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MC3/SAINT-O-Somes, a novel liposomal delivery system for efficient and safe delivery of siRNA into endothelial cells

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ABSTRACT

Increased understanding of chronic inflammatory diseases and the role of endothelial cell (EC) activation herein, have urged interest in sophisticated strategies to therapeutically intervene in activated EC to treat these diseases. Liposome-mediated delivery of therapeutic siRNA in inflammation-activated EC is such a strategy. In this study, we describe the design and characterisation of two liposomal siRNA delivery systems formulated with the cationic MC3 lipid or MC3/SAINT mixed lipids, referred to as MC3-O-Somes (MOS) and MC3/SAINT-O-Somes (MSS). The two formulations showed comparable physicochemical properties, except for better siRNA encapsulation efficiency in the MSS formulation. Antibody-mediated VCAM-1 targeting (Ab_{VCAM-1}) increased the association of the targeted MOS and MSS with activated EC, although the targeted MOS showed a significantly higher VCAM-1 specific association than the targeted MSS. Ab_{VCAM-1} MSS containing RelA siRNA achieved significant downregulation of RelA expression, while Ab_{VCAM-1} MOS containing RelA siRNA did not downregulate RelA expression in activated EC. Additionally, Ab_{VCAM-1} MSS containing RelA siRNA showed low cytotoxicity in EC and at the same time prohibited endothelial inflammatory activation by reducing expression of cell adhesion molecules. The Ab_{VCAM-1} MSS formulation is a novel siRNA delivery system based on a combination of the cationic lipids MC3 and SAINT, that shows good physicochemical characteristics, enhanced endothelial cell association, improved transfection activity, low toxicity and significant anti-inflammatory effect, thereby complying with the requirements for future in vivo investigations.

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Cationic lipids; liposomes; siRNA delivery; endothelial cells; endothelial cell activation; inflammation

1. Introduction

Chronic inflammatory diseases, such as cardiovascular diseases, are identified as a leading cause of death worldwide (Roth *et al.* 2020). It has been shown that endothelial cell (EC) activation triggers a cascade of inflammatory responses in the initiation and progression of chronic inflammatory diseases (Sun *et al.* 2020). EC activation is characterised by the upregulation of cell adhesion molecules, production of cytokines and chemokines, and increased interaction with leukocytes, leading to a proinflammatory environment of the blood vessels (Zhu *et al.* 2018). Regulation of EC activation is mediated by multiple inflammation-related signaling pathways, such as the nuclear factor-κB (NF-κB) pathway (Rahman *et al.* 2011). Therefore, inhibiting endothelial activation by reduction of NF-κB activation is considered a powerful therapeutic strategy for treating inflammatory diseases. This intervention strategy can be achieved by treatment with chemical inhibitors or RNA therapeutics-based RNA interference (RNAi). Small interfering RNA (siRNA) are efficient mediators of RNAi, however, *in vivo* delivery of naked siRNA therapeutics is highly restricted, due to the poor pharmacokinetics, such as a short half-life in the circulation and easy degradation by serum RNases (Khalil *et al.* 2018). Lipid-based carriers, such as liposomes and lipid-based nanoparticles (LNP), have been widely developed for siRNA delivery (Rietwyk *et al.* 2017). These lipid-based carriers, comprised of functional lipid components, possess many advantages as siRNA delivery systems, such as good biocompatibility, limited toxicity and ease of modification and large-scale preparation (Yonezawa *et al.* 2020).

The efficiency of siRNA delivery into cells depends on the cell type and the design of the liposomal formulation, such as the selection of cationic lipid(s), which is directly related to the transfection efficacy (established as gene silencing ability in our study) of lipid carriers (Semple *et al.* 2010, Ponti *et al.* 2021, He *et al.* 2022). Significant progress has been made in the design of cationic lipids for transfection purposes, since N-[1–(2,3-dioleoyloxy)propel]-N,N,N-trimethylammonium (DOTMA) was reported for lipofection-based transfection (Felgner *et al.* 1987, Zhi *et al.* 2013, Ponti *et al.* 2021). Cationic pyridinium-derived lipids have been proven to be suitable for delivering nucleic acids (NAs) (Zhi *et al.* 2013, Kowalski *et al.* 2015, Parvizi-Bahktar *et al.* 2016). Of

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these, SAINT-C18 (SAINT) lipid was formulated into various siRNA carriers by our research group for EC-specific delivery, including SAINT-based lipoplexes (SAINTarg and its derivatives) and SAINT-based liposomes (SAINT-O-Somes) (Adrian *et al.* 2010, Ásgeirsdóttir *et al.* 2010, Kowalski *et al.* 2013, Kowalski *et al.* 2014, Leus *et al.* 2014). SAINT-O-Somes (SOS) showed improved physicochemical properties compared to conventional liposomes, such as high siRNA loading, good stability and intracellular release of siRNA cargo (Adrian *et al.* 2010). In addition, RelA specific siRNA containing SOS displayed *in vitro* and *in vivo* therapeutic potential when specifically targeted to inflamed EC by effectively silencing NF-κB activation (Kowalski *et al.* 2014, Choi *et al.* 2017). However, there is still room for improvement of the transfection efficacy of the SOS. Increasing the cationic lipid content of formulations is assumed as one of the strategies to obtain better transfection. However, for SOS, 20% SAINT is the maximum molar ratio for obtaining homogeneous, stable and nanosized liposomes (Adrian *et al.* 2010). Another cationic lipid named DLin-MC3-DMA (MC3), one of the benchmark cationic lipids for LNP formulations, may be a suitable alternative for SAINT to form a stable liposomal formulation at higher cationic lipid contents, because of its small amine headgroup. MC3 LNP have been successfully applied to achieve *in vivo* hepatic gene silencing, showing excellent gene knockdown results (Jayaraman *et al.* 2012). In the current study, we introduced MC3 into the original SOS formulation to reach 30 mol% of total cationic lipids, aiming at obtaining improved transfection activity in EC without changing the favourable characteristics of SAINT-based liposomes. For this, two liposomal formulations encapsulating RelA siRNA were prepared, where the MC3-O-Somes (MOS) formulation contained 30 mol% of MC3 and the MC3/SAINT-O-Somes (MSS) contained a combination of SAINT and MC3 in a ratio of 18 mol% of SAINT and 12 mol% of MC3. Based on these two formulations, EC-specific liposomal counterparts were prepared by surface-modification with anti-VCAM-1 antibodies, referred to as Ab_{VCAM-1} MOS (Ab-MOS) and Ab_{VCAM-1} MSS (Ab-MSS), respectively. We assessed the physiochemical properties, cytotoxicity and the EC-association of non-conjugated and Ab_{VCAM-1} conjugated liposomes. The transfection efficacies and the anti-inflammatory effects of Ab-MOS and Ab-MSS were examined in activated EC.

2. Materials and methods

2.1. Materials

Lipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), distearoyl-sn-glycero3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]-maleimide (DSPE-PEG-Mal) were obtained from Avanti Polar Lipids (Alabaster AL, USA). The cationic lipid 1-methyl-4-(cis-9-dioleyl) methyl-pyridinium-chloride (SAINT-C18) was bought from Synvolux Therapeutics Inc. (Leiden, The Netherlands), and dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA) was purchased from Med Chem Express (Monmouth Junction, NJ, USA). Cholesterol (Chol) and N-succinimidyl-S-acetylthioacetate (SATA) were obtained from Sigma (St. Louis MO, USA). The lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) and the nucleic acid stain Hoechst33342 were purchased from Molecular probes (Leiden, The Netherlands). 6-(p-toluidino)-2-naphthalenesulfonic acid sodium salt (TNS), 2-[N-morpholino]ethanesulfonic acid (MES) and other chemicals were purchased from Sigma. Lipofectamine™ 2000 reagent was bought from Invitrogen (Breda, The Netherlands). Control siRNA (AllStars Negative Control siRNA) and RelA specific siRNA (Hs_RELA_7 FlexiTube siRNA, 5′-CCGGATTG AGGAGAAACGTAA-3′) were from Qiagen (Venlo, The Netherlands). E-1/6-aa2 anti-human VCAM-1 monoclonal antibody (mouse IgG1)-producing hybridomas were kindly provided by Dr. M. Gimbrone (Harvard Medical School, Boston, MA, USA).

2.2. Preparation of liposomes

Liposomal formulations were prepared by a thin-film hydration method and they were named MC3-O-Somes (MOS) and MC3/SAINT-O-Somes (MSS). The MOS was formulated with MC3, POPC, Chol, DSPE-PEG, DSPE-PEG-Mal in the molar ratio of 30: 25: 40: 4: 1, while the MSS was comprised of MC3, SAINT, POPC, Chol, DSPE-PEG and DSPE-PEG-Mal in a molar ratio of 12: 18: 25: 40: 4: 1. The lipids were mixed with DiI at 0.1 mol% of Total Lipids (TL) for fluorescent-labeling of the lipid bilayer. The lipid mixtures were dried under a constant stream of nitrogen gas and further dehydrated at 37 °C in a vacuum pump for 60 min to form a lipid thin-film. The lipid layer was then hydrated with 50 mM citrate buffer (pH 4) containing RelA siRNA ($siRNA_{RelA}$) or control siRNA ($siRNA_{ctrl}$), at a ratio of 1 nmol siRNA/μmol TL for 2h at 40 °C or overnight at 4 °C. After hydration, freezing-thawing of mixtures was performed 10 times, and the obtained liposome products were extruded through polycarbonate filters with pore sizes of 200 nm (5 times), 100 nm (10 times), and 80 nm (10 times), using the Lipex high-pressure extruder (Vancouver, Canada). After extrusion, the liposomes were dialysed against HN buffer (10 mM HEPES, 135 mM NaCl, pH 6.9) overnight at 4 °C. SATA-modified anti-human VCAM-1 antibodies were conjugated to the surface of liposomes via a sulfhydryl-maleimide method, as described previously (Kamps *et al.* 1996). Antibody-conjugated liposomes were purified by ultracentrifugation and subsequent dialysis against HN buffer (pH 7.4). Non-conjugated and anti-human VCAM-1 antibody- conjugated SOS were included for comparison where indicated, which were prepared as described previously (Kowalski *et al.* 2014).

2.3. Characterisation of liposomes

2.3.1. Phosphorus assay, Peterson-Lowry assay, Ribo-Green assay and DLS measurement

The liposomes were characterised by determining the phospholipid content using the phosphorus assay to calculate the TL, and by measuring the concentration of conjugated antibodies using the Peterson-Lowry assay with mouse

IgG as a standard (Bottcher 1961, Peterson 1977). The siRNA content was measured in the presence or absence of 1% (*v/v*) Triton X-100 in samples using Quant-iT ™ Ribo-Green® assay (Invitrogen, Breda, The Netherlands) in compliance to the protocol of the manufacturer. Based on these assays, siRNA encapsulation efficiency and the amount of encapsulated siRNA per TL were calculated. The amount of anti-VCAM-1 antibodies coupled per liposome was calculated according to the method described by Niwa et al (Niwa *et al.* 2018). Particle size and Polydispersity Index (PDI) of liposomes were measured using dynamic light scattering (DLS) in the volume weighing mode (NICOMP 380 ZLS Particle Sizer, Santa Barbara, USA) (He *et al.* 2022). Zeta potential measurement was measured on a Malvern ZEN3690 (Malvern Instruments Ltd., Malvern, UK).

2.3.2. pKa measurement by TNS assay

A TNS assay was performed to measure the apparent acid dissociation constant (pKa) values of the three liposomes (MOS, SOS and MSS). The pKa represents the ionisation status of liposomes (Patel *et al.* 2021). In brief, buffer solutions (10 mM MES, 10 mM HEPES, 10 mM ammonium acetate, 130 mM NaCl) were adjusted to obtain a series of pH values ranging from 3.5 to 9 in increments of 0.5 units, using 0.1 N HCl and 0.1 N NaOH. Hundred microliter of each pH buffer solution was added to each well of a black 96-well plate. TNS stock solution (300 μM) was then added into the buffer to the final concentration of 6 μM. Formulated liposomes were subsequently added to the above mixture at the final TL concentration of 25 μM. The fluorescence intensity was measured with a FLUOstar Omega plate reader (BMG Labtech, Chicago, IL, USA) at an excitation wavelength of 325 nm and emission wavelength of 435 nm. In the series of measurements, each measured fluorescence was subsequently normalised by (fluorescence – minimum value of fluorescence)/(maximum value of fluorescence – minimum value of fluorescence) (Kim *et al.* 2021). The normalised fluorescence averages were plotted with a sigmoidal best fit analysis, resulting in an 'S'-shaped titration curve. The pKa of each liposome formulation was calculated as the pH value at the point of half-maximal fluorescence intensity, which reflects 50% ionisation (protonation).

2.4. Long-term size stability and siRNA protection capability in serum

To determine the stability of both liposomal formulations during long-term storage under argon gas at 4 °C, particle size and PDI of liposomes were measured with DLS at day 1, 7, 14, and 28 post-preparation.

To investigate the siRNA protection capability of MOS and MSS, liposomes containing 100 ng of siRNA were incubated at 37 °C for 0, 0.5, and 3 h, in the absence or presence of 40% (*v/v*) Foetal Bovine Serum (FBS, Thermo Fisher, Waltham USA). The same amount of naked siRNA was used as control and incubated under the same condition as the liposome groups. At the end of incubation, all samples were mixed with 1% (*v/v*) Triton X-100 and 1% (*v/v*) gel loading dye (Biolabs, Leiden, The Netherlands), and ran in a 2% agarose gel at 110 V for 15 min. The siRNA bands were visualised with a Chemidoc™ XRS system (Bio-rad, Veenendaal, The Netherlands).

2.5. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza Bioscience (Breda, The Netherlands) and cultured by the Endothelial Cell Facility of the UMCG. Cells were grown in EBM-2 medium supplemented with EGM-2 MV SingleQuot Kit Supplements & Growth Factors (Lonza) in an incubator containing 5% CO_2 at 37 °C. HUVEC from passage 5 ∼ 7 were placed on various culture plates (Costar, Corning, USA) or Lab-Tek™ Chamber Slides Nunc (Rochester, USA) to reach a confluency of ∼60–90%.

2.6. Cell association of MOS and MSS formulations with HUVEC

2.6.1. Fluorescence microscopy

The EC association of different liposomes was investigated using fluorescence microscopy. HUVEC were cultured on Lab-Tek™ Slides to ∼70% confluency, and then they were or were not activated by 10 ng/mL of TNF-α (Beromun®, Boeringer Ingelheim, Denmark), 2h prior to addition of liposomes. Next, cells were incubated for another 3h with 80 nmol TL/mL non-conjugated or antibody-conjugated liposomes, labelled with DiI. The nuclei of the cells were stained during the last 30 min of the incubation, using 20 μg/mL of Hoechst 33342. The cells were washed with serum-free medium and imaged with a Leica DM/RXA fluorescence microscope (Wetzlar, Germany), at excitation/emission wavelengths of 350/461 nm for Hoechst 33342 and 550/570 nm for DiI.

2.6.2. Flow cytometry

The liposome-cell association was quantified by flow cytometry. Cells were seeded in 12-well plates to ∼90% confluency, activated with TNF-α (10 ng/mL, 2h) and further incubated with liposomes (80 nmol TL/mL, 3h). Cells were then washed using PBS and detached from their substrate using 0.025% trypsin/EDTA (Sigma, Ayrshire, UK), after which cells were transferred to tubes containing FACS buffer (5% (*v/v*) FBS in PBS). HUVEC were centrifuged (1,500 rpm, 5 min), followed by one more wash step, and finally resuspended in 0.2 ml of FACS buffer for flow cytometry analysis using the ACEA NovoCyte™ flow cytometer (ACEA Biosciences, USA). Analysis of data was performed using Kaluza Flow analysis V2.1 software (Beckman Coulter, Brea, USA).

2.7. In vitro liposome-mediated siRNA transfection

To investigate the gene silencing mediated by the different liposomal systems, *in vitro* transfection experiments were performed in activated HUVEC. HUVEC were cultured in 24-well plates to ∼60% confluency. They were activated with

TNF-α (10 ng/mL) for 4 h prior to adding liposomes, and then incubated with Ab-MOS or Ab-MSS containing $siRNA_{RelA}$ or $siRNA_{ctrl}$ for 5 h at an siRNA concentration of 3 or 4.5 μg/mL, as indicated, followed by further incubation for 19 h under diluted liposome condition by adding 0.5 ml of extra medium. After 24 h incubation with liposomes, cells were washed and incubated in fresh medium without liposomes and TNF-α for another 24 h. During the last 4 h of incubation, 1 μg/mL of LPS (Escherichia coli, serotype O26:B6 (15,000 EU/g), Sigma-Aldrich, St. Louis, MO, USA) was added to re-challenge the EC. The SOS-based transfection was performed as described previously (Kowalski *et al.* 2013).

2.8. Gene expression analysis by RT-qPCR

For gene expression analysis, RNA of HUVEC was extracted using TRIzol® reagent (Invitrogen, #15596–018) after the 48h transfection process, according to the instructions of the manufacturer. The purity and concentration of isolated RNA were measured using the NanoPhotometer® N60 (Implen, München, Germany). RNA samples with a OD260/OD280 value between 1.8 and 2.0 were taken for following processing. Reverse transcriptase-based cDNA synthesis and quantitative PCR (RT-qPCR) were performed as described previously (Dayang *et al.* 2019). The Assay-on-Demand primers for human RelA (Hs00153294_m1), E-selectin (Hs00174057_m1), VCAM-1 (Hs00365486_m1), ICAM-1 (Hs00164932_m1), MCP-1 (Hs00234140_m1), IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1) and GAPDH (Hs99999905_m1) were purchased from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands). qPCR was executed in a ViiA 7 PCR system (Applied Biosystems). Duplicate analyses for each sample were performed and the averaged threshold cycle values (CT) of duplicates were used for calculation. Target genes were normalised to housekeeping gene GAPDH to yield the ΔCT value, and the relative mRNA expression of a specific gene was calculated by $2^{-\Delta CT}$.

2.9. Cytotoxicity assay

Cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8, HY-K0301, MedChemExpress, Monmouth Junction, NJ, USA). To investigate the *in vitro* cytotoxicity of different cationic liposomes, HUVEC were cultured in a 96-well plate to ~70% confluency and then exposed to antibody-conjugated liposomes at a series of lipid concentrations (50, 150, 300, 450, and 900 μM) for 24 h. After 24 h incubation, 10 μl of the CCK-8 solution was added into each well, followed by an another 3 h incubation, and absorbance at 450 nm was measured using a microplate reader (BioTek, BioSPX, The Netherlands). The absorbance of resting HUVEC without the addition of liposomes was considered as 100% cell viability.

To evaluate the cytotoxicity of liposome-mediated siRNA transfection on activated EC, HUVEC were transfected with antibody-conjugated liposomes containing $siRNA_{ctrl}$ for 5 h in the presence of TNF-α (10 ng/ml), at siRNA concentrations of 3 and 4.5 μg/mL. A lipofectamine-mediated transfection (5 h) was performed in activated HUVEC for comparison, according to the protocol provided by the manufacturer. The cytotoxicity assessment was done by the above-mentioned CCK-8 assay. Here, the absorbance of TNF-α activated HUVEC without liposomes or lipofectamine was taken as 100% cell viability.

2.10. Statistical analysis

Statistical analysis of data was performed by One-way ANOVA followed by Bonferroni Post-hoc analysis to compare multiple groups. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Preparation and characterisation of MOS and MSS

We formulated liposomes called MOS and MSS. In addition, VCAM-1 was selected as the cellular target since inflammation activated EC overexpress VCAM-1 protein (Glassman *et al.* 2020). Endothelial-targeted liposomes were prepared by conjugating antibodies against VCAM-1 on the surface of the liposomes, referred to Ab-MOS and Ab-MSS. The molecular structures of two cationic lipids and the schematic diagram of the resulting liposomes are shown in Figure 1. The physiochemical characteristics of both liposomes and their antibody-conjugated forms are displayed in Table 1. Both MOS and MSS formulations were successfully coupled with antibodies, with a comparable surface density of antibodies. The particle sizes of the liposomes were comparable, 119 nm for the MOS and 120 nm for the MSS, respectively. Both Ab_{VCAM-1} conjugated forms were bigger than their non-conjugated counterparts, showing an average size of 143 nm for the Ab-MOS and 160 nm for the Ab-MSS. The corresponding PDI for each liposomal formulation was lower than 0.2, indicating that all liposomes were homogeneous and monodisperse. The siRNA encapsulation efficiency of MOS and MSS was 69 and 89%, respectively. Both Ab_{VCAM-1} conjugated forms showed over 90% siRNA encapsulation efficiencies. The siRNA loading ability, which is represented by the amount of encapsulated siRNA per TL (μg/μmol), was comparable in all formulations. To determine whether siRNA in liposomes is protected against RNases, both MOS and MSS containing siRNA were incubated with 40% serum at 37 °C (Figure 2(A)). In the presence of serum, the naked siRNA (control) was completely degraded within 3 h, whereas the siRNA formulated into MOS or MSS kept their integrity for at least 3h. Moreover, we demonstrated that all prepared liposomes could be stored at 4 °C under argon for at least 28 days without or with minor changes in particle size, indicating long-term stability of both formulations (Figure 2(B)).

3.2. Coupling Ab_{VCAM-1} increases MOS and MSS association with TNF-α activated HUVEC

EC association of different liposomes was investigated in resting (−) and TNF-α stimulated (+) HUVEC. The specificity and extent of DiI-labelled liposome-endothelial association

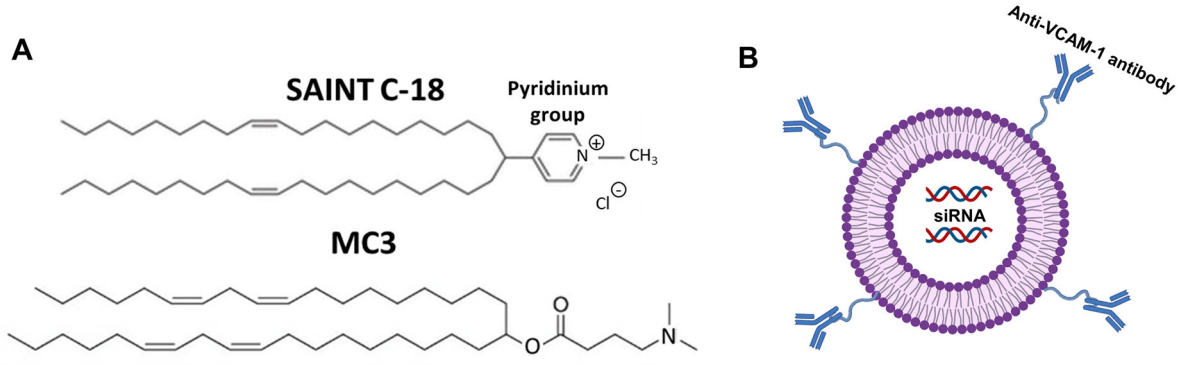

Figure 1. (A) Molecular structures of SAINT-C18 and MC3 lipids. (B) Schematic presentation of MC3 or MC3/SAINT containing liposomal siRNA delivery systems conjugated with anti-VCAM-1 antibodies.

Table 1. Physicochemical properties of non-conjugated and antibody-conjugated MOS and MSS containing siRNA.

	Formulation of different liposomes				
MOS	POPC : MC3 : Chol : DSPE-PEG : DSPE-PEG-Mal = 25 : 30 : 40 : 4 : 1				
MSS	POPC : MC3 : SAINT : Chol : DSPE-PEG : DSPE-PEG-Mal = 25 : 12 : 18 : 40 : 4 : 1				
Sample Name	Size [nm]	Polydispersity Index (PDI)	siRNA encapsulation efficiency (EE,%)	siRNA loading (μg siRNA/μmol TL)	Ab conjugated mol/liposome
MOS	119 ± 21	0.17 ± 0.08	69 ± 18	1.68 ± 0.39	n.a.
MSS	120 ± 18	0.18 ± 0.07	89 ± 9*	1.58 ± 0.51	n.a.
Ab-MOS	143 ± 29	0.17 ± 0.05	93 ± 3	1.53 ± 0.65	40 ± 27
Ab-MSS	160 ± 35	0.15 ± 0.06	95 ± 2	1.56 ± 0.40	50 ± 32

Data are presented as means of 7 preparations ± SD. Anti-human-VCAM-1 (Ab) was used as the conjugated antibody.
*$p < 0.05$, a significant difference between MOS and MSS.

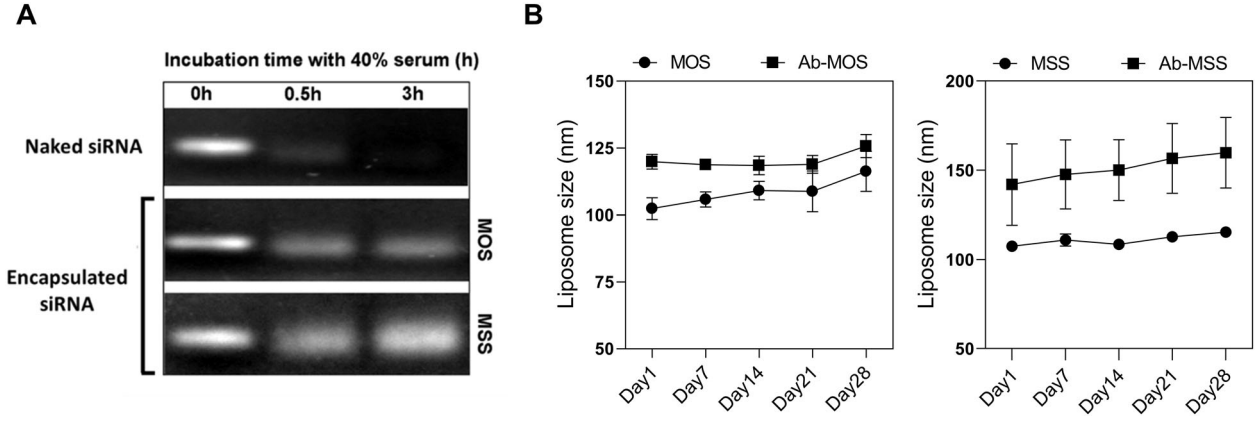

Figure 2. Characterisation of siRNA containing MOS and MSS formulations. (A) Agarose gel electrophoresis images of naked siRNA and siRNA encapsulated in the MOS and MSS. (B) Long-term size stability of MOS, MSS and their Ab_{VCAM-1} conjugated counterparts (Ab-MOS and Ab-MSS). Data presented show the means of 3 preparations ± SD.

were qualitatively evaluated by fluorescence microscopy (Figure 3(A)). The SOS formulation was included in this study for comparison. DiI fluorescence could be detected in MSS (+), Ab-MSS (−) and Ab-MSS (+) groups, indicating that all formulations exhibited non-specific cell association. The DiI fluorescence was only observed in the Ab-MOS (+) group and not in the MOS (+) and Ab-MOS (−) groups. SOS showed a similar pattern as the MOS groups, suggesting high specificity of the Ab-MOS formulation for activated EC. These findings were next quantified using flow cytometry. As shown in the histogram (Figure 3(B), left-side), an obvious peak shift occurred in the Ab-MOS (+) compared to the other groups, indicating increased cell association of the Ab-MOS to TNF-α activated HUVEC. In contrast, no or a little peak shift occurred in MOS (+) and Ab-MOS (−) groups, compared to control groups. Compared to control groups, obvious peak shifts were seen in all MSS formulations with the most prominent right-shift occurring in the Ab-MSS (+) group (Figure 3(B), right-side). Taken together, these data demonstrate that different liposomal formulations have different association specificities for EC, with the Ab-MOS formulation showing more specificity for TNF-α stimulated HUVEC. Both Ab_{VCAM-1} conjugated MOS and MSS showed increased association with inflammation-activated EC (Figure 3(C)).

3.3. In vitro transfection efficiencies of Ab-MOS and Ab-MSS containing RelA siRNA

To investigate if both targeted liposomes can downregulate RelA expression, TNF-α stimulated HUVEC were transfected

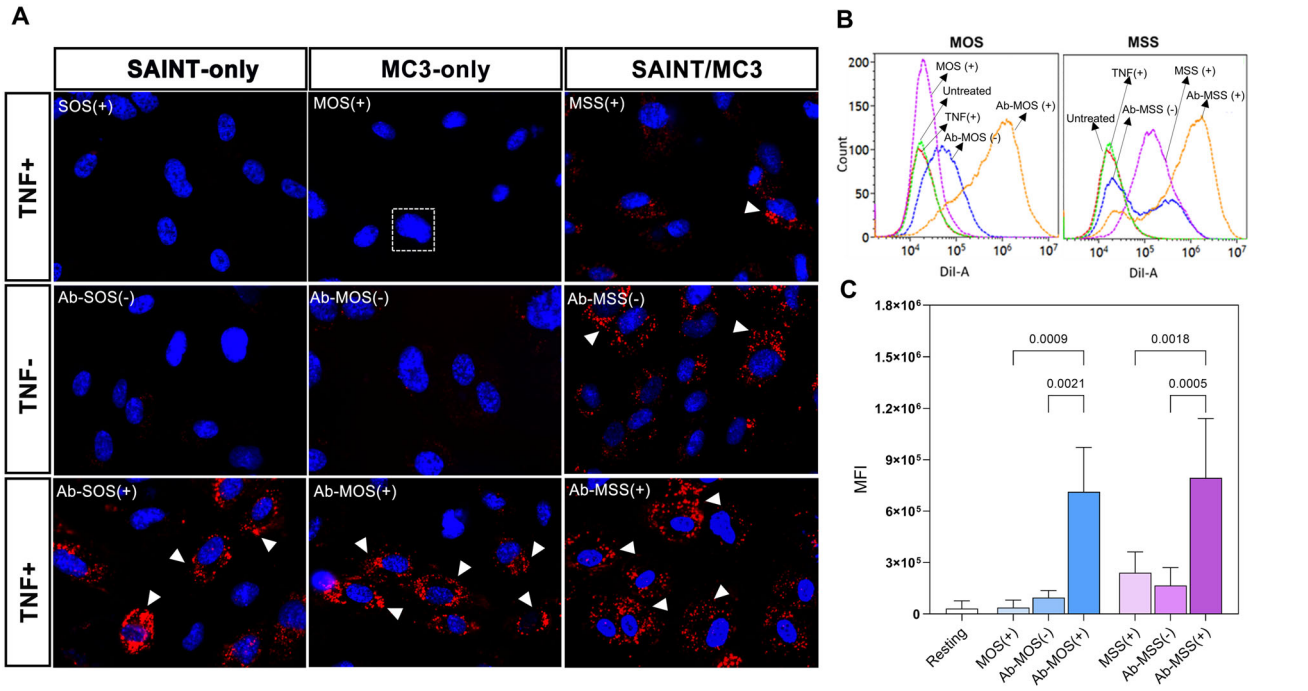

Figure 3. Endothelial cell association patterns of different liposome formulations. (A) Association of liposomes with HUVEC, visualised by fluorescence microscopy. Three non-conjugated liposomes (SOS, MOS and MSS) and their Ab_{VCAM-1} conjugated counterparts (Ab-SOS, Ab-MOS and Ab-MSS) were incubated for 3 h with quiescent (−) or/and TNF-stimulated (+) HUVEC. Liposomes were labelled with DiI (arrow head) and cell nuclei were stained with Hoechst (box). The images were taken at 400× magnification. Presented datasets are representative images of 3 independent experiments. (B) Representative flow cytometry histograms including the MOS (+) or MSS (+) groups, the Ab-MOS (+) or Ab-MSS (+) groups, and Ab-MOS (−) or Ab-MSS (−) groups. The untreated (−) groupsand TNF-α stimulated (+) groups are shown as controls. (C) Quantification of liposome-cell association, presented as Mean Fluorescence Intensity (MFI) ± SD of 3 independent experiments. Numbers represent *p* values between indicated bars.

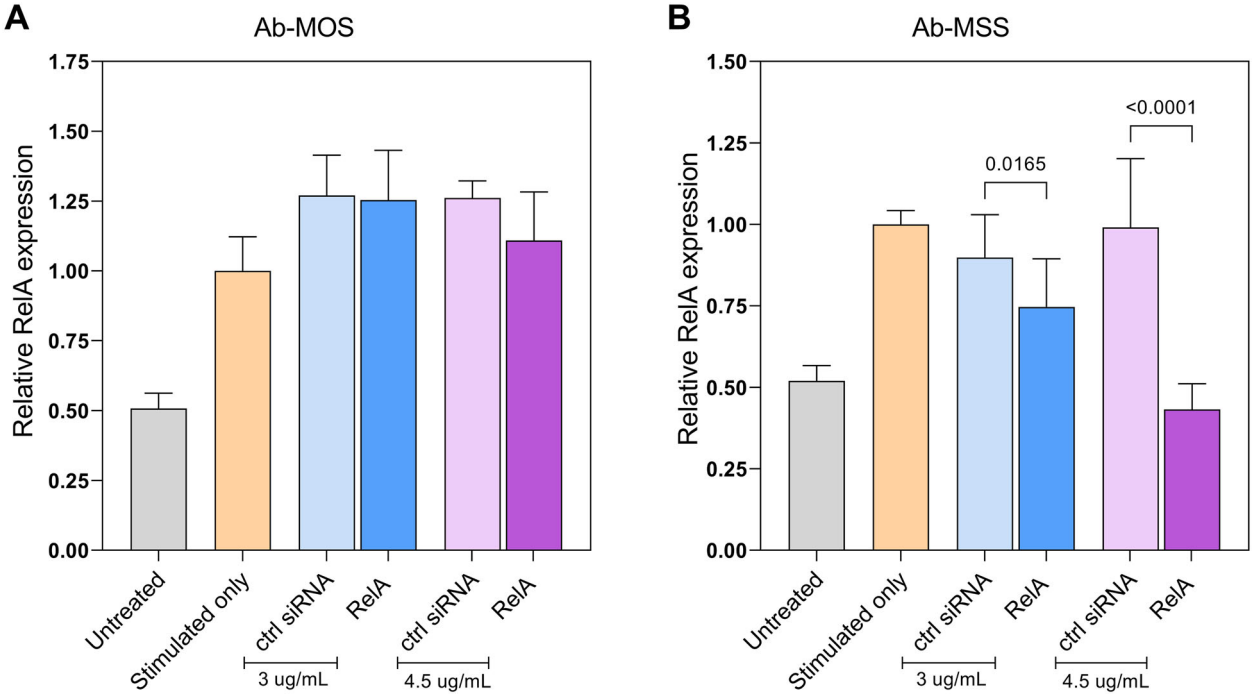

Figure 4. Relative RelA mRNA expression in activated EC treated with siRNA containing Ab-MOS and Ab-MSS. Activated HUVEC were transfected with Ab-MOS or Ab-MSS contained $siRNA_{ctrl}$ (ctrl siRNA) or $siRNA_{RelA}$ (RelA) at siRNA concentration of 3 or 4.5 μg/mL. Normalised data (relative to the stimulated control) are presented as means ± SD of three independent experiments. Numbers represent *p* values between indicated bars.

with Ab-MOS and Ab-MSS containing $siRNA_{RelA}$ or $siRNA_{ctrl}$ at the indicated siRNA concentrations. There was no RelA downregulation in HUVEC incubated with RelA siRNA containing Ab-MOS (Figure 4(A)). As shown in Figure 4(B), the RelA siRNA containing Ab-MSS groups showed significant down-regulation of RelA mRNA expression, with 17% knockdown at the siRNA concentration of 3 μg/mL, and 57% knockdown at 4.5 μg/mL. We also found that the transfection

efficiency of Ab-MSS is higher than that of Ab-SOS, since Ab-SOS did not downregulate RelA expression at 3 μg/mL and only resulted in 30% knockdown at a higher siRNA concentration (Figure S1).

It has been demonstrated that ionisation status of pH-sensitive delivery systems is a key determinant for effective siRNA delivery, which can be evaluated by the apparent pKa (Patel *et al.* 2021). Therefore, we measured the apparent pKa of the particles by TNS assay to better understand the difference in transfection efficacies of liposomes (Figure S2). All liposomes were ionisable, as demonstrated by their pKa values below 7. The formulation that contained MC3 only (MOS) showed a steeper "S"-shaped curve with a pKa of 6.44, while the SAINT/MC3 lipid-mixed formulation (MSS) exhibited a flatter "S"-shaped curve with a higher pKa of 6.84. This indicates that MOS and MSS have a different ionisation status at the same acidic environment, with the MSS showing a slightly higher positively charged profile, which was confirmed by zeta-potential measurements (Data not shown).

3.4. Ab_MSS containing RelA siRNA attenuates endothelial inflammation in vitro

To demonstrate anti-inflammatory effects of $siRNA_{RelA}$ containing Ab-MOS and Ab-MSS, expression of NF-κB driven pro-inflammatory genes after Ab-MOS or Ab-MSS-mediated RelA knockdown, was investigated in inflammation-activated EC. Compared to the non-stimulated control (resting HUVEC), no significant change in expression of pro-inflammatory genes was observed in cells treated with the $siRNA_{ctrl}$ containing Ab-MOS or Ab-MSS, indicating that the two liposomal formulations themselves did not aggravate endothelial activation (Data not shown). There was no significant difference between the $siRNA_{RelA}$ containing Ab-MOS group and the stimulated control, in mRNA expression level of all tested pro-inflammatory genes (Figure 5(A)). However, inhibition of upregulation of cell adhesion molecules (E-selectin, VCAM-1 and ICAM-1) was observed in activated HUVEC treated with $siRNA_{RelA}$ containing Ab-MSS at the siRNA concentration of 4.5 μg/mL (Figure 5(B)). Induced VCAM-1 expression was significantly inhibited by the Ab-MSS at 3 μg/mL of $siRNA_{RelA}$. No inhibition of other pro-inflammatory genes was seen in cells treated with $siRNA_{RelA}$ containing Ab-MSS at both siRNA concentrations.

3.5. Cytotoxicity

We first checked the cytotoxicity of liposomes in EC at a range of lipid concentrations. All liposome formulations were hardly toxic to primary EC at concentrations of 50–900 μM (Figure 6(A)). Additionally, we also evaluated the biocompatibility properties of different liposomes as transfection agents, by measuring the cytotoxicity of liposome-based transfection in activated EC. As shown in Figure 6(B), all three liposomes-mediated 24 h transfection cultures showed no or minor cellular toxicity at both siRNA transfection concentrations. Compared to the Ab-SOS, cell viability was significantly higher in the Ab-MSS mediated siRNA transfection. All liposome-transfected groups showed significantly higher cell viabilities compared to the lipofectamine-treated group.

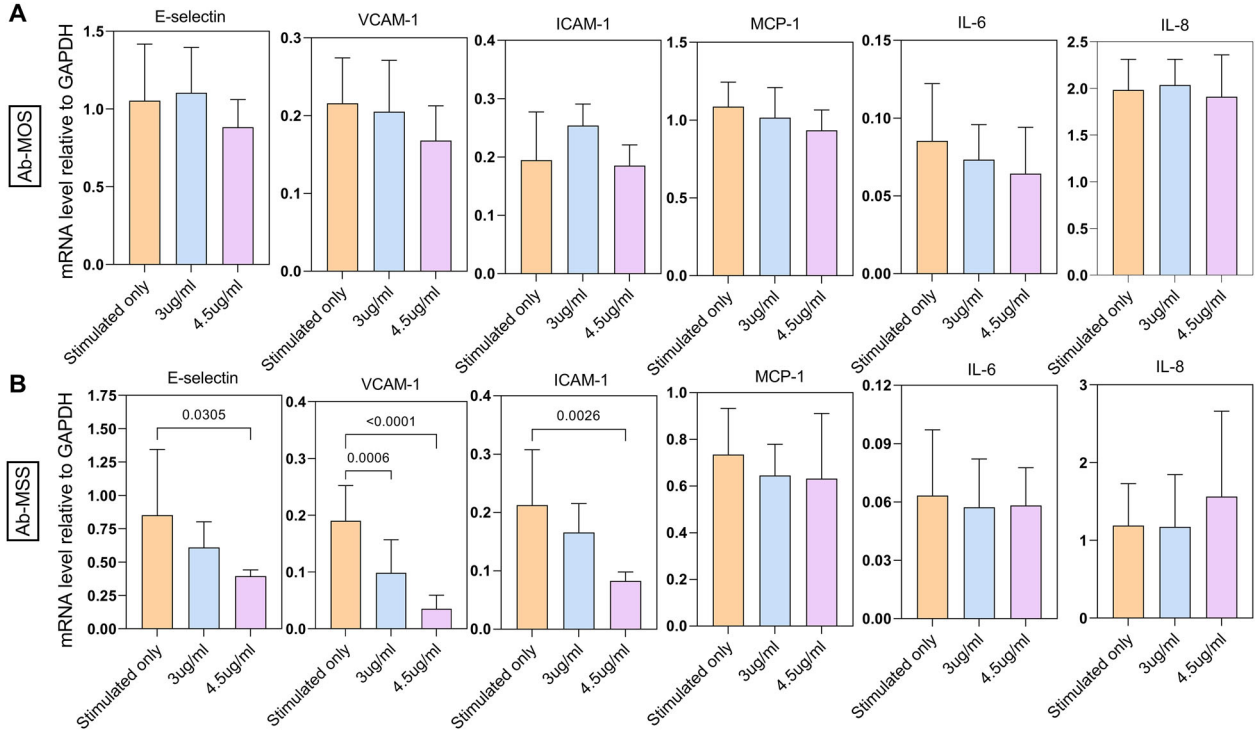

Figure 5. Evaluation of anti-inflammatory effects of $siRNA_{RelA}$ containing Ab-MOS or Ab-MSS in activated HUVEC. (A) No significant downregulation of pro-inflammatory genes was observed after Ab-MOS mediated RelA siRNA transfection. (B) Dose-dependent downregulation of cell adhesion molecules by $siRNA_{RelA}$ containing Ab-MSS. The graphs represent the means ± SD of three independent experiments. Numbers represent *p* values between indicated bars.

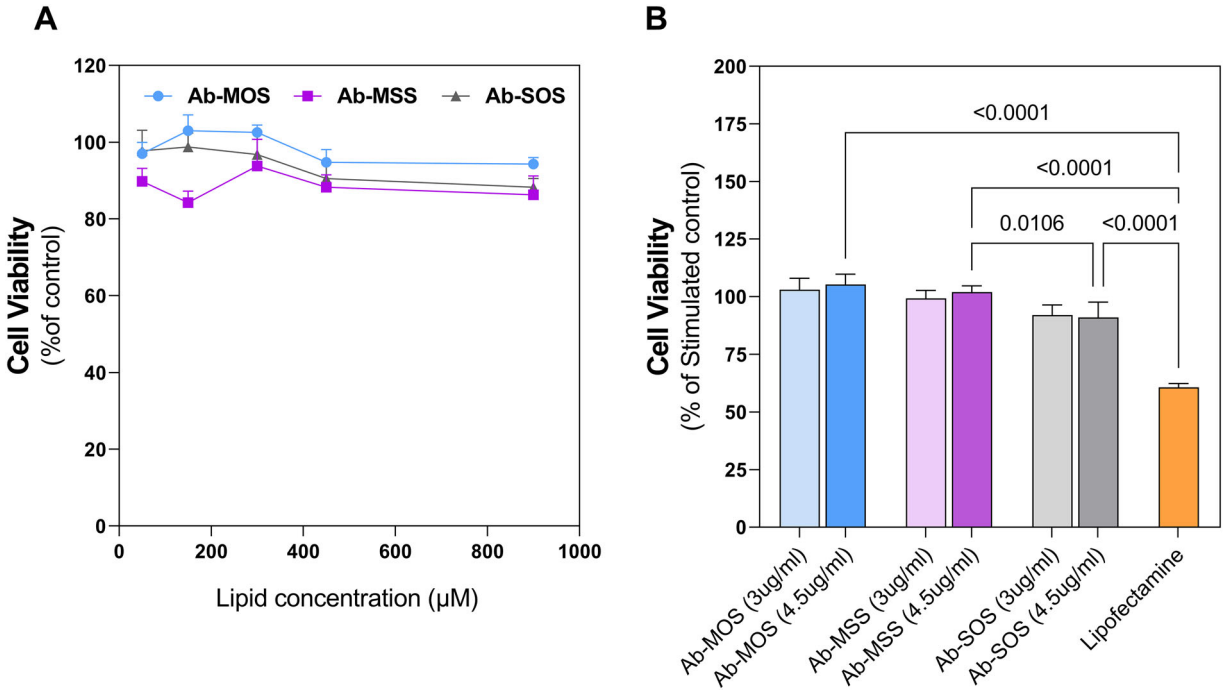

Figure 6. Evaluation of cytotoxicity of different liposomes and liposome-mediated siRNA transfection of (activated) HUVEC. (A) Viability of HUVEC treated with Ab-MOS, Ab-MSS and Ab-SOS for 4 h, at indicated lipid concentrations. (B) Viability of TNF-α activated HUVEC after 24 h transfection with Ab-MOS, Ab-MSS and Ab-SOS at indicated siRNA concentrations. $n = 4$. Numbers represent p values between indicated bars.

4. Discussion

The role of EC in inflammation and their accessibility for compounds from the blood make EC suitable target cells for pharmacological intervention in inflammatory diseases. However, achieving successful and effective drug delivery to EC is still a challenge (He *et al.* 2022). Previously, our team developed a SAINT-based liposome called SOS, which successfully delivered siRNA into activated EC to reduce endothelial cell activation (Adrian *et al.* 2010, Kowalski *et al.* 2013, Kowalski *et al.* 2014). This SOS formulation showed good physicochemical properties for in vivo application and relatively efficient cellular/intracellular processing of siRNA cargo by activated EC, however, its knockdown efficiency could still be improved. One of the possible reasons for low transfection efficacy might be the low amount of cationic lipids in the SOS formulation, since cationic lipids directly influence transfection activity (Adrian *et al.* 2010). In the current work, we formulated two liposomes inspired by SOS for advanced siRNA delivery to activated EC, by increasing the amount of cationic lipids in the formulation. Sizes smaller than 200 nm and good size stabilities were achieved with both 30 mol% MC3 containing MOS and 12 mol% MC3/18 mol% SAINT containing MSS. Formulation of 30 mol% SAINT containing SOS cannot be achieved, since the SOS become unstable when containing over 20% SAINT-C18 in the formulation (Adrian *et al.* 2010). Possibly, the small headgroup of the MC3 lipid allows for the formation of a tightly packed bilayer membrane at higher mol%, while the bulky headgroup of SAINT results in bilayer destabilisation in these conditions.

Compared to the MOS formulation, we found that the MSS has a significantly higher siRNA encapsulation efficiency with lower batch-to-batch variation. This indicates that the SAINT lipid in the formulation facilitates siRNA entrapment and retention in the aqueous core of the liposomes. Structurally, lipids with pyridinium headgroups (such as SAINT lipid) show delocalised positive charges on the heterocyclic moiety, leading to increased self-assembling capability of these amphiphiles (Savarala *et al.* 2013). In contrast, lipids bearing amine headgroups (such as MC3 lipid) exhibit relatively poor NA binding ability.

Typically, interactions between liposomes and cells are dictated by nanoparticle geometry and surface properties, which can be tuned by the lipid composition or adding targeting ligands (Verma *et al.* 2010, Zhao *et al.* 2019, Villanueva-Flores *et al.* 2020). Our data showed that mixing of the SAINT lipid with the MC3 lipid resulted in higher non-specific association with EC than their single cationic lipid-based formulations. This suggests that the specificity of lipid carriers for endothelial association is influenced by the cationic lipid composition, which was also shown by another study (He *et al.* 2022). Conjugating anti-VCAM-1 antibodies to the surface of the liposomes minimised the difference in cationic lipid-dependent cell association, and is a feasible strategy that can be applied to MOS and MSS formulations to achieve enhanced delivery of siRNA into activated EC.

We showed that increasing the cationic lipid content from 18 to 30 mol% improves siRNA transfection in EC, however, the transfection efficacy is also dependent on the cationic lipid composition. Cationic lipids with protonatable headgroups (e.g. tertiary amines and pyridinium) have been shown to be effective to mediate NA-based transfection, with different protonatable lipid headgroups leading to distinct transfection efficacies (Ponti *et al.* 2021). In our current work, the MOS formulation did not effectively silence RelA

gene expression, although MC3 lipid is considered as the standard amino-lipid in LNP settings for efficient siRNA-based gene silencing (Sato *et al.* 2021). This suggests that the high transfection potency of ionisable lipids (represented by MC3) might depend on the lipid carrier structure. Unlike the traditional liposome with a bilayer surrounding an aqueous core, the typical LNP formulation is hypothesised to possess an electron-dense core structure surrounded by a lipid monolayer (Evers *et al.* 2018). We speculate that MC3 lipids located in LNP structures somehow more easily exerts their membrane-destabilizing property at acidic pH by easier interacting with the anionic endosomal membrane lipid. This then forms an inverted hexagonal H_{II} phase, that triggers membrane destabilisation and further siRNA release (Semple *et al.* 2010). However, so far there is no experimental evidence to prove this structure-activity relationship. Compared to the Ab-MOS formulation, the Ab-MSS showed a higher transfection efficacy, and it downregulated the target gene expression in a dose-dependent manner. The diffused charge in pyridinium headgroup of SAINT may improve the NAs binding-release behaviour of the liposomes, resulting in improved transfection efficacy (Parvizi *et al.* 2014). Another possible explanation for higher transfection efficacy of MSS is that the overall liposomal pKa of MSS is closer to the optimum pKa for effective EC transfection. siRNA carriers with optimum pKa show a maximal gene silencing activity through their response to endosomal pH (Patel *et al.* 2021). The optimum pKa value for siRNA delivery depends on the cell type (Shobaki *et al.* 2018). A pKa in the range of $6.2 \sim 6.5$ is considered as the optimal value for in vivo gene silencing in hepatocytes (Jayaraman *et al.* 2012). Some studies reported that a slight pKa increase of particles, contributes to better siRNA delivery in liver sinusoidal endothelial cells (LSEC) (Sato *et al.* 2016, Shobaki *et al.* 2018). In our study, we found that the MSS with a higher pKa (6.84) showed higher uptake and better transfection activity in primary EC, compared to the MOS formulation with a lower pKa (6.44). This may suggest that a higher pKa is important for obtaining efficacious siRNA delivery in primary EC. But it was concluded that, the optimum pKa suitable for LSEC-specific sRNA delivery could not be applied to other types of EC *in vivo* (Sato *et al.* 2016). More studies are necessary to determine the optimum pKa of liposomes for effective siRNA delivery into EC.

As a therapeutic approach to silence endothelial activation, anti-inflammatory effects and biosafety of $siRNA_{RelA}$ containing liposomes are necessary to be assessed before in vivo application. Ab-MSS containing $siRNA_{RelA}$ exhibited anti-inflammatory effects on activated EC, as demonstrated by significant inhibition of upregulation of cell adhesion molecules (E-selectin, VCAM-1 and ICAM-1), which is in line with the results reported in the SOS study (Kowalski *et al.* 2014). However, a downregulation of other pro-inflammatory genes such as MCP-1 and IL-6 was not found after delivering RelA siRNA via Ab-MSS. This result may be caused by the limited maximum RelA knockdown efficiency (~57%) shown in this study, however which can be enhanced by using higher siRNA transfection concentration. The consequences of RelA knockdown mediated by Ab-MSS on the activation status of EC, should be further investigated to evaluate the therapeutic potential of this siRNA delivery system. In addition, all tested liposomes showed no or low toxicity to EC, which is a prerequisite for clinical translation. More importantly, at the effective siRNA transfection concentration, the MSS formulation exhibited significantly lower cytotoxicity than the SOS formulation, which suggests that the MSS formulation is more suitable for *in vivo* applications.

5. Conclusion

This work reports a novel liposomal formulation based on a combination of SAINT and MC3 lipid, which is suitable for delivery of siRNA to activated EC and that reduces endothelial cell activation in an *in vitro* evaluation setting. The MSS formulation is expected to offer enhanced *in vivo* delivery of therapeutic siRNA to diseased EC for the treatment of inflammatory diseases, although a thorough validation of this system with respect to safety and efficacy in *in vivo* models is a necessary next step to take.

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Data availability statement

Data are contained within this article.

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