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Targeted SAINT-O-Somes for improved intracellular delivery of siRNA and cytotoxic drugs into endothelial cells

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ABSTRACT

In non-phagocytic cells such as endothelial cells, processing of liposomes and subsequent release of drug content is often inefficient due to the absence of professional processing machinery, which limits pharmacological efficacy. We therefore developed a liposome based drug delivery system with superior intracellular release characteristics. The design was based on long circulating conventional liposomes that were formulated with a cationic amphiphile, 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chlorid (SAINT-C18). These so-called SAINT-O-Somes had a diameter of 100 nm, were as stable as conventionally formulated liposomes, and showed superior release of their content at pH conditions that liposomes encounter when they are endocytosed by cells. Attachment of anti-E-selectin specific antibodies to the distal end of surface grafted poly(ethylene glycol) resulted in immuno-SAINT-O-Somes that were as efficiently taken up by inflammation activated endothelial cells as conventional anti-E-selectin specific immunoliposomes. More importantly, intracellular release of calcein encapsulated in these targeted SAINT-O-Somes was 10 fold higher as compared to the release of calcein from conventional liposomes. For intracellular delivery siRNA into activated endothelial cells, formulation with SAINT-C18 was a necessity to induce a specific down-regulation of gene expression of VE-cadherin. Additionally, targeted doxorubicin loaded SAINT-O-Somes decreased endothelial cell viability significantly more than targeted conventional doxorubicin liposomes. SAINT-O-Somes therefore represent a new class of lipid based particles with superior drug release characteristics that can be applied for the efficacious intracellular delivery of hydrophilic drugs including siRNA.

1. Introduction

Drug delivery systems such as lipid based or polymer based nanoparticles show improved pharmacological and therapeutic properties compared to conventional drugs by changing the pharmacokinetics of these drugs. Today several drug delivery systems aimed at a variety of diseases have reached the clinic [1]. In most cases the delivery mechanism of liposomal drug formulations is based on passive targeting, i.e., extravasation of (long circulating) liposomes at sites of enhanced vascular permeability as a result of the so-called enhanced permeability and retention effect [2]. Leaky vasculature is a common feature of tumor growth and inflammatory reactions. Once small size liposomes have passed the endothelium into the diseased area the majority of the liposomes will be taken up by tumor cells, or tumor and inflammation associated macrophages [3–5]. These will efficiently degrade the liposomal drug carrier, resulting in release of the free drug at relatively high concentrations. Macrophages are professionals in scavenging and processing of particles and are therefore often the target of choice for liposomal drug delivery systems.

Increased insight in the role of endothelial cells in the pathology of cancer and inflammatory diseases has shifted the interest in the development of targeted drug delivery devices for pharmacological intervention of these cells [6,7]. Additionally endothelial cells are readily accessible for substances transported by the blood and endothelial cell heterogeneity allows for specific drug targeting approaches to subsets of endothelial cells that are vascular bed and/or disease specific [8]. However, endothelial cells are not equipped to process liposomes and the resulting inferior intracellular drug release properties make that therapeutic effects of targeted liposome formulations are limited.

A class of cationic pyridinium-derived lipids known as SAINT (Synthetic Amphiphile INTeraction), is successfully used as transfection

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reagent for cultured cells. When mixed with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), SAINT-C18 forms complexes with DNA that are characterized by high transfection efficiency and low toxicity [9]. Also siRNA and proteins [10] were shown to be efficiently delivered into the cytoplasma of cells with the SAINT:DOPE mixture.

We hypothesized that based on its properties, SAINT combined with established endothelial cell targeted long circulating liposomes that have high drug loading capacity would lead to hybrid drug delivery devices that have physicochemical advantages of both systems, i.e. stability in the blood circulation and efficient intracellular release of content after intracellular routing of the device. We incorporated SAINT-C18 (1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chlorid) into the lipid bilayer of targeted long circulating liposomes, resulting in particles we denominated SAINT-O-Somes. The influence of SAINT-C18 in the lipid membrane on particle stability was determined and the release of encapsulated material from SAINT-O-Somes was studied at different pH and in cultured endothelial cells using the fluorescent marker calcein. Anti-E-selectin antibody, covalently coupled to the distal end of surface grafted poly(ethylene glycol) furthermore created selectivity for inflammation activated endothelial cells and would route the immuno-SAINT-O-Somes into the cells via receptor mediated endocytosis [11]. Applying the anti-E-selectin targeted SAINT-O-Somes for delivery of siRNA and cytostatic drugs into activated endothelial cells, we demonstrate that incorporation of SAINT lipid into liposomes results in formation of stable particles which can serve as effective delivery vesicles of hydrophilic molecules for cells that do not have efficient processing machinery for conventional liposomes.

2. Materials and methods

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 (Mal-PEG_{2000}-DSPE), 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster AL, USA). 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chlorid (SAINT-C18) was purchased from Synvolux Therapeutics Inc. (Groningen, The Netherlands) [1α,2α(n)-^{3}H] cholesteryl oleyl ether (^{3}H-COE) and cholesteryl-[^{14}C]-oleate (^{14}C-COE) was from GE Healthcare Europe (Diagem, Belgium). Cholesterol (Chol) and N-succinimidyl-S-acetylthioacetate (SATA) were from Sigma (St. Louis MO, USA). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was from Molecular Probes (Leiden, the Netherlands).

The monoclonal rat anti-mouse E-selectin antibody (MES-1) was kindly provided by Dr. D. Brown, UCB Celltech, Slough, UK. The H18/7 (mouse IgG2a anti-human E-selectin) monoclonal antibody [12] producing hybridoma was kindly provided by Dr. M. Gimbrone (Boston, MA). H18/7 was isolated and purified as described before [11].

2.2. Liposome preparation

Lipids from stock solutions of POPC, Chol, DSPE-PEG_{2000} and DSPE-PEG_{2000}-Mal in chloroform:methanol (9:1), were mixed in a molar ratio of 55:40:4:1. Where indicated, SAINT-C18 was added to the lipid mixture at the indicated molar %, always at the cost of the amount of POPC. Where appropriate, trace amounts of [^{3}H]cholesteryloleyl-ether and cholesteryl-[^{14}C]-oleate were added to the preparation as non-degradable and degradable markers, respectively. To fluorescently label liposomes in the lipid bilayer, 0.5 mol% DiI was added to the lipid mixture. Next, the lipids were dried under reduced nitrogen pressure, dissolved in cyclohexane and lyophilized. The lipids were then hydrated in HN buffer (10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), 135 mM NaCl) pH 6.7 or where appropriate to incorporate a fluorescent cargo, in an aqueous solution of 0.4 M NaOH containing 100 mM calcein (Sigma), followed by 10 times rapid freezing (liquid N_2) and thawing (water bath at 40 °C). Formed liposomes were sized by repeated extrusion (13 times) at 40 °C through polycarbonate filters (Costar, Cambridge MA, USA), pore size 50 nm, using a high pressure extruder (Lipex, Vancouver, Canada). When calcein was encapsulated into liposomes, free calcein was removed by gel chromatography on a Sephadex G-50 column using HN buffer pH 6.7 as eluent. For doxorubicin (Tocris, UK) encapsulation the lipids were hydrated in 300 mM $(NH_4)_2SO_4$. After extrusion of the liposomes, doxorubicin was encapsulated through remote loading as described before [13,14]. The encapsulation efficiency for doxorubicin was, comparable for conventional liposomes and SAINT-O-Somes, more than 95%. Doxorubicin content was 117 ± 17 μg/μmol of total lipid and 113 ± 25 μg/μmol of total lipid for conventional liposomes and SAINT-O-Somes, respectively.

The monoclonal anti-E-selectin antibody was thiolated by means of SATA and coupled to a maleimide group at the distal end of the polyethylene glycol chain by sulfhydryl-maleimide coupling exactly as described before for albumin [15]. Liposomes without antibody (non-immunoliposome control) were prepared from the same lipid mixture, but instead of being incubated with antibody they were incubated with cystein in a molar amount, twice that of DSPE-PEG_{2000}-Mal to block reactive maleimido groups. The liposomes were characterized by determining protein concentration using mouse immunoglobulin G as a standard [16] and phospholipid phosphorus content [17]. Total liposomal lipid concentrations were adjusted for the amount of cholesterol and SAINT-C18 present in the liposome preparations. Particle size and Zeta-potential were analyzed by dynamic light scattering using a Nicomp model 380 ZLS submicron particle analyzer. Particle size was measured in the volume weighing mode (NICOMP particle sizing systems, Santa Barbara, CA, USA). The number of E-selectin molecules coupled to a liposome particle was calculated as described before [18]. Liposomes were stored at 4 °C under argon.

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

Cryo-TEM investigations were performed with a LEO 912 OMEGA electron microscope (Zeiss, Oberkochen, Germany) operating at 120 kV and 'zero-loss' conditions. After placing a droplet (approximately 5 μl, 10 μmol/ml) of the sample on a copper grid (Quantifoil S7/2 Cu 100 400 mesh, holey carbon films, Quantifoil Micro Tools GmbH, Jena, Germany), most of the liquid was absorbed by a filter paper, so that only a thin (100–500 nm) liquid film remained. The sample was then immediately shock-frozen by plunging into liquid ethane [19]. The vitrified sample was stored at −196 °C in liquid nitrogen until it was loaded into a cryogenic sample holder (D626, Gatan Inc, Pleasanton, USA). The specimen were examined at −174 °C. Digital images were recorded with a slow-scan CCD camera system (Proscan HSC 2, Oxford Instruments, Abington, USA) and at a minimal under-focus of the microscope objective lens to provide sufficient phase contrast [20]. Pictures were processed with software iTEM 5.0 ((Build 1054), Soft Imaging System GmbH, Münster, Germany).

2.4. Incorporation of siRNA into liposomes

Liposomes containing siRNA were prepared as described above. Dry lipids were hydrated with VE-cadherin siRNA (Mm_Cdh5_2_HP siRNA; target sequence AAGGATCAAGTCCAATCTAAA, (Qiagen Benelux, Venlo, The Netherlands)) or nonsilencing siRNA with no homology to any known mammalian gene (ALLStars negative control, Qiagen) in a ratio of 1 nmol siRNA per 1 μmol total lipid. siRNA was dissolved according to the protocol of the manufacturer. The concentration of siRNA encapsulated into liposomes was measured using Quant-iT™ RiboGreen® 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 siRNA in the absence and presence of 0.1% Triton X-100.

2.5. Calcein release study

For calcein release experiments liposomes were diluted 1:20 in a buffer (0.1–0.2 μmol TL/ml) of appropriate pH (range of pH 4.0 to 5.6 acetate buffer, pH 6.0 to 8.0 HN buffer). Measurement of the samples was performed in 96 well plates (Corning, Costar) using a Bio-Tek FL600™ fluorescence microplate reader (Bio-Tek Instruments, USA) with excitation and emission wavelengths of 485 nm and 530 nm, respectively. Fluorescence of each sample was measured in the absence and in the presence of 1% Triton X-100, which is supposed to cause total release of calcein. The calcein release R was calculated using

$$R = \frac{F_{-} - F_c\omega}{F_{+} - F_c\omega}.$$

In this formula, F_{-} represents the measured fluorescence in the absence and F_{+} in the presence of Triton X-100. F_c is the measured fluorescence of control liposomes, to correct for any background fluorescence unrelated to contained calcein. A scaling factor ω, the ratio of F_{+} and the fluorescence measured for control liposomes with added Triton X-100, compensate for any inherent differences introduced by the separate preparation of SAINT-O-Somes and control liposomes.

2.6. Size stability measurements

To determine the influence of temperature and serum on the size of SAINT-O-Somes, particles were incubated for 1, 4, 7 and 24 h at 37 °C and room temperature in the presence or absence of 10% fetal calf serum (FCS). The size of liposomes was measured as described above by dynamic light scattering.

2.7. Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from the Endothelial Cell Facility UMCG (Groningen, The Netherlands). Cells were isolated from umbilical cords as described before [21]. Isolated cells from at least two umbilical cords were pooled and cultured to confluence (i.e., approximately 60,000 cells/cm^2) in 25 cm^2 flasks (Corning, Costar) coated with 1% gelatin in a culture medium containing RPMI 1640 supplemented with 20% heat inactivated FCS, 2 mM L-glutamine, 5 U/ml heparine, 50 μg/ml endothelial cell growth factor, 100 μg/ml streptomycin and 100 IU/ml penicillin. For experiments, confluent HUVEC were detached by Trypsin–EDTA (0.5 mg/ml in PBS pH 7.4), diluted 1:3 and seeded in 1% gelatin coated 24 well plates or 96 well plates, resulting in 70% of cell confluency after an additional 24 h of culture. In all experiments passages 2 to 4 of HUVEC were used.

The mouse endothelial cell line H5V [22] was kindly provided by Dr. A. Vecchi (Instituto Mario Negri, Milan, Italy), H5V cells were cultured in DMEM supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin. One day before the experiment the cells were seeded in a 24 well plate at confluency 60–70%.

IC-21 cells (American Type Culture Collection, Rockville, MD), created by viral transformation of C57BL/6 murine peritoneal macrophages by simian virus 40, were cultured in RPMI 1640 supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin. One day before the experiment cells were seeded in a 24 well plate at confluency 60–70%.

2.8. Degradation of radioactively labeled liposomes by the cells

HUVEC were activated with 10 ng/ml TNF-α (Boehringer, Ingelheim, Germany) for the indicated time. Anti-E-sel immunoliposomes double labeled with [^{3}H]cholesteryloleyl-ether and cholesteryl-[^{14}C]-oleate, were added to the cells at a concentration of 80 nmol/ml and incubated for the indicated time in the presence of 5% FCS at 37 °C. Hereafter, the medium was removed and the cells were thoroughly washed 5 times with ice-cold PBS. Cells were then lysed with 0.4 M NaOH and cell associated radioactivity was measured by scintillation counting and normalized to the amount of cellular protein determined by protein assay [23] using bovine serum albumin as a standard.

From the ^{3}H and ^{14}C radioactivity the ^{3}H/^{14}C ratio was calculated at each time point and normalized to the initial ^{3}H/^{14}C ratio in the liposomes as a measure for liposome degradation [24]. Following lysosomal degradation the liposomal cholesteryl-[^{14}C]-oleate is hydrolyzed, resulting in the release of [^{14}C]-oleate into the medium. The non-degradable [^{3}H]cholesteryloleyl-ether will remain cell associated and thus the cellular ^{3}H/^{14}C ratio is a measure of liposome degradation.

2.9. Cellular uptake of liposomes by microscopy

For confocal laser scanning microscopy HUVEC were cultured on sterile cover glasses put into the wells of a 24 well plate. For fluorescence microscopy HUVEC were cultured on Lab-Tek Chamber Slides (NUNC, Rochester, NY). Before seeding the cells, glasses in the 24 well plate and Lab-Tek™ were coated with 1% gelatin for 45 min followed by 15 min fixation with 0.5% glutaraldehyde and extensive washing with PBS (2 times) and culture medium (6 times).

HUVEC were activated with 10 ng/ml TNF-α for 1 h and incubated with DiI and/or calcein labeled liposomes at a concentration of 80 nmol/ml for time periods indicated in the presence of 5% FCS at 37 °C. For fluorescent microscope images, nuclei of the cells were stained with Hoechst 33342 (Invitrogen, Leiden, The Netherlands).

At the end of the incubation the cells were placed on ice, washed 5 times with ice-cold serum free culture medium and placed in the dark until microscopy within 1 h after finishing the incubation. During this time, there was no change in cell morphology and images taken within this period were reproducible throughout the different independent experiments.

Confocal scanning images were taken with a confocal scanning laser microscope (True Confocal Scanner SP2; Leica, Heidelberg, Germany) equipped with argon-krypton and neon lasers and coupled to a Leica DM RXE microscope. 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 549/565 nm for DiI, 485/530 nm for calcein, and 350/461 nm for Hoechst 33342.

2.10. Flow cytometry

For flow cytometry experiments, HUVEC were seeded in 24 well plates. Where indicated 1 h before addition of liposomes cells were activated with 10 ng/ml TNF-α. The cytokine remained present in the medium during further incubation with liposomes. Liposomes fluorescently labeled with either DiI or calcein were added to HUVEC at 80 nmol lipid/ml and incubated 3 h. After 3 h cells were washed 2 times with PBS, after which HUVEC were detached from the surface by trypsin/EDTA and transferred immediately to tubes containing FCS and kept on ice. Next, cells were washed twice with 1 ml 5% FCS in PBS. Cells were resuspended in 0.16 ml 5% FCS/PBS and analyzed by flow cytometry (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.

2.11. Gene expression analysis by real-time RT-PCR

Mouse H5V endothelial cells were cultured as described above. After 1 h activation with recombinant mouse TNF-α (10 ng/ml)

(BioSource Europe, Nivelles, Belgium) anti-E-selectin SAINT-O-Somes containing VE-cadherin specific siRNA and scrambled siRNA were added to the cells at an siRNA concentration of 1000 pmol/ml, and incubated for 48 h. TNF-α was present in the medium during the whole incubation period. Next, liposomes were removed and cells were washed twice with PBS. Total RNA from H5V cells 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 Spectrophotometer (Wilmington, DE) and consistently found to be intact as analyzed qualitatively by gel electrophoresis. Subsequently, synthesis of first-strand cDNA from total RNA was performed with SuperScript™ III RNase H-Reverse Transcriptase (Invitrogen) and 40 U RNaseOut inhibitor (Invitrogen) in a volume of 20 µl containing 250 ng random hexamers (Promega, Leiden, The Netherlands). cDNA was diluted with Millipore water to 10 ng/µl and 1 µl was applied for each PCR reaction. The primers for VE-cadherin (Mm00486938_m1) and GAPDH (Mm99999915_g1) were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands) and used for real-time PCR. The PCR reaction was carried out in ABsolute QPCR Rox Mix (Thermo Scientific). The amplification reaction was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) using the following program 15 min 95 °C, 40 cycles 15 s 95 °C and 1 min 60 °C. For each sample, the real-time PCR reaction was performed in duplicate and the averages of the obtained threshold cycle values (C_t) were processed for further calculations according to the comparative C_t method. Gene expression levels were normalized to the expression of the housekeeping gene GAPDH giving the ΔC_t value. Then the average value of ΔC_t obtained from non-treated H5V cells was subtracted from the average of the ΔC_t value of treated cells and relative mRNA levels were calculated by $2^{-\Delta\Delta Ct}$.

2.12. Cell viability assay

Cell viability was determined using a [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] (MTS) assay (Promega, Madison WI, USA). TNF-α activated HUVEC were incubated in 96 well plates with anti-E-selectin immunoliposomes or anti-E-selectin SAINT-O-Somes containing doxorubicin or with free doxorubicin for 3 h in the presence of 5% FCS at 37 °C. Then the culture medium was discarded and replaced by fresh culture medium and after further incubation for 21 h, cell viability was measured.

2.13. Statistical analysis

Statistical analysis of differences was performed by a two tailed unpaired Student's *t*-test. Differences were considered significant when $P<0.05$.

3. Results

3.1. Conventional anti-E-selectin immunoliposomes are taken up but not efficiently degraded by primary endothelial cells

We previously showed that anti-E-selectin immunoliposomes are extensively taken up by TNF-α activated HUVEC [11,25]. TNF-α activated HUVEC incubated 24 h with DiI labeled anti-E-selectin immunoliposomes showed extensive liposomes accumulation in distinct intracellular vesicles. No indication of redistribution of the fluorescent label over cellular membranes was obtained, suggesting limited processing of the liposomes in these 24 h (Fig. 1A). Quantification of degradation of the anti-E-selectin immunoliposomes double labeled with the metabolically degradable cholesteryl [^{14}C]oleate ester and the non-degradable [^{3}H]cholesteryloleyl-ether in HUVEC showed that the ^{3}H/^{14}C ratio did not change upon prolonged incubation, indicating the absence of hydrolysis of cholesteryl-[^{14}C]oleate (the ^{3}H/^{14}C ratio after 24 h was 1.1 ± 0.02). In contrast, exposure of the macrophage cell line IC-21 to the same liposomal formulations demonstrated already after 3 h of incubation intracellular processing of the liposomes, which corroborates the acknowledged capacity of macrophages to degrade these conventional liposomal formulations (Fig. 1B).

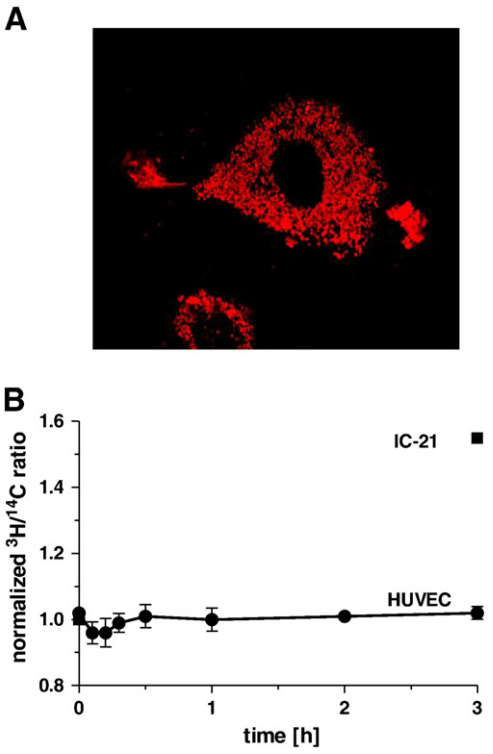

Fig. 1. E-selectin targeted liposomes are extensively taken up by HUVEC but hardly degraded inside the endocytic vesicles. A, TNF-α activated HUVEC were incubated for 24 h with DiI labeled anti-E-selectin immunoliposomes (red) and visualized using confocal laser scanning microscope as described in Materials and methods. Note the high intracellular accumulation of liposomes in vesicular structures. B, To quantify degradation of liposomes, anti-E-selectin immunoliposomes labeled with the metabolically degradable cholesteryl[^{14}C]oleate ester and the non-degradable [^{3}H]cholesteryloleyl-ether were incubated with TNF-α activated HUVEC and IC-21 macrophages for the indicated time. The degradation of liposomes by the cells was determined as described in Materials and methods. Data are presented as ± SD of 1 to 3 experiments.

3.2. Preparation and characterization of liposomes containing SAINT-C18 lipid

To design novel liposomal drug delivery formulations that display an improved intracellular processing by endothelial cells, we mixed the cationic amphiphile SAINT-C18 with conventional liposomal lipids (referred to as SAINT-O-Somes). With SAINT-C18 concentrations between 0 and 20 mol%, after extrusion through 50 nm filters, stable and homogenous particles with a size of around 100 nm were formed (Fig. 2). Higher amounts of SAINT-C18 incorporated into the lipid bilayer resulted in a significant increase in particle size and a non-homogenous size distribution of the particles.

Liposomes which contained up to 20% SAINT-C18 were characterized by good size stability in conditions that mimic the physiological situation (37 °C and in the presence of serum) (Fig. 3A, B). Also during storage at 4 °C we did not observe significant changes in the size of SAINT-O-Somes for at least one month (Fig. 3C).

To investigate whether SAINT-C18 lipid in the membrane of liposomes can improve release of encapsulated molecules we first used the fluorescent dye calcein as a model compound. At a high calcein concentration (100 mM) encapsulated inside the liposomes, the fluorescence of calcein is quenched. When the calcein is released e.g.,

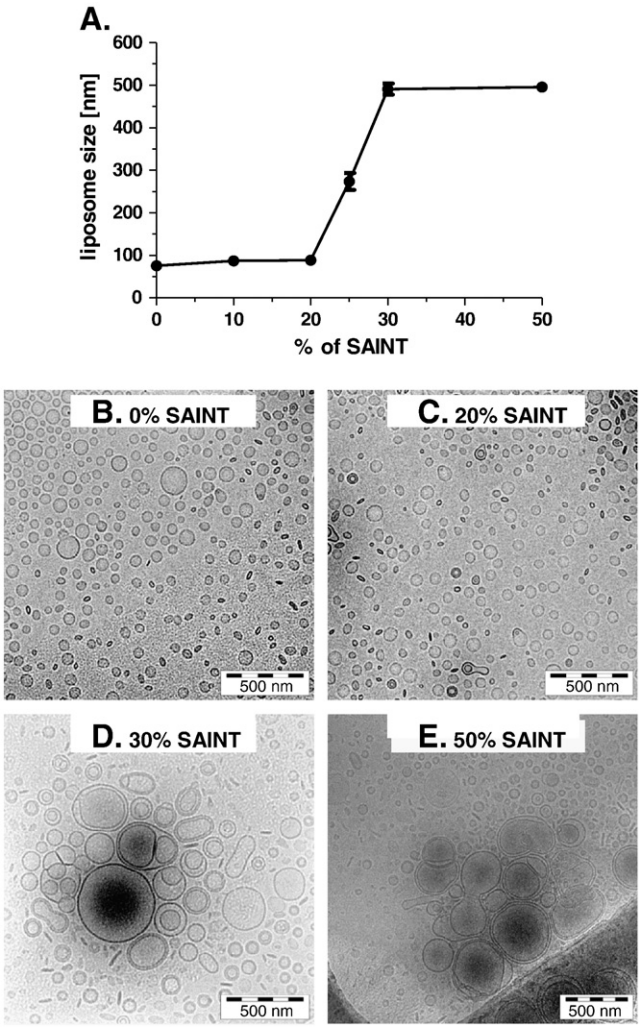

Fig. 2. Size and size distribution of liposomes containing increasing concentrations of SAINT-C18. Size of liposomes containing increasing concentration of SAINT-C18 was measured by dynamic light scattering using a Nicomp model 380 ZLS submicron particle analyzer in the volume weighing mode. Data are presented as diameter ± SD of 3 preparations (A). Photographs B to E depict Cryo-TEM images of liposomes containing 0% (B), 20% (C), 30% (D) and 50% (E) SAINT-C18.

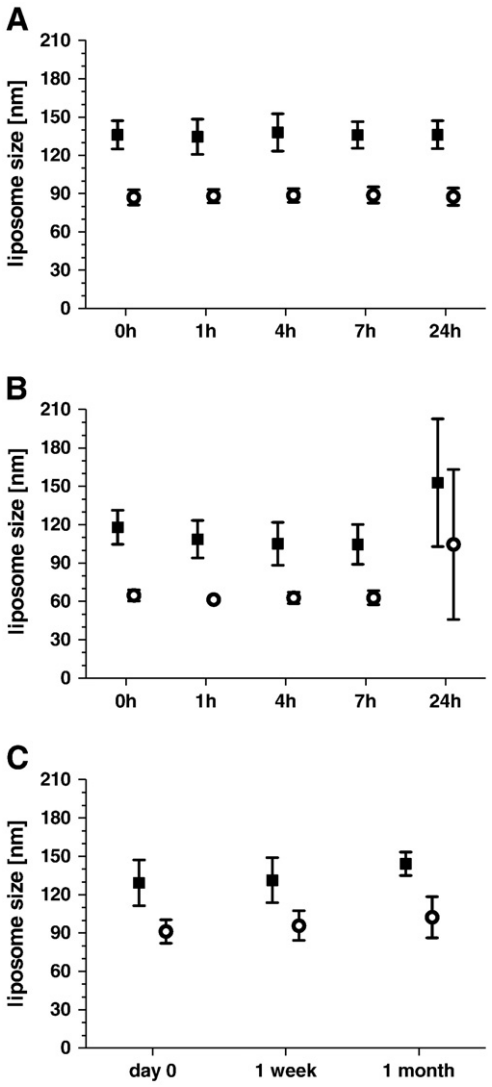

Fig. 3. Size stability of SAINT-O-Somes. Liposomes containing 0% (○) and 20% (■) SAINT-C18 were incubated for 1, 4, 7 or 24 h in the HN buffer pH 7.4 at 37 °C in the absence (A) or presence of 10% serum (B). The size stability of liposomes was also monitored during storage of the liposomes at 4 °C (C). The size of liposomes was measured as described in Materials and methods. Data are presented as diameter (nm) ± SD of 3 to 4 preparations.

upon membrane disruption, the consequent dilution of the calcein results in a fluorescence signal [26]. Using this property, the release of the model compound from the different formulations was compared at neutral pH 7.4 and acidic pH 5.0 which mimics the pH of endosomal compartments of the endocytotic pathway. While SAINT-O-Somes containing 10, 15 and 20% SAINT-C18 released only a limited amount of calcein at pH 7.4, at pH 5.0 a SAINT-C18 concentration dependent release of the dye was observed (Fig. 4A).

To further elaborate on the pH dependent release properties of liposomes containing SAINT-C18, the release of calcein from SAINT-O-Somes containing 20% SAINT-C18 in their bilayer was tested in the pH range from 4.6 to 8.0 and compared to liposomes containing DOTAP as cationic lipid. As presented in Fig. 4B only at a pH lower than 5.6 release of calcein was observed. The release profile of DOTAP liposomes was the same as for liposomes containing SAINT-C18, yet the total amount of released calcein at all tested pH values was significantly lower compared to that of the SAINT-O-Somes (Fig. 4B).

3.3. Uptake of liposomes containing SAINT-C18 by endothelial cells

To achieve the uptake of SAINT-O-Somes by activated HUVEC, a monoclonal antibody directed against E-selectin was covalently coupled to SAINT-O-Somes. The physicochemical properties of anti-E-selectin SAINT-O-Somes are presented in Table 1 and were compared to those of conventional anti-E-selectin immunoliposomes. The antibody density per particle surface area was comparable for both types of liposomes. The Zeta-potential was slightly negative for all liposome preparations.

The interaction of anti-E-selectin SAINT-O-Somes with HUVEC was determined using liposomes labeled either with DiI or calcein. The extent of uptake of anti-E-selectin SAINT-O-Somes by HUVEC was similar to that of anti-E-selectin immunoliposomes (Fig. 5A). The uptake was primarily driven by the anti-E-selectin/E-selectin interaction as no significant uptake was observed with non-targeted liposomes and E-selectin targeted liposomes and SAINT-O-Somes were only taken up by TNF-α activated endothelial cells.

While the uptake by TNF-α activated HUVEC was similar for anti-E-selectin SAINT-O-Somes and conventional anti-E-selectin liposomes, the calcein fluorescence detected was 10 fold higher when the cells were incubated with anti-E-selectin SAINT-O-Somes, indicating extensive intracellular release of the encapsulated calcein (Fig. 5B). A small though significant calcein fluorescence signal was

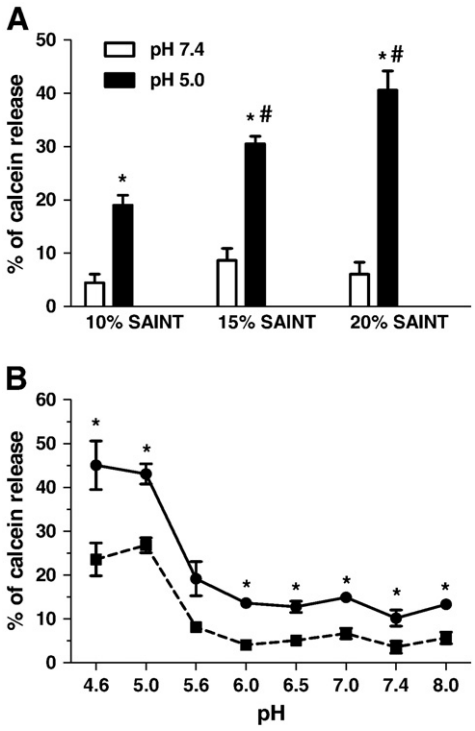

Fig. 4. Release of calcein from SAINT-O-Somes is pH sensitive. A, Release of calcein from liposomes containing increasing concentrations of SAINT-C18 was measured in buffer pH 7.4 or 5.0 as described in Materials and methods. Data are presented as relative calcein release ± SD of 3 to 4 preparations. *$P<0.05$ vs pH 7.4, #$P<0.05$ vs 10% SAINT pH 5.0. B, Release of calcein from liposomes containing 20% SAINT-C18 (●) compared to release of calcein from liposomes containing 20% DOTAP (■) in a buffer with pH ranging from 4.6 to 8.0. Data are presented as relative calcein release ± SEM of 3 to 4 preparations. *$P<0.05$ vs pH 7.4.

observed in non activated cells incubated with anti-E-selectin SAINT-O-Somes.

The binding and uptake of anti-E-selectin liposomes containing SAINT-C18 that were double labeled with DiI and calcein by activated HUVEC was also visualized by fluorescence microscopy (Fig. 5C). The fluorescent signal of calcein was already visible after 15 min incubation in the cells treated with anti-E-selectin SAINT-O-Somes, and continued to increase at 60 min, indicating a rapid release of the SAINT-O-Some content inside the endothelial cells. In contrast, in activated HUVEC treated with conventionally formulated anti-E-selectin immunoliposomes only a weak calcein signal was visible in the time frame studied (Fig. 5C (a–c)).

3.4. Superior content release by SAINT-O-Somes leads to effective delivery of siRNA

In order to investigate whether the improved content delivery capacity of the SAINT-O-Somes translates into superior effects of formulated drugs, we chose siRNA as a model drug. The uptake of siRNA by cells is limited due to its physicochemical characteristics and requires vehicles which introduce siRNA into the cytoplasm where its target mRNA is located. As siRNA is water soluble it is highly suitable for accommodation in the aqueous compartment of the liposomes.

Table 1
Characterization of SAINT-O-Somes.

		Anti-E-selectin liposomes		
Liposomes	Size [nm]	Size [nm]	μg Ab/μmol TL	Ab mol/liposome
0% SAINT-C18	80 ± 4	107 ± 10	105 ± 27	37 ± 15
20% SAINT-C18	101 ± 14	146 ± 18	106 ± 38	68 ± 31

Data are presented as means of 9 preparations ± SD. TL, total lipid, Ab, anti-E-selectin antibody.

The endothelial specific gene VE-cadherin [27] was selected as target gene for siRNA. The encapsulation efficiency of siRNA into anti-E-selectin SAINT-O-Somes was 63 ± 24% and significantly higher as compared to that of conventional immunoliposomes (3 ± 2%) which did not contain SAINT-C18 in their bilayer. Determination of the amount of siRNA associated in the presence or absence of triton X-100 revealed that 80% of the siRNA was in the SAINT-O-Some's interior. The amount of siRNA associated with conventional anti-E-selectin liposomes was almost 20 times lower than with anti-E-selectin SAINT-O-Somes (Fig. 6A).

Strong down-regulation of the VE-cadherin gene in TNF-α activated H5V endothelial cells was observed after 48 h incubation with anti-E-selectin SAINT-O-Somes containing specific siRNA for VE-cadherin (Fig. 6B). This effect was not observed in the cells incubated with liposomes containing scrambled siRNA. It was not possible to adequately compare siRNA effect of conventional liposomes with SAINT-O-Somes due to the large difference in siRNA encapsulation efficiency. Conventional anti-E-selectin liposomes containing siRNA did not affect VE-cadherin expression at liposome concentration similar to that of anti-E-selectin SAINT-O-Somes (result not shown).

3.5. Effect of doxorubicin containing SAINT-O-Somes on cell viability

Conventional liposomes and SAINT-O-Somes were remote loaded with doxorubicin after which anti-E-selectin was coupled to the particles. TNF-α activated HUVEC were incubated for 3 h with E-selectin targeted liposomes or SAINT-O-Somes allowing the cells to endocytose the lipid particles followed by further incubation of 21 h. Doxorubicin loaded anti-E-selectin SAINT-O-Somes decreased the cell viability by 79% as compared to non doxorubicin treated cells, which was comparable to the effect of free doxorubicin (74%) (Fig. 7). With conventional doxorubicin loaded anti-E-selectin liposomes viability of HUVEC was decreased by 42%, significantly less than with the targeted SAINT-O-Somes.

4. Discussion

Endothelial cells represent a cell type that is of interest for therapeutic intervention which does not have the intrinsic capacity to extensively degrade targeted delivery devices. In the present study we describe the design and characterization of a new high payload drug delivery device that has superior intracellular drug release properties with exquisite performance for endothelial cell specific drug delivery.

Efficient intracellular release of liposome content was induced by applying a class of cationic pyridinium-derived lipids called SAINT for the formation of the SAINT-O-Somes. When complexed with nucleotides or proteins, a mixture of SAINT:DOPE (1:1) acts as an efficient transfection agent for these molecules [9,10,28]. The limited stability of these SAINT complexes (lipoplexes) and the lack of cell specificity restrict them for *in vivo* applications. In this study we incorporated SAINT-C18 into the lipid bilayer of long circulating liposomes and characterized the stability and release properties. Selectivity for activated endothelial cells was created by introducing an anti-E-selectin monoclonal antibody to the SAINT-O-Some surface.

Homogeneous, small and unilamellar particles were formed when applying between 5 and 20 mol% of SAINT-C18 in the lipid bilayer. A further increase of the SAINT-C18 concentration in the membrane of liposomes resulted in the formation of large size particles (500 nm). Possibly the rather bulky structure of the SAINT-C18 lipid pyridinium ring influences formation of the tightly packed liposomal membrane at higher mol%. Additionally, SAINT-C18 lipid has two unsaturated alkyl chains which may affect fluidity of the membrane, as a result of which liposomes with high SAINT-C18 concentration may pass

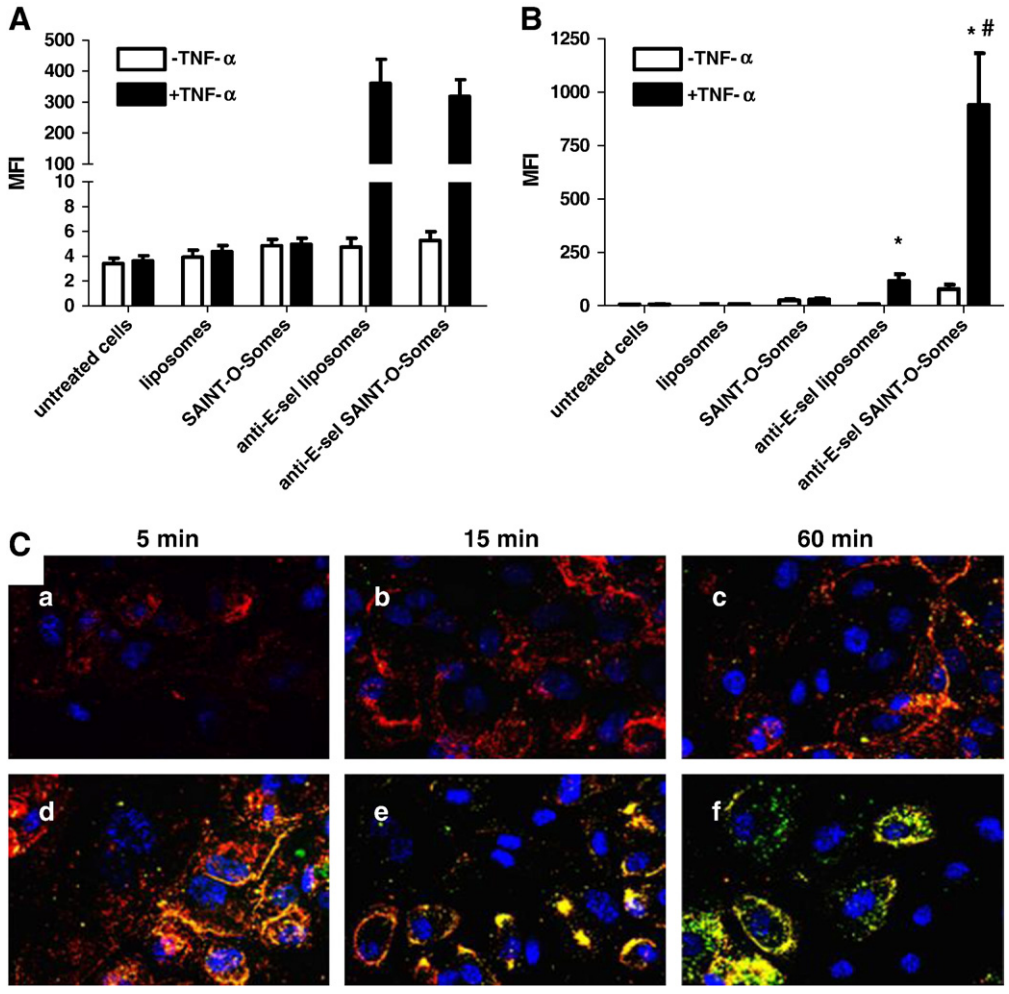

Fig. 5. Endothelial cell uptake of liposomes and SAINT-O-Somes and improved intracellular release of calcein by SAINT-O-Somes. Quiescent or TNF-α activated HUVEC were incubated for 3 h with non-targeted or E-selectin targeted liposomes or SAINT-O-Somes that were labeled with either DiI (lipid membrane marker) (A) or with calcein (water soluble encapsulated marker) (B). The uptake of liposomes and intracellular release of calcein was measured by FACS analysis as described in Materials and methods. Data are presented as mean ± SEM of three independent experiments. $^{*}P<0.05$ vs −TNF-α, $^{\#}P<0.05$ vs anti-E-selectin liposomes +TNF-α. C, The uptake of DiI (red) and calcein (green) labeled anti-E-selectin immunoliposomes containing 0% (a, b, c) or 20% SAINT-C18 (d, e, f) in cultured HUVEC were visualized by fluorescence microscopy. Photographs of DiI fluorescence and calcein fluorescence were taken after 5 (a, d), 15 (b, e) and 60 min (c, f) of incubation of liposomes with TNF-α activated HUVEC and are presented as merged pictures. The nuclei of the cells were stained using Hoechst (blue).Original magnification 400×.

through the polycarbonate filters during extrusion without reduction of their size.

Homogenous populations of SAINT-O-Somes with up to 20% SAINT-C18 were characterized by good size stability during storage as well as during incubation at conditions which mimic an *in vivo* situation (37 °C, the presence of serum), presenting opportunities to use these formulations for *in vitro* and *in vivo* applications.

Experiments in which we used calcein as a water soluble model molecule, demonstrated that incorporation of SAINT-C18 into the membrane of liposomes improved the release properties of these particles. A high release of calcein from the SAINT-O-Somes occurred at pH 5.0 which is similar to pH of endosomes in the endocytotic pathway. Improved release properties were also confirmed in experiments performed, using HUVEC. The amount of E-selectin targeted SAINT-O-Somes and conventional liposomes that were taken up by activated endothelial cells was comparable, yet SAINT-O-Somes released 10 fold more of the encapsulated calcein inside the cells. We also observed a small increase of calcein fluorescence in non activated endothelial cells incubated with anti-E-selectin SAINT-O-Somes which we cannot explain at the moment.

The mechanism by which SAINT-C18 triggers the release of liposomal cargo is not known. PH sensitive liposomes that also exhibit improved intracellular release characteristics at lower pH, generally require the presence of amphiphilic molecules in combination with DOPE [29], which is not present in SAINT-O-Somes. For SAINT it has been demonstrated that transfection efficiency of SAINT lipoplexes depends on the length and saturation degree of the alkyl chains [9], the molecular shape of SAINT [30], and the presence of helper lipid which facilitate formation of hexagonal morphology and determines endosomal escape of genetic material [30,31]. Some of these features of SAINT-C18 may also play a role in the improved release properties of SAINT-O-Somes but further studies are required to elucidate the precise mechanism.

The encapsulation efficiency of siRNA into SAINT-O-Somes was significantly higher compared to liposomes which did not contain SAINT-C18. Probably this is a result of charge interaction of negative siRNA molecules with SAINT-C18 lipids in the membrane of the liposomes. The siRNA that was encapsulated in the SAINT-O-Somes was efficiently delivered into endothelial cells and displayed biological functionality. In activated endothelial cells the constitutive expression of VE-cadherin gene could be reduced specifically by siRNA delivered by anti-E-selectin SAINT-O-Somes that are fully stable in a biological relevant milieu i.e. in the presence of serum, to an extent that has been described for SAINT based lipoplexes [32].

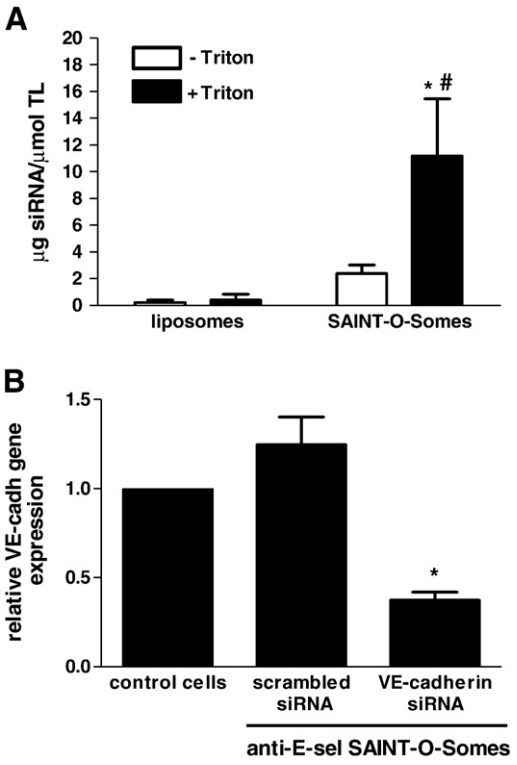

Fig. 6. Efficient siRNA encapsulation into SAINT-O-Somes results in effective delivery of siRNA into endothelial cells. A, The amount of siRNA formulated into liposomes and SAINT-O-Somes was measured as described in Materials and methods. Data are presented as mean ± SD of 3 preparations. *$P<0.05$ vs −Triton, #$P<0.05$ vs liposomes +Triton. B, Down-regulation of VE-cadherin by anti-E-selectin SAINT-O-Somes containing siRNA against VE-cadherin in H5V cells. Anti-E-selectin SAINT-O-Somes containing VE-cadherin siRNA or scrambled siRNA were incubated at the dose of 1000 pmol siRNA per ml with TNF-α activated H5V cells for 48 h. The expression of VE-cadherin was measured by real-time RT-PCR as described in Materials and methods. Data are presented as relative expression ± SD compared to non SAINT-O-Some treated cells. *$P<0.05$ vs anti-E-sel SAINT-O-Somes containing scrambled siRNA. TL; total lipid.

Coupling anti-E-selectin antibody to the surface of SAINT-O-Somes significantly increased the uptake of these particles by activated HUVEC. Similarly to conventional anti-E-selectin immunoliposomes this interaction was E-selectin specific. Recently, we showed that intravenous administered anti-E-selectin immunoliposomes localized in glomerular endothelial cells in the kidneys of the mouse suffering from glomerulonephritis [33]. *In vitro*, SAINT-O-Some cell interactions and stability parameters are comparable to that of conventional liposomes, suggesting its potential as an *in vivo* drug delivery platform aimed at cells that are not professionally equipped to process particles.

In the current work we demonstrated that SAINT-C18 can be successfully incorporated into membranes of liposomes and harnessed with target cell specificity, forming stable particles which show superior intracellular release properties of siRNA and a cytostatic drug by endothelial cells, resulting in selective gene knock down and cytotoxicity to the extent of the free drug, respectively. SAINT-O-Somes present the requirements of a versatile lipid based drug carrier system for the targeted delivery of drugs, *in vivo*, to cells that do not efficiently process particulate drug carriers.

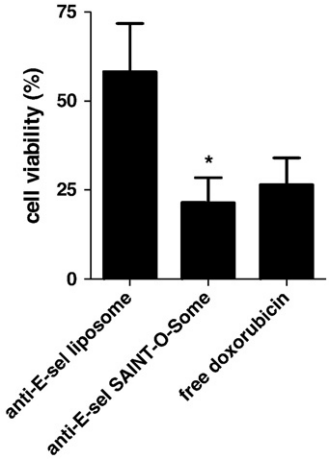

Fig. 7. Doxorubicin loaded anti-E-selectin SAINT-O-Somes decrease endothelial cell viability more efficient than conventional anti-E-selectin liposomes. HUVEC were incubated with 40 nmol/ml targeted liposomes or SAINT-O-Somes and cell viability was determined as described in the Materials and methods section. Free doxorubicin was added to the cells in an amount equivalent to the amount of encapsulated doxorubicin, i.e., 5 μg/ml. Data are presented as cell viability relative to control cells ± SEM ($n=5$). *$P<0.05$ vs anti-E-selectin liposomes.

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