SAINT:DOPE Based Liposome Polycation Particles for siRNA Delivery

siRNA induced Knockdown by SAINT:DOPE based LPD and LP Particles in Endothelial Cells

First research project (45 ECTS)

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Date: January-August 2012
Abstract

Since the discovery of RNA interference as post-translational gene silencing pathway via small RNA sequences, it gave rise to the idea to use this mechanism for pharmacological intervention. Short interfering RNA (siRNA) is one class of small RNAs that binds to target messenger RNA (mRNA) which leads to gene silencing by cleavage of this mRNA. Systemic administration of “naked” siRNA will lead to rapid excretion and degradation by serum RNases. siRNA is unable to cross cell membranes and therefore has to be delivered into the target cells. This can be achieved by formulating siRNA into a drug carrier. Drug delivery systems that are currently used for siRNA delivery in vitro and in vivo are lipid based drug delivery systems, like liposome polycation DNA particles (LPD). These nanoparticles developed by Huang et al. have a size of ±200 nm, contain an inner solid core consisting of siRNA, carrier DNA and protamine. This negatively charged core is wrapped by a supported bilayer, containing cationic lipids for membrane interaction and particle stability. LPD particles can be shielded with high concentrations of poly ethylene glycol (PEG) molecules to avoid clearance by the reticuloendothelial system.

Endothelial cells play a major role in many inflammatory (vascular) diseases, what makes them an interesting target for pharmacological intervention. By coupling anti-E-selectin to the PEG molecules of LPD, disease specific endothelial cells can be targeted.

Investigating the potency of LPD particles to deliver siRNA to endothelial cells (HUVECs), we formulated LPD with cationic SAINT:DOPE liposomes. These liposomes are known to effectively deliver siRNA and proteins into cells. Besides LPD, we came up with a new particle: liposome protamine particle (LP). We studied these particles for size, shielding by PEG, targeting potential with anti-E-selectin to TNF-α challenged HUVECs and targeting specific knockdown of model gene VE-cadherin in HUVECs.

The results show that LPD and LP particles can be shielded by PEGylation. SAINT:DOPE LP particles can be targeted to activated HUVECs with anti-E-selectin antibodies. Our data demonstrated that SAINT:DOPE LPD and LP particles deliver sufficient amounts of siRNA to HUVECs, causing knockdowns of more than 90% of VE-cadherin mRNA at siRNA concentrations of 600 and 300 pmol/ml, respectively. Unfortunately, this knock down was not anti E-selectin specific.
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1 Introduction

1.1 RNAi for therapy

RNA interference (RNAi) is a process in which small RNA molecules induce gene silencing by degradation of messenger RNA (mRNA). There are two main classes of small RNAs, endogenous micro RNA (miRNA) and short interfering RNA (siRNA) (1). Long double stranded RNAs (dsRNA) are converted in the cytoplasm by the Dicer complex into siRNA molecules, which are processed into RNA induced silencing complexes (RISC) (2). The active complex will bind to the target messenger RNA (mRNA), which generally results in a cleavage of the mRNA and thereby posttranscriptional gene silencing (3) (Fig. 1). A single siRNA molecule can induce cleavage of more than one mRNA (4). The main advantage of siRNA over other small RNAs is the homology to the target mRNA. The degree of this homology is related to the gene silencing capacity of RISC (5). The principle of gene silencing using small RNA gave rise to the idea of exploiting RNAi mechanism for pharmacological interventions (6). Moreover, siRNAs can be chemically synthesized for any target mRNA. This makes siRNA based therapy prominent for many kinds of diseases (7,8).

1.2 siRNA

siRNAs are double stranded RNA molecules that have a natural or a synthetic origin and size of around 21 to 23 base pairs (1). In contrast to natural occurring siRNA, synthetic siRNA is directly incorporated into RISC together with Argonaute 2 (AGO2) endonuclease. The passenger (sense) strand of siRNA will be cleaved, resulting in the presence of active RISC with the guide (anti-sense) strand (Fig. 1). Long double stranded RNA is a ligand for Toll like receptors and therefore it can induce a cellular interferon response. This response can be avoided by the usage of synthetic siRNA. Also, it is less likely that synthetic siRNA will interfere with the endogenous miRNA pathway, because it enters the gene silencing pathway at a later stage (6,9).

Figure 1. RNA interference via the siRNA silencing pathway: dsRNA is converted by the DICER complex into siRNA. The siRNA is rearranged into the RISC and AGO2 splices the passenger (sense) strand, so that only active RISC containing the guide (anti-sense) strand of siRNA remain. siRNA in active RISC will bind to the target mRNA and induces post-translational gene silencing by cleavage of the mRNA. Synthetic siRNA mimics the Dicer product. Therefore, synthetic siRNA enters the RNAi process in a later stage (1).

To use siRNA as pharmaceutical intervention, some hurdles have to be overcome. Systemic administration of “naked” siRNA will lead to rapid excretion in the urine by kidney filtration, due to its small size (10). Next to excretion, the siRNA will be degraded in the blood by serum RNases within ½-1 hour (11,12). Furthermore, the high molecular weight of siRNA, its poly anionic nature and high hydrophilicity make siRNA unable to cross cell
membranes and reach its site of action, the cytoplasm. To overcome these hurdles, siRNA as a drug has to be delivered to the target cells. This can be achieved by formulating siRNA into a drug carrier (13).

1.3 Drug delivery systems

Over the years, many different types of drug delivery systems (drug carriers) were developed, such as proteins, viruses, polymers and liposomes (14). A carrier that would be suitable for delivery of siRNA should not interfere with the gene silencing capacity and should be able to deliver sufficient amounts to the target site to achieve a therapeutic effect. Moreover, it has to avoid clearance by the reticuloendothelial system (RES) which consist of monocytes, macrophages, and specialized endothelial cells in the bone marrow, spleen, and lymph nodes (1). An example of drug carriers that are frequently used to deliver siRNA in vitro and in vivo are lipid based drug delivery systems, such as liposomes (13). Liposomes are spherical particles which consist of a lipid bilayer, containing an aqueous cavity. Liposomes are therefore a suitable drug carrier for hydrophilic drugs, such as siRNA (15). Moreover, cell specificity can be achieved by attaching ligands to the liposome, enabling targeted drug delivery. Targeting enables directing a drug to the target site, where it will accumulate in a higher concentration compared to the non-targeted drugs. This means that the effective dose of the drug needed for therapy will be lower and less side-effect will occur after systemic administration. With the use of e.g. monoclonal antibodies, nanobodies, and proteins as ligands, it is possible to target receptors and other proteins that are expressed on the surface of target cell.

Clearance of the carrier from the blood is an important factor that limits efficient drug delivery. To escape from the RES, the drug carrier has to have a certain size. Drug carriers which are smaller than 70nm or larger than 300 nm will be respectively taken up by the spleen or liver (16). Lipid based drug carriers can be sized to 150 nm in order to minimize clearances. Furthermore, shielding the carriers with polyethylene glycol (PEG) results in reduced elimination by the RES, which makes them a suitable tool for siRNA delivery (13).

1.4 Endothelial cells in inflammation

An interesting target for drug delivery is the endothelium. The inner layer of all blood vessels is lined with a monolayer of endothelial cells (ECs), forming this endothelium. It was thought that the only function of endothelium was to present a passive barrier between blood and tissues, but now it is known that endothelium plays many important roles in physiological regulatory processes. For instance, ECs are involved in regulation of blood pressure, blood vessel growth (angiogenesis) and absorption of a variety of components (17,18). Increased insights of the function of ECs showed that the endothelium also plays an important role in inflammatory diseases and cancer. This was described by many research groups (19-22).

There are many differences between ECs. For example, ECs in brain capillaries form the blood brain barrier consist of tight junctions to realize this barrier function. ECs in liver capillaries form less extensive interendothelial tight junctions and form more sinusoidal vessels, designed for clearance (18). In the kidney, ECs of the glomeruli are designed to form a network for filtration of the blood. These differences are caused by the micro-environmental differences of the ECs. As a result, ECs have their unique intrinsic behaviour, dependent
on their environment (23,24). The micro-environmental conditions are characterized by the stress caused by the blood flow, interaction with cellular components and the location of the ECs within the organ. All these factors contribute to heterogeneity of ECs (25).

ECs in inflammation recruit leukocytes to inflamed tissue (26,27). This mainly occurs by ECs that are located in the post-capillary venules (18). Here, the ECs generally do not have much organ specific functions, which makes the recruitment of leukocytes less obstructive. Besides, the interaction of the leukocytes and the cell membrane in the post-capillary veins is highly efficient. The diameter of the vessel is similar to that of the neutrophils. When an inflammation occurs, the ECs are activated and the expression of adhesion molecules on the cell surface is increased. The adhesion molecules interact with the leukocytes in the blood, resulting in rolling and braking of the leukocytes (26,27). Two mayor factors for activating ECs are cytokines interleukin-1 (IL-1) and tumour-necrosis factor α (TNF-α) (28).

Heterogeneity of ECs is also characterized by the differences in expression of adhesion molecules, like ICAM-1, VCAM-1 and E-selectin (29) (Fig 2). Adhesion molecules are not only expressed during inflammation, but also in normal health state.

This heterogeneity in adhesion molecule expression gave rise to the possibility of using adhesion molecules as possible target for drug delivery systems. Drug carriers targeted against E-selectin resulted in disease specific targeting (30,31), as E-selectin is prominently expressed by inflamed ECs.
1.5 Liposome polycation DNA particles

siRNA can be used to interfere with gene expression in different diseases. The problem of delivery of the therapeutics to the site of action is a bottle neck of this application. This may be overcome by using specific drug delivery systems. One of the systems that is developed for in vivo delivery of siRNA is the Liposome Polycation DNA particle, also known as lipid-protamine-DNA particle (LPD). LPDs appear with average size ranging from 100-200nm (16), dependent on the composition and ratio of the components. LPDs consist of negatively charged solid core, containing siRNA, carrier DNA and protamine. Carrier DNA is used to increase the negative charge of the nucleic acids pool and therefore conduct a greater interaction with protamine, compared to siRNA alone. By mixing these negatively charged nucleic acids with positively charged protamine, a solid core with a net negative charge will be formed (Fig 3). This core is then mixed with liposomes containing positively charged cationic lipids. This leads to the formation of a supported lipid bilayer wrapping the solid core by direct charge-charge interaction (32). The direct interaction leads to stability of the particle (1).

The stability that is formed by the supported lipid bilayer gives LPDs the ability to sustain high concentrations of PEG. Higher concentration of PEG molecules in the lipid bilayer allow better shielding of the particle from the interaction with the immune system and from clearance by the RES. Due to the relative strong curvature of the surface of nanoparticles, PEG tends to appear in its mushroom conformation with overlapping random coils of PEG. When PEG appears in this conformation, the density and the thickness of the PEG layer on the surface of the particles is not enough to shield the particle from opsonization. Increasing the density of PEG on the surface of the carrier favours formation of a brush-like conformation, due to steric hindrance. This conformation will provide better shielding of the particle. As PEG is a detergent-like surfactant, it can form micelles with other lipids. Because LPDs have a more stabilized lipid bilayer compared to liposomes, LPDs are capable of baring higher percentages of PEG-lipids. PEGylation results in a drop in surface charge of the particle from +40mV in unprotected LPD to nearly 0mV in 10mol% PEGylated LPD (1). A more neutral surface charge of
a particle leads to less opsonization of the particle by serum proteins and less uptake by phagocytotic cells of the RES and a prolonged circulation time (1). Generally LPDs are PEGylated to a density of 10 mol% of PEG to the total lipid concentration. In liposomes, the degree of PEGylation generally never exceeds 5%. This better shielding makes LPDs more promising for systemic delivery of siRNA (1,16). Furthermore, LPDs can be targeted to ECs by post-inserting PEG that is coupled to a specific antibody (e.g. against the E-selectin receptor expressed by inflamed ECs), as showed in this study. Delivery via receptor mediated endocytosis requires a drug carrier that can evade lysosomal degradation (Fig 4). This is especially important for the siRNA that cannot readily cross the endosomal membrane and is prone to degradation.

Several research groups have investigated LPDs as a targeted drug delivery system for siRNA to antigen presenting cells, tumour cells or angiogenic ECs in relation to tumour growth, in vitro and in vivo and showed successful delivery of siRNA. (12,16,33-36). These studies showed that after a single injection of cationic lipid containing nanoparticles (LNP) knockdown up to 90% in murine macrophages is realized (33). Also, mice injected with LPD-PEG-anisamide (LPD-PEG-AA) showed accumulation of 70-80% of the particle in the tumours (34,36), with 53% reduction of the tumour load (36).

1.6 Aim of the project

In this project we investigated LPDs as a lipid-based drug delivery system for targeted siRNA delivery to diseased ECs. Besides LPDs, we study liposome protamine (LP) particles which are newly developed in this project. LPDs are formulated with a cationic amphiphilic SAINT (1-methyl-4-(cis-9-dioleyl)methyl-pyridiniumchloride) lipid and neutral helper lipid DOPE (dioleoylphosphatidylethanolamine). As showed by Van der Gun, lipid based drug delivery systems containing SAINT and DOPE were able to deliver siRNA and protein to ECs, thus make an interesting candidate for LPD and LP formulation. We investigate the benefits of targeting...
SAINT:DOPE LPDs to activated ECs with E-selectin antibody and the efficacy of siRNA delivery to ECs in vitro. During the project, SAINT:DOPE particles were compared with DOTAP:Chol particles that were already described in the literature. Our research group previously formulated lipid based drug delivery systems containing SAINT as cationic lipid (e.g. SAINT-O-Somes and SAINTPEGargs) (13), which have shown endothelial specificity and siRNA-mediated gene silencing \textit{in vitro}, but have a limited efficacy of siRNA delivery (unpublished data). By developing SAINT:DOPE based LPDs for siRNA delivery, we aim to formulate a drug delivery system that shows greater gene silencing in ECs \textit{in vitro}, and will have potential for \textit{in vivo} application.

The possibility of shielding the carrier by post-insertion of PEG was investigated. Targeting possibilities were investigated by targeting the particles to activated ECs by post insertion of anti-E-selectin-Mal-PEG. As the SAINT:DOPE particles were created in this project for the first time, we aimed to optimize the particles size and surface charge of these LPD and LP particles. Furthermore, different methods of formulation SAINT:DOPE LP particles were studied.
2 Material and methods

2.1 Materials

Lipids 1,2-dioleyl-3-trimethylammonium-propane (DOTAP), dioleoylphosphatidylethanolamine (DOPE), 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]-maleimide (Mal-PEG) were purchased from Avanti Polar Lipids (Alabaster AL, USA). Cationic lipid 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chlorid (SAINT-C18) was purchased from Synvolux Therapeutics Inc. (Groningen, the Netherlands). Cholesterol (Chol) and N-succinimidyl-S-acetylthioacetate (SATA) were purchased from Sigma (St. Louis MO, USA). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarboxyline perchlorate (Dil) was obtained from Molecular Probes (Leiden, the Netherlands). All siRNAs and were purchased from Qiagen (Venlo, the Netherlands). Calf-thymus DNA (ct-DNA) was obtained from Sigma (St. Louis MO, USA) Phosphothiolated antisense FITC-ODN (FITC-ODN) (5'-ACTACTACACTAGACTAC-3’, FITC on 5’) was purchased from Biomers (Ulm, Germany). The monoclonal mouse anti-human E-selectin antibody was kindly provided by Dr. M. Gimbrone from Harvard Medical School (Boston MA, USA).

2.2 Cell cultures

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Walkersville inc. (CC2519, Lonza, Basel, Switzerland). Cells were cultured using EGM-2 MV medium, supplemented with EGM-2 MV Single Quot Kit Supplements & Growth Factors (catNo. CC-3202, Lonza). Cells were cultured on plastic tissue culture dishes plates (Costar, Corning, NY) at 37°C, 5% CO₂. Before seeding, dishes were pre-coated with culture medium for 15 min at 37°C. Cells from passage 5 up to passage 7 were used for the experiment. In all experiments, cells were seeded at a density of 1.8 x 10⁴ cells/cm², unless described otherwise.

2.3 Preparation of cationic liposomes and PEG-DSPE mixture

LPD and LP particles were formulated by preparing a negatively charged core containing nucleic acids and protamine. This core is mixed with cationic liposomes and due to liposomal collapse, the particles are formed. In this project, we used cationic liposomes containing DOTAP:Chol, or SAINT:DOPE. These liposomes were prepared by film hydration. The lipid stocks, dissolved in chloroform and methanol, were mixed in 1:1 molar ratios and dried, under a stream of nitrogen to form a lipid film. Subsequently, residual chloroform was removed by further drying under vacuum for at least 30 min at 30°C. The lipid film was hydrated with RNase free water (Qiagen) for at least 15 min.
at room temperature. Formed liposomes were subjected to 10 cycles of freezing and thawing and were vortexed at maximum speed after each cycle. Liposomes were sized by extrusion through polycarbonate filters (Whatman, Maidstone Kent, UK). First, liposomes were extruded 2-3 times through 100 nm pore filters, followed by 5-10 times through 50 nm filters. Extrusion was performed at 40°C, using a high pressure extruder (Northern Lipids, Vancouver, British Columbia, Canada). The liposomes were stored at 4°C in the presence of argon gas to prevent oxidation of the lipids. Liposomes were used up to 3 months after formulation. For fluorescence microscopy and TissueFax, liposomes were formulated with 0.05 mol% Dil for fluorescent tracking of the lipids. For DOTAP:Cho, the concentration of the total lipids was estimated based on 80% recovery, whereas for SAINT:DOPE, the concentration was determined by a phosphate assay.

For PEGylation of the particles, PEG micelles were prepared. Mal-PEG or PEG micelles were prepared by film hydration, which was done in the same way as described for the liposomes. The lipids were hydrated with RNase free water for at least 15 min and vortexed at maximum speed. The micelles were stored at 4°C in the presence of argon, to minimize oxidation of the lipids. The micelles were used up to one month after formulation.

Monoclonal anti-E-selectin antibodies were thiolated by means of SADA. The antibodies were coupled to the Mal-PEG micelles, which were prepared as described above. The antibodies were deacetylated by incubation with freshly made deacetylation reagent (0.5 M Hydroxylamine/HCL, 0.5 M HEPES, 0.025M EDTA, pH 7.4) and were gently mixed for 30 min. The deacetylated antibodies were added to Mal-PEG (1:10 molar ratio) and incubated and were gently mixed for 4 hours. To stop the coupling reaction, N-ethylmaleimide (80 mM in HN buffer pH 6.7) was added at room temperature, for 15 min, while gently mixed. Anti-E-selectin-Mal-PEG was stored at 4°C in the presence of argon used up to 1 month after formulation.

2.4 Formulation and characterization of LPD and LP

LPD particles were prepared as described by Li and Huang (34) with some modifications. Briefly, siRNA (0.08mg/ml), CT-DNA (0.08mg/ml), protamine (0.12mg/ml) and RNase free water were mixed in a 1.5 ml tube. The complex was allowed to stand at room temperature for 10 min before mixing the complex with cationic liposomes (3.98 μM). The LPD particles were kept at room temperature for another 10 min before further applications. Where indicated, particles were PEGylated by post-insertion with PEG (±1:10 (m/m) PEG to total lipid) at 50°C for 10 min, or with (±1:10 (m/m) PEG to total lipid) and anti-E-selectin-Mal-PEG 1:200 (m/m) to total lipid at 37°C for 1 hour. The particles were used for experiments within 1 hour after formulation. The 1:200 ratio of anti-E-selectin-Mal-
PEG to total lipid was used for fluorescent microscopy, TissueFax and gene expression analysis, unless it was described otherwise.

Liposome protamine particles (LP) were formulated from the same components as LPD excluding ct-DNA. To compensate for the loss in volume by leaving out ct-DNA from the mixture, extra RNase free water was added. Particle size and surface charge were measured using a Nicomp 380 ZLS particle sizer system.

Particle size measurement were performed in MilliQ water, using sufficient amount of particles to give a minimal intensity of 30 kHz (vesicle mode, volume weighting). To determine the surface charge of the particles we measured the ζ-potential of the particle in MilliQ water. MilliQ water was set as reference for the measurements.

2.5 Fluorescent microscopy

Cells were seeded on Lab-Tek Chamber Slides (NUNC, Rochester NY, USA) at a density of 1.6 x 10⁴ cells/cm², one day before the experiment. Cells were activated with TNF-α (10ng/ml) 2 hours before incubation with LPD or LP particles. TNF-α remained present in the medium during incubation with the particles. The cells were incubated for 4 hours with different formulations of LPD or LPs, containing siRNA that was labelled with an Alexa Fluor 488 tag (siRNA Alexa₄₈₈) at a concentration of 125 pmol/ml. Nuclei were stained using Hoechst 33342 (1:100 Hoechst stock to medium, 10 min) (Roche, Mannheim, Germany) for live cell imaging. Cells were washed 3 times with cold RPMI 1640 (Lonza) and kept on ice until imaging. Fluorescence images of the cells were taken with a Leica DM/RXA fluorescence microscope (Wetzlar, Germany) using Quantiment HR600 image analysis software (Leica). Images were taken at excitation/emission wavelengths of 490/520 nm for siRNA Alexa₄₈₈ and 350/461 nm for Hoechst 33342. Images were analysed and processed using ImageJ software v1.44.

2.6 Flow cytometry

For the flow cytometry experiments, cells were seeded in 24-wells, one day before the experiment. Cells were activated with TNF-α (10ng/ml) 2 hours before incubation with LPD or LP particles. TNF-α remained present in the medium during incubation with the particles. The cells were incubated for 4 hours with the anti-E-selectin LPD or LPs containing fluorescent labelled siRNA Alexa₄₈₈, at a siRNA concentration of 83.3 pmol/ml and formulated with different ratios of anti-E-selectin-Mal-PEG to total lipid (TL) (1:100, 1:200. 1:1000). After incubation, cells were washed twice with PBS and detached by 5 min incubation of Trypsin-EDTA. Next, the cells were immediately transferred to 3 ml ice-cold FACS buffer (PBS 1% fetal calf serum) and washed twice. The cells were fixed in 200 μl 0.5%
paraformaldehyde in PBS and stored at 4°C. During the experiment, the cells and particles were kept in the dark, to maintain the fluorescence. Cells were analysed by flow cytometry (Calibur, BD Bioscience, Franklin Lakes, NJ). The results were analysed with FlowJo software.

2.7 Anti-E-selectin specific release analysis of SAINT:DOPE LP particles with TissueFax.

Targeting specific release of FITC-ODN from SAINT:DOPE LP particles was investigated with TissueFax. Cells were cultured on Lab-Tek Chamber Slides (2 chambers) (NUNC) and activated with TNF-α (10 ng/ml) 2 hours before incubation with LPs. TNF-α remained present in the medium during incubation with LPs. The cells were incubated with anti-E-selectin LPs containing FITC-ODN for 4 hours at a concentration of 100 pmol/ml. E-selectin epitopes on the cell surfaces were blocked by adding 100x excess of anti-E-selectin antibody to the cells a few minutes before particles were added. Nuclei were stained using Hoechst 33342. Cells were imaged live and kept in a 37°C and CO₂ equipped chamber in the AxioObserver Z1 microscope (Zeiss, Germany). Images were taken every 30 min for 3 hours with TissueFAX acquisition software (TissueGnostics, Vienna, Austria). Images were taken at excitation/emission wavelengths of 490/520 nm for FITC-ODN and 350/461 nm for Hoechst 33342. Data was quantified and analysed with TissueQuest fluorescence analysis software (TissueGnostics).

2.8 Gene expression analysis by real-time RT-PCR

Cells were seeded in 12-well, one day before the experiment. After 2 hours of incubation with TNF-α (10 ng/ml), anti-E-selectin LPD and LPs containing VE-cadherin specific siRNA and negative control siRNA were added to the cells and incubated for 4 hours. siRNA concentrations in the particles were ranging from 25 to 600 pmol/ml. TNF-α remained present in the medium during further incubation with particles. Next, particles were removed and the cells were washed twice with PBS, subsequently fresh medium was applied. Cells were kept in culture up to 48 hours after incubation. Next, total RNA was isolated using the RNeasy® Mini Plus Kit (QiaGen) according to the protocol of the manufacturer. The amount of RNA was measured by Nanodrop® ND-1000 spectrometer (Wilmington DE, USA). Then, synthesis of cDNA from RNA was performed with SuperScript™ III RNase H-Reverse Transcriptase (#18080-044, Invitrogen, Breda, The Netherlands) and 40 U of RNaseOut inhibitor (#10777-049, Invitrogen) in a volume of 20 μl containing 250 ng random hexamers (#C1181, Promega, Leiden, the Netherlands), according to protocol provided by the manufacturer, with some modifications made by Peter Zwiers (EBVDT, Dept. Pathology and Medical Biology, UMCG, Groningen, The Netherlands) using a Polymerase Chain Reaction (PCR) apparatus (GeneAmp® PCR system 9700, Applied Biosystems) specified by the manufacturer. Synthesized cDNA was diluted with
RNase free water to 2-10 ng/ml and 1 μl of the dilution was used for each PCR reaction. The following primers, purchased from Applied Biosystems (Assay-on-Demand, Nieuwekerk a/d Ijssel, the Netherlands) were used for real-time PCR: VE-cadherin (Hs00174344_m1), CD31 (Hs00169777_m1), Tie2(Hs00176096_m1) and GAPDH (Hs99999905_m1). The PCR reaction was carried out in Absolute QPCR Rox Mix (CM-205, Thermo Scientific, Amsterdam, The Netherlands) with a final concentration of primers 250 nM in per sample. The amplification reaction was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems), using the program: 15 min 95°C, 40 cycles of 15 sec 95°C and 1 min 60°C. For each sample, the PCR reaction was performed in duplicate and the averages of the obtained threshold cycle values (Ct) were processed for further calculations. Gene expression values were normalized to the expression of the reference gene GAPDH giving the ∆Ct value (ΔCt = Ct\_target – Ct\_reference) and the average value of ΔCt obtained from non-treated cells was subtracted from the average value of the ΔCt of treated cells and calculated as relative mRNA levels by 2-ΔΔCt.

2.9 Particle size and surface charge optimization of SAINT:DOPE LPD and LP particles

To prepare particles with specific size and surface charge that are suitable for drug delivery, these particle formulations were optimized in three steps. In the first step the protamine to nucleic acid weight ratio was investigated. The ratio which gave the smallest particles and with the lowest dispersity of size was selected and used for optimization of the weight ratio of siRNA to total nucleic acid (only for LPDs containing ct-DNA). Here, the most suitable ratio was selected for step three, optimization of the molar ratio of PEG. The specific ratios investigated in this experiment were 0, 0.3, 0.6, 0.9, 1.2, 1.8 and 2.4 of protamine to nucleic acids. 0, 0.25, 0.5, 0.75 and 1 of siRNA to total nucleic acids for only LPD. Molar percentages 0, 5, 10, 20 and 30 of PEG to the total lipid concentration of the particles. After each step the particle sizes were measured and after step three when PEGylated with PEG also the ζ-potential was measured.

2.10 Formulation method study for LP particles with SAINT:DOPE

Two different methods of formulating LPDs are described in literature. Here, we investigated those two methods for the formulation of SAINT:DOPE LP particles. We refer to these methods as method A and B. Method A is described before in paragraph 2.3. and used throughout this project. In method B, two solutions were made (Fig 5). Solution I consisted out of siRNA and RNase free water, solution II consists out of liposomes and protamine. These mixtures were allowed to stand at room temperature for 10 min, before mixing together. This mixture of siRNA, liposomes and protamine was allowed to stand at room temperature for 10 min, before the particles were PEGylated. Particle sizes were measured by dynamic laser light scattering.
Throughout the experiment, particles were PEGylated by post insertion of PEG, 10 min after the liposomes were added to the mixture. These particles were put at 50°C for 10 min. We refer to this type of PEGylation as “conventional PEGylation”. Besides conventional PEGylation, we investigated PEGylation by post-insertion when PEG was directly added to the mixture, at the same time with the liposomes. The mixture was directly put at 50°C for 10 min. This way of PEGylation we refer to as “direct PEGylation” (Fig 5). Particle sizes were measured.

**Figure 5. Methods of formulating SAINT:DOPE LP particles.** (A) Method A: siRNA, protamine and RNase free water were firmly mixed and allowed to stand at room temperature for 10 min. Liposomes were added and gently mixed and allowed to stand at room temperature for 10 min. LPs were PEGylated by post-insertion with PEG and were gently mixed and put at 50°C for 10 min. (B) Method B: Solution I was made by firmly mixing siRNA and RNase free water. Solution II was made by firmly mixing liposomes with protamine. Both solutions were allowed to stand at room temperature for 10 min. The solutions were gently mixed together and allowed to stand at room temperature for 10 min. LPs were PEGylated by post-insertion with PEG and were gently mixed and put at 50°C for 10 min. For both methods, black arrows show the pathway of conventional PEGylation of LP particles. Green arrows indicate direct PEGylation of LP particles.

### 2.11 Statistical analysis

Statistical analysis was performed by a two tailed unpaired Student’s t-test, or one way ANOVA with Bonferroni’s Multiple Comparison Test. Excel (Microsoft Corporation, Redmond, USA) or Graphpad (Prism, La Jolla, USA) were used for statistical calculations. Differences were considered significant when \( P<0.05 \).
3 Results

3.1 Characterization of particles

We investigated the potency of SAINT based LPD particles and a novel formulation without ct-DNA called LP as siRNA carriers. As described in the “introduction”, particles size in a range of 70-300 nm is one of the criteria for such a drug carrier. From all particles formulated in this study, the mean diameter size was measured by dynamic light scattering. Data show that the mean size of non-targeted particles formulated with DOTAP:Chol liposome (LPD: 215 ± 50 nm; LP: 117 ± 24 nm) are smaller in size compared to particles formulated with SAINT:DOPE (LPD: 391 ± 86 nm; LP: 274 ± 56 nm). Moreover, LP particles are significantly smaller than LPD particles when formulated with DOTAP:Chol or SAINT:DOPE (Fig.6). Furthermore, particles size increases when particles were modified by post insertion with anti-E-selectin-Mal-PEG at a ratio of 1:200 of Mal-PEG to the total lipid concentration, for DOTAP:Chol LPD and LP (LPD: 509 ± 266 nm; LPD: 543 ± 311 nm) and SAINT:DOPE LP (574 ± 205) particles. There was no difference in size between non-targeted and targeted SAINT:DOPE LPD particles. There was no significant difference between LPD and LP particles for both DOTAP:Chol and SAINT:DOPE when the particles contained the same Mal-PEG ratios.

![Graph A](dotap-chol-particles.png)  
![Graph B](saint-dope-particles.png)

**Figure 6. Comparison of particle size between different formulations of LPDs and LPS.** Particles were formulated as described in “Material and Methods”. The core was mixed with cationic liposomes DOTAP:Chol (A) or SAINT:DOPE (B) to form LPDs and LPS. Targeted particles were formulated with several ratios of anti-E-selectin-Mal-PEG according to the total lipid concentration. Mean particle sizes were determined by dynamic light scattering. Data are presented as mean value of independent experiments ±SD (DOTAP:Chol LPD non-targeted, anti-E-selectin 1:100, 1:200, 1:1000 respectively n=11, 2, 5, 1; DOTAP:Chol LP non-targeted, anti-E-selectin-Mal-PEG 1:100, 1:200, 1:1000 respectively n=10, 1, 4, 1; SAINT:DOPE LPD non-targeted, anti-E-selectin-Mal-P 1:100, 1:200, 1:1000 respectively n=9, 3, 7, 4; SAINT:DOPE LP non-targeted, anti-E-selectin 1:100, 1:200, 1:1000 respectively n=10, 3, 8, 4; *P<0.05).
3.2 Influence of PEGylation and targeting on interaction of LPD and LP particles with endothelial cells.

It was demonstrated that LPD particles can withstand high concentrations of PEG molecules, resulting in shielding of the particle. To investigate the shielding effectiveness of the particles using post-insertion we formulated DOTAP:Chol LPD and LP particles with and without PEG and investigated interaction of the particles with ECs by fluorescence microscopy (Figure 7.A). We show that for both LPD and LP particles, PEGylation results in less interaction with quiescent HUVECs, which indicates shielding of the particles. Furthermore, overlapping patterns (orange-yellow) of the lipid membranes (red) and siRNA (green) fluorescence indicated co-localization of the lipids with siRNA. We observed in all condition some co-localization especially with LP particles without PEG. Furthermore we observed that fluorescent siRNA in PEGylated particles appear in a dotted pattern, where non-PEGylated particles show a diffused pattern of the siRNA.

Figure 7. PEGylated DOTAP:Chol particles are shielded from EC interaction while anti-E-selectin targeted particles show targeted specific interaction with ECs. (A) Fluorescence microscopy live images of quiescent HUVECs, incubated for 4 hours with LPDs and LPs containing siRNA Alexa 488 (green) at a siRNA concentration of 125 pmol/ml. Particles were formulated using DOTAP:Chol liposomes labeled with Dil (red). LPDs and LPs were PEGylated by post-insertion of 10% PEG micelles, or were prepared without addition of PEG. (B) LPDs and LPs were PEGylated by post-insertion of anti-E-selectin-Mal-PEG (ratio 1:200 to the total lipid concentration). In both experiments, the nuclei were stained with Hoechst (DAPI, blue). Data represent images from two independent experiments (A and B). Magnification of 100x or 200x.
To investigate whether particles can be targeted to ECs, DOTAP:Chol LPD and LP particles were formulated containing siRNA Alexa 488, Dil, and anti-E-selectin-Mal-PEG. Quiescent or with TNF-α activated HUVECs were incubated with the particles for 4 hours and subjected to live cell imaging (Fig. 7.B). Both DOTAP:Chol LPD and LP particles showed more interaction with activated HUVECs compared to resting cells. This indicates that post-insertion of anti-E-selectin-Mal-PEG to DOTAP:Chol LPD and LP particles led to more interaction of the particles with the activated ECs. Comparing LPD and LP, both quiescent and TNF-α activated HUVECs showed more interaction with LP particles than with LPD particles.

To determine an optimal ratio of post inserted anti-E-selectin PEG-micelles for the targeting of activated ECs LPD and LP particles were formulated with DOTAP:Chol and SAINT:DOPE liposomes with different ratios of anti-E-selectin-Mal-PEG micelles. Subsequently quiescent and activated HUVECs were incubated for 4 hours with the particles containing siRNA Alexa 488 and analysed by flow cytometry. The results showed that both LPD and LP particles formulated with DOTAP:Chol (Fig. 8.A) had more interaction with activated HUVECs for all ratios of anti-E-selectin-Mal-PEG. The targeting effect of LP particle with HUVECs was somewhat more pronounced compared to the targeting effect of LPD particles. SAINT:DOPE LPD and LP particles also showed a trend towards higher interaction with activated HUVECs (Fig.8.B). For SAINT:DOPE LP particles with a ratio of 1:200, we observed significant difference between the interaction to quiescent and activated cells. Significant differences in interaction between the different ratios were not observed.

Figure 8. Quantification of interaction of the particles incorporating different ratios of anti-E-selectin-Mal-PEG with endothelial cells. Quiescent and TNF-α activated HUVECs were incubated for 4 hours with LPDs and LPs, containing siRNA Alexa 488 at a concentration of 83.3 pmol/ml. The particles were formulated with (A) DOTAP:Chol and (B) SAINT:DOPE liposomes. LPDs and LPs were PEGylated by post-insertion of anti-E-selectin coupled Mal-PEG in ratios 1:100, 1:200 and 1:1000 to the total lipid concentration of the particles. The interaction of siRNA with ECs was measured by flow cytometry. Data are presented as mean fluorescence intensities, normalized to control non-targeted particles ±SD. Data set represent a single experiment for DOTAP:Chol based particles or three independent experiments for SAINT:DOPE; n=3 (SAINT:DOPE 1:100 /+ TNF-an = 2).
3.3 ODN release in ECs from targeted SAINT:DOPE LP particles, quantified by TissueFax.

By studying the delivery of FITC-ODN to HUVECs, we investigated the target specific release properties of the SAINT:DOPE LP particles (Fig 9). The results showed that in 1.2% and 2.2% of the cells release of FITC-ODN occurred, respectively with and without excess of free antibodies. As this experiment was only done once, no significance can be calculated.

![Figure 9. Targeting dependent release of ODN from SAINT:DOPE LP particles in activated HUVECs. TNF-α activated HUVECs were incubated for 4 hours with anti-E-selectin targeted (1:200 ratio) SAINT:DOPE LP particles containing FITC-ODN (100 pmol/ml). Cells were blocked with 100x excess free anti-E-selectin (+ anti-E-selectin). Nuclei were stained with Hoechst 33342. Live cell imaging was done every 30 min for 3 hours. Single positive (Hoechst 33342) and double positive cells (Hoechst 33342 and FITC-ODN) were quantified. Data are presented as percentage of double positive cells. Cell count: 6679 total amount with 149 double stained cells (–anti-E-selectin), 6142 total amount with 73 double stained cells (+anti-E-selectin).]

3.4 Efficacy of VE-cadherin down regulation by SAINT:DOPE based LPD and LP particles.

Here we studied the potency of the LPD and LP particles for functional siRNA delivery to diseased ECs. Quiescent and TNF-α activated HUVECs were incubated with DOTAP:Chol and SAINT:DOPE based LPD and LP targeted to E-selectin and containing scrambled or siRNA against VE-cadherin. Non-targeted particles containing siRNA against VE-cadherin were used as a control for targeted delivery (Fig.10. A-D). The knockdown of VE-cadherin mRNA was determined by real-time RT-PCR, normalized to the expression of housekeeping gene GAPDH and compared to untreated cells.

The results indicated that DOTAP:Chol LPD and LP particles containing VE-cadherin siRNA, at a siRNA concentration of 600 pmol/ml, do not lead to knockdown of VE-cadherin in both quiescent and activated HUVECs (Fig.10.A and B). Cells incubated with SAINT:DOPE LPDs and LPs at a siRNA concentration of 600 and 300 pmol/ml respectively showed knockdown of VE-cadherin mRNA. Cells treated with non-targeted SAINT:DOPE LPDs showed 82% knockdown of VE-cadherin by non-targeted LPDs and 91% of targeted LPDs. For non-targeted and targeted LPs, VE-cadherin knockdown was for both around 95%. There was no difference in the extent of knockdown between quiescent
and TNF-α activated HUVECs, for both targeted and non-targeted LPD and LP particles. This may indicate that the knockdown was anti-E-selectin targeting independent or that the administered concentration was too high to see targeting effect. No knockdown was observed in cells treated with particles containing negative control siRNA, thereby excluding non-specific down regulation of VE-cadherin.

Furthermore we investigated whether the effect of targeting could occur when lower concentrations of siRNA were administered. Quiescent and TNF-α activated HUVECs were incubated with anti-E-selectin targeted LPD and LP particles containing different concentrations of siRNA from 25 pmol/ml up to 600 pmol/ml for 4 hours (Fig.10.E and F). The results show that both LPD and LP particles display dose dependent knockdown of VE-cadherin, but no significant anti-E-selectin specific effect was observed. For LP particles, knockdown in activated HUVECs was higher than in quiescent HUVECs when incubated at a siRNA concentration of 100 pmol/ml, but no significance can be calculated from these results. Comparing knockdown caused by LPD and LP, incubation with LP particles lead to more pronounced knockdown of VE-cadherin compared with LPD. We measured the expression of CD31 and Tie2 in HUVECs mRNA, as controls for ECs integrity and off-target effect, respectively. We observed that cells treated with SAINT:DOPE or DOTAP:Chol LPD particles can have increased levels of the Tie2 mRNA, up to 3 times the level of the control cells. For the SAINT:DOPE LP dose-response study, we observed higher levels of Tie2 mRNA expression level for the conditions 50 pmol/ml –TNF-α in one of the experiment, which made us exclude this condition for the results of VE-cadherin mRNA expression. The expression levels of CD31 in quiescent cells from the second experiment with anti-E-selectin targeted SAINT:DOPE LP particles were decreased in all concentration of siRNA, which made us decide to exclude this experiment.
Figure 10. VE-cadherin mRNA knock-down with SAINT:DOPE LPDs and LPs. (A-F) Quiescent and TNF-α activated HUVECs were incubated for 4 hours with anti-E-selectin or non-targeted LPDs and LPs, containing negative control siRNA or siRNA against VE-cadherin. Cells were kept in culture for 48 hours after incubation. mRNA levels of VE-cadherin were determined by real-time RT-PCR as described in “Material and Methods”. Cells not treated with particles were used as a control (ctr). A-B Cells were treated with anti-E-selectin targeted (1:200 ratio) LPDs and LPs formulated with DOTAP:Chol liposomes and containing scrambled or VE-cadherin siRNA at a concentration of 600 pmol/ml. C-D LPDs and LPs formulated with SAINT:DOPE were used at a siRNA concentration of 600 and 300 pmol/ml respectively. E-F Quiescent and activated HUVECs were incubated for 4 hours with SAINT:DOPE LPDs and LPs, containing siRNA against VE-cadherin. Data sets represent a single experiment (C, D and E), or mean value of two independent experiments ±SD (A, B). F Data set present single value or mean value from independent experiments ±SD, with conditions –TNF-α 25, 50, 100, 150 and 300 respectively n = 0, 0, 1, 1, 2. Conditions +TNF-α 25, 50, 100, 150 and 300 is respectively n = 1, 2, 2, 2, 2.
3.5. Size and surface charge optimization of LPD and LP particles based on SAINT:DOPE liposomes

SAINT:DOPE LPDs and LPs are novel particles formulated for the purpose of this study. Thus we optimized and characterized the formulation to achieve optimal size and surface charge of the particles. We formulated LPD and LP particles with different ratios of protamine to nucleic acids (Fig. 11.A and B) and different percentages of PEG. We prepared LPD particles with different ratios of ct-DNA to total nucleic acids. The mean diameter of the particles was measured by dynamic light scattering.

We have shown that increased ratios of protamine to nucleic acids lead to smaller sizes of the particles. For both particles, ratio 1.8 was selected as the most suitable ratio, as those were the particles with the small size and low dispersity. With this ratio, we continued optimization for LPD by formulating particles with different ratios of ct-DNA to total nucleic acids (Fig. 11.C). Sizes of the particles increased with increasing ratio of ct-DNA. We found that a ratio of 0.25 is most suitable for LPD particles. Then, LPD and LP particles were formulated with different ratios of PEG (Figure 11 D and E). For both type of particles, no correlation with size of the particles was observed using different PEG ratios. For LPD and LP particles, respectively 20 and 30 mol% of PEG resulted in the smallest size of the particle. We anticipated that increased amounts of PEG would neutralize the surface charge and therefore the ζ-potential of the particles was measured (Fig11.F and G). However, we found the most neutral ζ-potentials for LPD and LP particles that were PEGylated with 30 and 10 mol% PEG respectively. For all ratios in both LPD and LP we measured a positive ζ-potential. Only for 10 and 20 mol% PEGylation in LP we found a neutral and negative ζ-potential, respectively.

According to the literature smaller and less polydispersed particles were considered to be more suitable for siRNA delivery (16). Standard deviations and Chi squares from the data are a measurement for dispersity. These calculations showed that for most LPD particles, the dispersity of the particles was high. Particles of acceptable dispersity were considered to have a chi square of 4 or lower. The standard deviation and chi square of LP particles are generally smaller compared to LPD particles. The dispersity of the particles of the selected ratios was acceptable, according to the chi squares. Data is not shown.
Figure 11. Particle size and surface charge optimization of SAINT:DOPE LPDs and LPs. To optimize average size of LPD (A) and LP particles (B) particles were formulated with different ratios of protamine to nucleic acids. Mean sizes of particles were determined by dynamic light scattering. (C) LPDs were formulated with different ratios of ct-DNA to nucleic acids from 0 to 1 (0 = no ct-DNA, 1 = only ct-DNA). For both type of particles the influence of different ratios of PEG (0 to 30% to total lipid concentration of the particle) on mean sizes and ζ-potential (surface charge) were investigated as described in "Materials and Methods". Data are presented as mean size or ζ-potential of a single experiment (A, B, C, E, F, G) or as mean value of the measured mean sizes of two independent experiments (D).
3.6. Comparison of different methods of formulating SAINT:DOPE LP particles

As described by Li, Gao and Vader (16,34,37), DOTAP:Chol LPD particles were formulated via different methods. To study the influence of different methods of formulating LP particles on the size of particles, we formulated these using two methods described in the literature and measured the mean size of the particles (Fig.12). Both methods are described in “Material and methods” (Fig 5). Obtained results show that sizes of the particles are not significantly different between the two methods.

Furthermore, we investigated the influence of PEGylation of LP particles on the mean size of the particles. We compared this conventional PEGylation with direct PEGylation (Fig 5). The results show that for both method A and B there was a tendency that conventional PEGylation leads to smaller SAINT:DOPE LP particle size, compared to direct PEGylation.

Figure 12. Comparison different methods to formulate LP particles. SAINT:DOPE LP particles were produced with optimized formulation as described in “Material and Methods”. The particles were produced via two different methods (method A and B) and with conventional and direct PEGylation (Fig 5). Mean sizes were measured by dynamic light scattering and data are presented as mean values ±SD of two independent experiments.
4. Conclusions and discussion

In this study we investigated SAINT:DOPE based liposome polycation DNA (LPD) and liposome protamine (LP) particles as a potential candidate for siRNA delivery to inflamed ECs. As already described in several studies, targeted LPD particles seem to be a promising system for siRNA delivery (1,16,34,35,37-40). Due to their small size (100-200 nm) and high mol% of PEG, particles are less likely to be taken up by the RES (1). However, originally developed LPD particles formulated with DOTAP:Chol liposomes show variable release of siRNA into the cytoplasm among different cells (41). We investigated a novel LPD formulation using SAINT:DOPE liposomes, which have previously been successfully used for delivery of nucleic acids and proteins to various types of cells including ECs (42). Furthermore, we have observed that the absence of ct-DNA in the formulation of LPD leads to particles with more favourable features for siRNA delivery compared to conventional LPDs. We have called those particles LP, as mentioned before.

4.1 Conclusions

We can conclude from our findings that we formulated a drug delivery system that can deliver siRNA to ECs, for post-translational gene silencing. Both SAINT:DOPE LPD and LP particles showed to efficaciously induce knockdown of VE-cadherin mRNA in ECs. Fluorescent microscopy study showed that particles can be shielded by post-insertion of PEG. Furthermore, the particles can be targeted to activated ECs via E-selectin, which was showed by the flow cytometry studies. This targeting effect was also observed when we studied the anti-E-selectin specific release of FITH-ODN from SAINT:DOPE LP particles.

The knockdown of VE-cadherin in ECs by both SAINT:DOPE LPD and LP particles was more than 90% at highest dose (600 and 300 pmol/ml siRNA, respectively). Comparing this to the findings with DOTAP:Chol LPD and LP particles in ECs, a much better efficacy of gene silencing was possible with SAINT:DOPE particle. With DOTAP:Chol particles, no knockdown of VE-cadherin was observed for both targeted and non-targeted particles. It seems that SAINT:DOPE were more suitable lipids for siRNA release from LPD and LP particle, compared to DOTAP:Chol.

The results from the fluorescence microscopy showed that interaction of the DOTAP:Chol LPD and LP particles with ECs is less when particles are PEGylated. From this we assume that due to PEGylation by post-insertion, particles can be shielded at a density of 10 %mol PEG to total lipid concentration of the particle as described in the literature (16,34). Fluorescence microscopy showed that the anti-E-selectin targeted DOTAP:Chol LPD and LP particles are more interactive to ECs, when challenged with TNF-α. Unfortunately, the control where activated ECs were incubated with non-targeted particles
was not obtained. Therefore, we could not exclude if this effect was caused by TNF-α alone. Results from the same experiment with SAINT:DOPE LP showed also more interaction with activated ECs. The level of interaction of anti-E-selectin targeted DOTAP:Chol and SAINT:DOPE particles with ECs was quantified with flow cytometry. The results indicated that all particles have more interaction with activated ECs than with quiescent ECs.

Investigating targeted specific delivery of ODN by anti-E-selectin targeted SAINT:DOPE LP particles to activated ECs, we observed that ODN is two times more efficient delivered to the cells. Not only can we conclude that delivery is anti-E-selectin specific, but also that the content of these particles is actually delivered to the target site. However, only 1% and 2% of the cells stained double positive, indicating that only a small part of the cells was susceptible for drug delivery.

4.2 Discussion

We were able to formulate LPD and LP particles containing SAINT:DOPE liposome and they proved to be very effective in siRNA delivery to primary ECs, in vitro. These cells displayed the physical features that suggest their potential for in vivo application. However, we were not able to show significant anti-E-selectin specific knockdown in ECs with both SAINT:DOPE LPD and LP particle. Even at a lower dose, no significant targeting effect was observed. Personal communication with Dr. Radu Stan learned us that cell adhesion molecule mediated endocytosis (CAM-ME) of nanoparticles usually occurs when particles are in a size range of 100-300nm (43). E-selectin seems to undergo clathrin-mediated uptake (CME), meaning that endocytosis only occurs with particles smaller than 200 nm in size (44). Formulation of targeted particles led to increase in size, compared to non-targeted particles. Moreover, the size of the particle seems to get larger with increasing ratio of post-inserted anti-E-selectin-Mal-PEG. This was also described in literature, where Chen et al. found that particle size increases when the particles were targeted with RGD peptides (45).

Knockdown of VE-cadherin was observed with SAINT:DOPE LPD and LP particles, that were in a size range from 450 to 650 nm. This would mean that internalization of these particles may occur via a different endocytotic pathway. As described by Rejman, particles exceeding a size of 500 nm may enter the cell by caveolae mediated endocytosis (CavME) (44). Rejman describes that endocytosis via CavME evade degradation by lysosomes (46). It may be that our SAINT:DOPE LPD and LP particles will not be internalized by CAM-ME or CME, but via CavME, and therefore no targeting effect on gene silencing is observed and high knockdown was established, possibly due to evading lysosomal degradation. Still, a lot needs to be clarified regarding the mechanisms of endocytosis. Different endocytotic routes can be studied by using endocytosis inhibitor. Results of such studies may clarify the mechanism of internalization of the particles. Besides that, the results from the experiment
regarding SAINT:DOPE LP particles were based on two independent experiments. The first experiment did show a modest anti-E-selectin antibody specific knockdown at lower doses. This was not reproducible in the second experiment, from which we had to exclude data. Specific ECs markers were altered and made the integrity of these ECs arguable. More experiments need to be performed to find out if incubation with SAINT:DOPE LP particles may or may not lead to targeted specific knockdown.

Another theory of how our SAINT:DOPE LPD and LP particles could deliver siRNA to the endothelial cells is via direct interaction of SAINT:DOPE with the cell membrane. In this way, our delivery system works as transfection reagent. Van der Gun showed that transfection of siRNA and proteins is not dependent on the size of the SAINT:DOPE liposomes (42), which could indicate that this was notregulated by endocytosis. 4 hours of incubation in static conditions causes the particles to sediment on the ECs, resulting in direct interact with the ECs membrane. This could explain why the targeting effect was not visible for VE-cadherin down regulation. If gene silencing would be studied under flow conditions, particles would stay in the suspension and targeting effect on gene silencing may be enhanced. Also, we observed that knockdown of VE-cadherin is higher in ECs treated with SAINT:DOPE LP, compared to ECs treated with LPD. These differences may be explained by the fact that LP particles have a higher surface charge compared to LPD particles, which will result in more interaction with the ECs membrane. This may lead to a higher transfection efficacy and improved release from endosomes. However, Zuhorn et al. showed that transfection with SAINT:DOPE containing lipoplexes was endocytosis dependent (47).

As mentioned above, we observed effective knockdown with SAINT:DOPE particles, but no knockdown with DOTAP:Chol particles. The reason for that may be found in the characteristics of amphiphilic cationic lipid SAINT itself, which shows high efficacy in cargo release in lower pH environments like endosomes (15). Especially with helper lipid DOPE, which facilitate the inverted hexagonal phase of SAINT. By establishing this phase, the endosomal membrane is disturbed and the content of the endosome is released into the cytoplasm. Knockdown with SAINT:DOPE LP particles was greater compared to the knockdown with LPD. Greater efficacy in knockdown could be caused by the smaller size of LP particle, which makes them more subjective to endocytosis, and therefore more siRNA delivery. It could also be that the higher surface charge of the LP particle leads to more interaction between the particle and the cellular membrane or the endosome of the ECs with better siRNA release in the cell.

It is unclear why no knockdown with DOTAP:Chol LPD and LP particles was observed in our study. The inability to escape the endosome may be one of the reasons. When particles were PEGylated, green
fluorescence labelled siRNA appeared as many pixels. This may indicate that siRNA in PEGylated particles was not released into the cytoplasm. However, hardly any co-localization of the Dil with the siRNA is observed. Dil is known to diffuse into cell membranes (48) and could have migrated into the cell membrane during incubation. However, the results show that Dil is localized around the nucleus, and not in the cell membrane.

4.3 Characterization, formulation and particle optimization.

During this study we used a well described protocol based on DOTAP:Chol LPDs formulation, to prepare all particles. We compared LPD and LP particles by measuring sizes and ζ-potentials. We showed that LPD formulated with DOTAP:Chol had similar qualities as described in other studies (16). Sizes of LPD were ±200 nm and ζ-potential varied from +5 to +40 mV (data not shown). We found that LP particles formulated with DOTAP:Chol (±110 nm) were significantly smaller than LPD particles. We observed a lower ζ-potential for LPD compared to LP particles (data not shown).

In comparison, SAINT:DOPE based LPD and LP particles displayed sizes around ±390 and ±270nm, respectively. This allowed us to conclude that SAINT:DOPE particles are larger in size than DOTAP:Chol particles. As described by Adrian, liposomes formulated with increased mol% of SAINT, increase in size. This is probably due to the rather bulky pyridinium ring of SAINT. Additionally, the two unsaturated alkyl chains might influence the fluidity of the liposomal membrane and are therefore less subjected to sizing during extrusion (15).

Because SAINT:DOPE LPD and LP particles were newly developed in this study, we aimed to optimize the formulation to obtain reproducible and small particles. We prepared LPD and LP particles by varying ratios of protamine and ct-DNA and siRNA (for LPD particles), comparable to DOTAP:Chol LPD optimization by Vader. Because particle size is important for prolonged systemic availability (49-51) and for internalization into ECs (42-44,46), we selected ratios that led to a size of the particle, which is most suitable for drug delivery. This means that we selected particles with the smallest diameter and as little dispersity of size as possible.

For SAINT:DOPE LPD particles, an optimal ratio of protamine to nucleic acids was 1.8 (w/w) and DNA to total nucleic acids 0.25 (w/w). Vader described that for DOTAP:Chol LPD particles with higher ratio of protamine lead to more condensed and smaller particles, but higher ratios than 0.6 did not lead to significant smaller particles with less varying size (16). Furthermore, too much protamine could lead to undesired high surface charge of the particle. Since we only performed one experiment to optimize the ratios of different components, no statistics can be calculated. However, we did not observe drastic variations in sizes of the particles. Vader described that increasing ratio of DNA to
siRNA leads to smaller sizes and less distribution of the particle size. In our study, we found that increasing ratios of DNA did not lead to smaller, but larger sizes of the particles. This opposed result to Vader’s findings led to a ct-DNA to total nucleic acid ratio in SAINT:DOPE LPD particles of 0.25. The reason for this opposed result is unknown and should be studied more thoroughly to understand these findings.

For LP particles based on SAINT:DOPE, the same protamine ratio of 0.25 was selected. SAINT:DOPE particles have a positive surface charge which may lead to improved uptake by endothelial cells (52). Also, it may lead to binding of serum proteins and opsonization of the particle, resulting in uptake by the RES (53,54). It was demonstrated that by shielding the particles with PEG, a steric barrier is formed and less binding of serum proteins occurs (55). In the study performed by Vader, increasing amount of PEG led to decrease in ζ-potential of the particles and had no influence on the particle sizes. At a PEG density of 15 mol%, they observed an almost neutral ζ-potential and with higher PEG densities, the ζ-potential became more negative. In our study for both SAINT:DOPE based LPD and LP we did not find any correlation between size and ζ-potential and the different ratios of PEG. This may be due to the fact that the particles were not mono-dispersed, meaning that particles of many different sizes were formulated. However, we only have done two independent experiments.

The literature described different methods of formulating DOTAP:Chol LPD particles (16,34,56). Method A is based on the mixing nucleic acids with protamine, before mixing it with liposomes (34). Method B is based on mixing nucleic acids with premixed liposomes and protamine (37). We investigated these methods for preparation of SAINT:DOPE based LP particles, and we did not observe any differences in size between both methods. Furthermore, we investigated different ways of PEGylation. For both methods we described them as direct and conventional PEGylation (see Material and methods 2.10.). We came to the conclusion that conventional PEGylation led to smaller sizes of the particles, compared to direct PEGylation. This might occur due to the chance of PEG disturbing the formation of the supported bilayer when PEG is added to the mixture at the same time as the liposome. Transmission electron microscopy of the particles may elucidate the influence of PEG on the structure of the supported bilayer. We conclude that PEGylation is therefore more suitable after formation of the supported bilayer.

During this study, some difficulties were observed in formulating reproducible DOTAP:Chol and SAINT:DOPE LPD and LP particles, especially when the particles were targeted. These particles varied in size between individual experiments. Particle size and surface charge are important criteria for suitable drug delivery systems, but reproducibility and dispersity of the particle as well. During this project, the particle sizes were always measured before use. When sizes of the particle populations
are widely distributed, it is difficult to conclude what kind of particle is responsible for the observed results. During this project mean particle sizes ranging from ±80 to ±900 nm were measured. Sometimes the particle populations were more mono-dispersed, but often populations contained a variety of particles of divergent sizes. Improvement in reproducibility of particles was necessary to acquire results that can be appointed to specific characteristics of the particles. The mechanism behind the formation of the particles should be studied more intensively in order to understand how these particles are being formed. The basis of the particle is the nucleic acids and protamine containing core. When this core can be prepared in a reproducible way with satisfying characteristics, a much more suitable drug delivery system for experimental use may be formed. Moreover, further research should be done to get an insight in the mechanism of the delivery of siRNA to ECs. This study has shown that the particles can be targeted to ECs, but targeting specific knockdown was not observed. Further investigation on the internalization of the particle and the release of siRNA to the cytoplasm may elucidate the actual mechanism of the drug delivery. To study the potency of the therapeutic intervention of siRNA delivery by SAINT:DOPE particles, in vivo experiments should be performed. In the in vivo studies, siRNA against disease related genes can be used, in disease animal models, to show the therapeutic possibilities and toxicity of the particles.
Acknowledgements

First of all, I would like to thank Ingrid Molema, for letting me into the EBVDT group and giving me the opportunity to do my first research project at such a nice lab. Also I would like to thank her for all feedback I got during the EFRO meetings and in personal conversations. It was very interesting to observe what being a member of the university staff is all about. I would like to thank her for giving me a look into the scientific world and for treating me to a trip to the Cardio Vascular Conference, which was really nice to experience. Secondly, I would like to thank Jan Kamps, for all his support during this project. Every meeting he was there and always gave good advice, asked important questions and showed much interest and passion for the work we are doing. Then, I would like to thank Rianne Jongman, Peter Zwiers and Henk Moorlag for all their fun and support at the lab. Furthermore, I would like to thank Marcel Ruiters and Ed Talman of Synvolux, who were always critical but supportive during the EFRO meetings. I would like to thank Hienriette Morselt, Titia van Woudenberg-Vrenken, Roel van der Heijden and Niek Leus for all discussion we have had about lipid based drug delivery systems and every other subject. Most of all I would like to thank Piotr Kowalski for being my daily supervisor. As a student it is really nice to be taught by someone that is so enthusiastic and passionate about his work. Besides that, I have to thank Piotr for all the effort he has put in this project. Showing me around at the lab and teaching me how to think and act in a more scientific way was not only instructive, but also a lot of fun.
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