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Interactions of antitumour Sialyl Lewis X liposomes with vascular endothelial cells

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ABSTRACT

Recently, we showed that tetrasaccharide selectin ligand SiaLe^X provided targeted delivery of liposomes loaded in the bilayer with melphalan lipophilic prodrug to tumour endothelium followed by severe injury of tumour vessels in a Lewis lung carcinoma model. Here, we study the impact of SiaLe^X ligand on the interactions of liposomes with human umbilical vein endothelial cells (HUVEC) using flow cytometry, spectrofluorimetry and confocal microscopy. Liposomes composed of egg phosphatidylcholine/yeast phosphatidylinositol/1,2-dioleoyl glycerol ester of melphalan, 8:1:1, by mol, and varying percentages of lipophilic SiaLe^X conjugate were labelled with BODIPY-phosphatidylcholine. The increase in SiaLe^X content in liposomes led to a proportional increase in their uptake by cytokine-activated cells as opposed to non-activated HUVEC: for 10% SiaLe^X liposomes, binding avidity and overall accumulation increased 14- and 6-fold, respectively. The early stages of intracellular traffic of targeted liposomes in the activated cells were monitored by co-localisation with the trackers of organelles. Endocytosis of SiaLe^X liposomes occurred mostly via clathrin-independent pathways, which does not contradict the available literature data on E-selectin localisation in the plasma membrane. Using dual fluorescence labelling, with rhodamine-labelled phospholipid and calcein encapsulated at self-quenching concentrations, we found that SiaLe^X liposomes undergo rapid (within minutes) internalisation by activated HUVEC accompanied by the disruption of liposomes; non-activated cells consumed a negligible dose of liposomes during at least 1.5 h. Our data evidence the selective effect of SiaLe^X formulations on activated endothelial cells and indicate their potential for intracellular delivery of melphalan lipophilic prodrug.

1. Introduction

Clinically useful antitumour effect produced by systemic administration of approved liposomal formulations of a specific size range (100–200 nm) generally relates to liposome accumulation in tumours owing to the leaky vasculature and impaired lymphatic drainage characteristic of most pathological tissues (enhanced permeability and retention effect, EPR) [1]. Further gain in selective delivery of cargo to tumour cells and expectable rise of therapeutic efficacy of a liposomal drug could be achieved through ligand-mediated targeting. A wide range of ligands were proposed for targeting of liposomes to tumour cell–specific receptors, primarily monoclonal antibodies to tumour antigens (immunoliposomes) [2]. However, only a few examples of therapeutic advance of targeted over non-targeted liposomes have been reported, all being related to the treatment of haematological malignancies, micrometastases and tumour vasculature, where vascular and interstitial barriers do not impede the delivery of nanosized vehicles (reviewed in [1]). Moreover, differentiation of tumour cells into various clones of malignant cells, including those resistant to the drug, results in temporal and spatial heterogeneity of tumour and thus failure of therapy specifically targeted to primary tumour cells. Targeting the angiogenic vasculature, which ensures the survival and growth of tumour tissue, is assumed a promising approach to overcome these shortcomings ([3] and references therein). For targeting vascular endothelial cells, sterically stabilized liposomes bearing RGD peptide covalently linked to the polyethylene glycol (PEG) termini (to target αv-integrins [4,5]) or VEGFR2 antibody (to target VEGF receptor [6]) were proposed.

E-selectin is recognized as another challenging target for delivery to tumour endothelium [7,8]. Selectins – carbohydrate-binding cell

Abbreviations: BODIPY-PC, 1-palmitoyl-2-[7-(Me$_4$-BODIPY-8)heptanoyl]-*sn*-glycero-3-phosphocholine; DPBS, PBS with calcium and magnesium salts; EDTA, ethylenediamine tetraacetate; HUVEC, human umbilical vein endothelial cells; Mlph-DOG, *rac*-1,2-dioleoylglycerol ester conjugate of melphalan; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PEG, polyethylene glycol; PFA, paraformaldehyde; PI, phosphatidylinositol; Rhod-PE, L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl); SiaLe^X, Sialyl Lewis X; SiaLe^X-PEG$_{8-15}$-DOG, *rac*-1,2-dioleoyl-3-carboxymethylene[poly(8–15)oxyethylene]oxyacetylamidopropionylglycerol; TNF-α, tumour necrosis factor alpha

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adhesion molecules – are expressed on the luminal surface of activated endothelial cells (E- and P-selectins), circulating leukocytes (L-selectin) and activated platelets (P-selectin). Selectin ligands comprise a variety of sialylated and fucosylated carbohydrates containing tetrasaccharide Sialyl Lewis X (SiaLe^X, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ) as a common epitope [9]. Selectins play key roles in a wide variety of physiologically important processes, including the migration of leukocytes to areas of inflammation and metastasis of cancer cells, through mediation of leukocyte tethering and rolling on activated endothelium [10,11]. The dominant role of selectins in cancer progression has recently been confirmed in several preclinical knock-down experiments in SCID mouse models. The crucial contribution of E- and P-selectins to spontaneous metastasis formation was demonstrated in a clinically relevant model of colon cancer [12], and E-selectin has been proven a potent target for inhibition of melanoma angiogenesis and growth [13].

Not so many studies have been reported on drug-loaded liposomes targeted to tumours and inflammation foci by the engrafted ligands of E-selectin – monoclonal antibodies (immunoliposomes) or SiaLe^X (glycoliposomes) (reviewed in [7]). For example, anti-E-selectin PEG-immunoliposomes encapsulating dexamethasone were shown to be a powerful strategy for the treatment of glomerulonephritis in a murine model [14]. The first SiaLe^X-conjugated liposomes, stabilised by PEG coating, were proposed in [15]; this study in a feline model demonstrated the anti-inflammatory cardioprotective effect of the drug-free formulation. Further studies demonstrated the inhibition of both cell adhesion to immobilised E-selectin in a concentration-dependent manner [16] and tumour cell adhesion to vascular endothelium in vitro [17] by the SiaLe^X liposomes. The feasibility of similar SiaLe^X-bearing formulations for the delivery of drugs to activated endothelial cells was demonstrated in vitro [18]. Another type of glycoliposome was equipped with a large portion of ganglioside GM1 linked to SiaLe^X ligand through human serum albumin and also bore hydrophilic coating with Tris molecules to prevent opsonisation [19–21]. These fluorescently labelled liposomes were shown to target inflammatory and tumour sites in vivo [19] and, after encapsulation of cisplatin, exhibited moderate yet reliable gain in tumour growth inhibition over SiaLe^X-free counterparts in a mouse xenograft model of lung carcinoma [20]. Recently, a study employing SiaLe^X-bearing formulations loaded with colloidal gold [21] evidenced their potential to deliver encapsulated contents into inflammatory cells around synovial blood vessels in arthritic joints.

Earlier, we showed the significant therapeutic advantage of liposomes loaded with a lipophilic prodrug of sarcolysin (D,L-melphalan) in the bilayer and supplemented with a mono-alkyl conjugate of SiaLe^X over SiaLe^X-free formulations in a mouse breast cancer model [22]. The pronounced superiority of cytotoxic SiaLe^X-modified liposomes might be ascribed not only to killing malignant cells but also to the blockage of tumour vascularisation. Recently, we demonstrated that liposomes loaded with melphalan diglyceride ester (Mlph-DOG; Fig. 1) and equipped with the diglyceride conjugate of SiaLe^X caused rigorous injuries of tumour vessels in a Lewis lung carcinoma model [23]. Non-targeted liposomes were shown to extravasate in tumour tissue, while liposomes equipped with SiaLe^X adhered to the endothelium of tumour vessels and produced antivascular effect through apoptotic cell death [23]. We assume that the effect is realised through internalisation of SiaLe^X liposomes by endothelial cells leading to cell disruption due to the cytotoxic action of melphalan generated from the prodrug intracellularly. The goal of this work was to study the details of the interactions between endothelial cells and liposomes loaded with Mlph-DOG.

The cell cycle non-specific alkylating agent melphalan is still indispensable in the treatment of multiple myeloma and metastasizing tumours and continues to be the core of many combination therapy regimens (e.g. [24]). However, attempts to efficiently encapsulate melphalan per se in a nanoparticulate carrier, which would reduce its severe side effects, have failed. We produced stable 100-nm liposomes with satisfactory loading capacity (cargo-to-lipid molar ratio of 1:9) by incorporation of the drug in the lipid bilayer in the form of an appropriately designed lipophilic prodrug Mlph-DOG [25,26]. The bilayer is composed of natural fluid-phase phospholipids and contains phosphatidylinositol as a stabilizing component to reduce premature withdrawal of liposomes from the circulation by the cells of the reticuloendothelial system [27]. Phosphatidylinositol presumably decreases opsonisation of liposomes, since inositol moieties generate highly hydrated coating on the surface of the bilayer, which shields the lipid membrane [28] similarly to PEG chains. In this study, to compare the influence of SiaLe^X targeting ligand on the interactions of our formulation with activated and non-activated endothelium, we used primary cultures of human umbilical vein endothelial cells (HUVEC). Keeping in mind that Mlph-DOG is a component of the lipid bilayer, we examined binding and internalisation of liposomes by HUVEC using several fluorescence techniques and revealed some details of the endocytosis process.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC) from egg yolk and phosphatidylinositol (PI) from *S. cerevisiae* were obtained from Reakhim (Russia). A conjugate of a tetrasaccharide Sialyl Lewis X 3-aminopropyl glycoside and *rac*-1,2-dioleoyl-3-carboxymethylene[poly(8-15)oxyethylene] oxyacetylamidopropionylglycerol (SiaLe^X-PEG$_{8-15}$-DOG) [29], *rac*-1,2-dioleoylglycerol ester conjugate of melphalan (Mlph-DOG) [30] and 1-

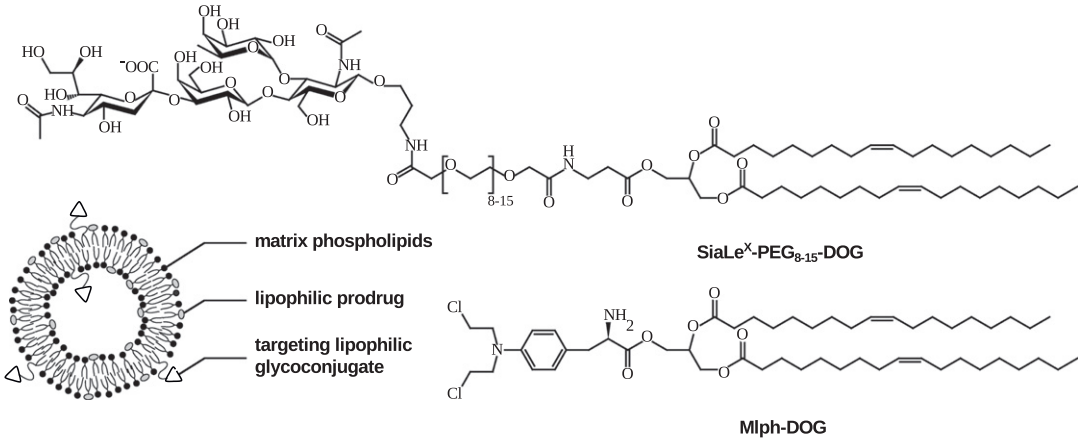

Fig. 1. Schematic representation of a liposome loaded with a lipophilic prodrug and a targeting tetrasaccharide conjugate. Chemical structures of the lipophilic conjugates of melphalan (Mlph-DOG) and Sialyl Lewis X (SiaLe^X-PEG$_{8-15}$-DOG) used in this work are shown as well.

palmitoyl-2-[7-(Me$_4$-BODIPY-8)heptanoyl]-*sn*-glycero-3-phosphocholine (BODIPY-PC) [31] were synthesized as previously reported.

Fluorescent probes and trackers used in the study were the following: BODIPY-PC; Rhod-PE (L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) obtained from Avanti Polar Lipids, #810146); ER-Tracker™ Red (Molecular Probes, #E34250, ~1 μM working solution); Transferrin Alexa Fluor® 555 conjugate (Molecular Probes, #T35352, 20 μg/mL working solution); wheat germ agglutinin (WGA) Alexa Fluor® 555 conjugate (Molecular Probes, #W32464, 10 μg/mL working solution); and Alexa Fluor® 555 F(ab′)2 fragment of goat anti-mouse IgG (H + L) secondary antibody (Molecular Probes, #A21425, 1:2000 dilution). Mouse monoclonal anti-human E-selectin antibody was purchased from Santa Cruz Biotechnology (#2Q780, 1:100 dilution). Human recombinant tumour necrosis factor alpha (TNF-α) was a kind gift from Dr. L.N. Shingarova (IBCh RAS).

2.2. Liposome preparation

Liposomes composed of PC–PI–Mlph-DOG, 8:1:1(by mol), either equipped with 2–10 mol.% glycoconjugate SiaLe^X-PEG$_{8-15}$-DOG or not (control), were prepared as described earlier [23]. Briefly, dry lipid films were hydrated in PBS (1.5 mM KH_2PO_4, 1.1 mM $NaH_2PO_4{}^{\circ}2H_2O$, 6.3 mM Na_2HPO_4, 2.7 mM KCl and 136.8 mM NaCl; pH 7.2) and subjected to 6–10 cycles of freezing/thawing (liquid nitrogen/+40 °C). The suspension was then extruded at ambient temperature through two stacked polycarbonate membrane filters with pore sizes of 100 nm (Nucleopore), 10 times, on a Mini-extruder (Avanti Polar Lipids). The size of the liposomes was controlled by dynamic light scattering using a 90Plus (Brookhaven Instruments Corp.) equipment in at least three runs per sample. Mean liposome diameter was in the range of 90–100 nm. To visualize liposomes, 0.5 mol.% of BODIPY-PC was added at the stage of lipid film formation, except for spectrofluorimetry experiments, where 2 mol.% of BODIPY-PC was used. Formulations were stored at 4 °C and used for biological experiments within 10 days.

To control lipid bilayer integrity on incubations in different media, liposome samples encapsulating calcein at the self-quenching concentration were prepared. Lipid films were hydrated in PBS with 80 mM calcein and processed as described above; after extrusion, non-encapsulated calcein was removed by size exclusion chromatography on a Sephadex G-50 column equilibrated in PBS. To control the final liposome concentration, calcein and Mlph-DOG absorbance peaks were registered upon liposome disruption with at least 20-fold volume of ethanol (calcein: λ_{max} 497 nm, ε ~74,000 M^{-1} cm^{-1}; Mlph-DOG: λ_{max} 258 nm, ε ~19,700 M^{-1} cm^{-1}) on an SF-256-UVI two-beam spectrophotometer (LOMO Fotonika, Russia). Leakage of calcein from the liposomes and its dilution results in the dequenching of the fluorophore and increase in the fluorescence signal. The percentage of calcein released was calculated according to the equation: $(I_i/I_T - I_0/I_T) / (1 - I_0/I_T) \times 100\%$, where I_i is the fluorescence intensity at given time point, I_0 is the intensity at the beginning of the measurement and I_T is the totally dequenched calcein fluorescence after the addition of Triton X-100 (1% by vol.). The measurements were performed on a GloMax®-Multi instrument (Promega) using the blue fluorescence optical kit (λ_{ex} 490 nm, λ_{em} 510–570 nm).

2.3. Cell culture

Individual donor endothelial cells (HUVEC) were isolates from human umbilical cords according to the methods of Jaffe [32] and Scheglovitova [33]. Briefly, fresh umbilical veins were cannulated and filled with dispase solution (2 mg/mL) (Gibco) and incubated at 37 °C for 30 min. Then, the veins were perfused with PBS. Cells were collected from the perfusate by centrifugation at 1000 rpm for 10 min, resuspended in Medium 199 (Gibco) supplemented with 10% fetal calf serum (HighClone), 200 μg/mL endothelial growth factor (Sigma), 100 μg/mL heparin (Moscow endocrine plant), 50 μg/mL gentamycin (KRKA) and seeded into 6-well plates. Cells were cultured in humidified atmosphere of 5% CO_2 at 37 °C. The confluent primary monolayers were washed and trypsinised (0.05% trypsin + 0.02% EDTA, Gibco). The cells were resuspended into complete medium, seeded on 24-well plates (120,000 cells/mL) (Costar), and cultured for four days. For experiments, only the first subcultures were used.

Umbilical cords were obtained after normal parturition from healthy donors following informed written consent.

2.4. Confocal microscopy studies

Four days 2-passage HUVEC were seeded on cover slides (~0.5×10^6 cells/mL) pre-coated with 0.2% w/v gelatin (Sigma) and incubated for 24 h in humidified atmosphere of 5% CO_2 at 37 °C. Then, cells (with/without activation) were incubated with Hoechst 33342 (Sigma) for 30 min to stain nuclei, washed with DPBS (PBS with calcium and magnesium salts, PanEco, Russia) and co-incubated for the stated time period at 37 °C with the trackers of intracellular organelles and liposomes added to a final lipid concentration of 50 μM in the serum-free medium. Liposomes contained none, 2 or 5 mol.% of SiaLe^X conjugate. Before being fixed with 1% paraformaldehyde (PFA) solution, cells were washed twice with DPBS. For tracking of early endosomes (Transferrin), Golgi apparatus (WGA) and endoplasmic reticulum (ER-tracker), co-incubations were performed for 15 min.

Prior to examination on a Nikon TE 2000 Eclipse confocal microscope, slides were mounted with a polymerizing agent Mowiol 4.88 solution (Calbiochem). Image analysis was carried out using ImageJ 1.48 software.

2.5. Flow cytometry studies

Cell suspensions were diluted with equal volumes of 1% BSA solution in PBS and 0.3 μg/mL propidium iodide solution in PBS (the latter being used to assess cell viability) and analysed on FACScan flow cytometer (Becton Dickinson), equipped with a 488 nm argon-ion laser. Fluorescence emission signals were detected in FL1 (515–545 nm spectral window), FL2 (565–610 nm) and FL3 (>650 nm) channels, duplicate measurements with 10,000 events were recorded for each sample. Side/forward scatter and propidium iodide fluorescence signals were used to gate the cell subsets of interest and eliminate debris, dead cells and cell aggregates. Data were analysed using CELLQuest software.

2.6. E-selectin expression

E-selectin expression by HUVEC in the range of TNF-α concentrations was assessed by FACS analysis and confocal microscopy. Cells were activated with 0.1–50 ng/mL TNF-α for 4 h at 37 °C. For confocal microscopy, cells were incubated with anti-human E-selectin antibodies (1:100) for 1 h at 37 °C under gentle shaking, washed with DPBS and fixed in 1% PFA for 15 min at 37 °C. Cells were then washed with DPBS 3 times, stained with Alexa Fluor® 555 anti-mouse secondary antibodies for 1 h at 37 °C, washed with DPBS 3 times and examined under a microscope. For FACS analysis, activated cells were washed with DPBS, detached with EDTA solution, resuspended in PBS with 1% BSA and incubated with anti-human E-selectin antibodies (1:100) for 1 h at 4 °C. Cells were than washed twice in PBS/BSA by centrifugation at 500 *g* for 5 min and stained with FITC (488 nm) anti-mouse secondary antibodies for 1 h at 4 °C (detection in FL1 channel).

2.7. Accumulation of liposomes by HUVEC

To assess the specificity of SiaLe^X liposomes, activated (50 ng/mL TNF-α, 4 h, 37 °C) HUVEC were pre-treated with excess anti-human E-selectin antibodies or non-specific IgG as a control (10 μg/mL, 1 h at 37 °C) followed by treatment with liposomes.

To reduce unspecific binding and membrane fusion, low-temperature (4 °C) incubations were applied at certain steps. For confocal microscopy, after incubation with mAbs, cells were chilled to 4 °C, washed with cold DPBS and incubated for 1 h on ice with liposomes (2 mol.% SiaLe^X conjugate, 25 μM total lipid in serum-free medium). Cells were then washed 3 times with cold DPBS, fixed in 1% PFA at 4 °C overnight, washed with DPBS and examined under a microscope.

For FACS analysis, after incubation with mAbs or control IgG, cells were rinsed with DPBS and incubated with 10% SiaLe^X liposomes (50 μM of the total lipid in serum-free medium) for 1 h at 37 °C. Cells were then rinsed 3 times with DPBS, detached with EDTA solution (10 min, 37 °C) and analysed by FACS.

Relative binding avidity was assessed according to [34] as follows: cells were chilled to 4 °C, washed with cold DPBS and incubated for 1 h on ice with 2% SiaLe^X or control liposomes (50 μM total lipid, 200 μL serum-free medium), then rinsed 3 times with DPBS, treated with 500 μL of complete medium and incubated for 1 h at 37 °C. Cells were then rinsed with DPBS, detached with EDTA solution (10 min 37 °C) and analysed by FACS.

Overall accumulation of liposomes in the range of SiaLe^X conjugate concentrations in the bilayer was characterized by FACS analysis and confocal microscopy; incubations were set up at 37 °C for 60 min with 50 μM of the total lipid liposomes in serum-free medium.

In the histograms of FACS analysis, when double peak patterns appeared, high-intensity peaks were taken into consideration (see Supplementary data).

2.8. Quantification of membrane-bound and internalised liposomes

HUVEC in monolayers in 24-well plates were activated with 10 ng/mL of TNF-α for 4 h at 37 °C, then incubated in 200 μL serum-free medium for 30 min at 37 °C on a shaker, and then the medium was replaced with the liposome dispersion (200 μL, 2% SiaLe^X or control liposomes, 3 mM total lipid with 2% BODIPY-PC) and incubated for 1 h at 37 °C under mild shaking. Then, cells were washed three times with PBS and treated with 0.5 mL of 0.05% trypsin/EDTA solution for 1 or 30 min at 37 °C. 400 μL of short time–treated cells were collected and solubilised with 20 μL of 10% Triton X-100 and sonicated for 30 min, resulting in total cell-associated liposomes. Long time–treated cells were centrifuged at 1000 *g* (4 °C, 20 min) to separate precipitated internalised fractions of liposomes and membrane-bound fractions in the supernatant [35]. After solubilisation with Triton X-100, the fluorescence of BODIPY-PC in the resulting solution was measured in a quartz cuvette under constant stirring using Hitachi F4000 spectrofluorimeter with excitation and emission band-pass 5 nm, λ_{ex} 490 nm and λ_{em} 506 nm. The fluorescence intensity was converted to the amount of cell-associated Mlph-DOG prodrug using calibration curves obtained with BODIPY-PC-labelled liposomes.

2.9. Double-labelled liposomes (calcein–rhodamine assay)

To assess liposome uptake by cells and intracellular liposome disruption, we used a technique described in [36]. Liposomes were labelled with 1 mol.% Rhod-PE probe in the bilayer and contained 80 mM calcein tetrasodium salt in the water interior. Non-encapsulated calcein was removed by gel filtration using a Sephadex G-50 column equilibrated in PBS. Liposome final concentration was controlled by Rhod-PE (λ_{max} 556 nm, ε ~82,000 M^{-1} cm^{-1}), calcein and Mlph-DOG absorbance peaks. Upon interactions with cells, mean rhodamine fluorescence value reflects the extent of binding and uptake of liposomes, whereas mean calcein fluorescence reflects the intracellular dequenching of the dye.

To calibrate and compensate rhodamine (detection in FL2 channel) and calcein (detection in the FL1 channel) fluorescent signals in FACS experiments, single-labelled liposomes were also prepared. When double peak patterns appeared, high-intensity peaks were accounted (see Supplementary data).

Resting or activated HUVEC monolayers in 24-well plates (50 ng/mL TNF-α, 4 h, 37 °C) were rinsed with DPBS and incubated with liposomes (5% SiaLe^X, 50 μM total lipid, 200 μL) for the stated time period at 37 °C. Control of binding was done by incubating the cells with liposomes at 4 °C, to prevent uptake and processing of liposomes. Cells then were washed 3 times with cold DPBS, treated with EDTA solution and kept on ice before FACS analysis.

For confocal microscopy, cells were cooled to 4 °C and incubated with liposomes (5% SiaLe^X, 50 μM in serum-free medium) for 30 min on ice, washed three times with DPBS and incubated for various time periods at 37 °C in complete medium. Cells were then fixed with 1% PFA for 15 min at 37 °C and examined under a microscope.

3. Results

3.1. Liposomes

As assessed by dynamic light scattering, cell medium or temperature of incubation had no effect on the size of non-targeted liposomes and those with 2 mol.% SiaLe^X conjugate (Table 1). Exactly the same formulations were used in our recent study in vivo [23] (see Introduction). The integrity of liposomes was also retained during 1-h incubations according to the calcein leakage assay (Fig. 2). Slight initial release of calcein (3–4%) may occur due to the dissolution of trace amounts of calcein adsorbed on the surface of liposomes due to 60-fold dilution of the samples before measurements. The averaged release in PBS was somewhat higher than that in the cell medium, probably due to differences in the compositions of the dispersing solutions (in addition to inorganic salts, Medium 199 contains nucleotides, nucleosides and vitamins).

The increase in SiaLe^X conjugate content in lipid bilayer from 2 to 10 mol.% did not influence the size of the liposomes, as assessed by dynamic light scattering (Supplementary data, Table S1).

3.2. E-selectin expression

Being primary cells from an individual donor, HUVEC may exhibit unique activity upon inflammatory cytokine activation. We have observed several instances in which there was a slight original expression of E-selectin (data not shown), but these cell samples were not used in further experiments. We explored the expression of E-selectin in response to TNF-α activation in a range of concentrations (from 0.1 to 50 ng/mL) by flow cytometry and confocal microscopy. Without activation, no detectable amount of E-selectin appeared, while activation with increasing TNF-α concentrations led to an exponential increase in its expression (Fig. 3A). Importantly, according to flow cytometry data, three populations of cells with different levels of E-selectin

Table 1
Effect of the medium and temperature on the size of liposomes after 1 h incubation.[a]

Conditions	Non-targeted liposomes		2% SiaLe^X liposomes	
	D (nm)	PDI	D (nm)	PDI
PBS, 4 °C	96 ± 1	0.062	91 ± 3	0.055
PBS, 37 °C	98 ± 1	0.085	90.4 ± 0.4	0.052
Serum-free medium, 4 °C	99 ± 2	0.074	92 ± 1	0.062
Serum-free medium, 37 °C	102 ± 1	0.069	91 ± 1	0.094

[a] According to dynamic light scattering data: D, mean diameter; PDI, polydispersity index; mean ± SE of the measurements.

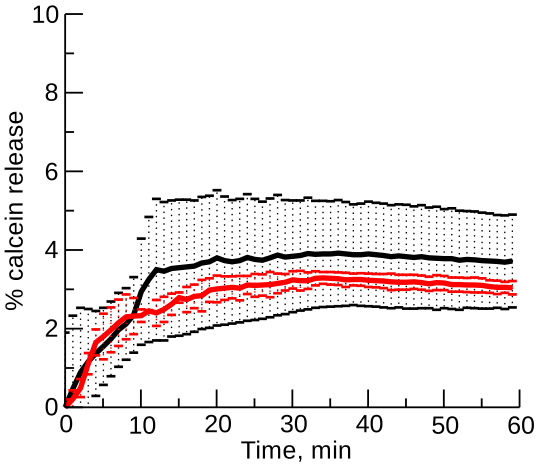

Fig. 2. Liposome stability. Calcein leakage from liposomes during 1 h incubation in PBS (black) or serum-free medium (red), mean ± SD, $n = 3$.

expression—arbitrarily high, middle and low—were distinguished in the majority of studied cell batches. Fig. 3B displays a typical FACS analysis histogram. High-intensity population proportion increased with growing TNF-α concentrations, but even at 50 ng/mL TNF-α, a population with low level of E-selectin expression was present. For our study, we employed this concentration of TNF-α, since it provided maximal up-regulation without visible cytotoxic effects. The confocal microscopy data (Fig. 3C) evidence massive expression of E-selectin molecules after activation with the maximal studied cytokine concentration.

3.3. Accumulation of liposomes by HUVEC

Initially, relative binding avidity of HUVEC for targeted and non-targeted liposomes in the range of the ligand concentrations was explored according to [34]. Activated or non-activated cells were incubated with the formulations under endocytosis-blocking conditions (at 4 °C); then, the cells were allowed to consume surface-bound liposomes (at 37 °C) and were analysed by FACS. Non-activated HUVEC bound targeted liposomes with low avidity approximately equal to that one exhibited by the activated cells towards $SiaLe^X$-free liposomes (Fig. 4A, blank bars). Only in the case of both activated cells and targeted liposomes did double peak histograms appear, with high-intensity fluorescence peaks evidencing specific interactions between $SiaLe^X$ ligand of liposomes and E-selectins on the cell surface (shown by the example of 10% $SiaLe^X$ liposomes in Fig. 4B; for other histograms, see Supplementary data). The lowest concentration of $SiaLe^X$ conjugate in liposomes (2 mol.%) generated a four-fold increase in fluorescence signals over non-activated cells, while the growth of ligand concentration up to 10% resulted in a 14-fold increase (Fig. 4A, filled bars).

Next, we explored the impact of $SiaLe^X$ ligand on the overall accumulation of liposomes by functioning HUVEC (at 37 °C). At physiological temperature, non-activated cells consumed about 10 times more liposomes than at 4 °C (Figs. 5A and 4A). The increase in $SiaLe^X$ content in liposomes from 2 to 10 mol.% led to about 6-fold increase in the liposome accumulation by activated cells, while non-activated HUVEC retained initial levels of liposome binding and uptake (Fig. 5A). According to confocal microscopy, all formulations exhibited diffuse staining in the case of non-activated cells (as shown by the example of 5% $SiaLe^X$ liposomes; Fig. 5B). In the case of activated cells, targeted liposomes appeared in the cytoplasm as bright spots, while $SiaLe^X$-free ones

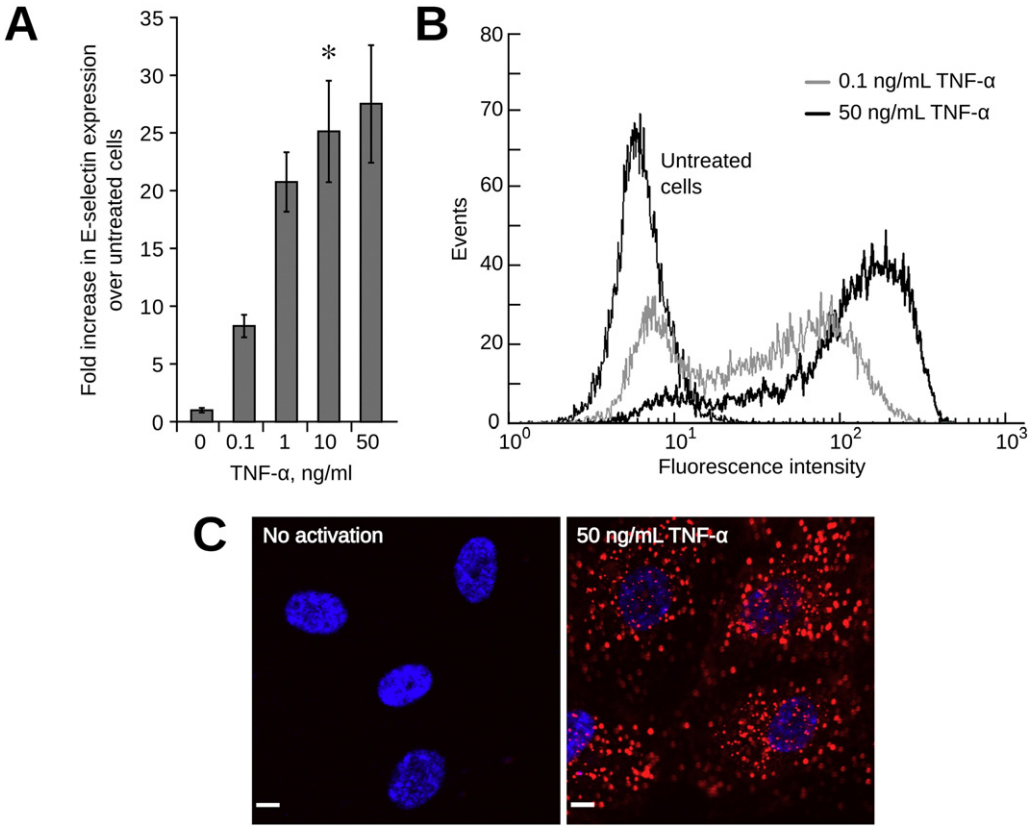

Fig. 3. E-selectin expression on the HUVEC endothelial cells. Cells were activated with different TNF-α concentrations for 4 h, then detached with EDTA solution, incubated with anti-E-selectin mAb for 1 h at 4 °C, stained with FITC-labelled secondary Ab for 1 h at 4 °C and analysed by flow cytometry; average data on the total population in three independent experiments, mean ± SD; *, data for one experiment, mean ± SE (A). Representative flow cytometry histograms; populations with no, middle and high response to activation were observed for all tested TNF-α concentrations (B). HUVEC monolayers with or without activation were incubated with E-selectin mAb for 1 h at 37 °C, stained with Hoechst for nuclei (blue), fixed and stained with Alexa-555-labelled secondary Ab (red) for 1 h at 37 °C and examined under a microscope; the bar is 5 μm (C).

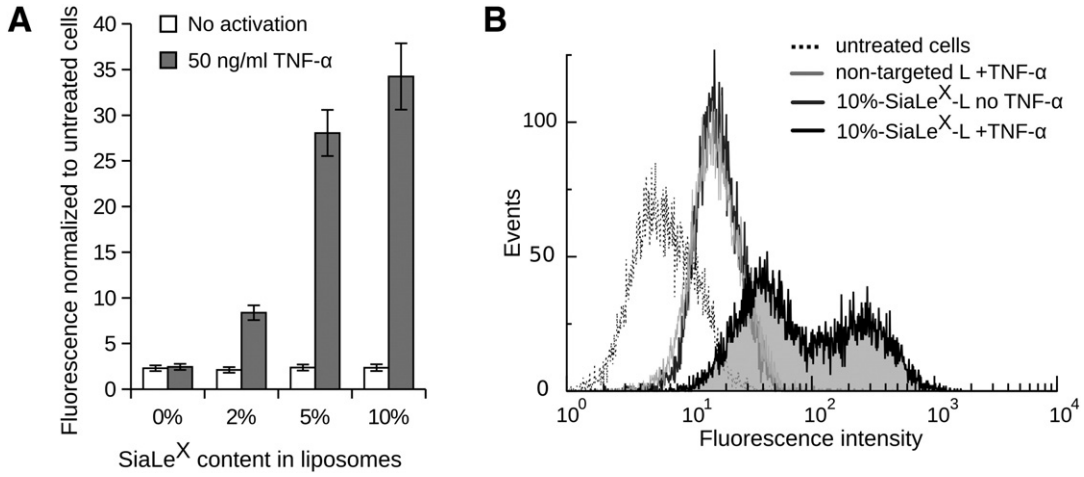

Fig. 4. Liposome (L) avidity. Resting or activated with TNF-α (50 ng/mL), HUVEC were incubated for 1 h on ice with BODIPY-PC-labelled liposomes (50 μM total lipid) bearing 0, 2, 5 or 10 mol.% SiaLe^X conjugate (10% SiaLe^X-L), rinsed and incubated in complete medium for 1 h at 37 °C; then, cells were detached with EDTA solution and analysed by flow cytometry; data of a representative experiment, mean ± SE, are reported (A). An example of typical FACS histograms for binding of liposomes with HUVEC: highly intensive population represents cells that bound liposomes mostly via E-selectin–SiaLe^X interactions (B).

resembled the pattern of non-activated cells. These data agree with the results of FACS analysis and evidence the internalisation of SiaLe^X liposomes by the activated HUVEC.

The specificity of SiaLe^X liposome interactions with activated HUVEC was established by competitive inhibition of binding with anti-E-selectin mAb. First, the activated cells were treated with the excess of anti-E-selectin mAb (or non-specific IgG). Further incubation of cells with 10% SiaLe^X liposomes at 37 °C allowed registering reliable differences in flow cytometry experiments. Control IgG practically did not change the overall accumulation of liposomes by cells, while specific mAb inhibited the consumption of liposomes by 70%, diminishing liposome accumulation practically to the level of non-activated HUVEC (Fig. 6A). To observe the inhibition of specific liposome binding with activated HUVEC by confocal microscopy (Fig. 6B), we used 2% SiaLe^X liposomes and low-temperature incubations at 4 °C to reduce non-specific binding and membrane fusion. Indeed, in the presence of excess specific mAb, bright punctuated patterns of bound SiaLe^X liposomes contouring plasma membranes (Fig. 6B, central image) changed to the weak diffuse

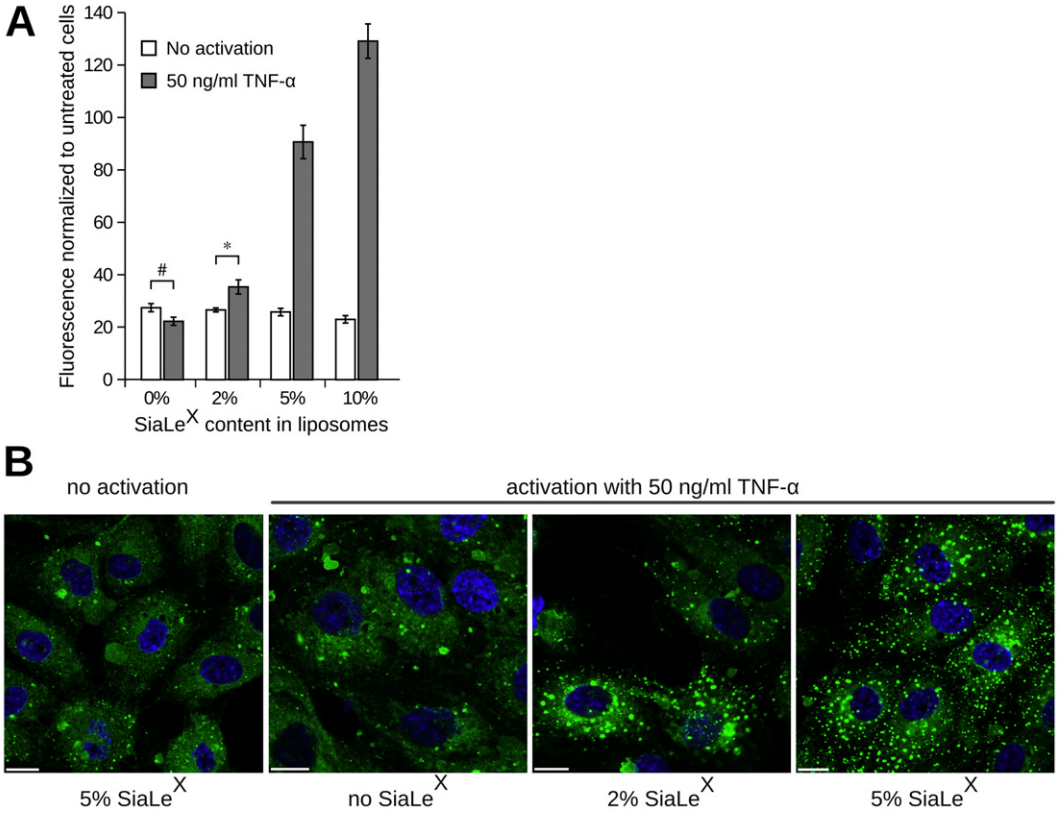

Fig. 5. Overall accumulation of liposomes by HUVEC in function of activation and the content of SiaLe^X conjugate in liposomes. Resting or activated with TNF-α (50 ng/mL) cells were incubated for 1 h at 37 °C with BODIPY-PC-labelled liposomes (green fluorescence, 50 μM total lipid) and rinsed. Then, (A) the cells were detached with EDTA solution and analysed by FACS; data of a representative experiment are presented (mean ± SE), # $p > 0.01$, * $p < 0.01$; or (B) the cells were stained with Hoechst for nuclei (blue), fixed with 1% PFA overnight at 4 °C and analysed by confocal microscopy. The bar is 10 μm.

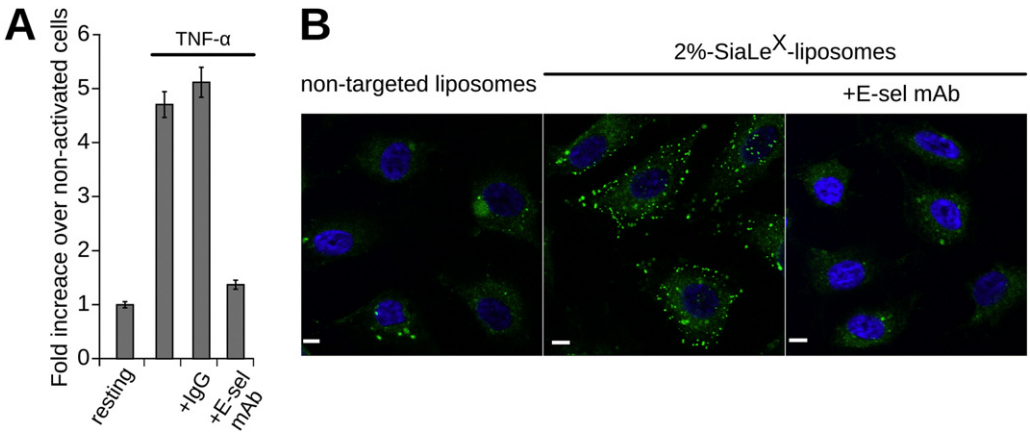

Fig. 6. The specificity of binding of SiaLe^X liposomes with HUVEC. Activated (50 ng/mL TNF-α) or resting cells were pre-treated with the excess of E-selectin mAb (10 μg/mL, 1 h at 37 °C) or non-specific IgG as a control, with subsequent treatment with BODIPY-PC-labelled liposomes (green fluorescence). (A) Cells were incubated with 10% SiaLe^X liposomes (50 μM total lipid, 1 h at 37 °C), rinsed, detached with EDTA solution and analysed by flow cytometry. Data of a representative experiment (mean ± SE) are reported. (B) Activated cells were incubated with non-targeted or 2% SiaLe^X liposomes (25 μM total lipid, 1 h at 4 °C) with or without mAb pre-treatment, stained with Hoechst for nuclei (blue), fixed with 1% PFA for 15 min at 37 °C and analysed by confocal microscopy. The bar is 5 μm.

staining (Fig. 6B, right image) characteristic of SiaLe^X-free liposomes (Fig. 6B, left image). Non-activated HUVEC showed low amounts of bound liposomes and diffuse patterns of staining, similar to the case of SiaLe^X-free liposomes and activated cells, independently of the presence of SiaLe^X ligand (data not shown).

3.4. Quantification of membrane-bound and internalised liposomes

Incorporation of as little as 2 mol.% of SiaLe^X conjugate in the bilayer of liposomes in the in vivo experiment [23] resulted in a pronounced antivascular effect and exhibited a different distribution in the tumour tissue as compared to the SiaLe^X-free counterpart. It was interesting to explore whether this difference was caused by an increase in the consumption of liposomes or in their binding.

To quantify membrane-bound and internalised liposomes after incubation with cells, we used spectrofluorimetry. According to [35], the membrane-bound fraction of liposomes can be separated by prolonged treatment of cells with trypsin (30 min at 37 °C instead of the ordinarily 1–5 min used for the detachment of cells from cultural plates), which results in glycocalyx shedding and liposome dissociation from the cell surface. After centrifugation, the supernatant contains membrane-bound liposomal fraction, and the pellet contains cells with internalised liposomes.

According to fluorescence spectroscopy data (Fig. 7), both processes—binding and internalisation of liposomes by activated HUVEC—were 40–50% more efficient in the case of SiaLe^X liposomes if compared to the non-targeted formulation. The effect of targeting ligand on liposome total accumulation by activated cells as compared to non-activated ones (~8% increase, Fig. 7) is not as pronounced as the effect exhibited in the flow cytometry experiments (~33% increase for 2% SiaLe^X liposomes; Fig. 5A). There are at least two reasons for this. First, to ensure reliable sensitivity of measurements, we increased the concentration of liposomes in the incubation media from 50 μM (as used in the flow cytometry experiments) to 3 mM of total lipids. This increased non-specific interactions of liposomes with cells to the level exceeding/beating/surpassing the impact of specific interactions between SiaLe^X ligand and E-selectin. Second, in contrast to fluorescence spectroscopy, where lysates from the whole pool of cells were analysed, in FACS analysis, we evaluated specific subsets of cells. In any case, spectrofluorimetry data evidence that 2% SiaLe^X liposomes bind and enter activated HUVEC more efficiently than SiaLe^X-free formulations. Based on the measured content of Mlph-DOG in cell fractions (4×10^{-11} mol; Fig. 7), the theoretical number of Mlph-DOG molecules per 100 nm liposome (~8000) and the average number of assayed cells of about 2×10^5, one may recalculate that in medium with an excess of liposomes, activated HUVEC accumulate approximately 15,000 liposomes per cell within an hour.

3.5. Intracellular delivery

To monitor the early stages of intracellular traffic of targeted liposomes in the activated HUVEC, we evaluated co-localisation of liposomes with the intracellular organelle trackers. Here, we used liposomes with intermediate amounts of SiaLe^X conjugate (5 mol.%) to obtain reliable results and avoid flaring of images. Within 15 min of incubation, liposomes were observed inside the cells (Fig. 8). Staining with the wheat germ agglutinin (WGA) clearly defined the cellular outline of the glycocalyx and the bright spots of the Golgi apparatus (Fig. 8B). Diffuse green staining mostly co-localised with the area of endoplasmic reticulum (Fig. 8A), while bright green spots corresponded to Golgi and, partly, to the early endosomes (Fig. 8C). Interestingly, co-

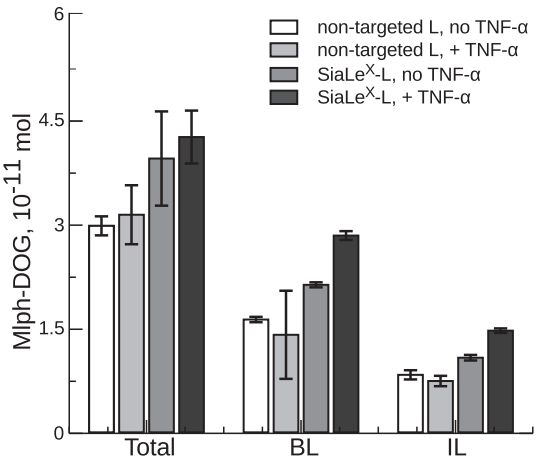

Fig. 7. Spectrofluorimetry of cell fractions. Liposomes (3 mM total lipid, 2% BODIPY-PC, 2% SiaLe^X conjugate) were incubated with HUVEC for 1 h at 37 °C. Then, the cells were rinsed and treated with trypsin/EDTA solution for 1 or 30 min at 37 °C. Shortly treated cells were solubilised with 0.5% Triton X-100 solution with subsequent sonication for 30 min at 37 °C; these comprised the total liposomes accumulated by the cells (Total). Long-term treated cells were centrifuged with subsequent Triton X-100 and ultrasound solubilisation of the precipitate (IL, internalised liposomes) and supernatant (BL, bound or membrane-associated liposomes). BODIPY-PC fluorescence signal was measured and converted to Mlph-DOG content using the calibration curve. Mean ± SD for two independent experiments are presented.

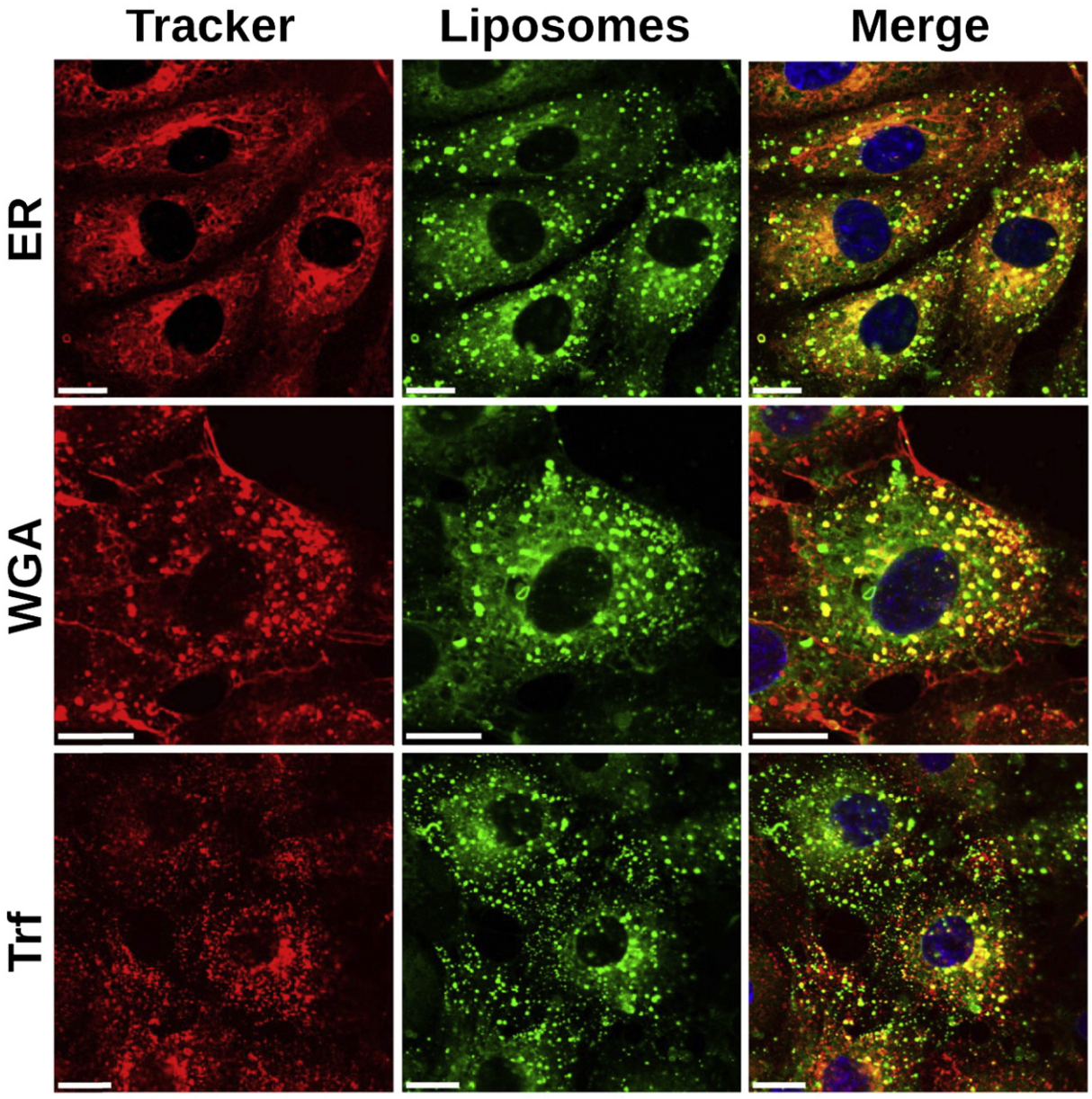

Fig. 8. Intracellular delivery of targeted liposomes labelled with BODIPY-PC in the membrane (green). Co-localisation with endoplasmic reticulum (ER), early endosomes (Trf), glycocalyx and Golgi apparatus (WGA, wheat germ agglutinin) trackers (red). Activated with 50 ng/mL TNF-α, HUVEC were prestained with Hoechst for nuclei (blue), co-incubated with 5% SiaLe^X liposomes (50 μM total lipid) and the trackers for 15 min at 37 °C and then fixed with 1% PFA for 15 min at 37 °C. Cells were visualised using confocal microscopy and are presented as single z-scans; the bar is 10 μm.

localisation with the latter, originating from the clathrin-mediated endocytosis pathway (transferrin molecule enters the cell through a receptor localised in classical coated pits), was only partial (Fig. 8C). Thus, the presence of SiaLe^X conjugate in liposomes brought an additional, clathrin-independent internalisation pathway. Non-targeted liposomes did not show such punctuated patterns of staining (Fig. 5B). Diffuse character of staining indicates rather the passive fusion of liposomes with the cell membrane than the active transport of SiaLe^X liposomes mediated through E-selectin molecules.

Thus, the preliminary picture of the early stages of internalisation of targeted liposomes by activated endothelial cells may be as follows: after binding with E-selectin molecules, liposomes undergo endocytosis followed by sorting and transport to Golgi and finally approach the endoplasmic reticulum. Yet, what is the state of the liposomes during these events? Do they retain the integrity being dispersed in water compartments of intracellular organelles? Or does the liposomal bilayer fuse with the cell membranes and thereby unload the lipophilic prodrug facilitating its presentation to intracellular esterases?

3.6. Kinetics of liposome binding, uptake and intracellular disruption

To follow the binding of targeted liposomes by HUVEC, their uptake and intracellular disruption, we used FACS analysis involving dual fluorescence labelling, as suggested in [36]. We prepared double-labelled 5% SiaLe^X liposomes bearing lissamine rhodamine B fluorophore in the bilayer (Rhod-PE probe) and encapsulating calcein in the water interior at a self-quenching concentration. Accordingly, the total accumulation of liposomes in cells was monitored by measuring rhodamine fluorescence and liposome disruption by fluorescence of calcein. Non-activated HUVEC showed very low accumulations of both targeted (Fig. 9A) and non-targeted (data not shown) formulations until up to 90-min of monitoring; calcein release was also negligible (Fig. 9B). On

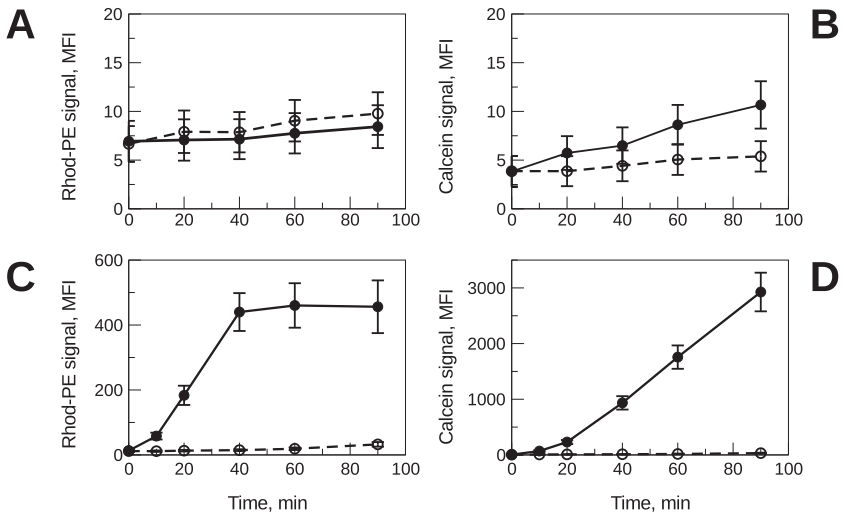

Fig. 9. Binding (Rhod-PE signal) and dynamics of intracellular disruption (calcein signal) of 5% SiaLe^X liposomes (50 μM total lipid) upon incubation with resting (A and B) and TNF-α-activated (C and D) HUVEC at 37 °C (solid line) or 4 °C (dashed line). The membrane of liposomes is labelled with Rhod-PE and calcein is encapsulated at self-quenching concentration. At 37 °C, activated HUVEC bound SiaLe^X liposomes with saturation after ~40 min and the binding was accompanied by rapid (within minutes) internalisation process resulting in liposome membrane disruption. Under low temperature, binding was drastically reduced, while liposome membranes remained intact. Non-activated cells consumed a negligible amount of liposomes at both temperatures. Flow cytometry data of a representative experiment (mean ± SE) are presented.

the contrary, activated cells consumed SiaLe^X liposomes, with saturation after ~40 min (Fig. 9C), the uptake being accompanied by rapid (within minutes) liposome membrane disruption (Fig. 9D). The data of confocal microscopy agrees with the results of flow cytometry (Fig. 10): indeed, images show intact liposomes bound to plasmalemma as small red spots after low-temperature incubation (0 min) with activated HUVEC; after 40/90 min incubation at physiological temperature, liposomes were detected in the cytosol as large green and yellow (due to the overlap of red and green fluorescence) areas.

4. Discussion

The study compares SiaLe^X-equipped liposomes loaded with lipophilic prodrug of an anticancer agent melphalan and SiaLe^X-free formulation regarding the interactions with activated and non-activated endothelial cells. We were interested in the exploration of both the targeting potential of our specifically designed formulation and the mechanism of its uptake by the cells, which determines subsequent intracellular traffic of liposomes and release of the cargo. As the prodrug may be considered a lipidic diglyceride molecule, well fitted and stably included in the lipid bilayer, we suppose that labelling of liposomes with phospholipid fluorescent probes (BODIPY-PC, Rhod-PE) allows monitoring the fate of lipophilic prodrug Mlph-DOG at least until liposomes retain their integrity. The study was performed using a number of fluorescent techniques and methodologies.

Endothelial cells transiently express E-selectin and present it on the surface in response to stimulation by inflammatory molecules [37], such as tumour necrosis factor (TNF-α) and interleukin-1. After cell exposure to cytokines, E-selectin expression temporally changes, reaching its maximum 4 h after stimulation and then rapidly decreasing—E-selectin is no longer detectable on cell surface after 24 h [37] due to constitutive internalisation by endocytosis [38]. For this reason, studies on targeting E-selectin in vitro are performed after 3–4 h incubations of HUVEC with the cytokines [39,40].

For our experiments, we selected activating concentration of TNF-α basing on the maximal level of E-selectin expression obtained within the studied range of concentrations (0.1–50 ng/mL). It is unlikely that further increase of cytokine concentration could result in the substantial increment of E-selectin expression, exponential increase of which ended shortly after reaching TNF-α concentration of 1 ng/mL (Fig. 3A). Stimulation by TNF-α generally led to high levels of E-selectin expression in a major population of cells; however, even at the maximal level of expression, a fraction not responding to activation persisted among the cells (Fig. 3B).

The potential of targeting of our liposomal formulation was evaluated in the function of the concentration of SiaLe^X conjugate. Here, we used the advantage of FACS analysis and specifically took into consideration only the fluorescence signal of the highly intensive cell populations. The contribution of SiaLe^X ligand (10 mol.%) to interactions of liposomes with activated HUVEC assessed by the relative binding avidity assay (Fig. 4A), in which non-specific interactions with the

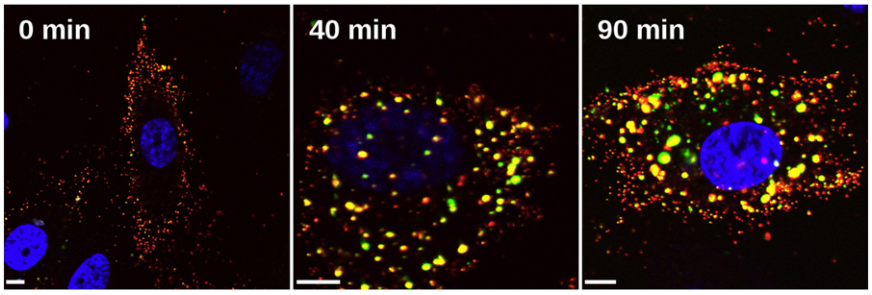

Fig. 10. Confocal microscopy images of the uptake of targeted liposomes by activated HUVEC. The liposome membrane is labelled with Rhod-PE (red) and the inner volume contains calcein (green) at self-quenching concentration; cell nuclei are stained with Hoechst (blue). Prior to the experiment, cells were cooled to 4 °C and incubated with liposomes (5% SiaLe^X, 50 μM) for 30 min on ice, washed and incubated for various time periods at 37 °C in a complete medium. The bar is 5 μm.

cytoplasmic membrane are minimized due to endocytosis blockage, resulted in a 14-fold increase in liposome binding over non-targeted formulations. The formulation with 2 mol.% SiaLe^X ligand provided only a four-fold increase; nevertheless, even for this formulation, we have shown an undoubtable targeting to tumour endothelium in vivo [23]. The liposomes are negatively charged due to the relatively high content of phosphatidylinositol (10 mol.%) in the bilayer: their zeta potential is around −30 mV [23]. Logically, this would hamper the interactions between liposomes and cells, most of which express negatively charged (mainly sulfated) proteoglycans on the surface [41]. The gradual addition of SiaLe^X conjugate up to 10 mol.% should only further increase the negative charge of liposomes due to the non-protonated carboxyls of the sialyc acid residues. However, these formulations exhibited the growth of avidity to HUVEC, thus evidencing the contribution of specific polyvalent interactions between SiaLe^X ligands of liposomes and multiple copies of E-selectin at the cell surface. The persisting low level of binding of liposomes with non-activated cells, independently of the presence of SiaLe^X conjugate, also proves the contribution of specific interactions with E-selectin (Fig. 4). At physiologically relevant temperatures, the effect of SiaLe^X ligand on liposome accumulation by HUVEC showed the same tendency: an approximately 6-fold increase of accumulated quantities of 10% SiaLe^X liposomes in activated cells against the retained level in the case of non-activated HUVEC (Fig. 5A). Here, the effect is less pronounced due to the involvement of a diversity of non-specific interactions in the course of endocytosis. Finally, both the inhibition of targeted liposome accumulation in activated HUVEC with anti-E-selectin mAb and the absence of such inhibition with non-specific IgG confirm the impact of SiaLe^X ligand on specific interactions with E-selectin (Fig. 6A).

The specificity of binding of SiaLe^X liposomes with activated chilled HUVEC is clearly visualised by confocal microscopy (Fig. 6B). Further, the microscopy patterns obtained for functioning cells (at 37 °C) not only corroborate the dependence of liposome accumulation upon SiaLe^X concentration but also demonstrate quite a different type of intracellular distribution of targeted formulations, implying their inclusion in the intracellular organelles of activated HUVEC (Fig. 5B).

To quantitatively discriminate between membrane-bound and internalised (consumed) liposomes, fluorescence spectroscopy was used. The technique permits the assessment of values averaged over the whole pool of cells under study, without gating cell subsets of interest afforded by FACS analysis. As opposed to the commonly used malignant cell lines, HUVEC do not proliferate but simply survive, so their consuming activity is moderate. To meet the lower sensitivity of the spectrofluorimetry technique, the concentration of liposomes in the incubation media was taken 60 times higher than that used in the experiments with flow cytometry and confocal microscopy. This could actually elevate the contribution of non-specific interactions to the level that downplays the effect of high-affinity polyvalent interactions between SiaLe^X ligand and E-selectin. Upon a balance, good agreement between the quantitative results of the influence of 2 mol.% SiaLe^X conjugate in liposomes on their overall accumulation by the activated HUVEC obtained with two different fluorescence methods (about 35%, Figs. 5A and 7 in a set of bars “total”) is a lucky chance. Another matter is that spectrofluorimetry yet evidences more efficient binding and internalisation of targeted liposomes by activated cells as compared to both SiaLe^X-free formulations and non-activated cells. Also, the technique revealed no essential differences between the bound-to-internalised liposome ratios: for all four studied combinations of formulations and cells (targeted/non-targeted liposomes and activated/non-activated cells) the ratio was close to 2 (Fig. 7), that is, only 50% of bound liposomes entered the cells. This is not that far from the results obtained in a study by Kessner et al. [39], who used the so-called dithionite quenching technique with *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-labelled liposomes initially bound on the cell surface at 4 °C and then allowed it to be consumed at 37 °C. Regardless of the presence or the specificity of the coupled antibodies, 70-nm liposomes equipped with anti-E selectin mAb or IgG in a varying manner—at the distal end of PEG-2000 derivatised phosphatidylethanolamine or near the polar head group of the phospholipid—demonstrated internalisation of not more than 20–25% of bound liposomes by activated HUVEC (about 3100 liposomes per cell).

Nevertheless, different patterns of intracellular distribution of BODIPY-labelled PC after 1-h incubations of targeted/non-targeted liposomes with activated/non-activated cells (Fig. 5B; SiaLe^X-free liposomes in the resting cells exhibited weak diffuse staining, data not shown) indicate the implementation of active transport in the uptake of SiaLe^X liposomes by activated HUVEC. Monitoring the early stages of intracellular traffic (Fig. 8), we found that endocytosis of SiaLe^X liposomes occurred only partially via clathrin-dependent pathway, which does not contradict the available literature data on E-selectin localisation in plasmalemma. Indeed, it has been demonstrated that E-selectin clusterises in both clathrin-coated pits and lipid rafts of endothelial cells (although its internalisation has been shown to proceed only through clathrin-coated pits) [43]. Moreover, in an early study by von Asmuth et al. [38], who immunostained E-selectins in the electron microscopy cryosections of TNF-α-activated HUVEC with colloidal gold, the observed number of clathrin-coated pits and clathrin-coated vesicles was too small to allow for a conclusion about the involvement of these structures in the process of E-selectin internalisation. In general, our targeted liposomes seem to follow the endocytosis track through endosomes and sorting to Golgi followed by entering endoplasmic reticulum, as it is outlined in [42] for both clathrin-dependent endocytosis and fluid phase uptake.

It was demonstrated by pyranine spectroscopic [39] and microscopic [40] assays that E-selectin-targeted immunoliposomes of various compositions underwent, at least in part, endosome/lysosome pathway appearing in acidic perinuclear vesicles 2–4 h after binding to the cell surface [40]. At the same time, microscopic investigation showed that liposomes also fused with the cell membrane and the entrapped calcein was released into the cytoplasm in 90-min incubations [39]. Further, the authors [18] found that SiaLe^X-conjugated liposomes composed mainly of egg phosphatidylcholine and loaded with antisense oligonucleotides down-regulated protein expression in activated HUVEC without affecting mRNA expression, which evidences that hybridization with the target mRNA proceeds in the cytoplasm but not in the nucleus. Using dual fluorescence labelling and FACS analysis, we found that our SiaLe^X formulations—liposomes loaded with melphalan lipophilic prodrug in the bilayer—undergo rapid internalisation by activated HUVEC accompanied by the disruption of liposomes, which starts practically immediately after their uptake and continues for at least 90 min (Fig. 9D). This process is visualised by the patterns of confocal microscopy (Fig. 10). We may hypothesize that liposomal membranes fuse with endosomal membranes at the very early stages and undergo trafficking preferably to the endoplasmic reticulum, since the most extensive colocalisation was observed exactly with this organelle, which accounts for the intracellular transport of membrane-bound molecules (Fig. 8). If so, SiaLe^X liposomes under study have a fair chance to escape lysosomal degradation prior to presentation of the lipophilic prodrug to esterases for the release of the cytotoxic drug, so that it can reach the nucleus and induce specific DNA inter-strand cross-linkages in the endothelial cells. This assumption agrees with the apoptotic cell death–mediated antivascular effect exhibited by these formulations in vivo in a mouse model of lung carcinoma, which resulted in tumour growth inhibition [23]. Another possible route for SiaLe^X liposomes is to enter an endothelial cell through caveolae-mediated pathway and fuse with the so-called membrane-bound vesicles. The lipophilic prodrug then could undergo transport from one side of the cell to the other within these vesicles avoiding any endosomal compartment (such transcytosis is characteristic of endothelial cells) [44]. Furthermore, the lipophilic prodrug or the released melphalan itself can exit the cell from the abluminal surface and further actuate the cytotoxic effect towards tumour cells or their microenvironment. However, both hypotheses should be the subject of further research.

Noteworthy is the fact that non-activated cells consumed negligible doses of liposomes during at least 1.5 h (Fig. 9A, B). The apparent mismatch of this result with the data on overall accumulation of BODIPY-labelled liposomes by resting cells (Fig. 5A) should be related to the different photophysical characteristics of rhodamine and BODIPY fluorophores used. In frames of a single experimental approach with the same fluorescent label, non-activated and activated HUVEC demonstrated drastic contrast in the rate of binding and internalisation of SiaLe^X formulations (Fig. 9A, C). This finding is the primary one in view of therapeutic potential of the liposomes since it promises good selectivity towards inflamed endothelium inherent to tumour microenvironment after intravenous administration of the targeted formulations.

In conclusion, in this study, we demonstrate that binding and internalisation of SiaLe^X liposomes loaded with melphalan lipophilic prodrug in the bilayer by cytokine-activated endothelial cells are essentially more efficient if compared to non-activated cells. This selective effect is realised through specific cell–liposome interactions upon cell stimulation and E-selectin exposure on the cell surface. The endocytosis of SiaLe^X liposomes occurs mostly via clathrin-independent pathways, which increases the possibility of evading lysosomal degradation. After internalisation, the liposome membrane undergoes rapid destabilization presumably allowing a facilitated esterase hydrolysis of the cargo with the release of the cytotoxic agent. The influence of dynamic conditions of capillary blood flow on the binding capacity of SiaLe^X formulation and endothelial cells is the subject of ongoing studies.

Declaration of interest statement

No conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.01.016.

References

[1] T.M. Allen, P.R. Cullis, Liposomal drug delivery systems: from concept to clinical applications, Adv. Drug Deliv. Rev. 65 (2013) 36–48. http://dx.doi.org/10.1016/j.addr.2012.09.037.
[2] R.R. Sawant, V.P. Torchilin, Challenges in development of targeted liposomal therapeutics, AAPS J. 14 (2012) 303–315. http://dx.doi.org/10.1208/s12248-012-9330-0.
[3] T. Lammers, F. Kiessling, W.E. Hennink, G. Storm, Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress, J. Control. Release 161 (2012) 175–187. http://dx.doi.org/10.1016/j.jconrel.2011.09.063.
[4] S. Cressman, I. Dobson, J.B. Lee, Y.Y.C. Tam, P.R. Cullis, Synthesis of a labeled RGD-lipid, its incorporation into liposomal nanoparticles, and their trafficking in cultured endothelial cells, Bioconjug. Chem. 20 (2009) 1404–1411. http://dx.doi.org/10.1021/bc900041f.
[5] P. Vader, B.J. Crielaard, S.M. van Dommelen, R. van der Meel, G. Storm, R.M. Schiffelers, Targeted delivery of small interfering RNA to angiogenic endothelial cells with liposome-polycation-DNA particles, J. Control. Release 160 (2012) 211–216. http://dx.doi.org/10.1016/j.jconrel.2011.09.080.
[6] A. Wicki, C. Rochlitz, A. Orleth, R. Ritschard, I. Albrecht, R. Herrmann, G. Christofori, C. Mamot, Targeting tumor-associated endothelial cells: anti-VEGFR2 immunoliposomes mediate tumor vessel disruption and inhibit tumor growth, Clin. Cancer Res. 18 (2012) 454–464. http://dx.doi.org/10.1158/1078-0432.CCR-11-1102.
[7] E. Jubeli, L. Moine, J. Vergnaud-Gauduchon, G. Barratt, E-selectin as a target for drug delivery and molecular imaging, J. Control. Release 158 (2012) 194–206. http://dx.doi.org/10.1016/j.jconrel.2011.09.084.
[8] C. Ehrhardt, C. Kneuer, U. Bakowsky, Selectins—an emerging target for drug delivery, Adv. Drug Deliv. Rev. 56 (2004) 527–549. http://dx.doi.org/10.1016/j.addr.2003.10.029.
[9] C. Foxall, S.R. Watson, D. Dowbenko, C. Fennie, L.A. Lasky, M. Kiso, A. Hasegawa, D. Asa, B.K. Brandley, The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis(x) oligosaccharide, J. Cell Biol. 117 (1992) 895–902 (PMID: 2289454).
[10] S.R. Barthel, J.D. Gavino, L. Descheny, C.J. Dimitroff, Targeting selectins and selectin ligands in inflammation and cancer, Expert Opin. Ther. Targets 11 (2007) 1473–1491. http://dx.doi.org/10.1517/14728222.11.11.1473.
[11] C.A. St Hill, Interactions between endothelial selectins and cancer cells regulate metastasis, Front. Biosci. (Landmark Ed.) 16 (2011) 3233–3251 (PMID: 21622232).
[12] S. Köhler, S. Ullrich, U. Richter, U. Schumacher, E-/P-selectins and colon carcinoma metastasis: first in vivo evidence for their crucial role in a clinically relevant model of spontaneous metastasis formation in the lung, Br. J. Cancer 102 (2010) 602–609. http://dx.doi.org/10.1038/sj.bjc.6605492.
[13] Z.-J. Liu, R. Tian, Y. Li, W. An, Y. Zhuge, A.S. Livingstone, O.C. Velazquez, Inhibition of tumor angiogenesis and melanoma growth by targeting vascular E-selectin, Ann. Surg. 254 (2011) 450–457. http://dx.doi.org/10.1097/SLA.0b013e31822a72dc.
[14] S. Asgeirsdottir, J. Kamps, Site-specific inhibition of glomerulonephritis progression by targeted delivery of dexamethasone to glomerular endothelium, Mol. Pharmacol. 72 (2007) 121–131. http://dx.doi.org/10.1124/mol.107.034140.
[15] T. Murohara, J. Margiotta, L.M. Phillips, J.C. Paulson, S. DeFrees, S. Zalipsky, L.S.S. Gucl, A.M. Lefer, Cardioprotection by liposome-conjugated sialyl Lewis x-oligosaccharide in myocardial ischaemia and reperfusion injury, Cardiovasc. Res. 30 (1995) 965–974. http://dx.doi.org/10.1016/S0008-6363(95)00157-3.
[16] S.A. DeFrees, L. Phillips, L. Guo, S. Zalipsky, Sialyl Lewis x liposomes as a multivalent ligand and inhibitor of E-selectin mediated cellular adhesion, J. Am. Chem. Soc. 118 (1996) 6101–6104. http://dx.doi.org/10.1021/ja954122g.
[17] R. Zeisig, R. Stahn, K. Wenzel, D. Behrens, I. Fichtner, Effect of sialyl Lewis X-glycoliposomes on the inhibition of E-selectin-mediated tumour cell adhesion in vitro, Biochim. Biophys. Acta 1660 (2004) 31–40. http://dx.doi.org/10.1016/j.bbamem.2003.10.014.
[18] R. Stahn, C. Grittner, R. Zeisig, U. Karsten, S.B. Felix, K. Wenzel, Sialyl Lewis(x)-liposomes as vehicles for site-directed, E-selectin-mediated drug transfer into activated endothelial cells, Cell. Mol. Life Sci. 58 (2001) 141–147 (PMID: 11229813).
[19] M. Hirai, H. Minematsu, N. Kondo, K. Oie, K. Igarashi, N. Yamazaki, Accumulation of liposome with Sialyl Lewis X to inflammation and tumor region: application to in vivo bio-imaging, Biochem. Biophys. Res. Commun. 353 (2007) 553–558. http://dx.doi.org/10.1016/j.bbrc.2006.12.060.
[20] M. Hirai, H. Minematsu, Y. Hiramatsu, H. Kitagawa, T. Otani, S. Iwashita, T. Kudoh, L. Chen, Y. Li, M. Okada, D.S. Salomon, K. Igarashi, M. Chikuma, M. Seno, Novel and simple loading procedure of cisplatin into liposomes and targeting tumor endothelial cells, Int. J. Pharm. 391 (2010) 274–283. http://dx.doi.org/10.1016/j.ijpharm.2010.02.030.
[21] A. Maehara, K. Nishida, M. Furutani, E. Matsumoto, A. Ohtsuka, Y. Ninomiya, T. Oohashi, Light and electron microscopic detection of inflammation-targeting liposomes encapsulating high-density colloidal gold in arthritic mice, Inflamm. Res. 63 (2014) 139–147. http://dx.doi.org/10.1007/s00011-013-0682-4.
[22] E.L. Vodovozova, E.V. Moiseeva, G.K. Grechko, G.P. Gayenko, N.E. Nifant'ev, N.V. Bovin, J.G. Molotkovsky, Antitumour activity of cytotoxic liposomes equipped with selectin ligand SiaLeX, in a mouse mammary adenocarcinoma mode, Eur. J. Cancer 36 (2000) 942–949. http://dx.doi.org/10.1016/S0959-8049(00)00029-0.
[23] N.R. Kuznetsova, E.V. Stepanova, N.M. Peretolchina, D.A. Khochenkov, I.A. Boldyrev, N.V. Bovin, E.L. Vodovozova, Targeting liposomes loaded with melphalan prodrug to tumour vasculature via the Sialyl Lewis X selectin ligand, J. Drug Target. 2330 (2013) 242–250. http://dx.doi.org/10.3109/1061186X.2013.862805.
[24] M.V. Mateos, J. San-Miguel, Treatment of newly diagnosed myeloma in patients not eligible for transplantation, Curr. Hematol. Malig. Rep. 6 (2011) 113–119. http://dx.doi.org/10.1007/s11899-011-0080-3.
[25] N. Kuznetsova, A. Kandyba, I. Vostrov, V. Kadykov, G. Gaenko, J. Molotkovsky, E. Vodovozova, Liposomes loaded with lipophilic prodrugs of methotrexate and melphalan as convenient drug delivery vehicles, J. Drug Deliv. Sci. Technol. 19 (2009) 51–59 (http://www.jddst.com/index.php?controller=product&id_product=707).
[26] N.R. Kuznetsova, C. Sevrin, D. Lespineux, N.V. Bovin, E.L. Vodovozova, T. Mészáros, J. Szebeni, C. Grandfils, Hemocompatibility of liposomes loaded with lipophilic prodrugs of methotrexate and melphalan in the lipid bilayer, J. Control. Release 160 (2012) 394–400. http://dx.doi.org/10.1016/j.jconrel.2011.12.010.
[27] A. Gabizon, D. Papahadjopoulos, Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 6949–6953 (PMID: 3413128).
[28] M. Muller, O. Zschornig, S. Ohki, K. Arnold, Fusion, leakage and surface hydrophobicity of vesicles containing phosphoinositides: influence of steric and electrostatic effects, J. Membr. Biol. 192 (2003) 33–43 (PMID: 12647032).
[29] E.L. Vodovozova, G.V. Pazynina, N.V. Bovin, Synthesis of diglyceride conjugate of selectin ligand SiaLeX as a vector for targeting of drug-loaded liposomes, Mendeleev Commun. 21 (2011) 69–71. http://dx.doi.org/10.1016/j.mencom.2011.03.002.
[30] E.L. Vodovozova, P.I. Nikol'skiĭ, I.I. Mikhalev, I.G. Molotkovskiĭ, Lipid derivatives of sarcolysin, methotrexate and rubomycin, Bioorg. Khim. 22 (1996) 548–556 (PMID: 8992961).
[31] I.A. Boldyrev, X. Zhai, M.M. Momsen, H.L. Brockman, R.E. Brown, J.G. Molotkovsky, New BODIPY lipid probes for fluorescence studies of membranes, J. Lipid Res. 48 (2007) 1518–1532. http://dx.doi.org/10.1194/jlr. M600459-JLR200.
[32] E.A. Jaffe, R.L. Nachman, C.G. Becker, C.R. Minick, Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria, J. Clin. Invest. 52 (1973) 2745–2756. http://dx.doi.org/10.1172/JCI107470.
[33] O.N. Scheglovitova, Y.A. Romanov, E.V. Maksianina, V.A. Svintsitskaya, A.G. Pronin, Herpes simplex type I virus infected human vascular endothelial cells induce the production of anti-viral and proinflammatory factors by peripheral blood leukocytes in vitro, Russ. J. Immunol. 7 (2002) 115–122 (PMID: 2687253).

[34] J. Kusunose, H. Zhang, M.K.J. Gagnon, T. Pan, S.I. Simon, K.W. Ferrara, Microfluidic system for facilitated quantification of nanoparticle accumulation to cells under laminar flow, Ann. Biomed. Eng. 41 (2013) 89–99. http://dx.doi.org/10.1007/s10439-012-0634-0.
[35] Y. Li, J. Wang, Y. Gao, J. Zhu, M.G. Wientjes, J.L.-S. Au, Relationships between liposome properties, cell membrane binding, intracellular processing, and intracellular bioavailability, AAPS J. 13 (2011) 585–597. http://dx.doi.org/10.1208/s12248-011-9298-1.
[36] S. Simões, V. Slepushkin, N. Düzgünes, M.C. Pedroso de Lima, On the mechanisms of internalization and intracellular delivery mediated by pH-sensitive liposomes, Biochim. Biophys. Acta 1515 (2001) 23–37 (PMID: 11597349).
[37] M.P. Bevilacqua, S. Stengelin, M.A. Gimbrone, B. Seed, Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins, Science 243 (1989) 1160–1165 (PMID: 2466335).
[38] E.J. von Asmuth, E.F. Smeets, L.A. Ginsel, J.J. Onderwater, J.F. Leeuwenberg, W.A. Buurman, Evidence for endocytosis of E-selectin in human endothelial cells, Eur. J. Immunol. 22 (1992) 2519–2526. http://dx.doi.org/10.1002/eji.1830221009.
[39] S. Kessner, A. Krause, U. Rothe, G. Bendas, Investigation of the cellular uptake of E-Selectin-targeted immunoliposomes by activated human endothelial cells, Biochim. Biophys. Acta 1514 (2001) 177–190 (PMID: 11557019).
[40] D.D. Spragg, D.R. Alford, R. Greferath, C.E. Larsen, K.D. Lee, G.C. Gurtner, M.I. Cybulsky, P.F. Tosi, C. Nicolau, M.A. Gimbrone, Immunotargeting of liposomes to activated vascular endothelial cells: a strategy for site-selective delivery in the cardiovascular system, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 8795–8800 (PMID: 9238057).
[41] K.A. Mislick, J.D. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene transfer, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 12349–12354 (PMID: 8901584).
[42] L. Johannes, C. Lamaze, Clathrin-dependent or not: is it still the question? Traffic 3 (2002) 443–451. http://dx.doi.org/10.1034/j.1600-0854.2002.30701.x.
[43] H. Setiadi, R.P. McEver, Clustering endothelial E-selectin in clathrin-coated pits and lipid rafts enhances leukocyte adhesion under flow, Blood 111 (2008) 1989–1998. http://dx.doi.org/10.1182/blood-2007-09-113423.
[44] P.L. Tuma, A.L. Hubbard, Transcytosis: crossing cellular barriers, Physiol. Rev. 83 (2003) 871–932 (www.prv.org.).