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Lipopeptide Delivery of siRNA to the Central Nervous System

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Abstract

RNA interference is a relatively new tool used to silence specific genes in diverse biological systems. The development of this promising new technique for research and therapeutic use in studying and treating neurological diseases has been hampered by the lack of an efficient way to deliver siRNA transvascularily across the blood–brain barrier (BBB) to the central nervous system (CNS). Here we describe a method for delivering siRNA to the CNS by complexing it to a peptide that acts as a neuronal address by binding to acetylcholine receptors (AChRs). Adding cationic liposomes to the complex protects it from serum nucleases and proteases en route. When injected intravenously, these liposome–siRNA–peptide complexes resist serum degradation, effectively cross the BBB, and deliver siRNA to AChR-expressing cells to suppress protein expression in the CNS.

Keywords

RNAi; siRNA; Delivery; Central nervous system; Blood–brain barrier; Liposomes; Peptides; Complexes; Prions

1. Introduction

Ribonucleic acid interference (RNAi) is a relatively new discovery in cell biology that diverse organisms use to help regulate the amount of messenger RNA (mRNA) available for translation. Dicer, an endogenous mammalian protein complex that mediates RNAi, cleaves double-stranded or short hairpin RNA (shRNA) into functional, small interfering RNA (siRNA). The RNA-induced silencing complex (RISC) guides siRNA to its complementary mRNA target, which is then digested by argonaut, an endonuclease present in the RISC. A concomitant reduction in protein expression ensues. Researchers have begun exploiting RNAi as a tool for silencing specific genes in biological systems (1–4). RNAi has shown

¹⁶LSPCs can be monitored in live mice by injecting them with fluorochrome-labeled LSPCs, which can be visualized using a Xenogen (Ivis) or a similar live animal imager. We observed LSPC delivery in as little as 2 min using this technique (Fig. 2) and detected LSPCs up to 10 days after injection (11).

¹⁷LSPC localization can be observed in the CNS by fluorescent microscopy (11). Euthanize injected mice and remove and snap-freeze their brains in OCT (Tissue Tek) in liquid nitrogen. Using a cryostat cooled to –20°C (Leica), cut 8-μm-thick brain sections and mount them on glass slides. Fix in ice-cold acetone for 10 min and air-dry overnight. Slides can be stained with additional markers (the protein knockdown target, for example) or DAPI before mounting in ProLong Gold (Invitrogen) or a similar anti-fade mounting media. Visualize LSPCs in brain sections using a microscope capable of detecting the fluorochromes used (Fig. 3a–t).

¹⁸Quantitative analysis of LSPC delivery can be performed on brain cells isolated from injected mice by flow cytometry (11). Sacrifice mice and remove their brains. Strain an approximately 1-mm-thick brain section through a 45 μm nylon mesh (Fisher Scientific) to produce a single cell suspension. Cells can be stained with additional cell marker of interest (the protein knockdown target) and analyzed using a Cyan (BD Pharmingen) or a similar flow cytometer (Fig. 3u–w).

potential efficacy in treating several diseases including hepatitis (5, 6), cancer (7), ocular disorders (8), chronic pain (9), and neuropathologies induced by viruses (10) and prions (11). Initial attempts to use RNAi in the central nervous system (CNS) primarily used intracranial (ic) injections of lentiviral vectors encoding shRNA. Lentivirus encoding shRNA against mRNA transcripts has demonstrated decreased expression of the cognate protein (12, 13), but several aspects of this method limit its efficacy. Inadequate neuroinvasion by lentiviral vectors across the blood–brain barrier (BBB) necessitates their direct delivery to the CNS via stereotactic intracranial injection of shRNA lentivirus directly into the brain. Even then, lentiviral infection and shRNA expression were limited to a small area around the injection site, resulting in spatially limited knockdown of protein expression. This method also lacks desired temporal control of protein expression, since once brain cells are infected with lentivirus, it will likely irreversibly suppress protein expression in these cells. Moreover, despite improvements in lentiviral vector technology and construction (14–17), concerns over the oncogenic capacity of lentiviral delivery systems remain (18–20).

Nonviral strategies to deliver siRNA molecules to cells include using cationic peptides capable of crossing plasma membranes (21–23). Conjugating siRNA to these cell-penetrating peptides via thiol linkages allows for efficient dissociation of siRNA–peptide complexes by reduction of the disulfide bond in the cytoplasm (24, 25). While this delivery method holds therapeutic promise, it still does not solve two important problems of drug delivery to the brain: cell specificity and transport of its cargo across the BBB. Kumar et al. developed a transvascular method to deliver siRNA across the BBB specifically to neuronal cells in the brain via intravenous (iv) injection (10). This method involves complexing siRNA to a short peptide derived from the rabies virus glycoprotein that binds specifically to acetylcholine receptors (AChRs) on neuronal cells (26, 27). Adding nine D-Arginines to the carboxyl terminus of this peptide (RVG-9r) enabled it to electrostatically interact with siRNA and specifically deliver siRNA to neurons in mouse brains to suppress protein expression and protect against fatal viral encephalitis. This significant advance in siRNA delivery to the CNS was tempered by the lack of direct detection of siRNA in the brains of a majority of treated mice, suggesting that these naked complexes may have degraded significantly in the blood during transport. Efficient peptide-mediated delivery of siRNA to the brain after iv injection relies on protecting the complex from serum nuclease and protease degradation en route.

Complexing or encapsulating siRNA with liposomes has been shown to protect siRNA from degradation and improve delivery through the vasculature (28–33). Adding polyethylene glycol (PEG) to lipids increases bioavailability and circulation time in blood (34–36) by decreasing immune clearance (37). PEGylated liposomes increased delivery of doxorubicin and cisplatin in experimental tumor models (38, 39) and have been used in clinical trials to treat a variety of cancers (40), including AIDS-related Kaposi’s sarcoma (41, 42) and advanced malignant solid tumors (43), including those found in ovarian (44) and breast (45) cancer.

While protecting therapeutic agents from serum degradation represents a crucial step in transvascular delivery to target sites in the brain, liposomes alone do not provide cell-specific delivery, nor do they ensure transport across the BBB. Coupling a monoclonal

antibody against glial fibrillary acidic protein (GFAP) to PEGylated liposomes successfully targeted them to astrocytes in cell culture, but still failed to deliver them across the BBB to astrocytes in mouse brains (46). An effective, efficient delivery system must do all three: (1) provide a molecular address to the agent for delivery to neuronal cells, (2) gain access to the brain by crossing the BBB, and (3) protect the agent from serum degradation en route. Using siRNA as a therapeutic agent and prion diseases as models, we have developed a therapeutic delivery system that addresses all of these issues. By complexing siRNA targeting mRNA transcripts encoding PrP^C to cationic liposomes, and then adding the RVG-9r peptide, we have created liposome–siRNA–peptide complexes (LSPCs) that effectively cross the BBB and deliver PrP siRNA to AchR-expressing cells to suppress PrP^C expression in vivo.

2. Materials

2.1. LSPC Reagents

1. siRNA: Designed using siRNA scales (47) and synthesized, pre-annealed, and lyophilized by Qiagen (see ^{Note 1}). Resuspend in sterile RNase-free water (supplied by Qiagen) to 20 μ M according to the manufacturer's directions. Store in single-use aliquots at -70°C (see ^{Note 2}).
2. Peptides: RVG-9r and control RVM-9r peptides (Table 1) were synthesized and purified by high-performance liquid chromatography by Global Peptide (www.globalpeptide.com). Resuspend in sterile phosphate-buffered saline (PBS), pH 7.4 to 400 μ M, or approximately 2 mg/mL. For example, resuspend 100 mg of peptide in 50 mL PBS. Store at -70°C in single-use aliquots (typically 400 μ L, see ^{Note 2}).
3. 10% Sterile sucrose: Weigh 1 g of sucrose and mix into 9 mL of sterile deionized water.
4. Liposomes: Prepare cationic liposomes by mixing *N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTAP, Sigma-Aldrich, St. Louis, MO) and cholesterol (NOF America, Irvine, CA) in chloroform at 1:1 molar ratio to a final concentration of 2 mM. Weigh and mix 14 mg of DOTAP and 7.7 mg of cholesterol into 10 mL of chloroform (see ^{Note 3}) in a round-bottom, 15-mL glass tube. Place the tube in a vacuum desiccator and dry overnight to a thin film (see ^{Note 4}).

Rehydrate lipids to a final concentration of 10 mM in sterile 10% sucrose. Add 2 mL of sucrose solution to the glass tube containing the lyophilized lipids and mix by pipetting.

¹We have used siRNA prepared for us by several biotechnology companies, but have observed optimal knockdown using siRNA prepared from Qiagen. Additionally, Qiagen can label siRNA with Alexa fluorochromes to monitor LSPC trafficking for determining delivery kinetics (see ^{Note 15}).

²We typically treat cages of five mice at a time and therefore aliquot 1 mL of 200 μ M stock siRNA, enough to treat five mice. Aliquots should be adjusted to meet researchers' experimental requirements.

³Accurately weighing these amounts requires a microbalance. Alternatively, prepare a 20 mM stock by mixing 140 mg of DOTAP and 77 mg of cholesterol into chloroform to a final volume of 10 mL, and then dilute 1 mL of this solution into 9 mL of chloroform to a final concentration of 2 mM. Store the 20 mM stock at room temperature protected from air and light for up to several months.

⁴Desiccation time may vary. Most importantly, the lipids must be completely dry. For faster desiccation, the solution can be split into several tubes.

Incubate for 1 h at 50°C, and then at room temperature for 2 h. Mix and serially filter through 1, 0.45, and 0.2 µm pore filters (Pall Life Sciences). Store in sterile tubes at 4°C for up to several months until ready to use.

Dilute liposomes to 200 µM working concentration 1:100 in sterile PBS immediately prior to use. For example, briefly mix stock liposomes by pipetting and add 2–198 µL sterile PBS. Mix by pipetting and put 100 µL into two 200 µL thin-walled PCR tubes (VWR). Seal tubes shut with parafilm and place into the tube holder of MP4000 horn sonicator (Qsonica). Sonicate liposomes for 40 s at 75% maximum power (see ^{Note 5}). The liposomes can be used immediately for complexation, or stored for several weeks at 4°C (see ^{Note 6}).

2.2. LSPC Delivery Supplies

1. Mice: Choose mouse lines and strains appropriate for the experimental design. Various strains of wild-type mice can be purchased from Charles River or Jackson labs (see ^{Note 7}).
2. 26–30 Gauge insulin syringes.
3. Heat lamp.
4. Rodent anesthesia machine dispensing Isoflurane/Oxygen (Parkland Scientific, see ^{Note 8}).

3. Methods

3.1. Preparing LSPCs

Complex siRNA, liposomes, and peptides using filter barrier tips and sterile technique in a cell culture hood. The following preparation will produce enough LSPCs to treat five mice.

1. Thaw 1 mL of stock siRNA and 400 µL stock peptides on ice.
2. Add 100 µL of 200 µM stock liposomes (sonicate first if necessary, see ^{Note 6}) to 1 mL of 20 µM siRNA and mix gently by pipetting.
3. Incubate at room temperature for 10 min (Fig. 1).
4. Add 500 µL of peptide, mix gently by pipetting, and incubate at room temperature for 10 min (see ^{Note 9}).

3.2. Injecting LSPCs

1. Warm mice in their cage by placing it 4–6 in. below a heat lamp for 5–10 min (see ^{Note 10}).

⁵Do not sonicate more than 100 µL per tube using this sonicator. Other sonicators may be used, but volumes may have to be increased significantly if a probe sonicator is used. Horn sonicators produce the best, most consistent results with the small volumes used here.

⁶If used later, sonicate liposomes again prior to complexation.

⁷White mice (FVB, C3H, CD-1, for example) tend to be much easier to inject intravenously in the tail than brown or black mice (129sv, C57/B16).

⁸The machine should have an incubating chamber and a nose cone. We recommend isoflurane, but alternative anesthesia, such as ketamine, can be used. While tail vein injections can be done using physical restraint, we strongly recommend using chemical restraint to avoid sudden movements from the mouse that can compromise the injection and waste valuable time and reagents.

⁹LSPCs are ready to use in 10 min, but are stable for several hours at room temperature.

2. While warming the mice, turn on the anesthesia machine such that oxygen flows at a rate of 3–4 L/min. Turn on the isoflurane at 3 L/min.
3. When the tail vasculature is clearly visible, place a single mouse in the anesthesia chamber until its respiration visibly slows to 1–2 breaths per second (see ^{Note 11}).
4. Meanwhile, load 300 µL of LSPCs into a sterile insulin syringe (see ^{Note 12}).
5. Place the mouse on a flat surface with its face in the nose cone attached to the anesthesia machine (see ^{Note 13}).
6. Starting with the mouse laying on its ventral side (dorsal side up), locate one of the two lateral veins. Starting as far posterior as possible, rotate the tail slightly and insert the needle with the bevel up at a shallow 10–15° angle 1–2 mm into the vein. Slowly press the plunger to inject the LSPC solution (see ^{Note 14}).

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¹⁰This step is crucial for successful tail vein injections, especially in dark-colored mice. The cage should reach an ambient temperature between 32 and 35°C. The lateral tail veins and/or the ventral artery should be clearly visible before proceeding. This usually coincides with salivation, so look for moisture around the mouth and nose. Monitor mice under the heat lamp every 2 min for signs of distress or hyperthermia. Do not leave a mouse cage under the heat lamp for more than 15 consecutive minutes.

¹¹This should take 1–3 min. Isoflurane is very safe and accidental overdoses are very rare. Distressed mice can be given pure oxygen to help revive them if necessary.

¹²If bubbles appear, hold the syringe vertically with the needle pointing up and flick the syringe until the bubbles dislodge and float to the top of the syringe, toward the needle. Carefully keeping the needle tip above the syringe barrel, place it on the inside of the tube containing the LSPCs and gently push the syringe plunger until the bubbles are evacuated (i.e., when a small amount of liquid emerges from the needle). Load additional LSPC mixture into the syringe to 300 µL if necessary.

¹³Mice can be placed in physical restraints at this time if desired, but we recommend simultaneous chemical restraint.

¹⁴Curling the tail over a finger can help orient the tail in a position that facilitates inserting the needle at the shallow 10–15° angle required for entering the vein. You should, but not always, feel the needle entering the vein lumen. You should feel little or no resistance on the plunger if you are in the lumen, and you will often see the vein blanch as the solution displaces the blood. If you feel resistance or see the tail swell just anterior to the injection site, stop immediately. You are most likely not in the vein. Apply direct pressure to the injection site for a few seconds and then attempt the injection again further anterior to the previous site. One typically has three opportunities for a successful injection per vein. If the injection is unsuccessful in both veins, turn the mouse over and try injecting into the ventral artery. If this fails, or if the vasculature is no longer visible, stop. Allow the mouse to recover, proceed to the next mouse, and try injecting the former mouse after attempting the others.

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Fig. 1.
LSPC formulation.

Fig. 2. LSPC delivery to the CNS in live animals. Mice were injected iv into the tail vein with PBS alone (*middle mouse* in each panel), PrP siRNA complexed to the RVG-9r peptide (*first mouse* in each panel), or PrP LSPCs (*third mouse* in each panel). siRNA was detected in the brains of mice in as little as 2 min after injection. LSPCs targeted siRNA more specifically to the brain, where after at least 10 days post injection, we detected more LSPCs than siRNA complexed with peptide alone.

Fig. 3. LSPC delivery to PrP^C-expressing cells in vivo (11). FVB mice were intravenously injected with liposomes alone (panels a–e) or LSPCs containing DyLight 488-labeled peptides and Alexa 488-labeled siRNAs as follows: PrP siRNA-control RVM-9r (f–j), Control siRNA-RVG-9r (k–o), or PrP siRNA-RVG-9r (p–t). Mice were sacrificed 24 h later, dissected, and their brains snap frozen and 8 μ m sections thereof stained with DyLight 649-labeled anti-PrP antibody (a, f, k, and p) and DAPI (c, h, m, and r) and visualized by fluorescence microscopy. Panels (e), (j), (o), and (t) depict magnifications of the boxed areas in panels (d), (i), (n), and (s). Inset in panel (p) depicts a serial section stained with an irrelevant isotype control antibody. Scale bars, 50 μ m. (u and v) FACS analysis of PrP-RVG-9r LSPC delivery to brain cells (checked peak (u) and circle (v)), kidney cells (white peak and circle), splenocytes (dark grey peak and triangle), and hepatocytes (black peak and square). (w) Cumulative data showing relative PrP expression on brain cells treated with liposomes alone or with LSPCs containing PrP-RVM-9r, control-RVG-9r, or PrP-RVG-9r. Symbols associated with each bar indicate values for individual mice.

Table 1

Delivery peptide sequences

RVG-9r ^a	YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGrrrrrrrrrr
RVM-9r ^b	MNLLRKIVKNRRDEDTQKSSPASAPLDGGGGrrrrrrrrrr

^aLower case r denotes D-arginine racemer^bControl