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pH-labile PEGylation of siRNA-loaded lipid nanoparticle improves active targeting and gene silencing activity in hepatocytes

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ABSTRACT

Lipid nanoparticles (LNPs) are one of the promising technologies for the *in vivo* delivery of short interfering RNA (siRNA). Modifying LNPs with polyethyleneglycol (PEG) is widely used to inhibit non-specific interactions with serum components in the blood stream, and is a useful strategy for maximizing the efficiency of active targeting. However, it is a widely accepted fact that PEGylation of the LNP surface strongly inhibits fusion between LNPs and endosomal membranes, resulting in poor cytosolic siRNA delivery, a process that is referred to as the 'PEG-dilemma'. In the present study, in an attempt to overcome this problem, siRNA-loaded LNPs were modified with PEG through maleic anhydride, a pH-labile linkage. The *in vitro*, suppression of cationic charge, stealth function at physiological pH up to 1 h and the rapid desorption of PEG and restoration of fusogenic activity under slightly acidic conditions (within only 2 min) were achieved by PEG modification of the LNPs through maleic anhydride. *In vivo*, PEG modification through maleic anhydride resulted in a dramatic improvement in the targeting capability of the active targeting of ligand (*N*-acetyl-D-galactosamine)-modified LNPs to hepatocytes, with an approximately 14-fold increase in gene silencing activity in factor 7 model mice. Taken together, the maleic anhydride-mediated pH-labile PEGylation of the active targeting LNPs is a useful strategy for achieving the specific and efficient delivery of siRNAs *in vivo*.

1. Introduction

Since the discovery of short interfering RNA (siRNA) [1], which induces specific gene silencing through RNA interference (RNAi) [2], the focus of many researchers has been on realizing RNAi-based medicine for treating refractory diseases. Because of the characteristics of siRNA, which include hydrophilicity, a negative charge and high molecular weight, adequate delivery technology is essential for improving the bioavailability of siRNA.

Active targeting is a useful strategy for achieving the cell-specific delivery of payloads. Basically, active targeting involves the use of an appropriate ligand that recognizes the target cells. However, specific delivery sometimes cannot be achieved because of non-specific interactions of the vehicle used with biomolecules and elimination from the blood circulation by the reticuloendothelial system (RES), resulting in a decreased efficiency for ligand-mediated specific delivery to target cells. Therefore, minimization of non-specific recognition by RES is important to maximize the efficiency of an active targeting agent.

Polyethyleneglycol (PEG) modification (PEGylation) is an accepted strategy for inhibiting non-specific recognition by RES [3,4]. PEGylation of the surface of nanoparticle results in formation of a fixed aqueous layer, which inhibits the adsorption of serum proteins through electrostatic and hydrophobic interactions [5]. However, after internalization into target cells, the aqueous layer also strongly inhibits interactions of the nanoparticles with the endosomal membrane, which reduces the efficiency of cytosolic delivery of the cargo. This issue is sometimes referred to as the PEG-dilemma [6–8].

Many kinds of strategies have been developed to overcome the PEG-dilemma. One such strategy is the environment-responsive removal of the PEG from nanoparticles inside and/or outside of the target cells [7]. Among the various environments, the difference in pH between the blood circulation and endosomes/lysosomes is one of the most extensively investigated factors. PEGylation through a pH-labile linkage, such as an acetal, orthoester and hydrazone, is a useful strategy for adding a pH-sensitive functionality [9–14]. However, while, in most studies, the pH-sensitive functionalities of these pH-labile linkages were examined *in vitro*, information concerning the pH-labile linkages *in vivo* is much less extensive. In addition, among these pH-labile linkages, a maleic anhydride derivative has some advantages including that the modification can be done in water and that fact that a pH-sensitivity of

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the final product can be controlled by slight modification of the chemical structure. Maleic anhydride derivatives have been applied only in a few polymer-based siRNA delivery systems but not in siRNA-loaded lipid nanoparticles (LNPs) [15–17].

LNPs are one of the promising systems for delivering siRNA [18–22]. As the result of the recent development of novel cationic lipids by rational design and combinatorial screening approaches, the efficiency of siRNA delivery has been dramatically improved, especially in liver tissues [23–27].

In the present study, we report on the development of siRNA-loaded LNPs for targeting hepatocytes using a hepatotropic ligand, *N*-acetyl-D-galactosamine (GalNAc) [28,29] and modifying the LNPs with maleic anhydride. The use of maleic anhydride for PEG-modification resulted in a significant improvement in the hepatocyte targeting efficiency of the GalNAc-modified LNPs and in an improvement in factor 7 (F7) gene silencing activity. F7 gene silencing activity was further improved by optimizing the structure of the maleic anhydride (14-fold higher activity compared to that of the GalNAc-LNPs without PEGylation through maleic anhydride). These findings suggest that the strategy that involves a combination of a specific ligand and pH-labile PEG shielding would be useful for improving the active targeting efficiency of LNPs for siRNA delivery *in vivo*.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycerol-3-phosphatidylserine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) and 1,2-dimyristoyl-*sn*-glycerol methoxypolyethylene glycol (DMG-$_m$PEG$_{2k}$) were purchased from NOF Corporation (Tokyo, Japan). Cholesterol (chol), 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DOPE) and 1,2-dioleoyl-*sn*-glycero-3-phsphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rho-DOPE) were purchased from Avanti Polar Lipids (Alabster, AL). Carboxylated dimethylmaleic anhydride (CDM)-$_m$PEG$_{2k}$, carboxylated ethylmaleic anhydride (CEM)-$_m$PEG$_{2k}$ and chol-PEG$_{400}$-amine were synthesized in our laboratory (Supplementary information). YSK05 was synthesized as described previously [30]. Trivalent GalNAc ligand was synthesized according to the PCT publication WO 2009/073809. Ribogreen, 3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlate (DiI) were purchased from Molecular Proves (Eugene, OR, USA). Sodium 2,4,6-trinitrobenzensulfonate (TNBS) was purchased from Wako Chemicals (Osaka, Japan). FITC-conjugated Isolectin B4 was purchased from Vector Laboratories (Burlingame, CA). All siRNA samples were purchased from Hokkaido System Science Co. Ltd. (Sapporo, Japan). The siF7 sense and antisense strand sequences are 5′-GGAucAucucAAGucuuAcTsT-3′ and 5′-GuAAGAcuuGAGAuGAuccTsT-3′, respectively. The siGFP-Cy5 sense and antisense strand sequences are 5′-ACAUGAAGCAGCACGACuUTsT-3′ and 5′-AAGUCGUGCUGCUUCAUGUTsT-Cy5–3′, respectively. 2′-Fluoro-modified nucleotides represented in lower case, phosphorothioate linkage represented as s.

2.2. Animals

Female ICR mice, 4 weeks of age, were purchased from Japan SLC (Shizuoka, Japan). 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. Preparation of LNPs

A 90% *t*-BuOH solution containing YSK05/chol/chol-PEG$_{400}$-amine/DMG-$_m$PEG$_{2k}$ at a molar ratio of 70/30/15/1.5 were prepared at a concentration of 7.5 mM total lipid. In case of GalNAc modification, 0.25 to 0.5 mol% of trivalent GalNAc ligand was added to the above solution. These lipid solutions were mixed with 0.4 mg/mL siRNA solution to be N/P ratio of 8. LNPs were prepared by gradually adding this mixture to 20 mM Citrate buffer (pH 4.0) under vigorous mixing. The resulting LNP solution were diluted with 0.1 M HEPES buffer (pH 9.5) and ultrafiltrated using Vivaspin Turbo-15 (MWCO 100 kDa, Sartorius) twice for removal of *t*-BuOH, adjustment of pH. The size and ζ-potential of the LNPs were measured by a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Worcestershire, UK). The encapsulation efficiency and total concentration of siRNA were measured by a Ribogreen assay, as described previously [30].

2.4. Modification of LNPs with maleic anhydride derivatives

To modify the LNPs with maleic anhydride derivatives, LNPs at a concentration of 10 mM total lipid suspended in 0.1 M HEPES buffer (pH 9.5) was added to a dried powder of various (0 to 2.5) equivalents of CDM-$_m$PEG$_{2k}$ or CEM-$_m$PEG$_{2k}$. The modified LNPs were preserved at 4 °C until used in experiments.

2.5. Measurement of the efficiency of maleic anhydride modification

The efficiency of surface modification was measured by detecting remaining primary amino groups using TNBS. The modified LNP solution was diluted with 0.1 M borate buffer (pH 9.5) dissolving 0.4 mM TNBS and incubated for 30 min at 25 °C with gentle shaking (700 rpm). After the incubation, TritonX-100 was added to a final TritonX-100 concentration of 1.0 w/v%, and the absorbance at 420 nm was then measured and normalized by unmodified LNPs.

2.6. Measurement of hydrolysis rate

The rate of hydrolysis maleic acid amide was measured by detecting primary amino group using TNBS. Maleic anhydride-modified LNPs were diluted with 0.1 M Citrate buffer (pH 4.0), 0.1 M MES buffer (pH 6.0) or 0.1 M HEPES buffer (pH 7.5), and then incubated for the indicated times at 37 °C with gentle shaking (700 rpm). After the incubation, each LNPs was diluted with 0.1 M borate buffer (pH 10) dissolving 0.4 mM TNBS, and was incubated for 30 min at 25 °C with gentle shaking (700 rpm). After the incubation, this mixture were added with TritonX-100 to be a final TritonX-100 concentration 0.5 w/v%, and then absorbance at 420 nm was measured and normalized by that of unmodified LNPs.

2.7. Measurement of cellular uptake

HeLa cells stably expressing Firefly and Renilla luciferase (HeLa-dluc) were cultured in cell-culture dishes (Corning) containing DMEM supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and G418 (0.4 mg/mL) at 37 °C in 5% CO_2. HeLa-dluc cells were seeded at 1.0×10^5 cells per well in 6-well plates in growth media 24 h prior to transfection. The LNPs were diluted with growth media to reach 10 nM siRNA and added to the cells after the aspiration of spent media. The cells were washed with PBS(−) twice at the indicated times and collected by trypsin treatment. The cells were centrifuged and, after removing the supernatant, were resuspended with FACS buffer (PBS(−) containing 0.5% bovine serum albumin and 0.02% sodium azide). The cells were filtered through a nylon mesh and measured by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

2.8. Measurement of fusogenic activity by fluorescent resonance energy transfer (FRET)

Anionic liposomes containing DOPS/DOPC/NBD-DOPE/Rho-DOPE at a molar ratio of 70/30/1.0/0.5 were prepared by simple hydration methods. Thirty hundred microlitre of Anionic liposome at a