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Preparation of siRNA encapsulated nanoliposomes suitable for siRNA delivery by simply discontinuous mixing

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ABSTRACT

Previously a scalable and extrusion-free method has been developed for efficient liposomal encapsulation of DNA by twice stepwise mixing of lipids in ethanol and DNA solution using T-shape mixing chamber. In this study, we prepared nanoliposomes encapsulating siRNA by simply discontinuous mixing of lipids in ethanol/ether/water mixture and acidic siRNA solution without use of special equipment. The simple mixing siRNA/liposomal particles (siRNA/SMLs) prepared using ethanol/ether/water (3:1:1) mixture showed 120.4 ± 20.2 nm particle size, 0.174 ± 0.033 polydispersity and 86.5 ± 2.76% siRNA encapsulation rate. In addition, the SMLs almost completely protected the encapsulated siRNA from RNase A digestion. Coupling of anti-human epidermal growth factor receptor (EGFR) Fab′ to siRNA/SMLs enhanced EGFR-specific cell penetration of SMLs and induced siRNA dependent gene silencing. Unexpectedly, the Cy5.5-labeled Fab′ showed almost no in vivo targeting to the xenografted A549 tumors in SCID-NOD mice. However, multiple injection of the unmodified siRNA/SMLs accumulated in the tumors and induced siRNA-dependent in vivo gene silencing. These results demonstrate that the siRNA/SMLs can be used as a siRNA delivery tool for gene therapy.

1. Introduction

Currently Small interfering RNA (siRNA) composed short length (18–25 nucleotides) double strand RNAs have been focused on cancer therapeutic application [1,2]. siRNAs either inhibit translation initiation in ribosomes or destruct target mRNAs by cellular ribonucleases [3]. Due to sequence specific targeting of siRNA and its potential to inhibit protein translation; siRNA is considered as a potential drug for gene therapy and treatment of disease such as cancers [4]. However, there are two major obstacles for siRNA treatment to be resolve; first quick degradation and elimination of siRNA from blood stream and body fluids, and second less cellular uptake of siRNA by target cells [5,6]. Liposomes are considered as appropriate vectors to overcome these obstacles.

Liposomes composed of amphiphilic lipids including phospholipids make it possible to entrap hydrophilic or hydrophobic materials into hydrophilic interior or hydrophobic lipid bilayers, respectively [7]. Based on these properties, liposomes have been developed as delivery cargos for various drug materials, such as small drug molecules, nucleic acids including siRNA and proteins [7–10]. Drugs entrapped in liposomes has many advantages such as prevention of degradation, specific drug delivery to the target tissues, and reduction of drug side effects [10–12].

Systemic administrated liposomes must be stable for long circulation in blood to accumulate at the target tissue and to deliver effectively the entrapped drugs into the target cells. However, most liposomes can be rapidly cleared in blood by uptake into the phagocytic cells in the reticuloendothelial system (RES), predominantly in liver and spleen [13]. Several factors affect liposome elimination from blood circulation, such as liposome size, lipid composition and surface modification [14]. Particle sizes of liposomes are significantly related to the RES clearance. Large size liposomes are predominantly eliminated by the RES system in the liver and spleen in mice, whereas reduction of the liposome size offers modest improvement of circulation half-life and accumulation of liposomes at xenografted tumors in mice [15–17]. Opsonization is one of key processes of the RES clearance of particles. The opsonized particles bound with opsonins (serum proteins) are quickly absorbed into phagocytic cells, such as macrophages and Kupffer cells [18,19]. Surface modification of nanoparticles by polyethylene glycol (PEG) reduces the particle uptake into phagocytic cells because of protection of nonspecific absorption of serum proteins [20]. In fact, surface PEGylation of liposomes surfaces have been reported to reduce the RES

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clearance and increase their circulation-time in blood [21,22].

Several methods have been developed to prepared the PEGylated liposomes with efficient siRNA encapsulation [23]. An efficient method was developed for encapsulation of plasmid DNA by stepwise mixing of DNA in acidic buffer and lipids in ethanol solution using a T-shape connector [24]. Based on the method, the siRNA encapsulated nanoliposomes were successfully prepared and evaluated efficient knockdown of target genes in mice [8,25]. However, special equipment for stepwise mixing was required for preparation of nanoliposomes encapsulating siRNA. In this study, we found that the siRNA/liposome particles by simply discontinuous mixing of the siRNA and lipid solution in ethanol displayed higher polydispersity. In addition, relatively higher temperature was required for complete solubilization of lipids in ethanol solution. We solved the problems by using the diethylether/ethanol (1:3) mixture as a lipid solvent instead of ethanol only. We could prepared much more uniformed nanoliposomes with > 85% siRNA encapsulation efficiency using the lipid solvent by simply discontinuous mixing. We investigated in vitro and in vivo efficacy of the siRNA/nanoliposomes in the presence or absence of modification of the ligands for cell penetration and/or targeting. We believe that this method for preparation of nanoliposomes by simple mixing will be widely applicable for a scalable production of siRNA/nanoliposomes.

2. Materials & methods

2.1. Materials

All lipids, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (mPEG-PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (miPEG-PE), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and cholesterol (Chol) were purchased from Avanti Polar Lipids, Inc. (USA). RT-PCR kit was obtained from New England Biolabs (USA). Double-strand siRNAs, the fluorescein isothiocyanate (FITC)-labeled siRNA (FITC-siRNA) and Cy5-labeled siRNA (Cy5-siRNA) were purchased from Bioneer Co. (Korea). Dialysis membranes with molecular weight cutoff values of 10 K, 50 K, and 300 K were purchased from Spectrum Laboratories (USA). Anti-epidermal growth factor receptor (EGFR) humanized monoclonal antibody (EGFR-hmAb, Erbitux) was obtained from Merck Serono (Germany). The non-small cell lung carcinoma (NSCLC) cells, A549, NCI-H322 and NCI-H460, and NIH-3 T3 mouse cells were obtained from ATCC (USA). The A549-luc-C8 (A549luc) cells expressing firefly luciferase were purchased from Caliper Life Sciences (USA). NOD-SCID mice were supplied from the animal facility under guidance on the operation of animals of our institute.

2.2. Liposome preparation by simply discontinuous mixing

The lipid mixture composed of DSPC:DOPE:DODAP:DC-chol:Chol:mPEG-PE:miPEG-PE:Chol with the molar ratio of 4:4:5:4:4:0.8:0.2 (3.2 μmole total, 2.3 mg) were made a thin lipid film in a test tube by nitrogen gas blowing and freeze-drying. The lipid film was dissolved in 360 μL of one of the ethanol/diethylether mixtures (4:0, 3:1 and 2:2), and then added 90 μL of distilled water treated with diethyl pyrocarbonate (DEPC; Sigma-Aldrich, USA). The lipid solution was directly mixed with 450 μL siRNA buffer (100 μg siRNA in 150 mM citrate, pH 4.0) using a vortex mixer. The resulting solution containing liposomes was dialyzed three times against DEPC treated HBS (20 mM HEPES and 150 mM sodium chloride, pH 7.5) at 4 °C to remove the organic solvents. The liposomes prepared by the present method were named simple mixing liposomes (SMLs). The particle sizes and zeta potentials of the SMLs were analyzed with an ELS-Z zeta-potential and particle size analyzer (Photal Otsuka Electronics, Japan).

2.3. Analysis of siRNA encapsulation in SMLs

Encapsulation efficiency of siRNAs in SMLs was monitored by gel retardation assay of siRNA. The siRNA/SMLs were subjected on 4% agarose gel in the Tris/borate/EDTA (TBE) buffer containing 1 × GelRed nucleic acid stain (Biotium Inc., USA) in the absence and presence of 1% nonidet-P40 (NP40). After the electrophoresis, siRNA bands were monitored with a gel documentation system using an ultraviolet transilluminator (Bio-Rad, USA). The siRNA band intensities were analyzed using the ImageJ program (NIH).

2.4. Ribonuclease protection assay of siRNA in SMLs

The ribonuclease (RNase) protection of the siRNA encapsulated in SMLs were analyzed by the method as we described previously [11]. Briefly, 8 μL of 10 μg/mL RNase A was incubated with 8 μL of siRNA/SMLs or free siRNA in HBS containing the equivalent amount of siRNA. The mixtures were then incubated at 37 °C for up to 24 h. Agarose gel electrophoresis of the incubated mixtures was conducted in the presence of 1% NP40 to monitor the remaining siRNA.

2.5. Preparation and characterization of Fab′

The $F(ab')_2$ fragment for EGFR-hmAb was prepared by treatment of 0.1 mg/mL pepsin in acetate buffer (pH 4) and then neutralized by adding 1/10 volume of 1.5 M Tris-HCl (pH 8.8) buffer as described previously [11]. The $F(ab')_2$ fragment was reduced by adding 20 mM dithiothreitol (DTT) for 20 min at room temperature, and DTT was removed by dialysis of Fab′ in PBS (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4). The dialyzed solution was incubated at room temperature for overnight to induce Fab′ formation. After analyzing the Fab′ formation using nonreducing SDS-PAGE, 1/100 volume of 0.5 M ethylenediaminetetraacetic acid (EDTA) were then added to the Fab′ solution to prevent disulfide bond formation between the Fab′ fragments. One hundred microgram of Fab′ fragment in PBS-EDTA were incubated with 20 μg of fluorescein-5-maleimide (ThermoFisher Sci., USA) for 2 h at room temperature and then dialyzed three times in PBS using a 10 K dialysis membrane.

2.6. Analysis of ligand-dependent cellular uptake

To evaluate cellular uptake of SMLs, 100 μL of the FITC-siRNA/SML solution was sequentially incubated with 20 μg of cell penetrating TAT peptide with a sulfhydryl group at the C-terminus, GRKKRRQRRRPQ-GGC and 1/100 volume of 200 mM 2-mercaptoethanol. The unreacted peptide and 2-mercaptoethanol were removed by dialysis in PBS. The SMLs without or with peptide conjugation were incubated with the NCI-H460 cells in a 8-well chamber slide (ibide, Germany) for 1 day in DMEM supplemented with 10% fetal bovine serum (DMEM-FBS) for 1 day at 37 °C and 5% CO_2 in a CO_2 incubator (Nuaire, USA), and the cells were then washed with PBS. Fluorescein signals were observed using a confocal microscope (Carl Zeiss LSM 510 META, Germany).

To evaluate Fab′-dependent cellular uptake, the Fab′ fragments were conjugated to fluorescein-maleimide and Cy5-siRNA/SMLs.

Fifty microgram of Fab′ was incubated for overnight at 16 °C with 20 μg of fluorescein-maleimide in 100 μL PBS. The unbound fluorescein-maleimide was removed by dialysis using 10 K membrane.

Thirty microgram of Fab′ was incubated for overnight at 16 °C with 100 μL of the freshly prepared Cy5-siRNA/SML solution, and the unreacted maleimide groups were inactivated by adding 1/100 volume of 200 mM 2-mercaptoethanol. The unbound Fab′ was removed by dialysis using a 300 K dialysis membrane.

The Fab′-labeled fluorescein and Cy5-siRNA/SMLs were applied to the EGFR expressing NSCLC cells (A549, NCI-H322 and NCI-H460) and NIH-3 T3 mouse cells cultured in the 8 well chamber slide in DMEM-FBS for 1 day at 37 °C and 5% CO_2. The cells were fixed with 4%