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RESEARCH PAPER

Development of Liposomal Nanoconstructs Targeting P-selectin (CD62P)-expressing Cells by Using A Sulfated Derivative of Sialic Acid

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ABSTRACT

Purpose NMSO3, a sulfated derivative of sialic acid, is a specific inhibitor for P-selectin (CD62P)-mediated cell adhesion. We attempted to apply liposomes modified with NMSO3 for selective targeting of activated platelets.

Methods The binding of fluorescently labeled NMSO3-containing liposomes (NMSO3-liposomes) to CHO cells expressing P-selectin (CHO-P cells) and activated platelets were examined. The distribution of NMSO3-liposomes incorporated into the cells was observed by fluorescence microscopy.

Results The binding assay revealed that NMSO3-liposomes specifically bound to immobilized P-selectin and CHO-P cells in a dose-dependent manner. The binding of NMSO3-liposomes to CHO-P cells was much stronger than that to the parental CHO-K1 cells. Fluorescence microscopic observation showed that NMSO3-liposomes were incorporated into CHO-P cells after the binding and distributed throughout the cytoplasm of the cell. NMSO3-liposomes bound more strongly to thrombin-activated platelets than to resting platelets, as assessed by flow cytometry.

Conclusions These results suggest that NMSO3-liposomes can be applied for selective drug delivery to activated platelets.

KEY WORDS activated platelet · drug delivery · liposome · P-selectin · sulfated sialyl lipid

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ABBREVIATIONS

PBS	Phosphate buffered saline
PRP	Platelet-rich plasma
sLeX	Sialyl Lewis X

INTRODUCTION

Site-directed drug delivery has been recognized as a promising approach for the chemotherapeutic treatment of cardiovascular diseases. Platelet activation and subsequent aggregation primarily cause vascular thrombosis and occlusion. Cell adhesion molecules have recently been utilized as target molecules on activated platelets. For example, targeting of αIIbβ3 integrin (GPIIb-IIIa) by RGD-peptide-modified liposomes indicated that the ligand-modified liposomes can be used for the selective delivery of therapeutic agents to activated platelets (1,2). However, the RGD-peptide is also recognized by integrin molecules other than αIIbβ3 integrin. To improve the specificity in the binding to activated platelets, another cell adhesion molecule, P-selectin, was recently picked out as a target molecule on activated platelets. Modery and co-workers demonstrated that the simultaneous modification of the RGD-liposomes with a P-selectin-binding peptide (EWVDV) enhanced the targeting to activated platelets (3).

P-selectin (CD62P) is a member of the selectin family of adhesion molecules, and is specifically expressed on activated platelets and vascular endothelial cells. This adhesion molecule plays a critical role in the recruitment of leukocytes to hemorrhagic sites and inflammatory tissues (4). P-selectin possesses a C-type lectin domain and recognizes a variety of sialylated, fucosylated and sulfated glycans, including sialyl Lewis X (sLeX) epitope (sialic acidα2-3galactoseβ1-

4(fucoseα1-3)*N*-acetylglucosamine) (5). The physiological high-affinity ligand for P-selectin was identified and designated as P-selectin glycoprotein ligand-1 (PSGL-1) (6). PSGL-1 is a mucin-like transmembrane glycoprotein expressed on various types of leukocytes. The high-affinity binding of PSGL-1 to P-selectin requires sulfation at tyrosine residues in the *N*-terminal region of PSGL-1 in addition to sLeX glycans (7,8). Because P-selectin plays pivotal roles in various acute and chronic inflammatory processes, such as ischemia reperfusion (9,10), atherosclerosis (11), and rheumatoid arthritis (12), through the endothelium-leukocyte and platelet-leukocyte interactions, the targeting to P-selectin may contribute to a potential therapeutic strategy for these inflammatory diseases.

NMSO3 is a chemically synthesized sulfated derivative of sialic acid that was originally developed as an antiviral agent (13,14). We previously reported that NMSO3 acted as a specific inhibitor for P-selectin-mediated cell adhesion (15). We also found that NMSO3 was directly bound by a P-selectin/IgG chimera and that NMSO3 inhibited P-selectin-induced tumor necrosis factor-α secretion from monocytes. Because NMSO3 has two alkyl chains in the molecule (Fig. 1), it seems likely to stick into the lipid bilayer of liposomes. We therefore attempted to prepare liposomes containing NMSO3 and evaluate their potential application for selective drug delivery to activated platelets and P-selectin-expressing cells.

MATERIALS AND METHODS

Reagents

NMSO3, sodium [2, 2-bis(docosyloxymethyl) propyl-5-acetamido-3, 5-dideoxy-4, 7, 8, 9-tetra-*O*-(sodium oxysulfonyl)-D-glycero-α-D-galacto-2-nonulopyranosid]onate) (Fig. 1), was supplied by Nissin Central Research Institute (13). Serum-free medium ASF104 was purchased from Ajinomoto (Tokyo, Japan). Thrombin, egg phosphatidylcholine (PC), cholesterol and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were products of Sigma (St. Louis, MO). H-Gly-Pro-Arg-Pro (GPRP) peptide was purchased from Bachem (Bubendorf, Switzerland). Anti-P-selectin nonblocking antibody (2D7, IgG) was prepared in our laboratory (16). Anti-P-selectin blocking antibody (2T60, IgG) was kindly donated by Dr. K. Tanoue (Tokyo Metropolitan Institute of Medical Science) (17). Phycoerythrin (PE)-conjugated anti-P-selectin antibody (AK4) was purchased from Bay Bioscience (Kobe, Japan). Anti-PSGL-1 antibody (PL1) was from Serotec, Ltd. (Oxford, UK). Alexa Fluor 488-conjugated goat anti-mouse IgG antibody was from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma and Wako Pure Chemicals (Osaka, Japan) unless otherwise indicated

Cells

CHO-K1 cells were supplied by the Health Science Research Resources Bank (Osaka Japan). P-selectin-expressing CHO cells (CHO-P cells) were prepared by the transfection of CHO-K1 cells with P-selectin cDNA from a human erythroid leukemia cell line (HEL) (5). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) under a 5% CO_2 atmosphere. Human platelets were prepared from peripheral blood collected by venipuncture from healthy volunteers using heparin as an anticoagulant. Briefly, the heparinized blood was centrifuged at 800 rpm for 10 min to obtain platelet-rich plasma (PRP). After the addition of a 1/10 volume of acid-citrate-dextrose (ACD: 0.08 M citric acid, 0.12 M sodium citrate, 0.11 M D-glucose), PRP was centrifuged at 1,000 x g for 1.5 min and resuspended with PBS.

Preparation of Liposomes

NMSO3-containing liposomes were prepared by the dry film method as described previously except for the addition of NMSO3 (18,19). Briefly, PC, cholesterol, NMSO3, and DiI in a molar ratio of 7:3:1:0.04 were dissolved in an appropriate volume of chloroform. Control liposome was also prepared by the same formula as used for the lipid mixture except for the lack of NMSO3. The solvent was evaporated in a round-bottom flask, and the deposited lipid mixture was kept under

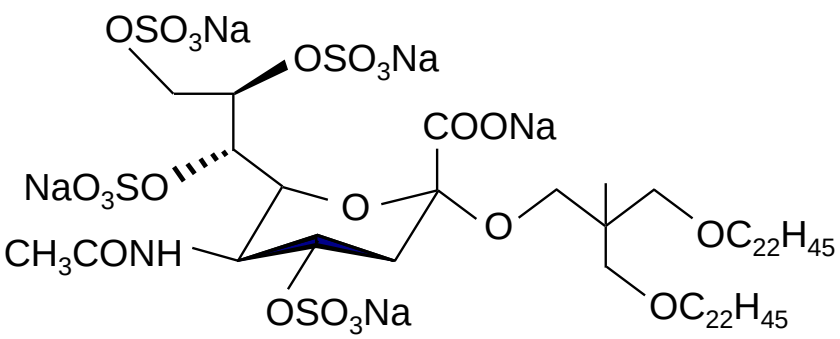


Fig. 1 Structure of NMSO3, a sulfated derivative of sialic acid. Molecular formula: $C_{60}H_{112}NO_{23}S_4Na_5$

a vacuum to give a dry lipid film. The lipid film was suspended in PBS, and the suspension was sonicated to obtain liposomes with a diameter of 200–300 nm. The particle size distribution and zeta-potential were measured by the dynamic light-scattering method and the electrophoresis light-scattering method, respectively (ELS-800; Otsuka Electronics Co., Ltd., Osaka, Japan) in water at 25°C. The zeta-potential of liposomes was measured to confirm that NMSO3 was incorporated into liposomes. The liposome suspension was filtered with a membrane filter (0.45 μm pore size) before use.

FLUOROMETRIC QUANTIFICATION OF LIPOSOME BINDING TO IMMOBILIZED P-SELECTIN

P-selectin was purified from human platelets as described previously (16). A 96-well culture plate was coated with purified P-selectin (0.25–4 μg/ml) in PBS at 4°C for 16 h. The well was washed with PBS three times and then blocked with 1% bovine serum albumin (BSA)/PBS at 4°C for 2 h. Fluorescently labeled liposomes (50 μmole lipid) were then added to each well of the plate, and the plate was incubated at 4°C for 1 h. After unbound liposomes were removed by washing three times with PBS, adhered liposomes were lysed with 1% Nonidet P-40 (0.1 ml). The amount of liposomes bound to immobilized P-selectin was determined by measuring the DiI fluorescence associated with liposomes using a fluorescence spectrophotometer (Model F-4010; Hitachi, Ltd., Tokyo, Japan; Ex=547 nm, Em=565 nm).

FLUOROMETRIC QUANTIFICATION OF LIPOSOMES BINDING TO CELLS

The parent CHO-K1 and the transfectant CHO-P cells were grown in RPMI 1640/10% FBS to a subconfluent monolayer in a 96-well culture plate. The culture medium was replaced with serum-free ASF104 medium, and the culture was continued for 16 h. Fluorescently labeled liposomes (50 μmole lipid) were then added to each well of the plate and incubated for 5–60 min at 4°C or 37°C. After unbound liposomes were removed by gentle washing three times with PBS, adhered liposomes were lysed with 5% Triton X-100 (0.1 ml). The quantity of adhered liposomes was calculated from the DiI-fluorescence associated with liposomes as described above.

FLOW CYTOMETRIC ANALYSIS OF P-SELECTIN EXPRESSION AND CELL-BOUND LIPOSOMES

The expression of P-selectin on platelets was analyzed by flow cytometry using phycoerythrin (PE)-conjugated anti-P-selectin antibody (AK4). Platelets were activated with 1 U/ml thrombin in the presence of 0.5 mM GPRP peptide at room temperature for 5 min. Aliquots of activated or resting platelet suspension (20 μl) were treated with PE-conjugated anti-P-selectin antibody (4 μl) for 10 min at room temperature and subjected to flow cytometry (FACS Calibur; BD Biosciences, San Diego, CA).

The CHO cell suspension (1 x 10^6 cells/ml in PBS) or platelet suspension (PRP) was incubated with various concentrations of DiI-containing liposomes at 4°C for 20 min (for CHO cells) or 10 min (for platelets), and then the cells were subjected to flow cytometry to quantify the DiI-labeled liposomes associated with cells. In the data acquisition, CHO cells and platelets were gated to distinguish from free liposomes or cell debris by a forward-scatter *versus* side-scatter plot. In some experiments, platelets were pretreated with blocking anti-P-selectin antibody (2T60, 20 μg/ml), non-blocking anti-P-selectin antibody (2D7, 20 μg/ml), or 5 mM EDTA at 4°C for 10 min before incubation with liposomes.

Fluorescence Microscopy

CHO-K1 and CHO-P cells were grown to subconfluence on Lab-Tek II chamber slide (Nalge Nunc International, Rochester, NY), and then incubated with DiI-labeled liposomes (50 μmole lipid) at 4°C for 10 min. The cells were fixed with 1% formaldehyde at room temperature for 10 min, and observed with a confocal laser scanning microscope (Radians 2100; Bio-Rad Laboratories, Hercules, CA). To visualize the cell surface P-selectin, fixed cells were stained with the

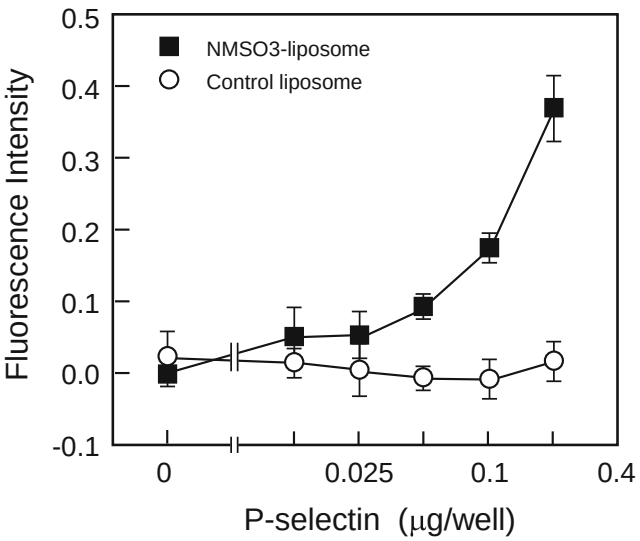


Fig. 2 Binding of NMSO3-containing liposomes to immobilized P-selectin. To a 96-well culture plate that had been coated with human P-selectin (0.0125–0.2 μg/well) in PBS for 16 h at 4°C, DiI-labeled NMSO3-liposomes (*closed square*) or control liposomes (*open circle*) (50 μmole lipid) were added, and the plates were incubated for 1 h at 4°C. After unbound liposomes were removed by gentle washing, the bound liposomes were lysed with 0.1 ml of 1% Nonidet P-40. The fluorescence intensity of the lysate was measured with a fluorescence spectrophotometer (Ex = 547 nm, Em = 565 nm)

combination of anti-P-selectin antibody (2D7) and Alexa Fluor 488-conjugated anti-mouse IgG antibody.

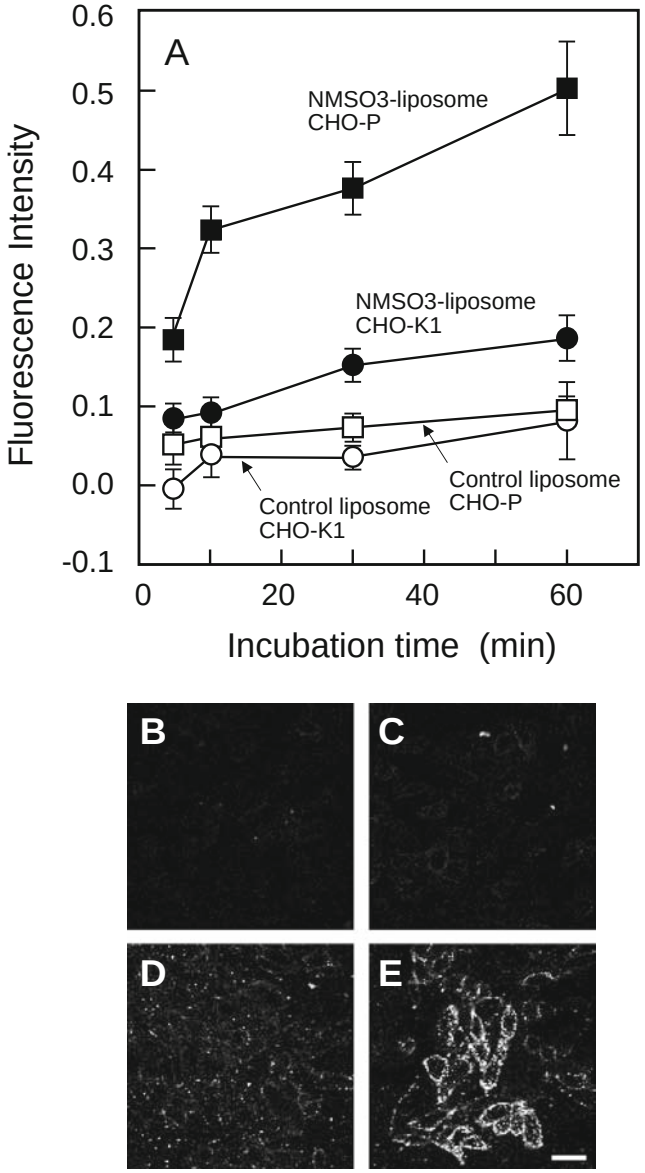


Fig. 3 Binding of NMSO3-containing liposomes to CHO cells expressing P-selectin. (**a**) DiI-labeled NMSO3-containing or control liposomes (50 μmole lipid) were added to a monolayer culture of CHO cells expressing P-selectin (CHO-P) or parental CHO-K1 cells in a 96-well culture plate, and the plate was incubated at 4°C for 5–60 min. After unbound liposomes were removed by gentle washing with medium three times, bound liposomes were lysed with 0.1 ml of 5% Triton X-100. The fluorescence intensity of the lysate was measured with a fluorescence spectrophotometer (Ex = 547 nm, Em = 565 nm). *Closed squares*, NMSO3-liposomes/CHO-P cells; *closed circles*, NMSO3-liposomes/CHO-K1 cells; *open squares*, control liposomes/CHO-P cells; *open circles*, control liposomes/CHO-K1 cells. (**b**)-(**e**) DiI-labeled control liposomes (**b** and **c**) or NMSO3-liposomes (**d** and **e**) were added to a monolayer culture of CHO-K1 cells (**b** and **d**) or CHO-P cells (**c** and **e**) and incubated at 4°C for 60 min. After unbound liposomes were removed by gentle washing with medium three times, the cells were fixed with 1% formaldehyde for 10 min and observed with a confocal laser-scanning microscope. *Scale bar*, 30 μm

RESULTS

Characterization of NMSO3-containing Liposomes

The average particle sizes of NMSO3-liposomes (NMSO3-liposomes) and control liposomes were 216.9 nm and 299.7 nm, respectively, indicating that these liposomes were not substantially different in size. However, the zeta-potential

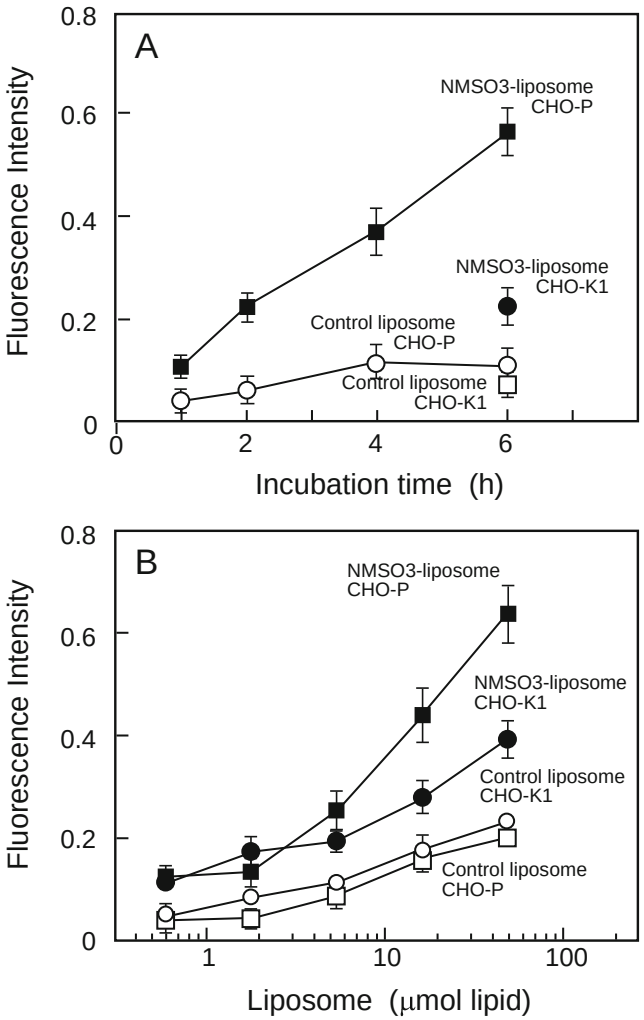


Fig. 4 Time- and dose-dependent incorporation of DiI-labeled NMSO3-containing liposomes into CHO cells expressing P-selectin. (**a**) DiI-labeled NMSO3-liposomes (*closed squares*) or control liposomes (*open circles*) (50 μmole lipid) were added to a monolayer culture of CHO-P cells in a 96-well culture plate, and the plate was incubated at 37°C for 1–6 h. After the well was washed with medium three times, the liposomes incorporated into the cells were lysed with 0.1 ml of 5% Triton X-100. The fluorescence in the lysate was measured with a fluorescence spectrophotometer (Ex = 547 nm, Em = 565 nm). (**b**) DiI-labeled NMSO3-liposomes or control liposomes (0.5–50 μmole lipid) were added to a monolayer culture of CHO-P or CHO-K1 cells in a 96-well culture plate, and the plate was incubated at 37°C for 4 h. The fluorescence associated with the incorporated liposomes was measured as described above. Values represent the mean ± SD (n = 3). *Closed squares*, NMSO3-liposomes/CHO-P cells; *closed circles*, NMSO3-liposomes/CHO-K1 cells; *open squares*, control liposomes/CHO-P cells; *open circles*, control liposomes/CHO-K1 cells

of NMSO3-liposome (−79.86 mV) was markedly lower than that of control liposome (−9.13 mV). The negative charge of the sulfate and carboxyl groups of NMSO3 was probably responsible for lowered zeta-potential of NMSO3-liposomes. The binding assay using fluorescently labeled liposomes revealed that NMSO3-liposomes bound to immobilized P-selectin (>0.05 μg/well, doses for plate-coating) more strongly than control liposomes, which showed no significant binding to immobilized P-selectin (Fig. 2).

Adhesion of NMSO3-liposomes to P-selectin-expressing CHO Cells

We next evaluated the adhesive properties of fluorescently labeled NMSO3-liposomes to CHO cells transfected with cDNA encoding P-selectin (CHO-P cells). NMSO3-liposomes adhered to a CHO-P cell monolayer in a time-dependent manner, and the adhesion to CHO-P cells was found to be much stronger than that to cells of the parent cell line, CHO-K1 (Fig. 3a). By contrast, control liposomes showed no significant adhesion either to CHO-K1 or CHO-P cells. The fluorescence microscopic observation confirmed the selective adhesion of NMSO3-liposome to a CHO-P cell monolayer (Fig. 3b-e).

Incorporation of NMSO3-liposomes into P-selectin-expressing CHO Cells

After NMSO3-liposomes and control liposomes were added to a CHO-P cell monolayer, NMSO3-liposomes were rapidly incorporated into the cells during incubation at 37°C up to 6 h; however, the uptake of control liposomes was quite limited (Fig. 4a). Furthermore, NMSO3-liposomes were more efficiently incorporated into CHO-P cells than into the parental CHO-K1 cells (Fig. 4b). Control liposomes showed only low-level incorporation into CHO-P cells or CHO-K1 cells. Fluorescence microscopic observation revealed that DiI-labeled liposomes were mainly distributed in the cytoplasms of cells after incubation for 1 or 4.5 h (Fig. 5).

Binding of NMSO3-liposome to Activated Platelets

We next compared the binding of liposomes to resting and activated platelets. Upon stimulation with thrombin, platelets expressed P-selectin on the cell surface as assessed by flow cytometry (Fig. 6a). NMSO3-liposome bound more strongly to activated platelets than to resting platelets (Fig. 6b). The geometric mean of fluorescence intensity associated with activated platelets was estimated to be approximately 15-fold stronger than that associated with resting platelets. Control liposomes showed no significant binding to platelets regardless of their activation status (data not shown). The binding of NMSO3-liposomes to activated platelets was found to depend on the dose of liposome added (Fig. 6c). We then examined the effects of antibodies against P-selectin and EDTA on the binding of NMSO3-liposome to activated platelets. The binding was inhibited by the pretreatment of platelets with the functionally blocking anti-P-selectin antibody (2T60) but not with the nonblocking antibody (2D7) (Fig. 6d). The inhibition by 2 T60 antibody was estimated to be ~95% by decrease in mean fluorescence intensity (log scale on the X-axis), indicating that NMSO3-liposomes bound to activated platelets in a P-selectin-dependent manner. Pretreatment of activated platelets with 5 mM EDTA moderately attenuated the binding of NMSO3-liposomes to activated platelets.

DISCUSSION

Because P-selectin is specifically expressed on activated platelets and vascular endothelial cells, this adhesion molecule is an attractive target for drug delivery to thrombotic and inflammatory tissues. In this study, we demonstrated that liposomes containing

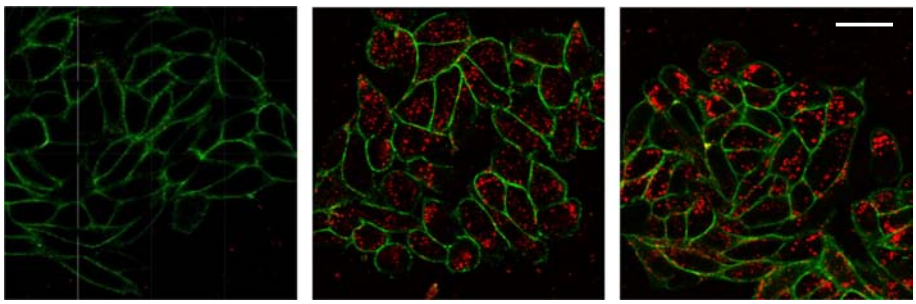
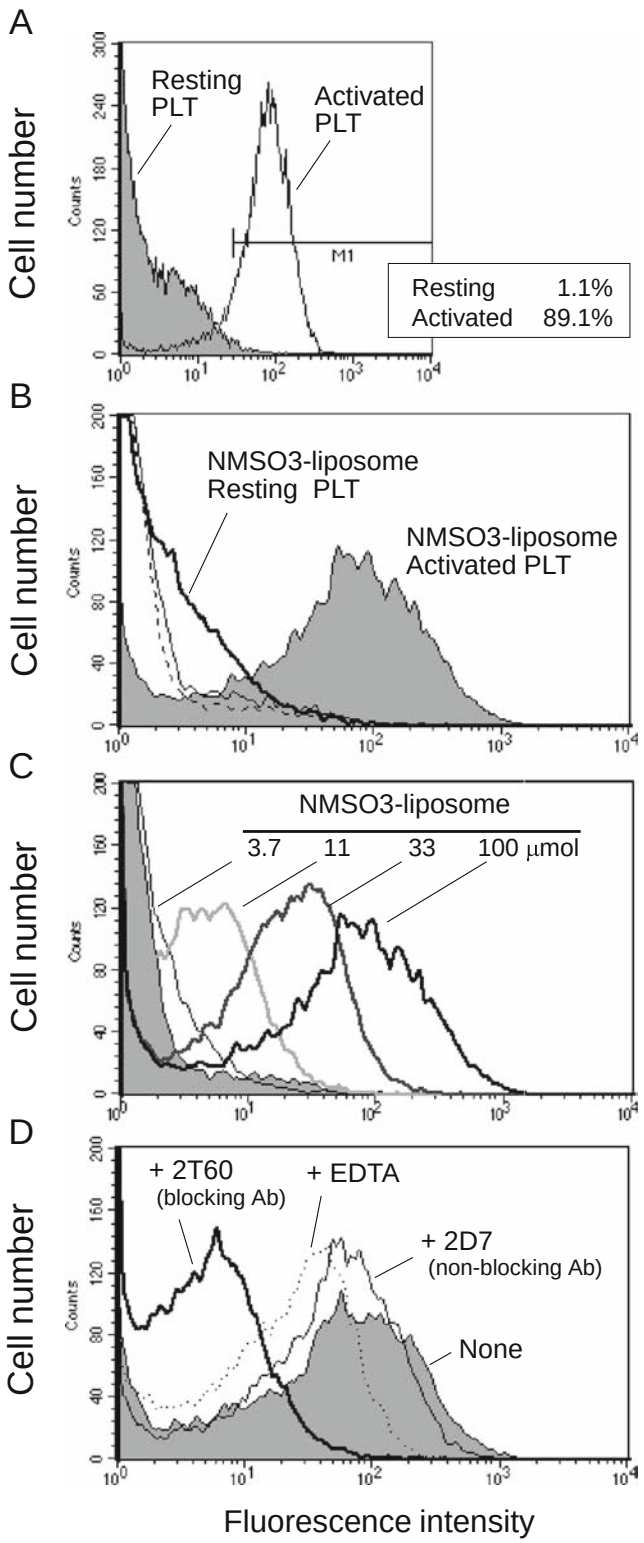


Fig. 5 Incorporation of NMSO3-containing liposomes into P-selectin-transfected cells. DiI-labeled NMSO3-containing liposomes (*red*) were added to a monolayer culture of CHO-P cells and incubated at 4°C for 10 min. The cells were washed with medium three times to remove unbound liposomes and further incubated at 37°C for 0 h (*left*), 1 h (*middle*) or 4.5 h (*right*). The cells were fixed with 1% formaldehyde for 10 min at room temperature, stained with anti-P-selectin antibody (2D7) plus Alexa Fluor 488-conjugated anti-mouse IgG (*green*), and observed with a confocal laser-scanning microscope. Scale bar, 30 μm



◀ **Fig. 6** Flow cytometric analysis of the binding of NMSO3-containing liposomes to thrombin-activated platelets. (**a**) Human platelets were treated with or without thrombin (1 U/ml) at room temperature for 5 min, and stained with PE-labeled anti-P-selectin antibody (AK4). The expression of P-selectin on the platelet surface was analyzed by flow cytometry. Data for 10,000 platelets were collected. (**b**) DiI-labeled NMSO3-containing liposomes (100 μmole lipid) were incubated with activated or resting platelets at 4°C for 10 min and analyzed by flow cytometry. The binding of DiI-labeled control liposomes to activated (*thin line*) or resting (*dotted line*) platelets was also analyzed. (**c**) Various doses (3.7–100 μmole lipid) of DiI-labeled NMSO3-containing liposomes were incubated with activated platelets at 4°C for 10 min and analyzed by flow cytometry. The binding of DiI-labeled control liposomes to activated platelets was also analyzed (*thin line with shading*). (**d**) Effects of anti-P-selectin antibody and EDTA on the binding of DiI-labeled NMSO3-containing liposomes to activated platelets were examined. Activated platelets were preincubated with anti-P-selectin antibody (2D7, non-blocking; 2T60, blocking) (20 μg/ml) or EDTA (5 mM) at 4°C for 10 min and mixed with DiI-labeled NMSO3-containing liposomes (100 μmole lipid). After incubation at 4°C for 10 min, the platelets were analyzed by flow cytometry. Data for 10,000 cells gated for platelets were collected

NMSO3 (a sulfated derivative of sialic acid) specifically bound to activated platelets in a P-selectin-dependent manner, indicating that NMSO3-liposome is a potentially promising agent for selective targeting to thromboses. P-selectin expressed on activated endothelial cells is involved in the recruitment of leukocytes to inflammatory sites and plays pivotal roles in various pathophysiological processes of acute and chronic inflammation, such as septic shock (20), ischemia reperfusion (9,10), atherosclerosis (11), and rheumatoid arthritis (12). The application of NMSO3-liposomes for targeting of activated endothelial cells may also be successful and contribute to anti-inflammatory therapy. For instance, although anti-tumor necrosis factor-α (TNF-α) antibody has been effectively applied to rheumatoid arthritis, the systemic administration of the antibody sometimes causes worsening of infectious diseases such as tuberculosis. Targeting the antibodies specifically to the inflammatory sites could improve such undesirable effects.

NMSO3 was originally developed as an antiviral agent against respiratory syncytial virus (13), adenovirus (14), human rotavirus (21), and human immunodeficiency virus (22,23), and later was found to be a potent inhibitor of P-selectin (15). Although the relationship between antiviral and anti-P-selectin activities is not clear, ligands for P-selectin and the viral receptors on the host cell may share analogous molecular structures that incorporate sulfated carbohydrates. Recently, Ding and co-workers (3) reported that the liposomes modified with the integrin-recognizing peptide (GSSSGRGDSPA) and the P-selectin ligand-mimicking peptide (DAEWVDVS) enhanced the target selectivity to activated platelets, suggesting that P-selectin is an effective target molecule for activated platelets. In a previous study, liposomes conjugated with carbohydrate epitopes such as sLeX were examined for their potential targeting to inflammatory tissues in an experimental arthritis model (24). The *in vivo* fluorescent imaging showed that the liposomes were co-localized with the vascular endothelial cell marker (CD31) and E-selectin, another member of the selectin family of adhesion molecules. The results suggested that sLeX-liposomes were accumulated in inflamed vascular endothelia. Another study using sLeX-liposomes also

revealed that the liposomes modified with the selectin-recognizing carbohydrate chains accumulated in tumor tissues in a mouse experimental model (25). It has been known that a variety of cancer cells ectopically express sLeX-related carbohydrate epitopes and that their interaction with selectin is involved in cancer invasion and metastasis (26–28). Therefore, NMSO3-liposomes could potentially be applied to chemotherapy for cancer metastasis. During the development of NMSO3 as an antiviral agent, its toxicity was examined in animal experiments, and no serious toxicity was found (13,21,22).

Although NMSO3-liposomes effectively bound to P-selectin-expressing cells, they also bound to control CHO cells somewhat more strongly than control liposomes did (Figs. 3a and 4b). This result suggests that NMSO3-liposomes tend to nonspecifically bind to CHO cells, but the binding of NMSO3-liposomes to resting platelets was almost negligible (Fig. 6b). One possibility is that CHO-K1 cells express siglec-like lectins that recognize acidic carbohydrates on the cell surface. We should further improve the physicochemical properties of the liposomes to minimize undesirable binding to cells. It is also important in the future study to search better formulation for liposome preparation, including lipid composition, NMSO3 content, and size of liposomes.

NMSO3-liposomes were incorporated into P-selectin-expressing CHO cells after their binding to the cell surface (Fig. 5). However, activated platelets may not have such high endocytotic potential, and we should consider the use of functional liposomes such as those with self-degrading activity or those with high capacity for fusion to the plasma membrane. In addition to targeting to activated platelets, the delivery of anti-inflammatory agents to inflamed vascular endothelia is another promising application of NMSO3-liposomes. In future studies, we will further examine the anti-inflammatory and immunosuppressive effects of NMSO3-liposomes through the control of leukocyte trafficking *in vivo*.

CONCLUSION

In this study, we indicated that liposomes modified with NMSO3, a sulfated derivative of sialic acid, strongly bound to P-selectin-expressing CHO cells and activated platelets. The results suggest that NMSO3-liposomes are potentially applicable for selective drug delivery to activated platelets and inflamed endothelia.

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The authors have no potential conflicts of interest to disclose.

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