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Research paper

SAINT-liposome-polycation particles, a new carrier for improved delivery of siRNAs to inflamed endothelial cells

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ABSTRACT

Interference with acute and chronic inflammatory processes by means of delivery of siRNAs into microvascular endothelial cells at a site of inflammation demands specific, non-toxic and effective siRNA delivery system. In the current work we describe the design and characterization of siRNA carriers based on cationic pyridinium-derived lipid 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chloride) (SAINT-C18) and the transfection enhancer protamine, complexed with siRNA/carrier DNA or siRNA only. These carriers, called SAINT-liposome-polycation-DNA (S-LPD) and SAINT-liposome-polycation (S-LP), have a high efficiency of siRNA encapsulation, low cellular toxicity, and superior efficacy of gene downregulation in endothelial cells *in vitro* as compared to DOTAP-LPD. Incorporation of 10 mol% PEG and anti-E-selectin antibody in these formulations resulted in selective siRNA delivery into activated endothelial cells. Furthermore, we showed that the physicochemical characteristics of S-LPD and S-LP, including size-stability and maintenance of the siRNA integrity in the presence of serum at 37 °C, comply with requirements for *in vivo* application.

1. Introduction

Nucleic acids such as short interfering RNAs (siRNA) are a promising new class of therapeutics, enabling specific interference with gene expression [1]. They can be therapeutically exploited for the inhibition of disease associated genes and provide the opportunity to address so far unmet therapeutic needs. However, unmodified or uncomplexed siRNAs (so-called "naked" siRNAs) are rapidly cleared from the circulation by the liver and renal filtration, and are sensitive to degradation by serum RNases, which limits their application *in vivo* [2]. Therefore for the development of clinically suitable siRNA therapeutics, safe and effective delivery systems that are specifically taken up by diseased cells, are crucial.

The pivotal role of endothelial cells in the pathology of inflammatory diseases and cancer along with the identification of disease-associated molecular targets (e.g., E-selectin, VCAM-1, $\alpha_V\beta_3$-integrins) on the endothelial cells [3] raised interest in the development of siRNA delivery devices for selective pharmacological intervention in the diseased endothelium. Systemic administration of siRNA via the bloodstream is a feasible route to reach the vascular endothelium, though only a few types of carriers suitable for *in vivo* siRNA delivery into endothelial cells have been developed so far [4]. We recently demonstrated specific delivery of siRNA to inflamed endothelial cells *in vivo* using two types of carriers based on cationic pyridinium-derived lipid 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chloride) (SAINT-C18), called SAINT-O-Somes [5,6] and SAINTPEGargs [7,8]. SAINT-C18 by itself is capable of delivering nucleic acids, and in combination with the helper-lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) it forms complexes with nucleic acids that are characterized by high transfection efficiency in the presence of serum and low toxicity *in vitro* [9]. Notably, administration of SAINT:DOPE (SD) also did not elicit any immune response or toxicity in mice [10,11]. For *in vivo* application addition of polyethylene-glycol (PEG) to an siRNA carrier is often essential, to avoid RES and improve carrier stability in the serum [12]. However, PEGylation of SD lipoplexes significantly reduces their transfection efficacy [7] and enforces formulation of the particles only with a low

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amount of PEG (2 mol%), resulting in short blood circulation times [8].

A major advantage of SD is the capacity to transfect not only siRNA or DNA but also proteins. Van der Gun et al. demonstrated that SD enables serum-insensitive protein delivery to various cell types, in contrast to other commercially available profection compounds [13]. Protamine is a small cationic protein with high arginine content that is FDA approved for parenteral administration [14]. Protamine aids DNA condensation and stabilization in sperm cells [15] and has attracted much attention as a nucleic acid transfection enhancer for gene delivery [14]. It was employed by Huang and coworkers to develop siRNA delivery systems called liposome-polycation-DNA (LPD) [16], that comprise of siRNA and carrier DNA complexed with protamine and lipids. These formulations show gene silencing at relatively low doses (0.15–0.45 mg siRNA/kg), display a more uniform size than lipoplexes, and could be grafted with up to 20 mol% of PEG allowing a substantial reduction of clearance by the RES [17].

In the current study we designed new carriers based on SAINT-C18 and protamine in order to improve the efficacy of gene silencing in endothelial cells and *in vivo* suitability of SAINT-based carriers. We formulated and characterized particles composed of a protamine complexed with siRNA/ctDNA or siRNA only, and encapsulated by SD liposomes, called SAINT-liposome-polycation-DNA (S-LPD) and SAINT-liposome-polycation (S-LP). These particles were examined for size, stability and influence of PEG grafting. Toxicity and VE-cadherin gene silencing efficacy in endothelial cells of S-LPD and S-LP was compared to liposome-polycation-DNA particles based on DOTAP: Cholesterol liposomes. When conjugated with anti-E-selectin antibodies, S-LPD and S-LP demonstrated selective siRNA delivery to activated endothelium. We showed that these novel SAINT-based systems allow efficient and specific siRNA delivery to inflamed endothelial cells and have physicochemical features that comply with demands for *in vivo* application.

2. Materials and methods

2.1. Materials

Lipids, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]-maleimide (DSPE-PEG_{2000}-Mal), and 2-distearoyl-sn-glycero-3-phosphoethanolamine-N [methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids (Alabaster AL, USA). The cationic lipid 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chloride (SAINT-C18) was obtained from Synvolux Therapeutics (Groningen, The Netherlands). Cholesterol (Chol), protamine (sulfate salt from Salmon), calf-thymus DNA (ct-DNA) and N-succinimidyl-S-acetylthioacetate (SATA) were purchased from Sigma (St. Louis, MO, USA). Nucleic acid stain DAPI was obtained from Roche Diagnostics (Manheim, Germany). All siRNAs were purchased from Qiagen (Venlo, The Netherlands).

The H18/7-acb (mouse IgG2a anti-human E-selectin) producing hybridoma was kindly provided by Dr. M. Gimbrone from Harvard Medical School (Boston, MA, USA).

2.2. Liposome preparation

Liposomes composed of SAINT and DOPE or DOTAP and cholesterol in a molar ratio of 1:1 were prepared by lipid film hydration. Lipids were dissolved in chloroform/methanol (9:1, v/v) and dried with nitrogen gas for 10 min followed by 30 min of vacuum. Dried lipid film was hydrated with RNase free water (Qiagen) for 10 min. Liposome size was reduced by repeated extrusion through a polycarbonate membrane (Whatman, Maidstone Kent, UK), pore size 100 nm, using a high pressure extruder (Lipex, Vancouver, Canada). The liposomes were stored at 4 °C under argon gas.

2.3. Preparation of LPD and LP particles

To prepare SAINT:DOPE based LPD and LP, referred to as S-LPD respectively S-LP, protamine (1 mg/ml) and nucleic acids (NA) were mixed at a ratio of 0.75 (w/w) and incubated for 10 min at room temperature (RT). A 1:1 (w/w) mixture of siRNA (0.3 mg/ml) and calf thymus DNA (1 mg/ml) in RNase free water was used to form LPD, while an equivalent total NA amount of siRNA only (0.3 mg/ml) was used for LP. Liposomes (10 mM total lipid (TL)) were added to the mixture at a ratio of 0.025 (μmol TL/μg NA). Samples were vortexed and incubated for 30 min at RT. DOTAP: Cholesterol based LPD were prepared as described by Li et al. [18] and are here referred to as DOTAP-LPD. Particles were PEGylated by postinsertion of DSPE-PEG_{2000} micelles (3.56 mM TL) for 10 min at 50 °C. DSPE-PEG_{2000} micelles were prepared by lipid film hydration, as described for liposomes in Section 2.2, and added to the particles at indicated mol% PEG ratios. LPD and LP were used for the experiments within 1 h after preparation.

Targeted S-LPD and S-LP were prepared by simultaneous post-insertion of anti-E-selectin DSPE-PEG_{2000} conjugate in a 1:4000 molar ratio of protein/TL and DSPE-PEG_{2000} micelles at indicated mol% PEG ratio. To prepare anti-E-selectin DSPE-PEG_{2000} conjugate, antibodies were thiolated by means of SATA and coupled to the maleimide group at the distal end of the polyethylene glycol (DSPE-PEG_{2000}-Mal) chain by sulfhydryl-maleimide coupling [19]. In brief, SATA-modified antibodies containing free sulfhydryl groups were added to DSPE-PEG_{2000}-Mal at a molar ratio of 1:20 and incubated at room temperature for 1 h. Excess of free DSPE-PEG_{2000}-Mal was removed by Zeba™ Desalt Spin Columns, 7K MWCO (Thermo Fisher Scientific, Rockford, IL, USA) [7]. Protein concentration of the anti-E-selectin DSPE-PEG_{2000} conjugate was determined by measuring absorbance at 280 nm using NanoDrop® ND 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.4. Characterization of S-LPD and S-LP particles

Particle size and ζ-potential were analyzed using a Nicomp 380 ZLS submicron particle analyzer (NICOMP particle sizing systems, Santa Barbara, CA, USA). Particle size was measured using dynamic light scattering (DLS) in the volume weighing mode. The polydispersity index (PDI) was analyzed using a Malvern Zetasizer Nano ZSP (Malvern Instruments Ltd., Worcestershire, UK). S-LPD and S-LP particles were freshly prepared using control siRNA (Qiagen, AllStars Negative Control, Cat No. 1027281) and, when indicated, formulated with various amounts of PEG-DSPE$_{2000}$. The siRNA encapsulation efficiency was measured using the Quant-iT™ Ribo-Green® assay (Invitrogen, Breda, The Netherlands). For gel retardation, particles containing 200 ng siRNA were mixed with 1% (v/v) Triton X-100 and 1% (v/v) SDS, and subsequently loaded on 2% agarose gel containing ethidium bromide (60 μg/ml). Electrophoresis was performed at 15 min at 110 V. Images of the gel were taken using the ChemiDoc XRS system (Bio-rad, Veenendaal, The Netherlands).

2.5. Cryo-transmission electron microscopy (Cryo-TEM)

The morphology of S-LPD and S-LP particles was determined by transmission cryo-EM. Samples were applied on glow discharged holey carbon-coated grids (Quantifoil 3.5/1), and the excess of