cells were regularly passaged and plated in 6-well culture plates for 16 h before transfection at 70% confluency. Cells were transfected with 1 mL per well of complex (siRNA-nanoparticles) for 4 h at 37°C. Efficiency of RNAi was determined as described previously.

3.3 *In Vivo* Silencing

All animal procedures were approved by the Institutional Animal Care and Use Committee (University of Massachusetts Medical School). Six- to eight-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were maintained under a 12 h dark cycle in a pathogen-free animal facility. Mice were administrated with either phosphate buffered saline pH 7.4 (PBS) or iNOP-7DS containing mismatch siRNA or perfect match siRNA at 1 mg kg^{-1} as bolus intravenous injection *via* the lateral tail vein. Forty-eight hours after the injection, liver, spleen, kidney, lung and plasma were collected and stored in -80°C until analysis.

3.4 Quantitative PCR

To determine mRNA levels in cell culture or mouse tissues after siRNA treatment, total RNA was extracted with TRIZOL (Invitrogen, Carlsbad, CA) and treated with TORBO DNA-free kit (Applied Biosystems, Foster City, CA) before quantification. In preparation for quantitative PCR, total RNA (400 ng) was reverse transcribed by using SuperScript II (Invitrogen, Carlsbad, CA) and random primers according to the manufacturer's protocol. The expression of mRNA was measured using ABsolute QPCR SYBR green mix (ThermoFisher Scientific, Epsom, Surrey, UK) normalized to GAPDH according to the manufacturer's instructions. Quantitative PCR was performed by using a Chromo4 Real-Time PCR Detection System (BioRad, Hercules, CA).

3.5 Western Blotting

Separation of serum proteins was accomplished by electrophoresis on 6% polyacrylamide/SDS gels. The separated proteins were electrophoretically transferred to PVDF membrane followed by incubation with a 1:1,000 dilution of goat polyclonal anti-ApoB antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The blot was then incubated with a 1:2,000 dilution of donkey anti-goat antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA), and antibody binding was detected by using an enhanced chemiluminescent detection kit (PerkinElmer, Waltham, MA). As a control, fibronectin was visualized by immunoblot using a polyclonal rabbit anti-fibronectin antibody (Sigma-Aldrich, St. Louis, MO).

3.6 Northern Blotting

RNA from mouse tissues was homogenized in TRIZOL (Invitrogen, Carlsbad, CA) and isolated according to the manufacturer’s instructions. Total RNA was separated on a 14% acrylamide/20% formamide/8 M urea gel, then electroblotted onto Hybond-XL nylon membrane (GE Healthcare, Piscataway, NJ). The probe with γ-^{32}P-labelled oligonucleotides for antisense of siRNA was hybridized to the membrane at 42°C. The blots were visualized by scanning in a FLA-5000 scanner (Fujifilm, Stamford, CT).

3.7 *In Vivo* Interferon Induction

To assess for any nonspecific immune response to injected siRNA complexed with nanoparticles, mouse liver RNA was analyzed for expression of the IFN-inducible genes by quantitative RT-PCR. Serum levels of mouse IFN- α were measured by using a sandwich ELISA kit according to the manufacturer's instructions (PBL Biomedical, Piscataway, NJ) (5).

References

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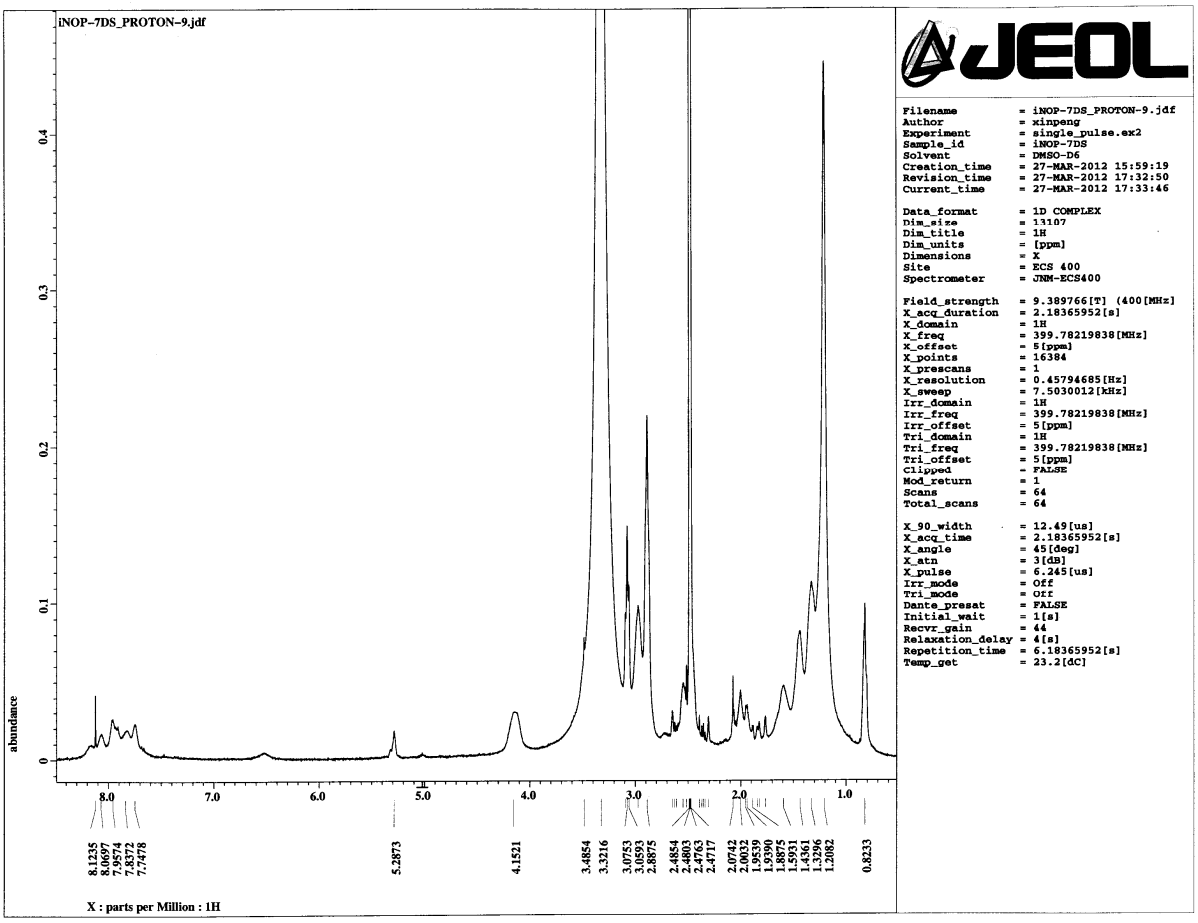
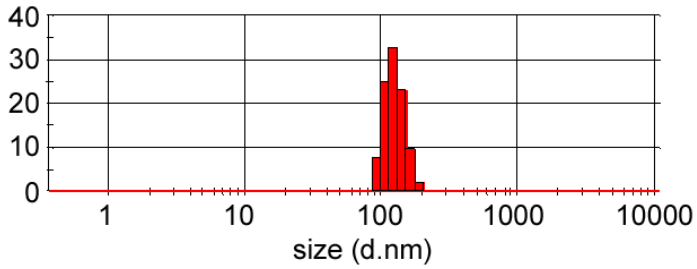
Supplementary Figure 1



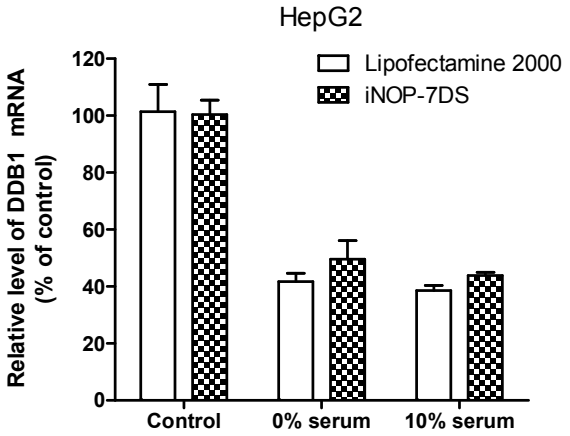
Supplementary Figure 1. ^{1}H-NMR spectrum of iNOP-7DS in DMSO-d6.

Supplementary Figure 2



Supplementary Figure 2. iNOP-7DS forms nanoparticles upon complex to siRNA. The size distribution of iNOP-7DS containing siRNA measured by dynamic light scattering. siRNA and iNOP-7DS were mixed at a weight ratio of 1:10 (N/P ratio 1:8) in HEPES buffer. The final concentration of siRNA and iNOP-7DS was 8.5 uM and 128 uM, respectively. The mixture was incubated at room temperature for 20 min before DLS measurement.

Supplementary Figure 3



Supplementary Figure 3. iNOP-7DS containing siRNA specifically silences DDB1 in HepG2 cells in the absence or presence of serum. Cells were treated for 3 h in a medium containing 0% or 10% FBS with iNOP-7DS complexed to nontargeting siRNA (siNT) or perfect match siRNA (siDDB1). DDB1 mRNA levels are expressed as percent of control.