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Enhanced depigmenting effects of N-glycosylation inhibitors delivered by pH-sensitive liposomes into HM3KO melanoma cells

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Synopsis

Delivery activity of pH-sensitive 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE):cholesteryl hemisuccinate (CHEMS) liposomes was assessed as an *in vitro* intracellular carrier system to increase the bioavailability of depigmentation actives. N-glycosylation inhibitors have a glycosylation-inhibiting effect, which is useful for the skin depigmentation that operates by interfering with the maturation of tyrosinase. However, an N-glycosylation inhibitor does not easily pass through skin or even cellular membranes due to its water-soluble property. Therefore, it should be transported to target cells by an efficient delivery carrier to reduce the glycosylated tyrosinase. Glycosylation-inhibiting and depigmentation effects of N-butyldeoxynojirimycine (NB-DNJ) and 1-deoxynojirimycine (DNJ)-loaded liposomes were evaluated using Western blotting and measurement of synthesized melanin. Interestingly, it was found that the pH-sensitive liposomes increased the glycosylation-inhibiting and thus, pigment-lightening effects of N-glycosylation inhibitors *in vitro*. In addition, cargo materials loaded in pH-sensitive liposomes were found to be much more efficiently delivered into the cytoplasm, as observed in fluorescent-activated cell sorting (FACS) and confocal laser-scanning microscopic (CLSM) analysis. These results indicate that pH-sensitive DOPE: CHEMS liposomes have a strong potential as a carrier system to promote delivery efficiency and to enhance the biological effects of water-soluble actives for applications in cosmetics, personal care products, and pharmaceutics.

INTRODUCTION

Classic liposomes reaching into a cytoplasmic target site are generally first recognized, taken up by endocytosis, and eventually delivered to lysosomes. Most of these liposomes

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and their cargo materials may be degraded by various hydrolases and peptidases in the lysosomes. The pH-sensitive liposomes have been designed to circumvent this lysosomal degradation by releasing their cargo contents prior to reaching the lysosomes or partly into the cytosol, where they can then diffuse to target sites (1,2). An amphiphilic stabilizer such as CHEMS incorporated in phosphatidylethanolamine (PE)-based liposomes is protonated, and their conformation is changed by acidic environments when they are delivered into endosomes (3,4). N-glycosylation inhibitors such as DNJ and NB-DNJ have an α-glucosidase-inhibiting effect, which is a useful property for enhancing pigment lightening on mammalian skin by interfering with the maturation of tyrosinase (5,6). However, it is difficult for these inhibitors to translocate through skin and even cellular membranes due to their hydrophilicity (7,8). Therefore, DNJ should be delivered across the skin and into the cytoplasmic active site by an efficient delivery carrier to facilitate biological activity for pigment-lightening effects with a minimum concentration *in vitro* and *in vivo.* In this study, we attempt to evaluate the N-glycosylation-inhibiting (*GI*) effects of the pH-sensitive liposomes containing N-glycosylation inhibitors on human melanoma cells, HM3KO, to see the possibility of cosmetic application as a delivery carrier of depigmentation active molecules. Furthermore, the *in vitro* delivery efficiency of pH-sensitive liposomes was examined to confirm the location of the intracellular-delivered pH-sensitive liposomes.

EXPERIMENTAL

PREPARATION OF LIPOSOMES

Liposomes were prepared according to lipid hydration methods. Molar ratios of lipid components of CHEMS were fixed at 3:2. Compositions of the prepared liposomes are listed in Table I. Briefly, a mixture of lipids in chloroform/methanol (95:5) was dried using a rotary evaporator under reduced pressure. Dried lipids were hydrated with PBS containing N-glycosylation inhibitors to be loaded. Hydrated lipid films were sonicated using a bath-type sonicator. The size distribution of the resulting liposomes was measured by dynamic light scattering (DLS) with a vertically polarized He-Ne laser (Zetasizer 3000HS, Malvern, UK). Turbidity was observed by the absorbence of a liposomal

Table I
Formulations of Prepared Liposomes

Number	Lipid composition
L_1	[a]DOPE:[b]CHEMS
L_2	[c]PC:CHEMS
L_3	DOPE:[d]fluorescein-DHPE:CHEMS
L_4	PC:fluorescein-DHPE:CHEMS
L_5	DOPE:fluorescein-DHPE:PEG-5 rapeseed sterol
L_6	DOPE:cholesterol

[a] 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine.
[b] Cholesteryl hemisuccinate.
[c] L-α-Phosphatidylcholine.
[d] N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine.

suspension at 500 nm using a Cary 3E UV-VIS spectrophotometer (Varian, Victoria, Australia) to evaluate the pH-sensitivity of the prepared liposomes (15).

To quantify intracellular delivery of pH-sensitive liposomes by FACS measurement, either a component of the lipid bilayer or a loading material was marked with fluorescence. To label the lipid bilayer, DOPE, CHEMS and 1% N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (fluorescein-DHPE) (w/w) (Ex/Em = 495/519 nm; Molecular Probes Inc., OR) was dissolved in chloroform/methanol, and then dried and hydrated (L_3 liposome). Fluorescein-encapsulated pH-sensitive liposomes were made by hydration of DOPE:CHEMS (3:2) film with 75 μM calcein (Ex/Em = 494/517) in PBS. L-α-Phosphatidylcholine (PC):CHEMS (3:2) was used as a control. After hydration, the procedure of liposome preparation was as previously described.

For the CLSM study, a dried film of DOPE:CHEMS (3:2) containing 1% fluorescein-DHPE (Ex/Em = 495/519 nm) (L_3 liposome) was hydrated with PBS containing 2.5 μM dextran-rhodamine B (10,000 mw (Ex/Em = 572/589 nm; Molecular Probes Inc., OR). Therefore, the lipid bilayer and loading materials were labeled simultaneously to trace the cellular uptake of the pH-sensitive liposomes. PEG-5 rapeseed sterol (Cognis GmbH, Düsseldorf, Germany) was used instead of CHEMS as an amphiphilic stabilizer for a control L_5 liposome. Unencapsulated fluorescence was separated from the liposomes by using MicroSpin™ G-25 columns (Amersham Biosciences Corp., NJ).

CELL CULTURE

HM3KO, a pigmented melanoma cell line (9), was cultured with minimum essential medium (MEM) supplemented with 1% (v/v) antibiotics (streptomycin, 10,000 μg/ml; penicillin, 10,000 IU/ml) and 10% (v/v) FBS (Gibco BRL, MD) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were subcultured every five days. For the cytotoxicity assay, the suspension of the cells was poured into a 96-well flat-bottomed plate. After adhesion to the plate, cells were incubated with a 2.8–360 μM lipid concentration of liposomes in 100 μl of culture media for an additional 48 hr, and the cytotoxicity was then evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (10).

Briefly, MTT was dissolved in phosphate buffer at 5 mg/ml and filtered for sterilization. Ten microliters of the MTT solution was added to the medium, resulting in a final concentration at 500 μg/ml, and then the cells were incubated for 4 h at 37°C. The purple formazan product was dissolved by 100 μl/well of 40 mN HCl in isopropyl alcohol. Absorbence was measured at 550 nm in a microplate reader (ELx800, Bio-TEK Instr. Inc.).

PIGMENT LIGHTENING

Pre-cultured HM3KO cells were incubated with culture media containing 1 to 200 μM of NB-DNJ or DNJ-loaded DOPE:CHEMS liposomes (L_1), for an additional five days. Concentration of the added liposomes was determined by the MIT assay evaluating the viability of the cells. The medium containing liposomes was renewed every two days.

After incubation with the liposomes for five days, the cells were washed with PBS and harvested with trypsin/EDTA.

The collected cells in 0.1 M of Tris-HCl (pH 7.2) buffer containing 1% Nonidet P-40, 0.01 % SDS, and protease inhibitors (Complete™ protease inhibitor mixture; Roche, Mannheim, Germany) were lysated by probe-type sonication. Synthesized melanin was separated from cell lysates by centrifugation. The quantity of melanin was measured by the absorbance at 490 nm. The amount of melanin obtained from the non-treated HM3KO cells was assumed as 100% melanin synthesis as a control. Thus, melanin synthesis of cells treated with liposomes or free N-glycosylation inhibitors was presented as a relative % to the control. Endoglycosidase H (EndoH) and peptide-N-glycosidase F (PNGaseF) digestion and Western blotting were followed as previously described (11). Proteins of the cell lysates (10 μg of protein in 5 μl of cell lysis buffer) were hydrolyzed with EndoH according to the manufacturer's instructions (New England Biolabs, MA). Carbohydrate cleavage of the proteins by Endo H was stopped with the sample buffer at 70°C for ten minutes, and the proteins were subjected to Western blotting. The concentration of hydrolyzed proteins was measured using a protein assay kit (Pierce Biotechnology, Inc., Rockford, IL) in comparison with bovine serum albumin as a standard.

Hydrolyzed proteins were separated on a 10% polyacrylamide gel by electrophoresis. Following the transblotting of polyacrylamide gels onto nitrocellulose membranes, the membranes were incubated with PEP7, a polyclonal antibody (1:1000 dilution) specific for humans, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, Bucks., U.K.; 1:1000 dilution). The immunoreactive bands were detected by ChemiLuminescence, using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

INTRACELLULAR DELIVERY

The cellular delivery of pH-sensitive liposomes was quantified by FACS measurements. Cells were incubated with pH-sensitive liposomes containing 1% fluorescein-DHPE (L_3) for examination of the cellular-delivered amount of carrier components. PC:CHEMS containing 1% fluorescein-DHPE liposomes (L_4), pH-insensitive liposomes, was used as a control. Furthermore, calcein was encapsulated in DOPE:CHEMS liposomes (L_1) to estimate the delivery efficiency of biologically active molecules loaded in pH-sensitive liposomes. Fluorescence intensity was compared with results from cells incubated with calcein-loaded PC:CHEMS liposomes (L_2). After one hour of incubation, the cells were washed with PBS three times, detached by treatment with trypsin/EDTA, and centrifuged at 1000 rpm for five minutes. The resulting cell pellet was resuspended in PBS and analyzed by FACS Vantage (Beckton Dickinson, NJ).

Intracellular delivery of the fluorescence-labeled pH-sensitive liposomes was monitored by a confocal laser-scanning microscope (CLSM). HM3KO cells grown in a Lab-Tek II chamber slide (Nunc Inc, IL) were incubated with dextran-rhodamine-B-loaded DOPE: CHEMS (L_3) or DOPE:PEG-5 rapeseed sterol (L_5) liposomes containing 1% fluorescein-DHPE for one hour. After incubation with the liposomes, the cells were washed with PBS three times and incubated with 2 μg/ml of DAPI (Ex/Em = 358/461) in PBS for two minutes to stain the nucleic acids. Then, the cells were washed with PBS. For fixation, pre-cooled methanol at −20°C was added to the cells for five minutes. After

removal of the methanol, the slides were air-dried for 30 minutes and treated with ProLong Antifade solution (Molecular Probes, Eugene, OR) to prevent photobleaching of the fluorescent materials. The mounted slides were kept in the dark at 4°C until microscopic observation was performed. Cells were examined on a CLSM (Radiance 2000/MP, Bio-Rad, UK).

RESULTS AND DISCUSSION

N-glycosylation inhibitors were encapsulated in pH-sensitive liposomes consisting of DOPE and CHEMS to increase the depigmentation effect on HM3KO melanoma cells. It was evaluated that DOPE:CHEMS liposomes efficiently delivered whitening active molecules into the cytosolic active site adjacent to the endoplasmic reticulum (ER) of human melanoma cells. The size distribution of the N-glycosylation inhibitor-loaded pH-sensitive liposomes was examined by DLS, as shown in Figure 1a. The mean diameters of the prepared liposomes listed in Table I were in the range of 150–300 nm. The pH-sensitive characteristics of the DOPE:CHEMS liposomes were evaluated at various pH levels by turbidity measurements. The turbidity of the suspension containing DOPE:Chol (L_6) liposomes was slightly increased between pH 6.0 and pH 5.0, and subsequently reached a plateau below pH 5.0 (Figure 1b). While DOPE:Chol liposomes did not show conformational changes by decreasing pH, the turbidity of DOPE:CHEMS (L_1) was suddenly increased below pH 6.0, which is the early endosomal pH in cytoplasm. Subsequently, the lipid components of pH-sensitive DOPE:CHEMS liposomes did not sustain a stable liposomal configuration. Thus, pH-sensitive liposomes collapsed below pH 5.0, a lower range of endosomal pH, and precipitated as shown in the lower part of Figure 1c. These results represents the pH sensitivity of DOPE:CHEMS liposomes, correlating with previous research (3,12). Note that CHEMS renders the vesicles pH-sensitive; it stabilizes the lipid vesicles above neutral pH, while inducing structural instability via its own protonation at acidic pH. The cytotoxicity of pH-sensitive and pH-insensitive liposomes was evaluated using the MTT assay. Lipid concentrations of pH-sensitive liposomes and pH-insensitive liposomes up to 100 μM showed negligible cytotoxicity (data not shown). Therefore, 90 μM, the concentration of the lipid composition, was used for the preparation of liposomes in all *in vitro* experiments.

After incubation with HM3KO melanoma cells, the enhanced N-glycosylation-inhibiting (*GI*) effects of NB-DNJ or DNJ by the pH-sensitive delivery carrier were evaluated by Western blotting. Pigment-lightening effects were also monitored via the measurement of melanin biosynthesis by absorbence at 490 nm. When appropriate amounts of N-glycosylation inhibitors are delivered into melanocytes, the process of making a glycosylated tyrosinase (80 kDa) is inhibited and, thus, immature tyrosinase cannot help to synthesize the melanin (13). Therefore, the brighter band thickness of 80 kDa in the result of Western blotting meant that maturation of tyrosinase was inhibited and that the amount of a glycosylated tyrosinase (80 kDa) was decreased. Figure 2a shows that the *GI* effect of 50 μM of NB-DNJ-loaded pH-sensitive DOPE:CHEMS (L_1) liposome was similar to that of the 200 μM of intact NB-DNJ, by comparison of the band thickness of 80 kDa. Concentrations at 100 μM and even 50 μM of NB-DNJ and DNJ showed the enhanced *GI* effect by the delivery carrier, DOPE:CHEMS (L_1) liposomes, when it was compared to that of each 100 μM of free NB-DNJ and DNJ (Figure 2b). Consequently, it could be demonstrated that the DOPE:CHEMS liposome can

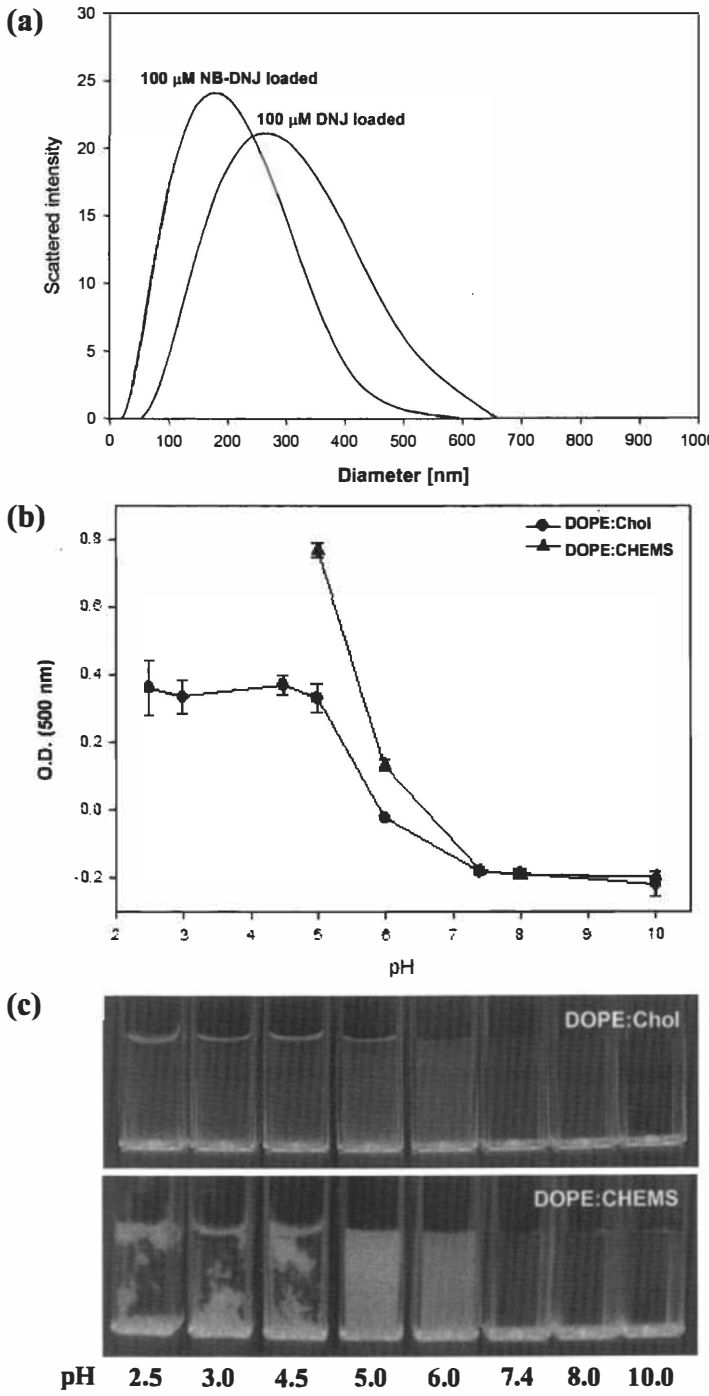

Figure 1. The size distribution of the glycosylation inhibitor-loaded pH-sensitive DOPE:CHEMS liposomes measured by DLS (a); turbidity measured using a suspension of DOPE:CHEMS (▲) and DOPE:Chol (●) liposomes (b); and conformational changes of DOPE:CHEMS (lower) and DOPE:Chol (upper) liposomes induced by a lowering pH (c).

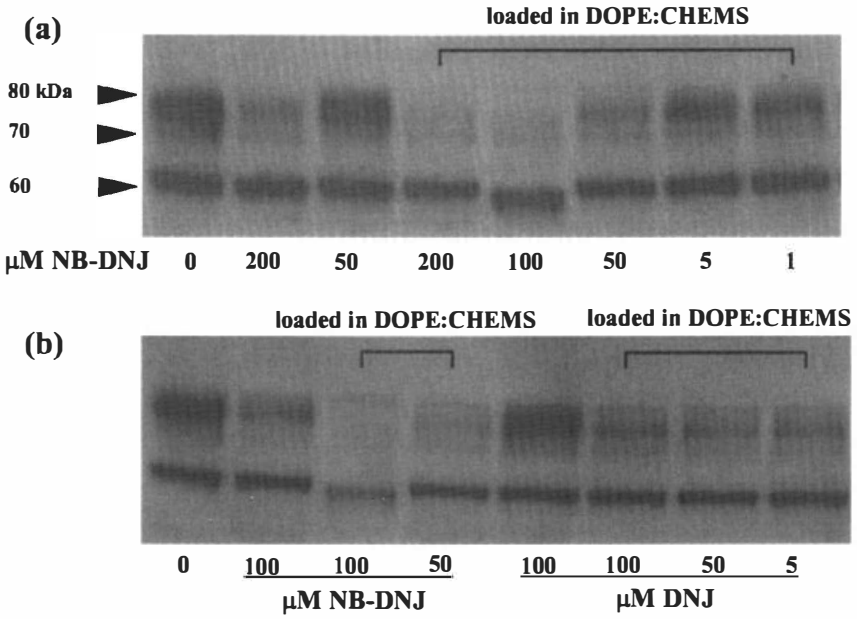

Figure 2. *GI* effects of glycosylation inhibitor-loaded pH-sensitive liposomes in comparison with the free N-glycosylation inhibitors evaluated by Western blotting: *GI* effects depending on various concentrations of NB-DNJ loaded or not loaded in DOPE:CHEMS liposomes (a); and enhanced *GI* effects by NB-DNJ-loaded or DNJ-loaded DOPE:CHEMS liposomes (b).

increase *GI* effects of N-glycosylation inhibitors by increasing the delivery efficiency. The measurement of synthesized melanin (Figure 3) correlates with the results from Western blotting (Figure 2). The amount of synthesized melanin from HM3KO cells incubated without N-glycosylation inhibitors was assumed as 100% of melanin synthesis. When cells were incubated with 100 μM of NB-DNJ-loaded pH-sensitive liposomes, 22.8 % of the melanin synthesis was reduced in comparison with 100 μM of free NB-DNJ. In the case of 100 μM of DNJ, 26.1% of the melanin synthesis was also decreased by the incorporation of pH-sensitive liposomes. Thus, it was found that the N-glycosylation inhibitor, which is loaded in pH-sensitive liposomes, efficiently reduced melanin biosynthesis in mammalian cells.

Intracellular delivery of two fluorescent molecules, fluorescein-DHPE incorporated in liposomes and calcein loaded in liposomes was evaluated by FACS. After incubation with 1% fluorescein-DHPE-embedded liposomes for one hour, HM3KO cells with pH-sensitive liposomes resulted in a relative strong fluorescent intensity value in comparison to cells with pH-insensitive liposomes (Figure 4a). Calcein, a membrane-impermeable molecule, also showed higher intensity when it was loaded in pH-sensitive liposomes, as presented in Figure 4b. It was found that the fluorescent intensities of fluorescein, a marker for components of pH-sensitive liposomes and calcein, and a tracer as a delivered cargo material in pH-sensitive liposomes, were significantly higher than the observed intensity from the cells treated with pH-insensitive PC:CHEMS liposomes.

In additional CLSM experiments, cells were incubated with 1% fluorescein-embedded liposomes (L_3) containing dextran-rhodamine B to better define the intracellular deliv-

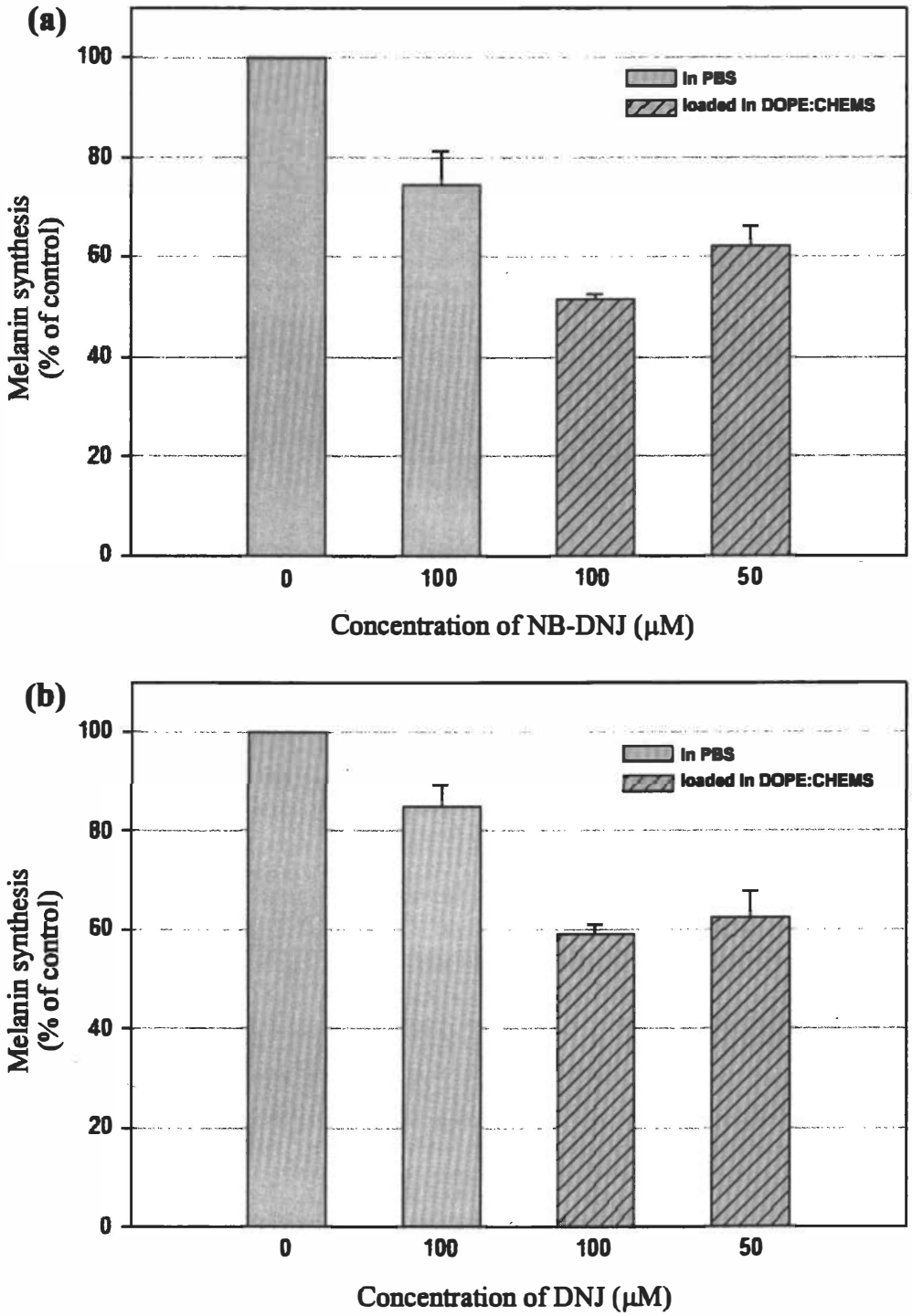

Figure 3. Melanin synthesis reduced by NB-DNJ loaded (a) and DNJ loaded (b) pH-sensitive liposomes in comparison with each of free N-glycosylation inhibitors.

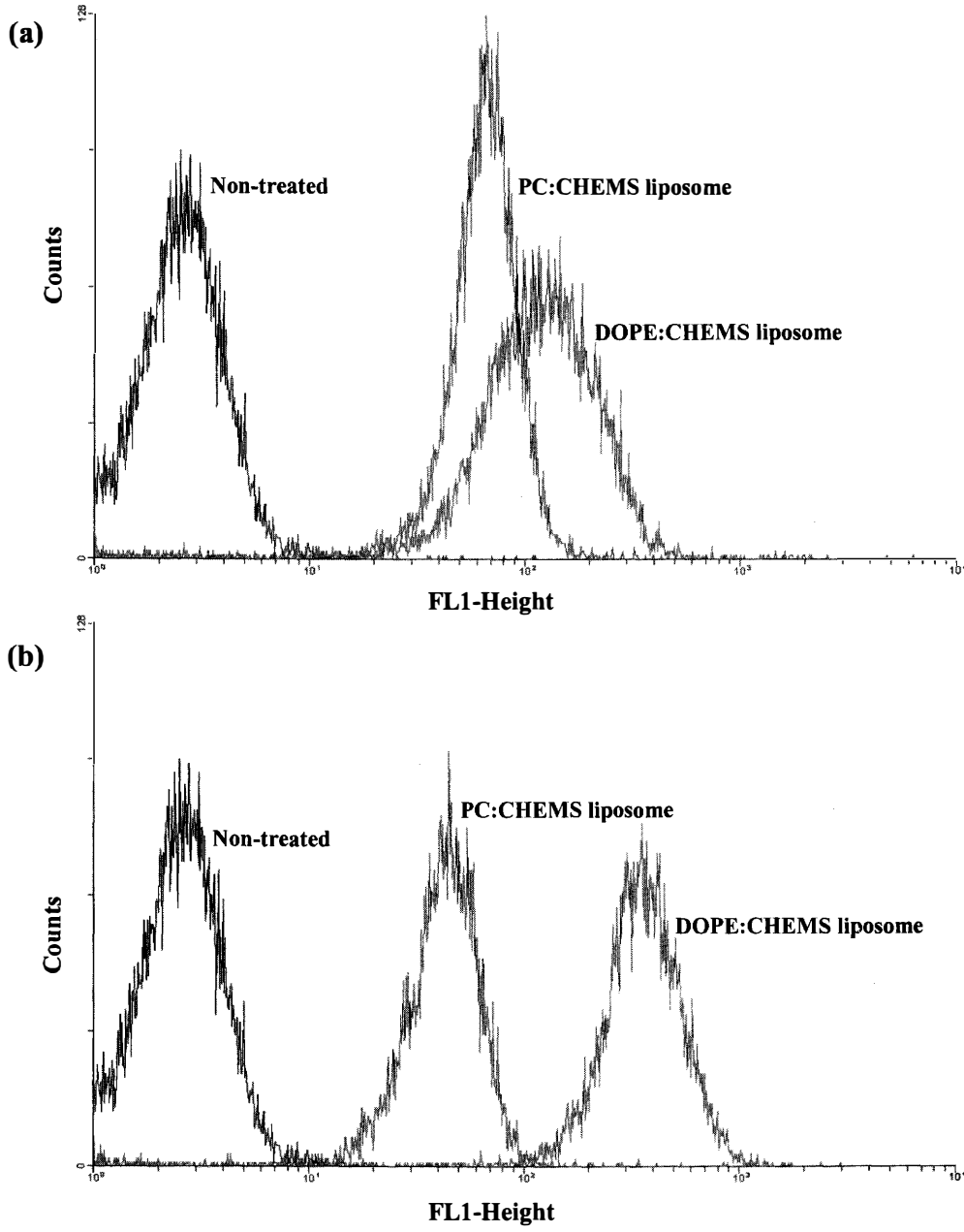

Figure 4. Histograms of cellular fluorescent intensity of HM3KO cells treated with PC:CHEMS and DOPE:CHEMS liposomes containing 1% fluorescent-DHPE (a) and treated with calcein-loaded DOPE:CHEMS and PC:CHEMS liposomes (b) as measured by FACS.

ery and the internalization mediated by pH-sensitive DOPE:CHEMS liposomes. DOPE:fluorescein-DHPE:PEG-5 rapeseed sterol (L_5) liposome was used as a control for pH-insensitive liposomes. Cellular uptake behavior of the pH-sensitive liposomes was monitored by using 1% fluorescein-DHPE incorporated in the liposomes. Simultaneously,

the release of molecules loaded in the pH-sensitive liposomes was evaluated by encapsulating dextran-rhodamine B in the liposomes. Figure 5 was obtained from a representative CLSM experiment following one hour of incubation of HM3KO cells with 1% fluorescein-embedded and dextran-rhodamin-B-loaded pH-sensitive and pH-insensitive liposomes. Fluorescent intensity from fluorescein and rhodamine B is significantly high in cells incubated with pH-sensitive liposomes (L_3) as compared with the control. The results of calcein and dextran-rhodamin B delivery by pH-sensitive liposomes correlate

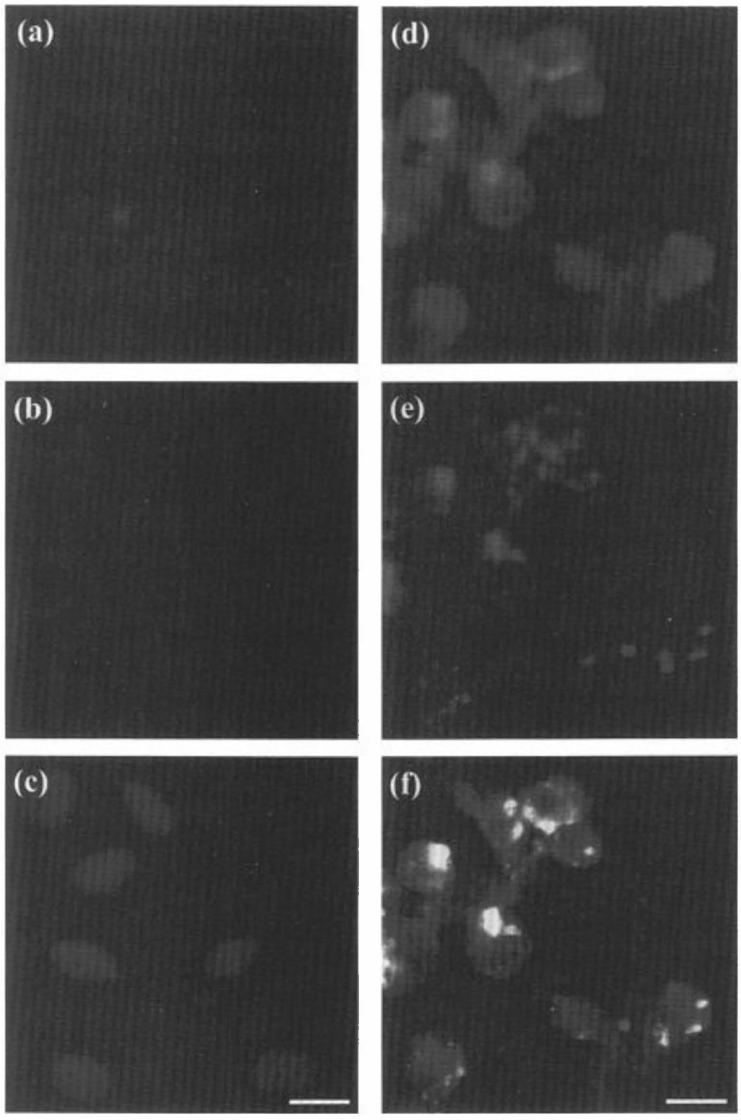

Figure 5. CLSM images of HM3KO cells after incubation with DOPE:PEG-5 rapeseed sterol (L_5) (a,b,c) and DOPE:CHEMS (L_3) (d,e,f) liposomes containing 1% fluorescent-DHPE at 37°C for one hour. Cells are under a fluorescein filter (a and d) and under a rhodamine filter (b and e). Part c is a composite of superimposed layers from a and b and an image under a DAPI filter. Part f is a composite of superimposed layers from d and e and an image under a DAPI filter. Scale bar = 20 μm.

with previous research findings, indicating that pH-sensitive EYPC liposomes modified with SucPG increased the delivery of calcein into the cytoplasm (14).

Therefore, it can be demonstrated that after cellular uptake through an endocytic pathway, the DOPE:CHEMS pH-sensitive liposomes transfer their contents into the cytoplasm. These results indicate that the improved efficacy of the N-glycosylation inhibitors loaded in pH-sensitive DOPE:CHEMS liposomes was induced because of the increased translocation efficiency into the cytoplasm.

CONCLUSIONS

We prepared pH-sensitive liposomes consisting of DOPE and CHEMS with diameters in the range of 150–300 nm to deliver water-soluble N-glycosylation inhibitors into HM3KO melanoma cells. The glycosylation-inhibiting (*GI*) and pigment-lightening effects of NB-DNJ or DNJ were significantly increased *in vitro* by the pH-sensitive liposomes. It was also found that the delivery efficiency of pH-sensitive DOPE:CHEMS liposomes was higher than that of control pH-insensitive liposomes determined by evaluation using FACS and CLSM study. These results demonstrate that the increased effects of N-glycosylation inhibitors *in vitro* can be attributed to facilitated delivery by the pH-sensitive DOPE:CHEMS liposomes into the target cytoplasmic site. Consequently, these results indicate that CHEMS-incorporated pH-sensitive liposomes may have a high delivery efficiency and thus can be used as an intracellular delivery system to transport biological actives to a target site in cytoplasm. DOPE:CHEMS liposomes show promise as a nano-sized and pH-stimulant delivery carrier to enhance the bioavailability of water-soluble compounds. This pH-sensitive liposome carrier may be directly applied as a powerful delivery system for therapeutic formulations to treat skin disorders and for applications in cosmetics, personal care products, and pharmaceutics.

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