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Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency

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Abstract

Cationic lipid-based gene delivery systems have shown promise in transfecting cells in vitro and in vivo. However, liposome/DNA complexes tend to form aggregates after preparation. Lyophilization of these systems, therefore, has become of increasing interest. In this study, we investigated the feasibility of preserving complexes as a dried preparation using a modified dehydration rehydration vesicle (DRV) method as a convenient and reliable procedure. We also studied storage stability of a lyophilized novel cationic gene delivery system incorporating sucrose, isomaltose and isomaltotriose. Liposomes were composed of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and L-dioleoylphosphatidylethanolamine (DOPE), plus sucrose, isomaltose or isomaltotriose. Lyophilized liposome/DNA complexes were stored at −20, 25, 40 and 50 °C and their stability was followed for 50 days. Liposome/DNA complexes with sucrose could be stored even at 50 °C without large loss of transfection efficiency. The transfection efficiency of formulations stored at various temperatures indicated that the stabilizing effect of sugars on plasmid DNA was higher in the following order: isomaltotriose < isomaltose < sucrose, which was inverse to the order of their glass transition temperature (T_g) values. It was concluded that we could prepare novel lyophilized liposome/DNA complexes with high transfection efficiency and stability, which might be concerned that sucrose stabilized plasmid DNA in liposomes by directly interacting with plasmid DNA rather than by vitrifying to a high T_g solid.

Keywords: Cationic liposome; Sucrose; Dehydration rehydration vesicle; Transfection; Storage stability; Lyophilization

1. Introduction

Cationic liposome-mediated transfer of DNA is a promising approach, because of low immunogenicity and toxicity, ease of preparation, and potential applications for active targeting. The disadvantages include poor efficiency of transfection in vivo. Therefore, many cationic lipid-based transfection reagents have been developed for the efficient delivery of DNA into cells (Gao and Huang, 1991; Vigneron et al., 1996). Commercially available cationic liposomes or particles are mixed with plasmid DNA, and tend to form large liposome/DNA aggregates in solution, especially at high DNA concentrations. They form as a result of electrostatic binding between cationic liposomes and negatively charged DNA, and are inherently difficult to manipulate, resulting in a decrease of transfection (Sternberg et al., 1994; Lai and van Zanten, 2002). Because of this problem, cationic liposome/DNA complexes have to be freshly prepared when they are used. This would make it demanding to prepare them, and make quality control very difficult due to the fact that preparation of cationic liposome/DNA complexes is a process that is poorly defined and difficult to control.

To produce stable gene delivery systems that avoid these problems, lyophilization is suitable for long-term storage. There are many studies about lyophilization of liposome vectors using sugars (Anchordoquy et al., 1997; Li et al., 2000; Molina et al., 2004). Disaccharides were used in most studies. Especially, sucrose, which has a high glass transition temperature (T_g), is known to be effective to maintain the stability of liposomes, presumably by forming glasses under the typical freezing conditions used for lyophilization (Molina et al., 2001). To develop lyophilized liposome complexes with plasmid DNA vector, we used a modified dehydration rehydration vesicle (DRV) method as a convenient and reliable procedure (Perrie and Gregoriadis, 2000). The technique of the DRV method, employing sucrose at

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the lyophilization stage, has been evaluated for a range of solutes (Zadi and Gregoriadis, 2000; Kawano et al., 2003) and plasmid DNA (Perrie et al., 2004). The effects of sugars on the stability of lyophilized liposomes, sizes of liposomes and entrapment efficiency of solutes using DRV methods have been reported (Zadi and Gregoriadis, 2000; Kawano et al., 2003), but there have been a few reports about the effect of sugars on the stability of plasmid DNA in liposomes during storage (Li et al., 2000).

In this study, we examined stability of lyophilized liposome/DNA complexes with sucrose, isomaltose or isomaltotriose at different temperatures over 50 days, and determined which sugars could inhibit aggregation and maintain the transfection activity of plasmid DNA during preservation at temperatures above T_g. We found that DRV/DNA complexes with sucrose could be stored even at 50 °C without a large loss of transfection activity. Isomaltose and isomaltotriose were selected as excipients because their T_g values were higher than that of sucrose and therefore, they were expected to exhibit a greater stabilizing effect.

2. Materials and methods

2.1. Materials

3β-[*N*-(*N*′,*N*′-Dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and L-dioleoylphosphatidylethanolamine (DOPE) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Lipofectamine 2000 was purchased from Invitrogen Corp. (Carlsbad, CA, USA). The Pica gene luciferase assay kit was purchased from Toyo Ink Mfg. Co. Ltd. (Tokyo, Japan). BCA protein assay reagent was purchased from Pierce (Rockford, IL, USA). All other chemicals used were of reagent grade. The plasmid DNA encoding the luciferase marker gene (pAAV-CMV-Luc) was supplied by Dr. S. Tanaka in Mt. Sinai School of Medicine (NY, USA). All reagents were of analytical grade. RPMI1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Grand Island, NY, USA).

2.2. Preparation of DRV

The preparation method has been reported previously (Perrie and Gregoriadis, 2000). Briefly, lipids (e.g., DC-Chol:DOPE = 3:2 and 1:2 mol/mol) were dissolved in chloroform and a dried film was formed by rotary-evaporation. The preparation was hydrated with filtered water and vortexed at room temperature. The resulting multilamellar vesicle (MLV) suspension was extruded through a series of polycarbonate membranes with pore sizes of 0.6 and 0.2 μm (Millipore, Billerica, MA) to yield about 200-nm-sized vesicles. A sugar/total lipid (w/w) of 5, and 12.5–100 μg of plasmid DNA at a charge ratio of (+/−) of 2 and 16 were carefully added to the vesicle suspension, and the mixture was transferred to polypropylene tubes (10 mm in diameter and 40 mm in length), frozen by immersing in liquid nitrogen for 10 min, and lyophilized (DRVs) using a Freezevac C-1 lyophilizer (Tozai Tsusho Co., Tokyo, Japan) at a vacuum level below 5 Pa. Shelf temperature was controlled at −40 °C for 12 h, at −20 °C for 12 h, at 0 °C for 8 h, at 20 °C for 4 h, and at 30 °C for 4 h. After lyophilization, dry nitrogen was introduced in the drying chamber, and vials of DRVs were sealed with screw caps in a nitrogen atmosphere. Water contents of formulations obtained were less than 0.5%, as determined by the Karl Fischer method.

Prior to the measurement of transfection efficiency, the dry cake of DRVs was rehydrated with milli-Q water (1 ml of water per vial) and ultracentrifuged at 45,000 rpm for 45 min to partition sugars from the liposome suspension. The supernatant was collected and then milli-Q water was added to the DRV pellets to achieve 100 μg DNA/ml (DRV pellet suspension).

2.3. Measurement of size

The mean particle size of the DRVs suspended in water was determined using a light scattering instrument (DLS-7000, Otsuka Electronics Co. Ltd., Osaka, Japan) by a dynamic laser light scattering method at 25 ± 1 °C. The reported particle size was the average value of two measurements.

2.4. Stability test

Vials of DRVs were transferred to vessels containing P_2O_5 and were stored at −20, 25, 40 and 50 °C for 50 days.

2.5. Measurement of T_g

A T_g of DRV formulation was measured by using a model 2920 differential scanning calorimeter (DSC) with a refrigerator cooling system (TA Instruments, Newcastle, DE, USA). Approximately 3 mg of DRV cake was put in an aluminum sample pan, dried in vacuum at 25 °C for 16 h and sealed hermetically in a nitrogen atmosphere in order to prevent water sorption during sample preparation. DSC traces were measured at a heating rate of 20 °C/min. An empty pan was used as a reference sample. Temperature calibration of the instrument was carried out using indium. T_g values reported were obtained for first heating scan. The T_g values and changes in the heat capacity at T_g of stored samples were similar to those before storage, indicating that crystallization of amorphous excipient in the formulations did not occur during stability studies.

2.6. Entrapment efficiency of plasmid DNA in DRV

The plasmid DNA in the supernatant after ultracentrifugation of the rehydrated DRV suspension at 45,000 rpm for 45 min was measured as free plasmid DNA using a PicoGreen dsDNA Quantitation Kit *200-2000 assays* (Molecular Probes, Inc., OR, USA).

2.7. Cell culture

Human cervical carcinoma HeLa cells were kindly provided by Toyobo Co. Ltd. (Osaka, Japan) and grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2