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Development of anionic bubble lipopolyplexes for efficient and safe gene transfection with ultrasound exposure in mice

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ABSTRACT

Anionic bubble lipopolyplexes have been developed as anionic ultrasound (US)-responsive gene delivery carriers with biocompatible compounds for efficient and safe transfection in mice. The particles of the anionic bubble lipopolyplexes were approximately 450–600 nm with an anionic surface charge. In the absence of US exposure, the bubble lipopolyplexes showed extremely low gene expression in the human vascular endothelial cell line EAhy926. The anionic bubble lipopolyplexes, however, delivered pDNA into cells without endocytosis and showed markedly high gene expression following US exposure. The anionic bubble lipopolyplexes showed little cytotoxicity in EAhy926 cells and little aggregation with erythrocytes. Following intravenous administration into mice, the anionic bubble lipopolyplexes showed high levels of gene expression in the liver, kidney, and spleen only after US exposure to the abdominal area. The level of gene expression in liver non-parenchymal cells was significantly higher than that in parenchymal cells. In addition, the anionic bubble lipopolyplexes did not show any severe hepatic toxicity and did not enhance the production of proinflammatory cytokines. Overall, we have succeeded in preparing anionic bubble lipopolyplexes for efficient and safe transfection with US exposure in mice.

1. Introduction

It is largely expected that gene therapy will provide a range of novel approaches for the treatment of genetic disorders and intractable diseases such as cancer [1–3]. Advances in this area, however, have been limited by the fact that naked plasmid DNA (pDNA) is readily degraded in vivo and barely taken up by cells. To date, a variety of different gene delivery methods have been developed to take full advantage of gene therapy, and these gene delivery methods can be categorized as either viral or non-viral methods [4,5]. Non-viral gene delivery methods have several advantages over the viral methods, such as providing the flexibility to design a vehicle with well-defined structural and chemical properties capable of mass production [6,7]. Non-viral gene delivery methods can also be split into two sub-categories, with one involving the use of gene delivery carriers such as cationic polymers and liposomes, and the other involving the use of naked pDNA molecules according to the electroporation, hydrodynamic, pressure, and suction methods [8–12]. Most of the gene delivery carriers deliver the pDNA into cells through endocytosis. Unfortunately, however, intracellular delivery from endosomes to the cytosol provides a major obstacle to gene expression [13].

Sonoporation, which is a combination of ultrasound (US) exposure and microbubbles containing US imaging gas, has recently been reported to deliver pDNA directly into the cytosol without endocytosis and show high levels of gene expression [14,15]. US exposure disrupts the microbubbles and generates cavitation energy [16], which can create transient pores in the cellular membrane that allow pDNA to be delivered into the cytosol [15,17]. In addition, some novel approaches have been developed to allow for the incorporation of non-viral gene delivery carriers with microbubbles using covalent binding, as well as avidin–biotin and electrostatic interactions [18–24].

The microbubbles are generally several micrometers in size, which generally prevents these materials from reaching the peripheral tissues following intravenous administration. Maruyama et al. recently succeeded in developing a novel formulation capable of producing bubble liposome particles of approximately 500 nm in size [25,26]. In our previous study, we reported the development of mannosylated bubble

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lipoplexes for the targeted delivery of pDNA and siRNA into macrophages [27–29]. With regard to the surface charge of the bubble lipoplexes, those possessing an anionic or neutral surface charge were suitable for clinical application because cationic charged lipoplexes have been reported to interact with biogenic substances such as serum albumin and erythrocytes [30]. Given that neutral bubble lipoplexes can readily aggregate through the neutralization of their electric charge, it was envisaged that the anionic bubble lipopolyplexes could be prepared without aggregation even under high concentration conditions. There have, however, been very few reports concerning the development or application of US responsive gene delivery carriers with an anionic surface charge. With all of this in mind, we prepared bubble lipopolyplexes as novel anionic US responsive gene delivery carriers from a ternary pDNA complex, cationic polymers, and anionic bubble liposomes with biocompatible compounds for efficient and safe transfection.

In this study, we have investigated the physicochemical properties, transfection efficiencies, and toxicity of the anionic bubble lipopolyplexes before and after US exposure.

2. Materials and methods

2.1. Chemicals

Protamine sulfate (PS), poly-L-lysine (PLL), poly-L-arginine (PLA), and polyethylenimine (PEI, branched form, average molecular weight 25,000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dendrigraft poly-L-lysine (DPLL) was purchased from Colcom (Montpellier, France). Distearoyl phosphatidylglycerol (DSPG), distearoyl phosphatidylic acid (DSPA), distearoyl phosphatidylserine (DSPS), distearoyl phosphatidylcholine (DSPC), and dioleoyl trimethylammoniumpropane (DOTAP) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol (Chol) and methoxypolyethyleneglycol 2000-distearoylphosphatidylethanolamine (PEG-DSPE) were purchased by Nacalai Tesque (Kyoto, Japan) and NOF Co. (Tokyo, Japan), respectively. All of the other chemicals used in the current study were purchased as the highest purity grades available.

2.2. Construction of pDNA

pCMV-Luc was used as described previously [31]. The pDNA was amplified using an EndoFree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany) and dissolved in sterile dH_2O. Fluorescein-labeled pDNA was prepared using a Label IT Tracker Fluorescein Kit (Mirus Co., Madison, WI, USA).

2.3. Preparation of the anionic bubble lipopolyplexes

DSPG, DSPC, and PEG-DSPE were mixed in chloroform in a molar ratio of 7:2:1 for the construction of the anionic liposomes (AL). The resulting lipid mixture was then dried by evaporation before being desiccated in a vacuum to give a lipid film, which was subsequently suspended in sterile dH_2O. Following a 30-min period of hydration at 65 °C, the dispersion was sonicated for 10 min using a bath sonicator, and then sonicated in a tip sonicator for 3 min to produce liposomes, which were sterilized using a 0.45-μm filter (Nihon-Millipore, Tokyo, Japan).

Various cationic polyplexes were prepared by gently mixing a pDNA solution with an appropriate amount of cationic polymer solution such as PS, PLL, PLA, or DPLL, with the resulting mixture being incubated for 15 min. The resulting cationic polyplexes were then mixed with the appropriate amount of the AL to allow for the construction of the anionic lipopolyplexes. Various anionic lipopolyplexes were also constructed at weight ratios of 1.0:1.5:17.6 (pDNA:PLL, PLA, or DPLL:AL) or 1.0:1.25:2.5 (pDNA:PS:AL). The isotonic properties of the solutions were then adjusted via the addition of 10× phosphate buffered saline (PBS). For the preparation of the bubble lipopolyplexes (pDNA/PS, PLL, PLA, and DPLL/BL), US imaging gas captured within the anionic lipopolyplexes using a method described previously in the literature [32]. Briefly, the anionic lipopolyplexes were added to 5-mL sterilized vials, which were subsequently capped and pressurized with 7.5 mL of perfluoropropane gas (Takachiho Chemical Industries Co., Ltd., Tokyo, Japan). To enable the US imaging gas to become encapsulated within the anionic lipopolyplexes, the vial was sonicated in a bath-type sonicator (AS ONE Co., Osaka, Japan) for 5 min. For the optimization of the anionic lipids, three anionic liposomes containing DSPG, DSPA, or DSPS were prepared and pDNA/PS/AL and pDNA/PS/BL were developed for each of the anionic liposomes.

2.4. Physicochemical properties of the anionic bubble lipopolyplexes

The particle sizes and zeta-potentials of the bubble lipopolyplexes were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom).

Ten-microliter aliquots of different complex solutions containing 1 μg of pDNA were mixed with 2 μL of loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 1% agarose gel. Electrophoresis (Mupid-2X; Cosmo Bio, Tokyo, Japan) experiments were carried out at 100 V in a running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM EDTA). The pDNA was visualized with a GelRed Nucleic Acid Gel Stain (Wako, Osaka, Japan) using an ImageQuant LAS4000 System (GE Healthcare Life Sciences, Fairfield, CT, USA). Transmission electron microscope (TEM) images of the pDNA/PS, pDNA/PS/AL, and pDNA/PS/BL were recorded on an H-7650 system (Hitachi Co., Tokyo, Japan) with negative staining using uranyl acetate.

2.5. In-vitro gene expression and intracellular distribution experiments

The human vascular endothelial cell line EAhy926 was purchased from American Type Culture Collection (Manassas, VA, USA) and the cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 100 μM non-essential amino acids at 37 °C in 5% CO_2. Following a pre-incubation period of 24 h, the culture medium was replaced with Opti-MEM I containing different complexes (10 μg pDNA). The EAhy926 cells were then exposed to US (frequency, 2.0 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2) for 20 s using a 6-mm diameter probe, which was placed in the well 10 min after the addition of the different complexes. The US was generated using a Sonopore-4000 sonicator (Nepa Gene, Co., Ltd., Chiba, Japan). The incubation medium was then replaced with the culture medium and the resulting mixture was incubated for an additional 24 h. Following the incubation period, the cells were suspended in lysis buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris; pH 7.8), and the lysate was mixed with luciferase assay buffer (Picagene, Toyo Ink Co., Ltd., Tokyo, Japan). The luciferase activity was then measured using a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany) and normalized with respect to the protein content of the cells using a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan).

The intracellular distribution of the pDNA was determined using fluorescein-labeled pDNA and LysoTracker Red DND-99 (Invitrogen, Carlsbad, CA, USA). Briefly, the cells were transfected with the pDNA/PS/BL or Lipofectamine 2000 (Invitrogen) using fluorescein-labeled pDNA. The cells were subsequently treated with LysoTracker Red DND-99 6 h after the transfection process, and then fixed using a 10% formalin solution before being stained with DAPI and observed under a confocal microscope (Nikon A1RMP, Nikon, Tokyo, Japan).

2.6. WST-1 assay

The cytotoxicities of the bubble lipopolyplexes towards the EAhy926 cells were determined using a WST-1 Cell Proliferation Reagent (Roche