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Anti-VCAM-1 and Anti-E-selectin SAINT-O-Somes for Selective Delivery of siRNA into Inflammation-Activated Primary Endothelial Cells

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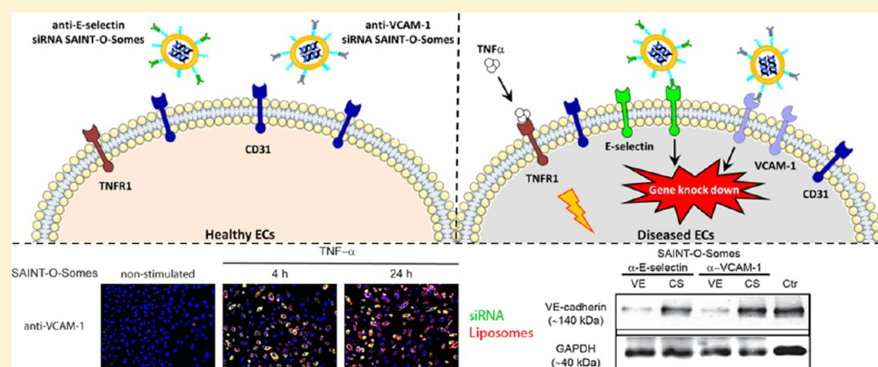
Supporting Information



ABSTRACT: Activated endothelial cells play a pivotal role in the pathology of inflammatory diseases and present a rational target for therapeutic intervention by endothelial specific delivery of short interfering RNAs (siRNA). This study demonstrates the potential of the recently developed new generation of liposomes based on cationic amphiphile SAINT-C18 (1-methyl-4-(*cis*-9-dioleyl)methyl-pyridinium-chloride) for functional and selective delivery of siRNA into inflamed primary endothelial cells. To create specificity for inflamed endothelial cells, these so-called SAINT-O-Somes were harnessed with antibodies against vascular cell adhesion protein 1 (VCAM-1) or respectively E-selectin and tested in TNF-α activated primary endothelial cells from venous and aortic vascular beds. Both targeted SAINT-O-Somes carrying siRNA against the endothelial gene VE-cadherin specifically downregulated its target mRNA and protein without exerting cellular toxicity. SAINT-O-Somes formulated with siRNA formed small particles (106 nm) with a 71% siRNA encapsulation efficiency. SAINT-O-Somes were stable in the presence of serum at 37 °C, protected siRNA from degradation by serum RNases, and after i.v. injection displayed pharmacokinetic comparable to conventional long circulating liposomes. These anti-VCAM-1 and anti-E-selectin SAINT-O-Somes are thus a novel drug delivery system that can achieve specific and effective delivery of siRNA into inflamed primary endothelial cells and have physicochemical features that comply with *in vivo* application demands.

KEYWORDS: *siRNA delivery, endothelium, adhesion molecules, Tumor Necrosis Factor, liposomes, SAINT*

1. INTRODUCTION

Gene silencing by means of RNA interference (RNAi) is a powerful technique with a potential for pharmacological application. Short interfering RNAs (siRNAs) are extensively investigated as a new class of molecular drugs due to their highly specific interference with gene expression. However, unmodified or uncomplexed siRNAs (so-called "naked" siRNAs) are subjected to rapid clearance from the circulation by the liver and by renal filtration which limits their usefulness as a therapeutic *per se*.[1,2] Moreover, the physicochemical properties of siRNAs, including their high molecular weight (~13−15 kDa), polyanionic nature (~40 negatively charged phosphate groups), and their susceptibility to degradation by serum RNases, enforce application of chemical modifications or proper formulation into a delivery system to create potent therapeutics.[3,4] siRNA carriers have proven to be able of knocking down targets in various diseases *in vivo* including hypercholesterolemia,[5] liver cirrhosis,[6] and cancer.[7] Moreover, first evidence of successful siRNA mediated gene silencing in humans by systemically delivered siRNA nanoparticles has been published recently.[8]

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Increased insight in the role of endothelial cells in the pathophysiology of cancer, inflammatory, and cardiovascular diseases has drawn great interest in pharmacological interventions toward the endothelial cells at diseased sites.[9,10] Their prevalence throughout the body and accessibility for intravenously administered compounds make them attractive targets for targeted drug delivery approaches. Interfering with molecular processes in diseased endothelial cells by means of siRNA will advance to create novel endothelial cells directed therapies. Santel and Aleku et al., for example, demonstrated that *in vivo* silencing of protein kinase N3 (PKN3) in the tumor vasculature by siRNA containing lipoplexes significantly inhibited growth of various types of tumors as well as formation of metastases.[11,12]

Despite successful attempts of siRNA delivery to tumor endothelium, targeted delivery to endothelial cells in inflamed sites is still ill-addressed. Upon pro-inflammatory stimulation, each organ displays a vascular bed specific pattern of cell adhesion molecules as E-selectin or vascular cell adhesion protein 1 (VCAM-1), providing challenging opportunities to deliver drugs or small RNAs to organ specific (micro)vascular endothelial subsets.[13] E-selectin expression is restricted to endothelial cells and dramatically upregulated during inflammation.[14,15] Also VCAM-1 is an attractive target as it is strongly upregulated upon inflammatory stimuli and during angiogenesis.[1,16] In previous studies, we successfully employed immunoliposomes targeted to E-selectin for delivery of the anti-inflammatory drug dexamethasone and the cytotoxic drug doxorubicine to inflamed endothelial cells.[17−19]

Limited processing of liposomes and subsequent release of their content displayed by primary endothelial cells remain a serious obstacle to achieve effective drug delivery.[18] To address this, we recently developed a new generation of liposomes called SAINT-O-Somes, based on formulation of conventional long circulating liposomes and the cationic amphiphilic lipid SAINT-C18 (1-methyl-4-(*cis*-9-dioleyl)methyl-pyridinium-chloride). SAINT-O-Somes showed superior intracellular release of their content in endothelial cells compared to conventional liposomes. Moreover, this system displayed the ability to efficiently encapsulate low molecular weight compounds and siRNA.[18] In the current study, we show that siRNA encapsulated in SAINT-O-Somes targeted to VCAM-1 or E-selectin can be successfully delivered into inflamed primary endothelial cells originating from distinct vascular beds (venous and arterial). We investigated the potential of SAINT-O-Somes to specifically deliver siRNA into activated endothelial cells taking into account their physicochemical features (size, stability, protection of siRNA) and target cell conditions as appear *in vivo*. In addition, *in vivo* pharmacokinetic (PK) behavior of siRNA SAINT-O-Somes was studied. Since little is known about the intracellular fate of siRNA delivery systems internalized *via* E-selectin and VCAM-1, we studied the intracellular trafficking of both siRNA and SAINT-O-Somes in the activated endothelial cells. Based on our results, we concluded that SAINT-O-Somes fulfill important requirements for successful targeted delivery of siRNA into inflamed endothelial cells and meet the requirements for *in vivo* application.

2. EXPERIMENTAL SECTION

2.1. Materials. Lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]-maleimide (DSPE-PEG$_{2000}$-Mal), and 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG$_{2000}$) were purchased from Avanti Polar Lipids (Alabaster AL, USA). The cationic lipid 1-methyl-4-(*cis*-9-dioleyl)methyl-pyridinium-chloride (SAINT-C18) was obtained from Synvolux Therapeutics (Groningen, The Netherlands). Cholesterol (Chol) and *N*-succinimidyl-*S*-acetylthioacetate (SATA) were obtained from Sigma (St. Louis MO, USA). Nucleic acid stain Hoechst 33342 (trihydrochloride), lipophilic tracers 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI), 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indodicarbocyanine perchlorate (DiD), and LysoTracker Green DND-26 probe were purchased from Molecular Probes (Leiden, The Netherlands). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Breda, The Netherlands). All siRNAs were purchased from Qiagen (Venlo, The Netherlands).

The H18/7-acb (mouse IgG2a antihuman E-selectin) and E1/6-aa2 (mouse IgG1 antihuman VCAM-1 antibody) monoclonal antibody-producing hybridomas were kindly provided by Dr. M. Gimbrone from Harvard Medical School (Boston, MA, USA).

2.2. Preparation of siRNA Containing SAINT-O-Somes. SAINT-O-Somes were prepared as described previously with slight modifications.[18] In brief, lipids from stock solutions of POPC, SAINT-C18, Chol, DSPE-PEG$_{2000}$, and DSPE-PEG$_{2000}$-Mal in chloroform−methanol (9:1) were mixed in a mol % ratio of 37:18:40:4:1. To fluorescently label the liposomal bilayer, DiI or DiD was added to the lipid mixture in a 0.25 mol % ratio of total lipid (TL). We prepared SAINT-O-Somes containing VE-cadherin siRNA (Hu_CDH5_2 FlexiTube siRNA, cat No. SI00028483) or control siRNA (AllStars Negative Control, cat No. 1027281), with no homology to any known mammalian gene, with or without a fluorescent label. siRNA was dissolved according to the protocol of the manufacturer and mixed with dried lipids at a ratio of 1 nmol siRNA per 1 μmol TL. After extrusion through polycarbonate filters (50 nm pore size) nonencapsulated siRNA was removed by ion exchange chromatography on a DEAE Sepharose CL-6B (Sigma, The Netherlands) column using HN buffer (135 mM NaCl, 10 mM HEPES) pH 6.7 as an eluent. The concentration of siRNA encapsulated in liposomes was measured using the Quant-iT Ribo-Green assay (Invitrogen, Breda, The Netherlands) according to the protocol of the manufacturer. The efficiency of siRNA encapsulation into liposomes was calculated based on the measurements of encapsulated siRNA in the presence or absence of 1% (v/v) Triton X-100 (Sigma-Aldrich, Zwijndrecht, The Netherlands). The monoclonal anti-E-selectin and anti-VCAM-1 antibodies were thiolated by means of SATA and coupled to a maleimide group at the distal end of the polyethylene glycol chain by sulfhydryl-maleimide coupling as described before for albumin.[20] SAINT-O-Somes without an antibody were prepared from the same lipid mixture, but instead of being conjugated to the antibody, they were allowed to react with cysteine in a molar ratio twice that of DSPE-PEG$_{2000}$-Mal to block reactive maleimide groups. The SAINT-O-Somes were characterized by determining the protein concentration using rat IgG as a standard and measuring the total lipid concentration by phosphorus assay.[21] The particle size and zeta potential were analyzed using a Nicomp model 380 ZLS submicrometer particle analyzer. Particle size was measured using dynamic light scattering (DLS) in the volume weighing mode (NICOMP

particle sizing systems, Santa Barbara, CA, USA). The polydispersity index (PDI) was calculated using a Malvern Zetasizer Nano ZSP (Malvern Instruments ltd., Worcestershire, UK). The number of antibody molecules coupled per liposome was calculated as described before.[22] SAINT-O-Somes were stored at 4 °C under argon gas and were used within 4 weeks.

2.3. Cryo-transmission Microscopy (Cryo-TEM). Cryo-TEM analysis of SAINT-O-Somes was performed as described previously.[18]

2.4. Cell Cultures. Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Breda, The Netherlands). Cells were cultured in EBM-2 medium supplemented with EGM-2 MV Single Quot Kit Supplements & Growth Factors (cat No. CC-3202, Lonza). In all experiments, cells from passages 5 to 7 were used, and they were plated on culture plates (Costar, Corning, NY) at a density of 1.8×10^4 cells/cm^2 one day before the experiment unless stated differently. Before seeding the cells, culture plates were incubated with EGM2MV medium for 30 min.

Human aortic endothelial cells (HAECs) were obtained from Cascade Biologics Invitrogen cell culture (Life Technologies, Bleiswijk, The Netherlands). Cells were cultured in Medium 200 (Life Technologies) supplemented with low serum growth component (LSGS, containing fetal bovine serum, 2% v/v; hydrocortisone, 1 μg/mL; human epidermal growth factor, 10 ng/mL; basic fibroblast growth factor, 3 ng/mL) and heparin 10 μg/mL, streptomycin 100 μg/mL, and penicillin 100 IU/mL). In all experiments, cells from passage 5 to 10 were used, and they were plated on culture plates (Costar) at a density of 2×10^4 cells/cm^2 one day before the experiment. All cell cultures were maintained by the endothelial cell facility of UMCG.

2.5. Influence of Serum on Particle Size Stability and siRNA Integrity. To determine the influence of temperature and serum on the size of siRNA SAINT-O-Somes, particles were incubated for 2, 4, 6, and 24 h at 37 °C in the presence or absence of 50% human serum. The size of liposomes was measured as described above by dynamic light scattering.

To investigate the integrity of encapsulated siRNA, SAINT-O-Somes containing 160 ng of control siRNA were incubated in the presence of 50% human serum for 0.5, 1, 4, 6, and 24 h at 37 °C. An equal amount of naked control siRNA was incubated with human serum for the same period of time as SAINT-O-Somes. At the end of the incubation, 1% (v/v) Triton X-100 and the gel loading dye (BioLabs, Leiden, The Netherlands) were added to the samples. Subsequently, samples were loaded on 2% agarose gel containing ethidium bromide and run for 15 min at 110 V. Bands were visualized using the ChemiDoc XRS system (Biorad, Veenendaal, The Netherlands). The integrity of encapsulated siRNA after eight weeks of storage at 4 °C under argon gas was also analyzed by agarose gel electrophoresis as described above.

2.6. Investigation of SAINT-O-Somes Uptake by Flow Cytometry and Fluorescence Microscopy. For flow cytometry experiments, HUVEC and HAEC were seeded in 24-well plates. The 10 ng/mL TNF-α (BioSource Europe, Nivelles, Belgium) was added to the cells 2 h before addition of 80 nmol TL/mL of SAINT-O-Somes containing AlexaFluor$_{488}$ siRNA. TNF-α remained present in the medium during further incubation with liposomes. To block E-selectin or respectively VCAM-1 protein, a 75-fold excess over the amount of antibodies coupled to the liposomes of anti-E-selectin or anti-VCAM-1 monoclonal antibodies was added to the cells, together with the liposomes. At 4 or 24 h after addition of SAINT-O-Somes, cells were washed twice with PBS and detached from the surface using trypsin/EDTA (Sigma, Ayrshire, UK) after which they were immediately transferred to tubes containing 5% FBS (fetal bovine serum, Thermo Scientific HyClone, Cramlington, UK) in PBS and kept on ice. Next, samples were centrifuged for 5 min at 500 g at 4 °C, followed by two washing steps with 3 mL of 5% FBS in PBS and resuspended in 0.2 mL PBS for flow cytometry analysis (Calibur, BD Biosciences, Franklin Lakes, NJ). When flow cytometry was performed the following day, cells were fixed with 0.5% paraformaldehyde in PBS and stored at 4 °C.

For fluorescence microscopy HUVEC were cultured on Lab-Tek Chamber Slides (Nunc, Rochester, NY, USA). Before seeding the cells, chamber slides were incubated with EGM2MV medium for 30 min. Cells were activated with TNF-α and incubated with liposomes in a similar way as described for the flow cytometry experiments. Targeted and nontargeted SAINT-O-Somes labeled with DiI and containing AlexaFluor$_{488}$ labeled siRNA were used at 80 nmol TL/mL. Nuclei of the cells were stained using Hoechst 33342. At the end of the incubation, cells were washed twice with ice-cold serum-free culture medium, placed on ice, and subjected to imaging within 45 min. Fluorescence images of cells were taken with a Leica DM/RXA fluorescence microscope (Wetzlar, Germany) using Quantimet HR600 image analysis software (Leica). Images were taken at excitation/emission wavelengths of 550/570 nm for DiI, 490/520 nm for siRNA AlexaFluor$_{488}$, and 350/461 nm for Hoechst 33342.

2.7. Investigation of Intracellular Processing of siRNA Containing SAINT-O-Somes by Confocal Microscopy. For confocal laser scanning microscopy (CLSM), HUVEC were cultured on sterile cover glasses (Menzel-Gläser, Braunschweig, Germany) placed into the wells of a 12-well plate. Before seeding the cells, the cover glasses were incubated with EGM2MV medium for 30 min. Prior to incubation with anti-E-selectin or anti-VCAM-1 SAINT-O-Somes, HUVEC were activated for 4 h with 10 ng/mL of TNF-α. Liposomes containing membrane label (DiD) and/or fluorescent siRNA were incubated with the cells at concentration of 80 nmol TL/mL for the indicated time periods. For staining of acidic organelles, cells were incubated with LysoTracker at a concentration of 75 nM for the last 20 min of the incubation. To stain late endosomal/lysosomal compartments cells were plated at a density of 1.4×10^4 cell/cm^2 and transfected with 2 μg/mL pLamp-1-RFP plasmid (obtained from Dr. Walther E. Mothes from Yale School of Medicine, New Haven, CT, USA) using Lipofectamine 2000, 48 h prior the experiment according to the manufacturer's protocol. At the end of the incubation, cells were washed twice with ice-cold serum-free culture medium, placed on ice, and subjected to imaging within 45 min. During this time, there was no change in cell morphology, and images taken within this period were reproducible throughout the different experiments. For studying the internalization, cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature (RT), and subsequently slides were mounted with Citi-Fluor AF1 (Citifluor Ltd., London, UK) and kept at 4 °C until analysis. Images were taken with a confocal laser scanning microscope (True Confocal Scanner SP2-AOBS; Leica, Heidelberg, Germany) equipped with argon (Ar) and helium−neon lasers (HeNe) and coupled to a LeicaDM RXE microscope using an HCXPL APO CS 63 × 1.40 oil immersion objective. Continuing scans were taken for fluorophore pairs AlexaFluor$_{488}$/DiD and LysoTracker green/DiD, while sequen-

tial scans were obtained for fluorophore pairs $AlexaFluor_{488}$/RFP to avoid bleed through. $AlexaFluor_{488}$ was excited using the 488 nm Ar laser line, $AlexaFluor_{546}$ and RPF were excited using the 543 nm HeNe laser line, and DiD was excited using the 633 nm HeNe laser line. All images were recorded in the linear range, avoiding local saturation, and at an image resolution of 1024 × 1024 pixels and with pinhole size of 1 Airy unit. A series of *xyz*-scans with a 0.3 μm step size were taken along the *z*-axis from top to bottom of the cells. Presented images show a single *z*-scan or maximum intensity projection of all *z*-scans from a single series as indicated. Images were analyzed and processed using Leica Confocal Software V2.61 and ImageJ V1.45s.

2.8. Gene Expression Analysis by RT-qPCR. For gene expression analysis, HUVEC and HAEC were seeded in 24-well plates. After 2 h of activation with TNF-α (10 ng/mL), anti-E-selectin or anti-VCAM-1 SAINT-O-Somes containing VE-cadherin specific siRNA or control siRNA were added to the cells at 1 μM siRNA concentration and incubated for 48 h. TNF-α was present in the medium during the entire incubation period. After 48 h total RNA was isolated using the RNeasy Mini Plus Kit (Qiagen, Venlo, The Netherlands) according to the protocol of the manufacturer. The amount of RNA was measured by a NanoDrop ND-1000 spectrophotometer (Wilmington, DE), and qualitative gel electrophoresis consistently showed intact RNA integrity. cDNA synthesis and quantitative (q) PCR, including data analysis, were performed as described previously.[18] The real-time PCR primers for human VE-cadherin (Hs00174344_m1) and GAPDH (Hs99999905_m1) were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands). Gene expression levels were normalized to the expression of the reference gene GAPDH and compared to cells treated only with TNF-α.

2.9. Protein Expression Analysis by Western Blot and ELISA. HUVEC and HAEC were seeded in 6-well plates, and after 2 h of activation with TNF-α (10 ng/mL), anti-E-selectin or anti-VCAM-1 SAINT-O-Somes containing VE-cadherin specific siRNA or control siRNA were added to the cells at 1 μM siRNA concentration. After 48 h of incubation cells were lysed using RIPA buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands), and lysates were used for Western blot and ELISA. Western blot analysis was performed as described previously,[23] with slight modifications. The membrane was horizontally cut through the 70 kDa prestained marker, and the upper part of the blot was incubated overnight at 4 °C with polyclonal rabbit antihuman VE-cadherin antibody (cat. no. #2158, Cell Signaling Technology, Inc., Leiden, The Netherlands). For loading control the lower part of the blot was incubated for 1 h at RT with monoclonal mouse anti-human GAPDH antibody (cat. no. mAbcam 9484, Abcam, Cambridge, UK). Antibody binding was visualized by horseradish peroxidase-conjugated anti-rabbit IgG (cat. no. 7074, Cell Signaling Technology, Inc., Leiden, The Netherlands) and anti-mouse IgG H+L (cat No. 1010-05 Southern Biotech, Birmingham, AL, USA). Chemiluminescence (Thermo Scientific, Rockford, IL, USA) signals were quantified by densitometric analysis using Quantity One software (Bio-Rad, Hercules, CA, USA). Protein expression from at least three independent experiments was quantified using DuoSet IC Human Total VE-cadherin ELISA (R&D Systems, Abingdon, UK), according to the manufacturer's protocol.

2.10. Investigation of Cellular Toxicity by MTS Assay. To investigate the influence of anti-E-selectin and anti-VCAM-1 siRNA SAINT-O-Somes on cell viability, HUVEC and HAEC were seeded in 96-well plates. Incubations with TNF-α and liposomes were performed as described for gene and protein expression analysis. After 48 h cells were washed twice with PBS followed by addition of 100 μL of fresh culture medium and 20 μL of CellTiter 96 AQ_{ueous} One Solution reagent (Promega, Leiden, The Netherlands). After 1.5 h, the absorbance at 490 nm was recorded with a Varioskan Flash Multimode reader (Thermo Scientific, Breda, The Netherlands). Absorbance of the activated endothelial cells without addition of SAINT-O-Somes was considered 100%.

2.11. Pharmacokinetics of (siRNA) SAINT-O-Somes. Male C57bl/6OlaHsd mice (18−23 g) were purchased from Harlan (Zeist, The Netherlands) and randomly divided into experimental groups. To induce an acute systemic inflammation mice were i.v. injected with 0.2 μg of recombinant mouse TNF-α (mrTNF-α; Gibco, Camarilo, CA, USA) in 0.9% NaCl, 2 h prior to injection of liposomes. Subsequently, animals received i.v. a single dose (10 μmol TL/kg, 3 mice/group) of $[^3H]$ cholesteryl hexadecyl ether (PerkinElmer, Shelton, CT, USA) labeled SAINT-O-Somes, empty or containing control siRNA. Blood was sampled at 10 min, 30 min, 1 h, 6 h, and 24 h. ^3H radioactivity was measured using a Packard Tri-Carb 2500 TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA). The total amount of radioactivity in the serum, separated from the blood by centrifugation, was calculated as described previously.[20] Pharmacokinetic parameters were calculated according to population analysis using the interactive two-stage Bayesian program.[24] All animal experiments were performed according to national guidelines and upon approval of the local Animal Care and Use Committee of Groningen University.

2.12. Statistical Analysis. Statistical analysis of the results was performed by a two-tailed unpaired Student's *t*-test, assuming equal variances to compare two replicate means, or one-way ANOVA followed by Bonferroni posthoc analysis to compare multiple replicate means. Differences were considered significant when $P < 0.05$.

3. RESULTS

3.1. Preparation and Characterization of SAINT-O-Somes Loaded with siRNA. In the present study we formulated SAINT-O-Somes containing 18 mol % of SAINT-C18 and investigated their potential for effective siRNA delivery into activated primary endothelial cells. The physicochemical properties of SAINT-O-Somes loaded with siRNA are given in Table 1. Using cryo-EM microscopy we demonstrated that siRNA SAINT-O-Somes mainly consist of

Table 1. Physicochemical Properties of siRNA Containing SAINT-O-Somes[a]

Formulation: POPC−SAINT−Chol−DSPE-PEG−DSPE-PEG-Mal, 37:18:40:4:1				
size [nm]	polydispersity index	zeta potential [mV]	Ab conjugated mol/liposome	siRNA encapsulation efficiency (%)
106 ± 48	0.17 ± 0.03	3.8 ± 5	24 ± 5	71 ± 15

[a]Data are presented as means of eight preparations ± SD. Ab, average amount for anti-E-selectin or anti-VCAM-1 antibody conjugated to liposome.

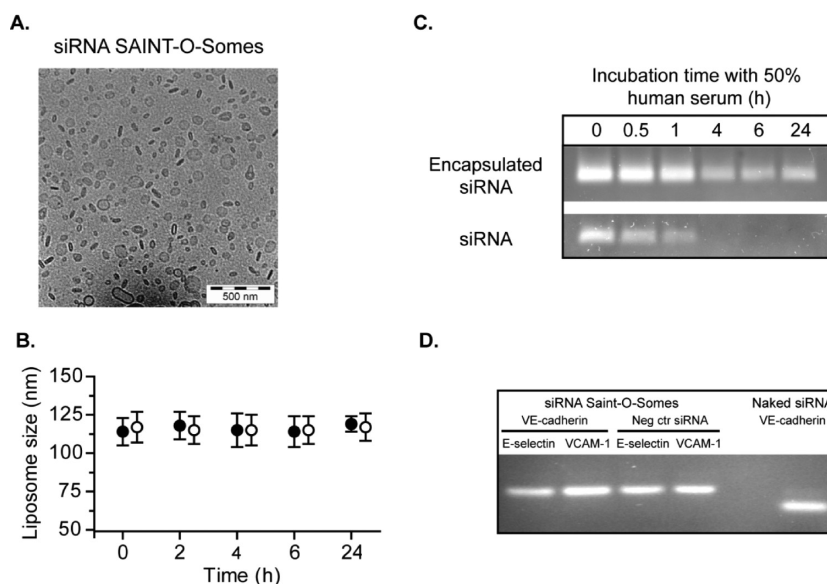

Figure 1. Characterization of siRNA containing SAINT-O-Somes. (A) Cryo-EM images of siRNA containing SAINT-O-Somes; (B) size stability of siRNA SAINT-O-Somes in the (●) presence or (○) absence of 50% serum. Particles were incubated for 2, 4, 6, and 24 h at 37 °C in HN buffer pH 7.4 or in 50% human serum. Data are presented as the diameter (nm) ± SD of three preparations; maintenance of siRNA integrity by encapsulation in SAINT-O-Somes was studied by comparison of equal amounts of nonencapsulated and encapsulated siRNA incubated in 50% human serum for 0.5, 1, 4, 6, and 24 h at 37 °C. (C) Agarose gel electrophoresis of nonencapsulated and encapsulated siRNA. (D) Agarose gel electrophoresis of siRNA encapsulated in anti-E-selectin or respectively anti-VCAM-1 antibody conjugated SAINT-O-Somes that were stored for 8 weeks at 4 °C; intact naked VE-cadherin siRNA was used as a control.

liposomes. Additionally, disk-like micelles were observed in the population (Figure 1 A), the formation of which could have been induced by the presence of DSPE-PEG_{2000} > 4 mol % TL in the liposome formulation as described by Johnsson and Edwards.[25,26] However, based on volume-weighted Gaussian distribution (data not shown) and the presented cryo-EM images, disc-like micelles comprise a minor proportion (<10%) of siRNA SAINT-O-Somes preparation. The average liposome size defined by DLS method was 106 nm with a corresponding polydispersity index of 0.17 which according to multiple sources[27,28] is considered acceptable for the sample to be homogeneous and monodisperse. siRNA SAINT-O-Somes showed good size stability in the presence of 50% serum and 37 °C (Figure 1B). The surface charge of targeted SAINT-O-Somes was neutral, and particles displayed a siRNA encapsulation efficiency of 71% ± 15 (Table 1). Based on the Ribogreen measurements we estimated that additional 5−6% of siRNA was electrostatically attached to the surface of liposomes. To investigate whether encapsulation into SAINT-O-Somes protects siRNA against RNases, we incubated particles with 50% human serum at 37 °C (Figure 1C). Nonencapsulated siRNA was entirely degraded in serum within 4 h, whereas formulation into SAINT-O-Somes preserved the integrity of siRNA for 24 h. Moreover, we demonstrated that SAINT-O-Somes could be stored for at least 8 weeks at 4 °C in HN buffer without loss of siRNA integrity (Figure 1D) and without change of the particle size (data not shown).

To demonstrate feasibility of SAINT-O-Somes for *in vivo* administration, we investigated the pharmacokinetic behavior of i.v. injected SAINT-O-Somes both empty and loaded with siRNA. The plasma concentration of the particles showed two-phase clearance kinetics with a short initial half-life ranging from 9 to 12 min and a secondary half-life of >11 h (Figure S1 and Table 2). siRNA SAINT-O-Somes showed a longer circulation time than empty SAINT-O-Somes, as indicated by a longer secondary half-life and significantly lower plasma clearance and steady state volume of distribution. Coupling of antibody to siRNA SAINT-O-Somes resulted in significantly prolonged secondary half-life from approximately 12 to 17 h and lower plasma clearance.

Table 2. Pharmacokinetic Parameters of siRNA SAINT-O-Somes[a]

parameter	SAINT-O-Somes empty	SAINT-O-Somes siRNA	SAINT-O-Somes siRNA + Ab
C_L, mL/h	2.87 ± 0.07	2.02 ± 0.11[b]	1.36 ± 0.04[c]
V_{ss}, mL/g	32.65 ± 0.53	25.92 ± 1.39[b]	26.09 ± 0.55
$t_{1/2}$ (1), h	0.15 ± 0.01	0.21 ± 0.02	0.21 ± 0.02
$t_{1/2}$ (2), h	11.33 ± 0.06	11.72 ± 0.01[b]	17.36 ± 1.67[c]

[a]Data are presented as mean values ± SD of 3 mice/group. C_L, plasma clearance; V_{ss}, steady-state volume of distribution; $t_{1/2}$ (1), initial half-life; $t_{1/2}$ (2), secondary half-life. [b]$P < 0.05$ empty vs siRNA. [c]$P < 0.05$ siRNA vs siRNA + Ab (antibody).

3.2. SAINT-O-Somes Targeted to VCAM-1 and E-selectin Specifically Deliver siRNA into TNF-α Activated Primary Endothelial Cells. To achieve specific delivery of siRNA to activated endothelial cells we conjugated SAINT-O-Somes with antibodies directed against E-selectin or VCAM-1. Primary HUVEC and HAEC were used to investigate specificity and efficacy of siRNA delivery. Cells were activated with TNF-α and incubated with targeted SAINT-O-Somes containing fluorescently labeled siRNA. As compared to resting cells, anti-E-selectin and anti-VCAM-1 SAINT-O-Somes showed a 35-fold increase in association (binding and uptake) of siRNA with activated HUVEC and a 25-fold increase with activated HAEC (Figure 2A, B). HUVEC and HAEC exhibited the highest association with anti-VCAM-1 SAINT-O-Somes after 24 h and comparable association between 4 and 24 h with anti-E-selectin SAINT-O-Somes, which corroborated the

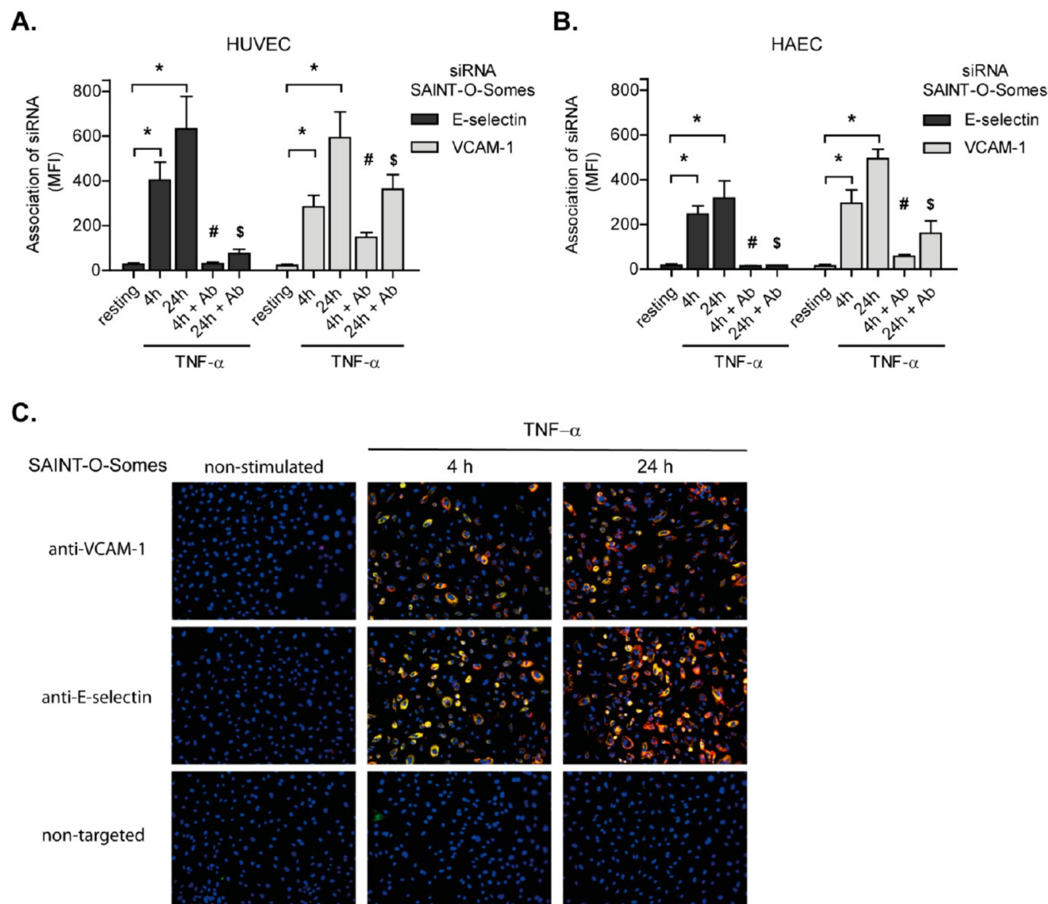

Figure 2. Selective delivery of siRNA to activated primary endothelial cells by targeted SAINT-O-Somes. Quiescent and TNF-α activated (**A**) HUVEC and (**B**) HAEC were incubated for 4 or 24 h with anti-E-selectin and anti-VCAM-1 SAINT-O-Somes containing AlexaFluor_{488} siRNA. Specificity of association to E-selectin respectively VCAM-1, was determined by coincubation with 75 times excess of anti-E-selectin or anti-VCAM-1 monoclonal antibodies together with the liposomes. The association of siRNA with the cells was quantified by flow cytometric analysis. Data are presented as mean florescence intensity (MFI) values ± SD of three independent experiments. * $P < 0.05$, # $P < 0.05$ - 4h vs 4h + Ab, \$ $P < 0.05$ - 24h vs 24h + Ab. (**C**) Fluorescence microscopy images of the uptake of targeted and nontargeted SAINT-O-Somes by cultured HUVEC. The liposome membrane was labeled with DiI (red) and encapsulated siRNA was labeled with AlexaFluor_{488} (green), the nuclei of the cells were stained using Hoechst (blue). The presented data set shows representative images, presented as merged pictures, of tree independent experiments. Original magnification 100x.

protein expression kinetics of both adhesion molecules (data not shown). In HUVEC, SAINT-O-Somes targeted to E-selectin or VCAM-1 showed comparable siRNA delivery, whereas in HAEC 50% more siRNA was delivered after 24 h by anti-VCAM-1 than by anti-E-selectin SAINT-O-Somes. To prove that association of siRNA in activated endothelial cells was mediated by E-selectin and VCAM-1, we coincubated cells with an excess of antibodies against both target proteins. This led to a significant decrease in the siRNA association with the activated cells (Figure 2A, B). Additionally, in the absence of targeting antibody on the surface of SAINT-O-Somes no association of siRNA was observed either in resting or in activated cells (Figure 2C and S2A, B). The uptake of targeted SAINT-O-Somes by activated endothelial cells was confirmed by fluorescence microscopy using DiI (red) labeled liposomes containing fluorescent siRNA (green) (Figure 2C). The two labels colocalized, indicating uptake of siRNA in conjunction with the carrier.

3.3. Intracellular Trafficking of Anti-VCAM-1 and Anti-E-selectin SAINT-O-Somes in Endothelial Cells. Uptake and intracellular trafficking of the anti-E-selectin and anti-VCAM-1 SAINT-O-Somes loaded with labeled siRNA in TNF-α activated HUVEC was investigated using CLSM. Alexa-Fluor_{546} siRNA (red) encapsulated in SAINT-O-Somes was binding to the surface of activated HUVEC at 4 °C (Figure 3A a1, a5) and was internalized at 37 °C (Figure 3A a2−a4, a6−a8). siRNA SAINT-O-Somes endocytosed via E-selectin and VCAM-1 localized in acidic compartments (late endosomes/lysosomes) within 4 h after start of the incubation with the cells (Figure 3B, C), as demonstrated by colocalization with LysoTracker (Figure 3B) or lysosomally expressed Lamp-1-RFP (Figure 3C). By tracking simultaneously labeled SAINT-O-Somes and the encapsulated siRNA we demonstrated colocalization of the two labels after 4 h and partial dissociation of the siRNA label from the lipid label after 24 h (Figure S3).

3.4. Anti-VCAM-1 and Anti-E-selectin SAINT-O-Somes Can Effectively Downregulate Target Genes in Primary Endothelial Cells. Using anti-E-selectin or anti-VCAM-1 siRNA SAINT-O-Somes we aimed to knock down a gene that is expressed in the endothelial cells and would pose a model for studying the effects of the targeted system. VE-cadherin is an endothelial gene regulating formation of adherent cell junctions,[29,30] the expression of which is restricted to endothelial cells and maintained under TNF-α stimulation

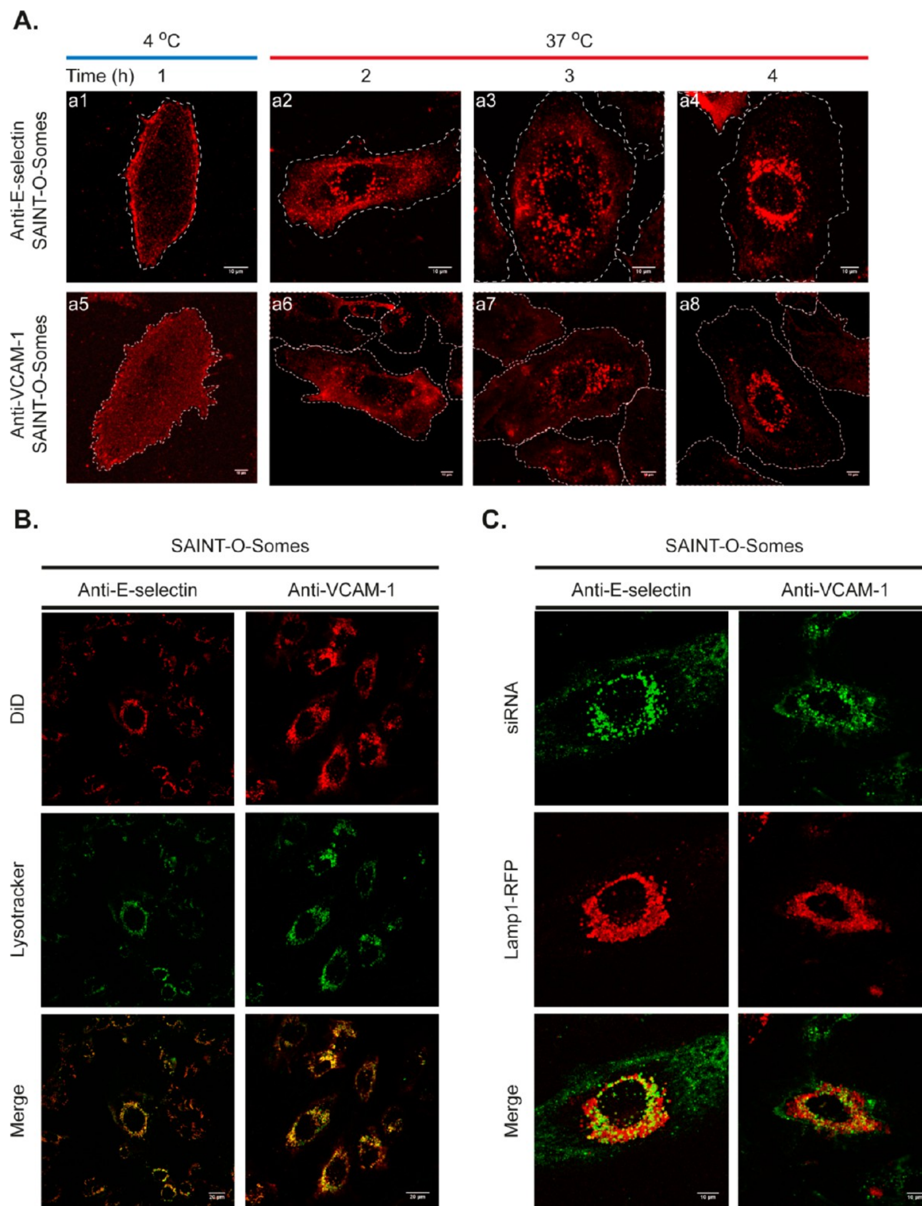

Figure 3. Anti-E-selectin and anti-VCAM-1 targeted SAINT-O-Somes loaded with siRNA directed to endolysosomal compartments. (A) Encapsulated siRNA labeled with $AlexaFluor_{546}$ (red) internalized via E-selectin or VCAM-1 displayed perinuclear localization in vesicular structures within 4 h after addition of liposomes (a1−a8). After addition of liposomes, cells were kept at 4 °C to allow binding but inhibit formation of endocytic vesicles (a1, a5). Borders of the cells (white dotted lines) were drawn based on deferential interference contrast (DIC) images. Images of the cells were taken using CLSM and are presented as a merge of x−y−z scans using maximum intensity projection; co-localization of SAINT-O-Somes and siRNA with acidic compartments (late endosomes/lysosomes) in activated HUVEC was demonstrated using (B) LysoTracker. After 4 h of incubation with targeted SAINT-O-Somes labeled with DiD (liposome membrane, red), LysoTracker (green) was added to the cells for 20 min. (C) Co-localization with late endolysosomal compartments was shown using cells transected with a plasmid expressing Lamp-1-RFP protein (red) prior to the experiment as described in the Experimental Section. After 4 h of incubation with targeted SAINT-O-Somes containing $AlexaFluor_{488}$ labeled siRNA (green), cells were visualized using CLSM and are presented as single z-scans. The presented data set shows representative images of three independent experiments.

(Supplementary Table 1), making it a suitable target gene for this study. TNF-α activated HUVEC and HAEC were incubated for 48 h with SAINT-O-Somes containing VE-cadherin specific siRNA (VE) or control siRNA at 1 μM concentration. SAINT-O-Somes loaded with VE-cadherin specific siRNA caused up to 60% downregulation of VE-cadherin mRNA in both HUVEC and HAEC, while uncoupled SAINT-O-Somes (data not shown) and SAINT-O-Somes containing control siRNA were devoid of an effect (Figure 4A, B). Moreover, VE-cadherin downregulation did not alter the expression of the unrelated endothelial genes CD31 or Tie2 in both HUVEC and HAEC (Figure S4A−D). Furthermore, reduction in VE-cadherin mRNA resulted in up to 50% decrease in protein expression in HUVEC and 25% in HAEC, as quantified by ELISA (Figure 4C, D), while no effects were observed with targeted SAINT-O-Somes containing control siRNA. Specific downregulation of VE-cadherin protein was also confirmed using Western blot analysis (Figure 4E, F).

The possibility that targeted siRNA SAINT-O-Somes influence VE-cadherin expression by exerting a toxic effect on

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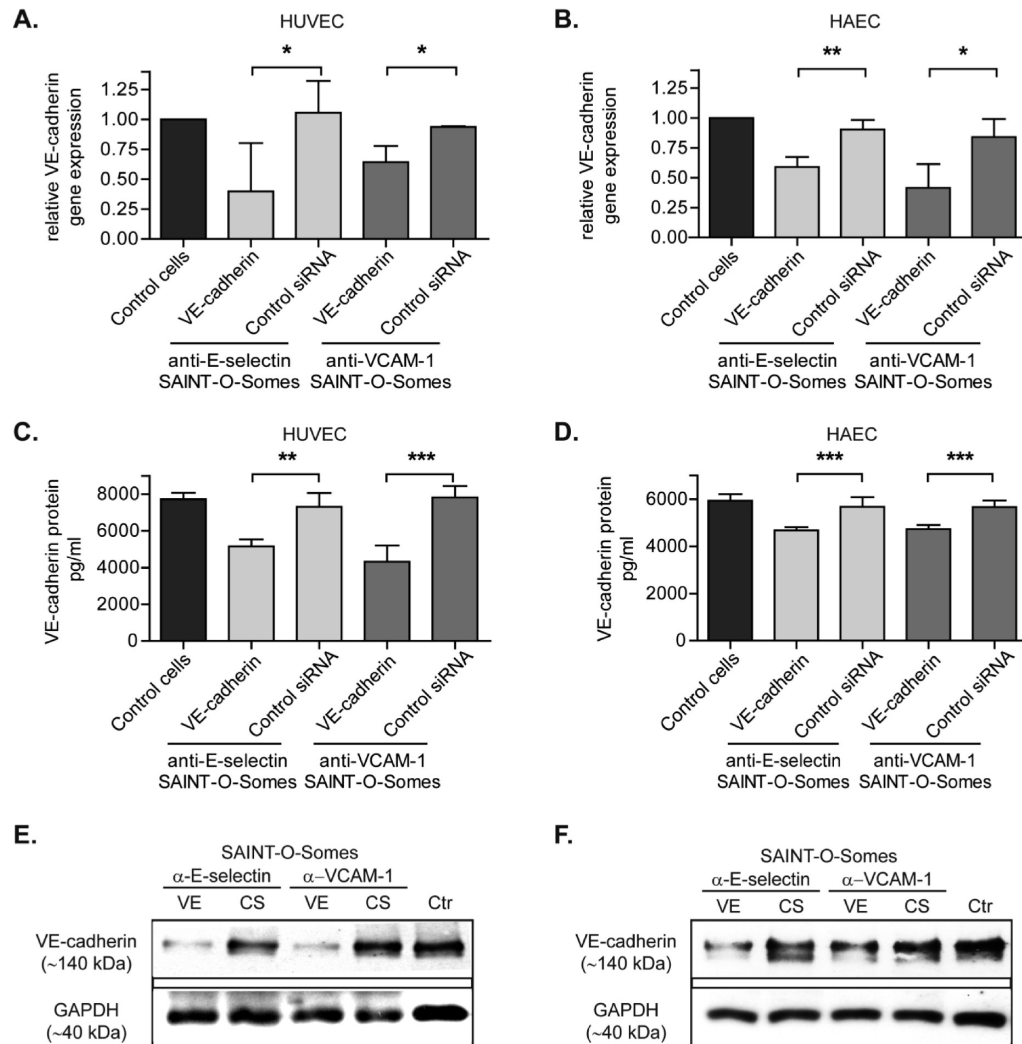

Figure 4. Effective downregulation of VE-cadherin in primary endothelial cells via targeted SAINT-O-Somes siRNA delivery. TNF-α activated HUVEC and HAEC were incubated with targeted SAINT-O-Somes containing VE-cadherin (VE) or control siRNA (CS) at 1 μM concentration for 48 h. After incubation, RNA or cell lysates were used for RT-qPCR (A,B), ELISA (C,D), and Western blot (E,F) as described in the Experimental Section. (A−B) Data are presented as relative expression ± SD, compared to cells treated only with TNF-α (Ctr), of a minimum of three independent experiments. *$P < 0.05$. (C−D) Data are presented as VE-cadherin protein expression ± SD of three independent experiments. (E−F) Images show a representative Western blot.

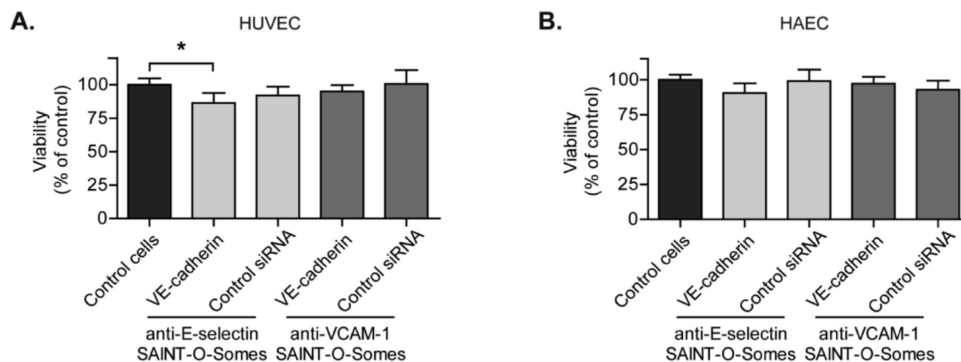

Figure 5. Targeted SAINT-O-Somes delivered siRNA do not affect cell viability. TNF-α activated HUVEC (A) and HAEC (B) were incubated with targeted SAINT-O-Somes containing VE-cadherin or control siRNA at a 1 μM concentration for 48 h. After the incubation viability of cells was investigated using CellTiter 96 AQ$_{ueous}$ One Solution reagent as described in the Experimental Section. The viability of activated endothelial cells without addition of SAINT-O-Somes was considered to be 100%. Data are presented as mean values ± SD of three independent experiments. *$P < 0.05$.

the cells was excluded by their negligible effect on endothelial cell viability (Figure 5A, B). TNF-α activated HUVEC and HAEC were incubated for 48 h with SAINT-O-Somes containing VE-cadherin or control siRNA at 1 μM concen-

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tration. A small or no decrease in cell viability was observed either in cells treated with SAINT-O-Somes containing VE-cadherin specific siRNA or in groups incubated with SAINT-O-Somes loaded with control siRNA.

4. DISCUSSION

Inflamed endothelial cells play a significant role in the pathology of cancer and inflammatory diseases and present an accessible target for systemically applied drug carriers.[13] As such, development of siRNA delivery devices for pharmacological intervention at the level of inflamed or angiogenic endothelium holds clinical potential. Development of a carrier which selectively delivers siRNA into endothelial cells at the site of inflammation or angiogenesis can enhance pharmacological efficacy and limit possible side effects of the treatment. In the current work we demonstrated that, by using SAINT-C18 based liposomes (SAINT-O-Somes) surface-modified with antibodies specific for the inflammatory adhesion molecules E-selectin or VCAM-1, we were able to selectively deliver siRNA into activated primary endothelial cells. We found that E-selectin and VCAM-1 mediated internalization guided SAINT-O-Somes to endolysosomal compartments and allowed the release of the encapsulated siRNA. Targeted SAINT-O-Somes containing VE-cadherin specific siRNA showed significant downregulation of both the target gene mRNA and protein without exerting cellular toxicity. Furthermore, we demonstrated that the physicochemical properties and *in vivo* pharmacokinetic behavior of SAINT-O-Somes renders them suitable for *in vivo* delivery of siRNA. From this study we conclude that anti-E-selectin and anti-VCAM-1 SAINT-O-Somes have the features that allows specific and effective delivery of siRNA to inflamed endothelial cells *in vivo*, which justifies further *in vivo* studies on the use of siRNA SAINT-O-Somes to interfere with inflammatory diseases.

SAINT-O-Somes formulated with siRNA and 18 mol % of SAINT-C18 in the liposomal bilayer form small and unilamellar particles. Reported disc-like micelles are not expected to contribute significantly to the physicochemical properties and findings reported in this manuscript since they comprise <10% of siRNA SAINT-O-Some preparation, that with a polydispersity index of 0.17 is considered homogeneous and monodisperse. The here-described SAINT-O-Somes are stable in the presence of serum and show an siRNA encapsulation efficiency of 71%, which is high compared to other liposomal delivery platforms incorporating cationic lipids.[31,32] Good stability and encapsulation efficiency are crucial features of *in vivo* siRNA delivery systems. For example, lipoplex formulations can reach >90% siRNA entrapment efficiency,[33] but their limited stability under physiological conditions restricts *in vivo* application.[34] Our results indicated that the integrity of siRNA encapsulated in SAINT-O-Somes is preserved under conditions that resemble the *in vivo* situation (presence of 50% serum and 37 °C) as well as upon long-term storage. We showed that i.v. injected SAINT-O-Somes display a two-phase PK with a long secondary half-life >11 h, comparable to the PK behavior of conventional long circulating liposomes, that have been successfully used to target endothelial cells *in vivo*.[17] Encapsulation of siRNA significantly influenced the PK parameters and resulted in delayed clearance from the circulation. This could be explained by lower surface charge of SAINT-O-Somes containing negatively charged siRNA. Coupling of antibody to siRNA SAINT-O-Somes prolonged the circulation time from approximately 12 to 17 h. Combined, our results justify use of siRNA SAINT-O-Somes in follow-up *in vivo* studies in the future.

In the current study we focused on primary endothelial cells as they more closely represent endothelial cells *in vivo* than cell lines. Previous studies demonstrated that these cells are difficult to transfect[23] and show limited processing capabilities,[18] which makes them a challenging target for our newly designed siRNA carrier. Recently, Whitehead et al.[35] demonstrated that, in contrast to cell lines, primary cells were found to yield the most predictive correlations between *in vitro* and *in vivo* siRNA delivery efficacy of lipid nanoparticles. Taking into account the well-established heterogeneity of endothelial cells *in vivo*,[36] we included primary cells from two distinct vascular beds in our investigation (HUVEC—venous, HAEC—arterial). Both VCAM-1 and E-selectin have been described as attractive targets for endothelial specific drug delivery;[13] however use of VCAM-1 as a target for siRNA delivery to activated endothelial cells has not been demonstrated before. Here we compared association of siRNA encapsulated into E-selectin or VCAM-1 targeted SAINT-O-Somes with activated HUVEC and HAEC. We demonstrated that both anti-E-selectin and anti-VCAM-1 SAINT-O-Somes allowed robust and specific siRNA delivery into activated cells and showed comparable downregulation of VE-cadherin mRNA without exerting any cellular toxicity, as often seen with siRNA delivery systems containing cationic amphiphiles.[37] Up to 60% downregulation of VE-cadherin mRNA was reached in both cell types which was associated by 50% decrease in protein expression in HUVEC and 25% in HAEC (Figure 4C−F). Sato et al.[6] demonstrated that 60% downregulation of a target gene mRNA and protein by siRNA-bearing liposomes targeted to hepatic stellate cells was sufficient to almost completely resolve liver fibrosis and prolonged survival of cirrhotic rats. Thus, the demonstrated capacity of targeted SAINT-O-Somes for gene silencing could be sufficient to interfere with the expression of disease-associated genes and requires further investigation in animal models. Differences in the efficacy of protein downregulation between HUVEC and HAEC in our study could not be attributed to differences in mRNA and protein expression levels (Table 2, Supporting Information). Since HAEC and HUVEC exerted a similar extent of uptake of siRNA and siRNA delivered by the SAINT-O-Somes did not affect cell viability, possibly the ability of both cells to process SAINT-O-Somes influencing siRNA release is responsible for the observed differences. Moreover, the efficacy of the siRNA delivery can be affected by different molecular aspects that are part of the concept of heterogeneity of endothelial cells.[38] We demonstrated that anti-E-selectin and anti-VCAM-1 SAINT-O-Somes can effectively deliver siRNA to primary endothelial cells originating from different vascular beds, which provides an opportunity for effective targeting of disease-associated endothelial cell subsets in different vascular segments.

Target epitopes residing at the membrane of the target cells should internalize upon ligand binding and allow intracellular release of the siRNA, which is a prerequisite for effective gene downregulation. It was previously shown that both E-selectin and VCAM-1 could mediate internalization of immunoliposomes conjugated with antibodies targeting these proteins.[15,39] Nevertheless little is known about the intracellular processing of siRNA delivered *via* this route. Therefore we studied the fate of anti-VCAM-1 and anti-E-selectin SAINT-O-Somes containing siRNA after binding to the endothelial cell surface applying confocal microscopy. siRNA endocytosed *via* E-selectin and

VCAM-1 localized in endolysosomal compartments in the perinuclear region within 4 h after the start of incubation of cells with SAINT-O-Somes. Our observations are in line with findings describing transport of VCAM-1 and E-selectin ligands mainly *via* clathrin-mediated endocytosis to lysosomes (summarized by Muro et al.[40]) although involvement of other pathways, such as macropinocytosis or cell adhesion molecule endocytosis cannot be excluded. Adrian et al.[18] reported that at low pH (pH < 5.5) the SAINT-O-Some bilayer is destabilized, which enhances content release. Within the endolysosomal compartments the low pH may thus be able to facilitate destabilization of the particles and enhance the release of siRNA. Furthermore, cationic lipids such as SAINT can destabilize the endosomal membrane by inducing "flipping" of anionic lipids in the endosomal bilayer which facilitates release of the cargo into the cytoplasm.[41] Under physiological conditions a polyethylene-glycol (PEG) coating limits interaction of particles with the cell membrane by masking their surface charge.[42] Destabilization of SAINT-O-Some bilayer may reduce the effect of PEG and allow SAINT to interact with the endosomal membrane. Moreover, we showed that SAINT-O-Somes and encapsulated siRNA followed the same route of internalization and partially dissociated after 24 h, which indicates limited release of siRNA from the carrier. Up to now, little is known about the relation between siRNA disassembly from the carrier and the efficacy of gene silencing. The first tools to study intracellular disassembly of siRNA nanocomplexes have been developed only recently.[43] Poor processing of liposomes by HUVEC, as reported by Adrian et al.,[18] may as well contribute to limited release of siRNA. Based on the intracellular trafficking of SAINT-O-Somes targeted to VCAM-1 and E-selectin, the inclusion of pH-sensitive components in the formulation (e.g., pH-sensitive PEG) might improve the efficacy of siRNA release.[32,44] Further studies focusing on the exact siRNA release mechanism involving SAINT-O-Somes will shed more light and facilitate the rational design of these SAINT-based carriers.

We here report a liposomal formulation that allows selective and functional siRNA delivery into inflammation activated primary endothelial cells, mediates significant downregulation of a target gene, and is suitable for *in vivo* application based on their physicochemical and pharmacokinetic features. We demonstrated that both VCAM-1 and E-selectin can serve as an efficient and specific entry route for siRNA delivery to inflamed endothelial cells. Liposomes based on cationic amphiphile SAINT-C18 (SAINT-O-Somes) are a suitable carrier for siRNA and when harnessed with anti-VCAM-1 or anti-E-selectin antibodies allow effective delivery of siRNA to activated primary endothelial cells from venous and aortic vascular beds. Anti-VCAM-1 and anti-E-selectin SAINT-O-Somes are thus a novel drug delivery system that provide an opportunity for siRNA delivery to disease-associated endothelial cell subsets in different vascular segments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 showing blood clearance of empty and siRNA loaded SAINT-O-Somes. Figure S2 showing the association of nontargeted SAINT-O-Somes with endothelial cells. Figure S3 showing CLSM images of intracellular colocalization of SAINT-O-Somes and siRNA in activated HUVEC. Figure S4 showing expression of endothelial genes CD31 and Tie-2 in HUVEC and HAEC after transfection with anti-E-selectin and anti-VCAM-1 SAINT-O-Somes containing VE-cadherin specific or control siRNA. Supplementary Table 1 showing basal expression of VE-cadherin, CD31, and Tie-2 in resting or TNF-α activated cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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