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One-step encapsulation of siRNA between lipid-layers of multi-layer polycation liposomes by lipoplex freeze-thawing

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ABSTRACT

Small interfering RNA (siRNA) has the potential to be a candidate as a cure for intractable diseases. However, an appropriate vector is required for siRNA delivery because of the low transfection efficiency of siRNA without a vector and its easy degradation *in vivo*. Here, we report a simple, only one step, and efficient method for siRNA encapsulation into a lipidic nanocarrier by freeze-thawing: siRNA was entrapped between the lipid layers of multi-layer liposomes by freeze-thawing of lipoplexes composed of polycation liposomes (PCLs) and siRNA. siRNA-holding capacity to the PCL was increased by repeating freeze–thaw of the lipoplex up to 5 cycles. Although siRNA in the conventional lipoplex was degraded after incubation in 90% fetal bovine serum for 72 h, siRNA in the frozen and thawed lipoplex was not degraded. Interestingly, we found that the lipoplex formed a "packed multi-layer" structure after the freeze-thawing of "single-layer" PCL and siRNA complex, suggesting that siRNA exists between the lipid layers working as a binder. The frozen and thawed lipoplex showed significantly higher knockdown efficacy compared with the conventional lipoplex. In addition, PEGylated freeze-thawed lipoplexes delivered a higher amount of siRNA to a tumor *in vivo* compared with the PEGylated conventional ones. These results provide an attractive strategy for "one-step" encapsulation of siRNA into liposomes by freeze-thawing.

1. Introduction

Small interfering RNA (siRNA), a nucleic acid having RNA interference activity, is a short double-stranded RNA composed of 21–23 base pairs. The siRNA interacts with and cleaves sequence-specific mRNA [1–3]. siRNA has been widely used for investigating specific protein functions in the field of scientific research. Because of its fewer side effects and its potential therapeutic effects, siRNA is expected to be a therapeutic agent for intractable diseases such as cancer. Since siRNA is degraded in the bloodstream by RNases and has little transfection efficiency owing to its low permeation through the plasma membrane [4,5], cationic liposomes, polycations and lipidic nanoparticles have been used as a siRNA delivery vector to overcome these problems [5–8]. The major approach for the delivery is the attachment of the siRNA on the lipidic vector surface to make a lipoplex. However, the cationic groups of these siRNA vectors tend to induce cytotoxic effect. For the purpose of siRNA delivery, we previously developed dicetyl phosphate-tetraethylenepentamine (DCP-TEPA) for preparing polycation liposomes (PCLs), and found that siRNA complexed with these PCLs induced a remarkable gene-silencing effect *in vitro* and *in vivo* [9–11]. Moreover, the PCLs showed fewer cytotoxic effects than the conventional cationic liposomes, although it is preferred that the cationic charges in the PCL should be decreased as much as possible.

It is well known that attaching siRNA onto the cationic liposome or PCL surface through electrostatic interaction is the major method for siRNA holding and delivery [7,9]. The method does not require any special techniques or machines, as lipoplexes can be prepared by simply incubating siRNA and the vector for several minutes. Although siRNA in the PCL-complex induces significant knockdown of target mRNA *in vitro*, binding affinity of siRNA to PCLs is not very strong. In fact, siRNA tends to detach from the PCL surface after polyethylene glycol (PEG)-modification [9]. Therefore, an additional interaction between siRNA and PCL, such as hydrophobic interaction by conjugation of cholesterol to the siRNA, should be required [9,12–14].

The other approach is the encapsulation of siRNA inside a liposome, lipidic nanoparticle or like vector [15,16]. The most important

Abbreviations: BCA, bicinchoninic acid; DAPI, 2-amidino-diphenyl-indole; DCP-DETA, dicetyl phosphate-diethylenetriamine; DEPC, diethylpyrocarbonate; DOPE, dioleoylphosphatidylethanolamine; EPR, enhanced permeability and retention; FITC, fluorescein isothiocyanate; lipoplex, liposome and siRNA complex; N/P, nitrogen/phosphorus ratio; PCLs, polycationic liposomes; PEG, polyethylene glycol; siRNA, small-interfering RNA; TEM, transmission electron microscope.

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advantage of this approach is that siRNA degradation in the bloodstream is prevented. Lipidic vectors encapsulating siRNA are suitable for manipulating surface charge and probe-modification for active targeting, as well as for protecting against degradation by plasma RNases. Therefore, this approach is a quite useful one for *in vivo* siRNA delivery. However, additional technologies or organic solvents are required for preparation of siRNA-encapsulated lipidic nanoparticles [17–20]. Therefore, an easier method for encapsulating siRNA, such as a one-step encapsulation method, is still awaited for efficient siRNA delivery.

The liposome is a closed vesicle composed of a lipid bilayer membrane. The liposome can encapsulate hydrophilic materials in its interior water phase and hydrophobic materials in the lipid membrane and, therefore, is widely used as a drug delivery tool. In fact, Doxil®, which is a doxorubicin (Dox)-encapsulated polyethylene glycol (PEG)-modified liposome, has been used clinically [21,22]. The Doxil® formulation reduces side effects and enhances the anticancer activity of Dox through enhanced permeability and retention (EPR) effect [23,24]. In general, a multi-layer structure is formed after hydration of a lipid film [25]. Since the multi-layer liposome is large heterogeneous in size, with a small volume of water phase per liposomal lipids, the single-layer liposome was developed by "freeze-thawing" of the multi-layer liposome [26]. The freeze-thawing method has also been used for the encapsulation of small hydrophilic molecules into the aqueous pocket of the liposome [27]. It is known that freeze-thawing of single-layer liposomes with calcein increases the encapsulation ratio of calcein into the "single-layer" liposome [28]. However, there has been no previous study on the influence of freeze-thawing on liposomes with siRNA.

In the present study, we investigated the effect of freeze-thawing on PCL-based lipoplexes and found that freeze-thawing of single-layer PCLs and siRNA complex formed multi-layer structures, suggesting that siRNA was effectively encapsulated between the lipid layers of these multi-layer PCLs. The freeze-thawed lipoplexes protected siRNA from RNases and enhanced the knockdown efficiency of target mRNA *in vitro*. In addition, the accumulation of siRNA in tumor was increased by systemic administration of PEGylated freeze-thawed lipoplexes compared with the PEGylated conventional ones. These results suggest that this freeze-thawing strategy can offer a novel approach to develop a "one-step" preparation of siRNA-encapsulated PCLs or cationic liposomes.

2. Materials and methods

2.1. Materials

Cholesterol, dioleoylphosphatidylethanolamine (DOPE), dipalmitoylphosphatidylcholine (DPPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were kindly donated by Nippon Fine Chemical Co. (Takasago, Hyogo, Japan). Dicetyl phosphate-tetraethylenepentamine (DCP-TEPA) was synthesized as described previously [9]. siRNAs for Luc2 were purchased from Hokkaido System Science Co. (Hokkaido, Japan). The nucleotide sequences of siRNA with a 2-nucleotide overhang (underline) were 5′-GGCUACGUCCAG GAGCGCACC-3′ (sense) and 5′-UGCGCUCCUGGACGUAGCCUU-3′ (anti-sense). Fluorescein isothiocyanate was conjugated with siRNA for preparing FITC-labeled siRNA. Alexa750 was also conjugated with siRNA at the 3′-end of the anti-sense strand for the *in vivo* study. All other reagents were of analytical grade.

2.2. Synthesis of DCP-DETA

Dicetyl phosphate-diethylenetriamine (DCP-DETA) was synthesized by using a procedure analogous to that reported previously [29,30]. Dimerized dicetyl phosphate anhydride (216 mg, 0.2 mmol) in anhydrous chloroform (0.5 mL) was added to a solution of diethylenetriamine (62 mg, 0.6 mmol) in 0.5 mL of anhydrous pyridine. The reaction mixture was stirred under nitrogen atmosphere for 4 h at room temperature. After removal of the solvent under reduced pressure, the residue was suspended in distilled water and then filtered to remove any unreacted diethylenetriamine. The residue was subsequently subjected to column chromatography using aminated silica gel (Chromatorex NH, Fuji Silisia Chemical LTD, Aichi, Japan) with an eluting solution of $CHCl_3$ and subsequent $CHCl_3$/MeOH (39/1, v/v), giving 73 mg (57%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.88 (t, 6H), 1.25 (s, 52H), 1.66 (m, 4H), 1.80 (br s, 4H), 2.68–2.83 (m, 5H), 2.99 (br m, 2H), 3.25 (br m, 1H), 3.98 (bm, 4H); MALDI-TOF-MS for $(C_{36}H_{79}N_3O_3P)^+$ calcd 632.99, found 632.53.

2.3. Preparation of lipoplexes

Dioleoylphosphatidylethanolamine (DOPE), cholesterol, and dicetyl phosphate-diethylenetriamine (DCP-DETA; 1:1:1 as a molar ratio) were dissolved in *t*-butyl alcohol and freeze-dried. PCLs were produced by hydration of the lipid mixture with DEPC-treated RNase-free water. Liposomes were sized by extrusion 10 times through a polycarbonate membrane filter having 100-nm pores (Nucleopore, Maidstone, UK). Liposomes and siRNA were mixed and incubated for 20 min at room temperature to form liposome/siRNA complexes. To prepare freeze-thawed lipoplex, the complex was frozen in liquid nitrogen and thawed in a water bath at 43 °C with vortexing. The particle size and ζ-potential of the complexes diluted with 0.5 mM PBS (pH = 7.4) were measured by using a Zetasizer Nano ZS (Malvern, Worcs, UK). For the *in vivo* study, lipoplexes were decorated with polyethylene glycol (PEG) by incubating them with DSPE-PEG6000 (10 mol% to total lipids) at 40 °C for 10 min. Dipalmitoylphosphatidylcholine (DPPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and dicetyl phosphate-tetraethylenepentamine (DCP-TEPA) liposomes (DOPE:cholesterol: DPPC, DOTAP or DCP-TEPA = 1:1:1) were also prepared by same procedure.

2.4. Electrophoretic assay

The amount of siRNA that was not or only loosely attached to lipoplexes was checked by performng15% polyacrylamide gel electrophoresis, where the siRNA in stable complexes did not enter the gel. The gel was stained for 30 min in GelRed, and siRNA was detected by using a LAS-3000 mini system (Fuji Film, Tokyo, Japan).

2.5. Stability of siRNA in the absence and presence of serum

Naked siRNA or lipoplexes were incubated in 90% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO) at 37 °C for 72 h. Then, the siRNA was extracted from the serum by using TRIzol reagent and subjected to 15% polyacrylamide gel electrophoresis for detecting intact siRNA, as described above.

2.6. LC–MS measurement of siRNA in conventional and freeze-thawed lipoplexes

The HPLC–IT–MS system consisted of a NANOSPACE SI-2 high-performance liquid chromatography (SHISEIDO, Tokyo, Japan) and a LCQ Fleet™ ion-trap mass spectrometer (Thermo Scientific™, MA, USA). An Asahipak ODP-50 2D column (5 μm, 250 mm × 2.0 mm i.d., Shodex) was used as the analytical column, and was maintained at 60 °C. The injection volume was 10 μL. The mobile phase, consisting of solvent A (0.01 M triethylamine in acetic acid buffer [pH 6.0]) and solvent B (acetonitrile), was delivered at the flow rate of 0.2 mL/min. The gradient elution was as follows: B% = 2–100 (0–15 min). The scan mode was used from *m/z* 1639.20–1644.20 (antisense strand), 1676.62–1681.62 (sense strand). The wavelength of detection was set at 260 nm.