Journal of Controlled Release 163 (2012) 267–276

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

A pH-sensitive cationic lipid facilitates the delivery of liposomal siRNA and gene silencing activity *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 22 May 2012
Accepted 13 September 2012
Available online 20 September 2012

Keywords:
Multifunctional envelope-type nano device (MEND)
siRNA delivery
pH-sensitive cationic lipid
Intracellular trafficking
Endosomal escape

ABSTRACT

Modification of liposomal siRNA carriers with polyethylene glycol, *i.e.*, PEGylation, is a generally accepted strategy for achieving *in vivo* stability and delivery to tumor tissue. However, PEGylation significantly inhibits both cellular uptake and the endosomal escape process of the carriers. In a previous study, we reported on the development of a multifunctional envelope-type nano device (MEND) for siRNA delivery and peptide-based functional devices for overcoming the limitations and succeeded in the efficient delivery of siRNA to tumors. In this study, we synthesized a pH-sensitive cationic lipid, YSK05, to overcome the limitations. The YSK05-MEND had a higher ability for endosomal escape than other MENDs containing conventional cationic lipids. The PEGylated YSK05-MEND induced efficient gene silencing and overcame the limitations followed by optimization of the lipid composition. Furthermore, the intratumoral administration of the YSK05-MEND resulted in a more efficient gene silencing compared with MENDs containing conventional cationic lipids. Collectively, these data confirm that YSK05 facilitates the endosomal escape of the MEND and thereby enhances the efficacy of siRNA delivery into cytosol and gene silencing.

1. Introduction

RNA interference (RNAi) can be used as novel therapeutic procedure through the specific *in vivo* silencing of therapeutically relevant target genes [1,2]. Small interfering RNA (siRNA) duplexes are promising candidates for therapeutic molecules that might be capable of achieving the sequence-specific inhibition of objective genes such as for oncogenes in carcinomas. The most significant issue for bringing out the potency of siRNAs is an efficient delivery system, because the high molecular weight (~13 kDa) and negative charge of siRNA molecules are serious limitations to the passive diffusion across the plasma membrane of most cells and susceptibility to enzymatic degradation in an *in vivo* environment [3,4]. Therefore, although delivering siRNA without a carrier may be possible in some cases [5,6], systemic delivery to various tissues, including tumors, demands a carrier to stabilize and transport siRNA to the target cells. Moreover, a carrier system is required, which can allow siRNA to avoid endosomal/lysosomal degradation and to be localized in the cytoplasm, where the RNAi machinery is located. Thus, a number of attempts to develop various nano carrier systems have been reported [7–12]. We also recently reported on the development of a unique siRNA delivery system, described as a multifunctional envelope-type nano device (MEND) [13,14].

It is generally accepted that prolonging the circulation time of a nano carrier will facilitate tumoral accumulation *via* the enhanced permeability and retention (EPR) effect [15]. Sterically stabilization of a lipid based nano carrier by poly(ethyleneglycol) (PEG) is the most popular method and is widely used to enhance circulation time by reducing nonspecific interaction between positively charged nano carriers and negatively charged serum components, leading to severe aggregation and rapid clearance from circulation by the reticuloendothelial system [16]. However, it is well-known that PEGylation leads to a severe decline of cellular uptake *via* endocytosis and the endosomal escape process of nano carriers, which results in the loss of efficacy for

Abbreviations: cDNA, Complementary DNA; CHE, Cholesteryl hexadecyl ether; Chol, Cholesterol; DMEM, Dulbecco's modified Eagle medium; DMG, Dimyristoyl-*sn*-glycerol; DSG, Distearoyl-*sn*-glycerol; DODAP, 1,2-Dioleoyl-3-dimethylammonium propane; DOPC, 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPE, 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, 1,2-Dioleoyl-3-trimethylammonium propane; DSPC, 1,2-Distearoyl-*sn*-glycero-3-phosphatidylcholine; EPR, Enhanced permeability and retention; FBS, Fatal bovine albumin; LF2k, Lipofectamine 2000; LNPs, Lipid nanoparticles; MEND, Multifunctional envelope-type nano device; MMPs, Matrix metalloproteases; mRNA, Messenger RNA; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PEG, Polyethyleneglycol; PLK1, Polo-like kinase 1; POPE, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; RACE, Rapid amplification of cDNA ends; RBC, Red blood cell; RISC, RNA-induced silencing complex; RNAi, RNA interference; siRNA, Short interference RNA; SNALP, Stable nucleic acid lipid nanoparticles; SOPC, 1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; TNS, 6-(p-Toluidino)-2-naphthalenesulfonic acid.

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http://dx.doi.org/10.1016/j.jconrel.2012.09.009

delivering siRNA into the cytoplasm [17,18]. In other words, PEGylation improves the pharmacokinetics but decreases the intracellular trafficking of the nanocarrier, a situation that we refer to as the 'PEG-dilemma' [19].

To resolve the PEG-dilemma, many groups have attempted to use functional devices, such as a cleavable PEG-lipid that is affected by an acidic or reducing condition [20,21]. We previously reported on the production of a functional PEG-lipid, PPD, that is affected by matrix metalloproteinases (MMPs), which are secreted by various tumor cells and succeeded in silencing marker gene expression in tumor tissue by the systemic administration of PPD modified MEND [22,23]. In addition, we reported that GALA, a pH-sensitive fusogenic peptide, modified MEND which could be applied to an *in vivo* intratumoral injection model and shGALA, a new shorter version of GALA, to improve the pharmacokinetics of the MEND, which was modified to achieve gene silencing in tumor tissue followed by systemic administration [23–26]. As other strategy, Semple S.C. et al. [27] reported that the pharmacokinetics of the lipid nanoparticles (LNPs) with an ionizable aminolipid, which is largely neutral but changes to a cationic form under acidic conditions, was improved compared to that with a conventional cationic lipid. The LNPs system also succeeded in tumor specific reporter gene expression, and gene silencing in orthotopic and subcutaneous tumors [28,29].

In the present study, we report on the development of a new pH-sensitive cationic lipid, YSK05, for improving the efficient intracellular trafficking and consequently the gene silencing activity of a MEND both *in vitro* and *in vivo* instead of peptide-based functional devices (PPD, GALA and shGALA). Our results suggest that by the suitable manipulation of intracellular trafficking, the successful delivery of siRNA can be achieved, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Anti-luciferase siRNA (sense: 5′-CCG UCG UAU UCG UGA GCA AdTdT-3′; antisense: 5′-UUG CUC ACG AAU ACG ACG GdTdT-3′) was purchased from Sigma (Ishikari, Japan). Anti-PLK1 siRNA (sense: 5′-AGA uCA CCC uCC UUA AAu AUU-3′; antisense: 5′-UAU UUA AGG AGG GUG AuC UUU-3′, 2′-OMe-modified nucleotides are in lowercase.) and Cy5-labeled anti-luciferase siRNA (sense: 5′-Cy5-GCG CUG CUG GUG CCA ACC CdTdT-3′; antisense: 5′-GGG UUG GCA CCA GCA GCA GCC CdTdT-3′) were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). Protamine was purchased from Calbiochem (San Diego, CA, USA). 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dioleolyl-3-dimethylammonium propane (DODAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-stearoyl-2-oleyl-*sn*-glycero-3-phosphatidylcholine (SOPC), 1,2-dioleyl-*sn*-3-phosphatidylcholine (DOPC) and cholesterol were purchased from Avanti Polar Lipid (Alabaster, AL, USA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1,2-distearoyl-*sn*-3-phosphatidylcholine (DSPC), 1,2-dimyristoyl-*sn*-glycerol, methoxyethyleneglycol 2000 ether (PEG-DMG), 1,2-distearoyl-*sn*-glycerol, and methoxyethyleneglycol 2000 ether (PEG-DSG) were purchased from NOF Corporation (Tokyo, Japan). ^{3}H-labeled cholesteryl hexadecyl ether (^{3}H-CHE) was purchased from PerkinElmer Life Science (Tokyo, Japan). 6-(p-Toluidino)-2-naphthalenesulfonic acid (TNS) was purchased from Wako Chemicals (Osaka, Japan). RiboGreen was purchased from Molecular Probes (Eugene, OR, USA). Lipofectamine 2000 (LF2k) and TRIZOL reagent were purchased from Invitrogen (Carlsbad, CA, USA). Dual-Luciferase Reporter Assay Reagent was purchased from Promega Corporation (Madison, WI, USA). HeLa human cervical carcinoma cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). OS-RC-2 human renal cell carcinoma cells were kindly provided by K. Hida (Hokkaido University, Sapporo, Hokkaido, Japan).

2.2. Experimental animals

Male ICR mice and BALB/cAjcl nude mice were purchased from Japan SLC (Shizuoka, Japan) and CLEA (Tokyo, Japan), respectively. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the guidelines for the care and use of laboratory animals.

2.3. MEND formulations

All MENDs were prepared using a cationic lipid, a phospholipid, cholesterol and PEG-DMG using a *t*-BuOH dilution procedure. The initial MENDs had a component molar ratio of 30/40/30/3 (DOTAP, DODAP or YSK05/DOPE/cholesterol/PEG-DMG) and optimized YSK05 MEND had 50/25/25/3 (YSK05/POPE/cholesterol/PEG-DMG). PEG-DMG was used to stabilize the lipid membrane during the formulation process and for preservation. Typically, 1.5 mM lipids were dissolved in 90% *t*-BuOH solution. siRNA was complexed with protamine at a nitrogen/phosphate ratio of 1.1 in 1 mM citrate buffer (pH 4.5) and was titrated slowly to lipid solution under vigorous mixing to avoid low local concentration of *t*-BuOH and diluted quickly with citrate buffer to final concentration of <20% *t*-BuOH. Ultrafiltration was performed to remove *t*-BuOH, replacing external buffer with phosphate buffered saline (PBS, pH 7.4) and concentrating the MENDs. To incorporate PEG-DSG, the MENDs were incubated at 45 °C for 45 min with PEG-DSG at 5.0 mol% total lipid under 10 v/v% ethanol conditions. Again, ultrafiltration was performed against PBS to remove EtOH and for concentration. An empty MEND with the same lipid composition was prepared by a similar procedure, with the exception that an equivalent volume of 1 mM citrate buffer was titrated to the lipid solution instead of siRNA/protamine complex. Radiolabeled MENDs were prepared by adding a trace amount of ^{3}H-CHE to the lipid-*t*-BuOH solution prior to mixing with the siRNA/protamine complex. The average diameter and zeta-potential of MENDs were determined using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments, Worcestershire, UK).

2.4. RiboGreen assay

To determine siRNA encapsulation efficiency and its concentration, RiboGreen fluorescence assay was performed. MENDs were diluted in 10 mM HEPES buffer at pH 7.4 containing 20 μg/mL dextran sulfate and RiboGreen in the presence or absence of 0.1 w/v% Triton X-100. Fluorescence was measured by Varioskan Flash (Thermo Scientific) with $\lambda_{ex} = 500$ nm, $\lambda_{em} = 525$ nm. siRNA concentration was calculated from siRNA standard curve. siRNA encapsulation efficiency was calculated by comparing siRNA concentration in the presence and absence of Triton X-100.

2.5. TNS assay

Thirty μM of MEND lipid and 6 μM of TNS were mixed in 200 μL of 20 mM citrate buffer, 20 mM sodium phosphate buffer or 20 mM Tris–HCl buffer, containing 130 mM NaCl, at a pH ranging from 3.0 to 9.0. Fluorescence was measured by a Varioskan Flash set up with $\lambda_{ex} = 321$ nm, $\lambda_{em} = 447$ nm at 37 °C. The pKa values were measured as the pH giving rise to half-maximal fluorescent intensity.

2.6. Stability of MENDs in mouse serum

Fresh mouse serum was collected from a male ICR mouse and mixed with free siRNA, a mixture of siRNA/protamine complex and the empty MEND, and MEND formulating siRNA followed by incubation at 37 °C for various periods. For disruption of the lipid bilayer of the MEND, 0.05 w/v% Triton X-100 was added to the incubation mixture. At selected time points, aliquots were frozen at −80 °C to stop siRNA degradation. Afterward, the samples were thawed at r.t. and