Chapter 29

Integrin-Targeted Nanoparticles for siRNA Delivery

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Abstract

Integrins are heterodimeric membrane glycoproteins composed of noncovalently associated α and β subunits. Integrins support cell attachment and migration on the surrounding extracellular matrix as well as mediate cell–cell interaction in physiological and pathological settings. Constant recycling of integrins from the plasma membrane to the endosome makes integrins ideal receptors for the delivery of drugs to the cell cytoplasm. RNA interference (RNAi) has evolved not only as a powerful tool for studying gene expression and validating new drug targets, but also as a potential therapeutic intervention. However, the major challenge facing the translation of RNAi into clinical practice is the lack of efficient systemic delivery to specific cell types. Utilizing integrins as delivery target, we have recently devised a strategy to target leukocytes termed Integrin-targeted and stabilized NanoParticles (I-tsNPs) that entrap high RNAi payloads and deliver them in a leukocyte-specific manner to induce robust gene silencing.

Key words: Leukocyte integrins, Gene silencing, Nanoparticles, Hyaluronan, siRNAs, Nanomedicine

1. Introduction

RNA interference is a cellular mechanism that can be induced either by synthetic small-interfering RNAs (siRNAs) or by vectors that express small hairpin RNAs (shRNA) (1). This mechanism mediates sequence-specific gene silencing by cleavage of the targeted messenger RNA or by suppression of the translation machinery (2, 3). To realize the potential of siRNAs for in vivo drug discovery and therapy, there is a need to overcome the considerable hurdles of systemic and intracellular delivery of payloads into the cell (4). siRNAs are subjected to a rapid renal clearance and can be degraded by serum RNases, shortening their half-life in vivo and thus, suitable nanocarriers that protect

Motomu Shimaoka (ed.), *Integrin and Cell Adhesion Molecules: Methods and Protocols*,
Methods in Molecular Biology, vol. 757, DOI 10.1007/978-1-61779-166-6_29, © Springer Science+Business Media, LLC 2011

the RNAi payloads need to be employed (5). Devising such carriers equipped with integrin ligands or their antibodies enable cell-specific delivery and intracellular release of siRNAs to the cell cytoplasm (6, 7). Herein, we describe a method that exploits integrin internalization to introduce siRNAs to the cytoplasm of leukocytes (7), which are known to be difficult to transduce with nucleic acids (8).

2. Materials

2.1. I-tsNPs Production

1. Lipids: L α-Phosphatidylcholine (PC Egg, Chicken), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine (DPPE), cholesterol (Chol) (Avanti polar lipids, Inc., Alabaster, AL). All lipids are stored as powders at −20°C freezer.
2. Rotary evaporator (Buchi Corporation, Switzerland).
3. Thermobarrel Lipex extruder™ (Lipex biomembranes Inc., Vancover, British Columbia, Canada).
4. Nucleopore membranes with 0.1–1 μm pore size.
5. 20 mM HEPES-buffered saline (HBS), pH 7.2.
6. 1 × 0.1 M MES buffer pH 6.0.
7. Hyaluronan (HA, 751 kDa, intrinsic viscosity, 16 dL/g, Genzyme Corp., Cambridge, MA): Stored as a powder at −20°C freezer in a desiccator.
8. 1 M Borate buffer, pH 9.0: Diluted to 0.1 M with H_2O.
9. 400 mmol/L 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride.
10. 400 mmol/L Sulfo-*N*-Hydroxysuccinimide.
11. Monoclonal antibody FIB 504.64 Rat anti-mouse IgG2a against β_7 integrin: Adjusted to 10 mg/mL.
12. 1 M ethanolamine hydrochloride, pH 8.5.
13. Size exclusion column with sepharose CL-4B beads.
14. Alpha 1-2 LDplus lyophilizer (Christ, Osterode, Germany).

2.2. Characterization of I-tsNPs

2.2.1. Lipid Mass Assay (7, 9)

1. ^{3}H-hexadecylcholesterol (Perkin Elmer, Boston, MA).
2. 5% (w/v) Ammonium molybdate.
3. Fiske & Subbarow 0.5 g in 3 ml DDW.
4. Perchloric acid (phosphorus free).
5. Phosphate standard.

2.2.2. Particle and Zeta Potential Analysis

1. Malvern Zetasizer nano ZS™ (Malvern Instruments Ltd, Southborough, MA).
2. 1× PBS, pH 7.4, at 20°C.

2.2.3. Binding to Cells and Transfection

1. Flow cytometer: We use FACScan flow cytometer or FACSCalibur (BD Biosciences).
2. FACS buffer: PBS, pH 7.4, 1% FBS, 0.01% sodium azide.
3. TK-1 cells (ATCC): Grown in RPMI1640 supplemented with 1% antibiotics (penicillin and streptomycin), 4 mM L-glutamine, and 10% fetal calf serum (FCS).
4. Primary antibodies: FIB 504.64 (BD Pharmingen) and isotype control rat IgG2a.
5. Secondary antibody: Alexa488-labeled anti-rat IgG2a antibody.
6. Fix-and-Perm Kit (Caltag Laboratories, Burlingame, CA): Used for intracellular staining.
7. Antibody to Ku70 (purified mouse anti-Ku70, Santa Cruz Biotechnology, Santa Cruz, CA): Used for intracellular staining.

2.2.4. siRNA Entrapment Efficiency in I-tsNPs

1. Ku70-siRNAs (Dharmacon Inc., Boulder, CO): The following four sequences are used in equimolar ratios.

   siRNA Sequence #1:

   Sense: 5′-GCUCUGCUCAUCAAGUGUCUGdTdT-3′

   Antisense: 5′-CAGACACUUGAUGAGCAGAGCdTdT-3′

   siRNA Sequence #2:

   Sense: 5′-UCCUUGACUUGAUGCACCUGAdTdT-3′

   Antisense: 5′-UCAGGUGCAUCAAGUCAAGGAdTdT-3′

   siRNA Sequence #3:

   Sense: 5′-ACGGAUCUGACUACUCACUCAdTdT-3′

   Antisense: 5′-UGAGUGAGUAGUCAGAUCCGUdTdT-3′

   siRNA Sequence #4:

   Sense: 5′-ACGAAUUCUAGAGCUUGACCAdTdT-3′

   Antisense: 5′-UGGUCAAGCUCUAGAAUUCGUdTdT-3′

   We recommend to use 2′-*o*-metheyl-modified siRNAs; pre-designed ON-TARGETplus siRNA SMARTpool, Gene ID 14375 for mouse Ku70 (Dharmacon Inc., Boulder, CO); or lock nucleic acid (LNA)-modified siRNAs (Life Technologies, Austin, TX).
2. Nuclease-free water (Ambion Inc., Austin, TX).
3. Human recombinant Protamine (Abnova, TaipeiCity, Taiwan).
4. Quant-iT RiboGreen RNA assay kit for percent entrapment efficiency (Invitrogen, Carlsbad, CA).

2.3. In Vivo siRNA Delivery Using I-tsNPs

1. C57BL/6 mice: Housed in a specific pathogen-free animal facility.
2. 30-gauge needle with a tuberculin syringe: Used to inject to a mouse tail vein.
3. Bath sonicator: Used to briefly sonicate the I-tsNPs suspension prior to intravenous injection to avoid aggregation.

3. Methods

3.1. I-tsNPs Production and Purification

I-tsNPs are nanometer-sized hyaluronan-coated neutral liposomes possessing targeting moieties (antibodies to integrins on leukocytes) on their surface. The preparation involves two critical steps: (1) preparation of stabilized nanoparticles (NPs) by chemical conjugation of hyaluronan that coats the surface of the liposomes and (2) introduction of targeting molecules (mAbs) on the surface of the stabilized NPs (Fig. 1).

1. Prepare multilamellar vesicles (MLV), composed of phosphatidylcholine (PC), dipalmitoylphosphatidylethanolamine (DPPE), and cholesterol (Chol) at molar ratios of 3:1:1 PC:Chol:DPPE using conventional lipid-film hydration method (10, 11). Weight the appropriate amounts of lipids to a final concentration of 40 mg/mL in a round-bottom flask.
2. Dissolve the lipids in the round-bottom flask with 20 mL of 96% ethanol by stirring for 30 min at 65°C. Then, evaporate the ethanol using rotary evaporator until complete dryness (usually takes ~1 h) and look for the appearance of a thin film layer on the round-bottom-flask. Upon completion of the drying process, blow nitrogen to the round bottle flask for 10 min (see Note 1).
3. Hydrate the lipid film with 20mM HEPES pH 7.4 to create MLV (40mg/mL). Thoroughly vortex until a thin milky liposome suspension is formed.

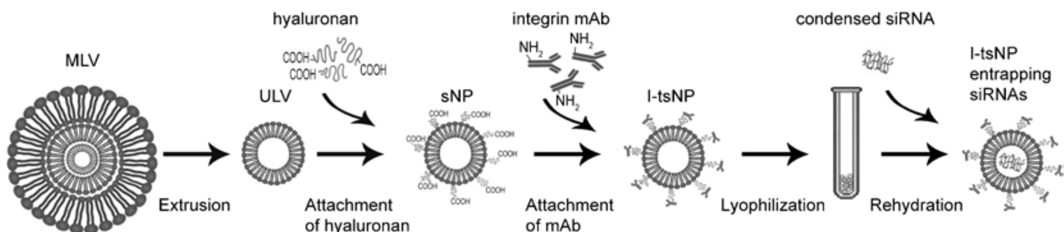

Fig. 1. The steps of producing Integrin-targeted and stabilized nanoparticles (I-tsNP). Multilamellar vesicles (MLV) prepared from neutral lipids are excluded to form unilamellar vesicles (ULV). ULV are surface-modified with a covalent attachment of hyaluronan that stabilized the particles (sNP). An antibody against integrin is then covalently attached to the hyaluronan scaffold, generating I-tsNPs. To encapsulate siRNAs, lyophilized I-tsNPs are rehydrated with a protamine-condensed siRNAs solution.

4. Incubate the MLV suspension in a shaker (~200 rpm) at 37°C for 2 h to ensure complete mixing and homogeneity (see Note 2).
5. Extrude the resulting MLV into small unilamellar vesicles (ULV) with a Thermobarrel Lipex extruder™ with a circulating water bath set at 65°C (above the transition temperature of the lipids) under nitrogen pressures of 300 to 550 psi.
6. Carry out the extrusion in a stepwise manner using progressively decreasing pore-sized membranes (from 1, 0.8, 0.6, 0.4, 0.2 to 0.1 μm), with ten cycles per pore-size.
7. ULV are surface-modified with high molecular weight hyaluronan (HA) (751 kDa, intrinsic viscosity: 16 dL/g) as described below.
8. Dissolve 20 mg HA in 10 mL of double distilled water. Adjust pH 4.5 with HCl. Add 400 mg EDAC and adjust the pH to 4.5. Stir at 37°C for 30 min to fully dissolve HA. Centrifuge the extruded ULV for 1 h in a high speed ultracentrifuge (640,000 × *g*, 4°C) and resuspend the pellet (with the same volume) in 0.1 M borate buffer pH 9.0. Combine the activated HA with the ULV suspension in a 1:1 volume ratio and adjust the pH to 7.4 then incubate for 2 h at incubate 37°C, with gentle stirring. Adjust pH to 8.6 with NaOH and incubate overnight. Separate the resulting HA-ULV (stabilized nanoparticles; sNP) from free HA by washing three times with 0.1 M MES buffer pH 6.0 by ultra-centrifugation (640,000 × *g*, 4°C, for 1 h). Make sure to resuspend to the original volume after the last washing step (see Note 3).
9. Perform the coupling reaction of sNP to mAbs using an amine-coupling method (7). Incubate 50 μL of sNP in 0.1 M MES buffer pH 6.0 with 200 μL of 400 mmol/L EDAC and 200 μL of 400 mmol/L sulfo-NHS (at this order) for 20 min at room temperature (RT) with gentle stirring. Make sure the pH is 6.0 if not adjust accordingly. Separate the sNP from unbound EDC and S-NHS by adding 2.5 ml of 10 mM PBS pH 7.4 and wash with Mini-ultracentrifugation (sorvall) (6.4×10^5 *g*, 40°C, for 1 h) resuspend in PBS to 450 μl. All cross-linkers must be freshly prepared from powder.
10. Mix the EDAC-NHS-activated sNP with 50 μL of mAb (10 mg/mL FIB 504 in HBS or PBS, pH 7.4) and incubate for 3 h at RT with gentle stirring. At the end of the incubation, add 20 μL of 1 M ethanolamine HCl, pH 8.5 to block the reactive residues and stir for 10 min at RT (see Note 4).
11. Purify I-tsNPs to remove uncoupled antibody using a size exclusion column packed with sepharose CL-4B or CL-6B beads equilibrated with HBS, pH 7.4. Test lipid mass and adjust the lipid concentration for in vitro or in vivo use.

12. Prepare the purified I-tsNPs suspensions for lyophilization. Snap freeze 0.2-mL aliquots in a mixture of 96% ethanol and dry ice for ~20–30 min; freeze the aliquots for 2–4 h at −80°C and lyophilize for 48 h using an alpha 1–2 LDplus lyophilizer (see Note 5).
13. Store lyophilized I-tsNPs at −20°C until further use.

3.2. I-tsNP Characterization

3.2.1. Lipid Mass Determination

Determination of phospholipids is preformed either by including a ^{3}H-hexadecylcholesterol trace in the particles membrane (7) or by using a modified Bartlett assay (9). The sensitivity of the assay is in a 0–200 nM phosphate range. The color produced in this assay is proportional to the concentration of phosphorous up to 1.5 μM in each sample.

1. To create a standard curve, add 0, 10, 20, 40, 80, and 100 nM phosphate from phosphate standard (sigma) to six tall glass tubes. Sample volume should not exceed 200 nM phosphate and volume of 200 μL.
2. Add 400 μL of 70% perchloric acid and place the tube in the oven heated to 180°C for 30 min. Use chemical hood to add the perchloric acid and perform all experiments inside a chemical hood.
3. After 30 min, let tubes cool down to RT and add 1.2 mL of H_2O and vortex well.
4. Add 200 μL of 5% (w/v) ammonium molybdate.
5. Add 50 μL of Fiske & Subbarow and vortex well.
6. Place tubes in 100°C water bath and boil for 7 min.
7. Let tubes cool down to RT.
8. Read O.D. values at 830 nm.

3.2.2. Particle and Zeta Potential Analysis

The diameter and surface charge (zeta potential) of nanoparticles are measured using a Malvern Zetasizer nano ZS™ (Table 1).

3.2.3. Binding Efficiency of I-tsNPs

Flow cytometry is used to ensure that conjugated antibodies are functional.

Table 1
Particle size and zeta potential measurements

Particle type	Hydrodynamic diameter (nm)	ζ potential (mV)
IgG sNPs	127 ± 13	−18.5 ± 1.2
β_7 I-tsNPs	139 ± 21	−23.7 ± 2.6

All measurements were done in pH 6.7 (with 10 mM NaCl) at 20°C in a Zetasizer nano ZS, Malvern. Data presented as an average ± SD from $n = 4$ independent experiments

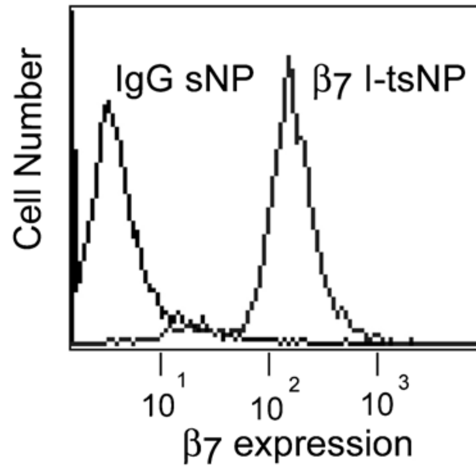

Fig. 2. FACS histograms showing binding of β_7 I-tsNPs to TK-1 cells.

1. Dispense TK-1 cells to FACS tubes at 0.5×10^6 cells per tube.
2. Add to each tube 1 ml of FACS buffer, centrifuge at $300 \times g$, 5 min, 4°C, and aspirate supernatant.
3. Add to each tube 100 μL of FACS buffer containing appropriate antibodies at 10 μg/mL concentrations.
4. Incubate samples on ice for 30 min.
5. Add to each tube 1 mL of FACS buffer, centrifuge at $300 \times$ g, 5 min, 4°C, and aspirate supernatant.
6. Add to each tube 100 μL of FACS buffer containing 1 μg/mL secondary antibody Alexa488-Anti-Rat Ab IgG2a.
7. Incubate on ice for 20–30 min.
8. Add to each tube 1 mL of FACS buffer, centrifuge at $300 \times g$, 5 min, 4°C, and aspirate supernatant.
9. Resuspend the cell pellets in appropriate volume of FACS buffer and analyze samples by FACS (Fig. 2).

3.2.4. siRNA Entrapment Efficiency in I-tsNPs

1. Mix siRNAs (e.g., Ku70-siRNAs) with full-length recombinant protamine in a 1:5 (siRNA:protein) mole ratio, in nuclease-free water in a total volume of 200 μL (per each tube) and incubate for 20 min at RT to form a complex.
2. For siRNA entrapment in I-tsNPs, rehydrate the lyophilized nanoparticles (e.g., 0.5–1.5 mg lipids for in vivo experiments and 10–100 μg lipids for in vitro experiments) (see Note 6) by adding 0.2 mL nuclease-free water containing protamine-condensed siRNAs (1–4 nM for in vivo experiments and 50–500 pM for in vitro experiments). Perform the entrapment procedure immediately before use in vitro transfection or intravenous tail-vein injection.

Table 2
Entrapment of siRNAs molecules in I-tsNPs

	siRNAs entrapment (No. of molecules)	Encapsulation efficiency of condensed siRNA
Nanoparticles type	Mean ± SEM	Mean ± SEM
IgG sNPs	3,750 ± 1,300	78 ± 10
FIB I-tsNPs	4,000 ± 1,200	80 ± 12

The amount of siRNAs that was used for encapsulation was known. Upon encapsulation a RiboGreen™ assay (Invitrogen) was preformed according to the manufacturer instructions to assess the amount of siRNAs that was entrapped

3. Determine siRNAs entrapment efficiencies by a Quant-iT™ RiboGreen™ RNA assay (Molecular Probes, Invitrogen) (Table 2).

3.3. Ku70-siRNA Delivery In Vitro in TK-1 Cells

1. Seed TK-1 cells in a six-well microtiter plate at 2.5×10^5 cells in 300 μL per well. Culture cells overnight at 37°C, 5% CO_2.
2. Add to each well 50 μL of β_7 I-tsNP entrapping Ku70-siRNAs. Appropriate controls should be included: cells with no treatment; cells with Ku70 siRNA alone; cells with negative control siRNA (e.g., silencer firefly Luciferase siRNA or scrambled siRNA).
3. Culture cells for 48–72 h at 37°C, 5% CO_2. Perform intracellular staining (as described below) to detect Ku70 protein expression.

3.3.1. Intracellular Staining and Flow Cytometry

1. Transfer TK-1 cells to 96-well V bottom plates.
2. Fix and permeabilize cells with Fix-and-Perm Kit™ (Caltag Laboratories, Burlingame, CA).
3. Add to cells 100 μL-aliquots of FACS buffer containing 10 μg/mL of Ku70 antibody.
4. Incubate samples on ice for 30 min.
5. Counter-stain cells with 1 μg/mL Alexa 488-conjugated goat anti-mouse IgG antibody.
6. Wash cells with FACS buffer and perform FACS analysis to measure Ku70 expression (Fig. 3).

3.4. Delivery of Ku70-siRNA In Vivo

1. Prepare mice for a tail vain injection. Pre-heat mice with a small U.V. lamp for 3–4 min in order to expose the tail veins. Alternatively, expose the mouse's tail to pre-warm water (37°C) until you clearly see the veins.

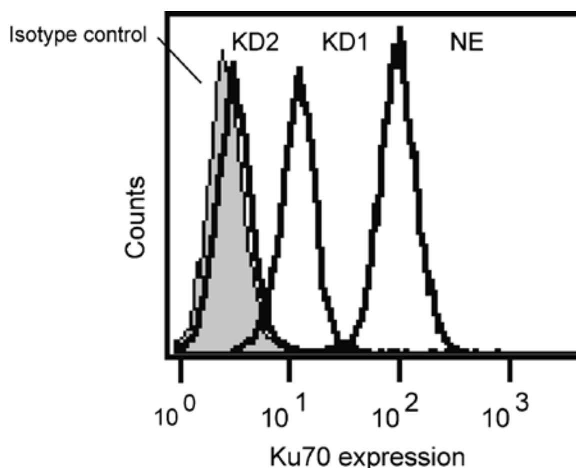

Fig. 3. FACS histograms showing knockdown of the DNA repair protein, Ku70 in mouse T-cell lymphoma (TK-1 cells). Ku70 expression in cells mock-treated (NE), treated with 100 pM Ku70-siRNAs delivered via β_7 I-tsNPs (KD1), and treated with 500 pM Ku70-siRNAs delivered via β_7 I-tsNPs (KD2).

2. Sonicate the I-tsNP suspension for 1.5 min in a bath sonicator to dissolve any potential aggregates.
3. Inject 150 μL of I-tsNP suspension to the tail vein with a 27-gauge needle with a tuberculin syringe (see Note 7). Initially inject 50 μg of siRNA entrapped in 500 μg of I-tsNPs per mouse as a starting dose. After checking the level of silencing, you may modify dosing.
4. At 48 or 72 h after injection, sacrifice the mice and harvest spleens.
5. Prepare a single-cell suspension of splenocytes.
6. Perform an intracellular staining with anti-Ku70 mAb as described in Subheading 3.3.

4. Notes

1. MLV preparation: a water bath of the rotary evaporator is set to 65°C and the chiller is set to be at least in −10°C to make the evaporation process more efficient. After the evaporation of ethanol, it is advisable to pass any inert gas such as argon or nitrogen for 10 min to completely remove traces of ethanol and prevent oxidation of lipids.
2. The MLV suspension can be stored at 4°C until extrusion procedure. However, prior to extrusion, the MLV should be pre-warmed to 37°C to enable easy extrusion process.

3. sNP can be stored at 4°C for 2 weeks. However, the sNP should be pre-warmed to RT before further modification is done.
4. mAb coupling reaction: EDAC/NHS-activated sNP with antibody reaction mixture is incubated overnight at RT and then blocked with 20 μL of 1 M ethanolamine-HCl, pH 8.5. Alternatively, coupling is made at lower pH 5.0 in 0.1 M acetate buffer. Antibody is in 0.1 M sodium acetate pH 5.0 at a concentration of 10 mg/mL. The sNP are in 0.1 M Borate buffer pH 9.0. Added antibody to the sNP at a molar ratio of 1:250. Then, add buffer and 500 molar equivalents of 50 mg/mL EDC made up immediately before use in ultrapure water. Mix the sample gently by pipetting. Centrifuged to remove air bubbles. Adjust pH to 5.0 with 0.1N HCl. Place the sample in a 37°C water bath while gentle shaking for 2 h.
5. Lyophilization of I-tsNPs: Prior to lyophilization of purified liposome fractions, I-tsNP suspensions should be tested for antibody-binding efficiency by flow cytometry. Pool only the fractions that give good binding. Add 0.2 mL aliquots of the I-tsNP suspension into amber glass vials prior to lyophilization.
6. We usually use the following lipid ranges in each vial. For in vitro transfection 10–100 μg lipids per vial. For in vivo delivery, 0.5–1.5 mg lipids per vial.
7. I-tsNPs are in saline containing 5% glucose for in vivo injection.

Acknowledgments

The authors would like to thank Prof. Motomu Shimaoka for his advice and encouragement and Dr. Yoshiyuki Morishita, and Dr. Charudharshini Srinivasan, for technical assistance. This work was supported by the European union – IRG (D.P.), the Alon Foundation (D.P.), and the Lewis Trust for Cancer Research (D.P.).

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