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Disaccharide-modified liposomes and their in vitro intracellular uptake

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ABSTRACT

Sterically stabilized liposomes (SSL) were known to be accumulated passively in cancer due to the effect of enhanced permeability and retention (EPR). However, drug delivery via SSL to cancer seemed to show an insufficient improvement of chemotherapeutic efficacy. Herein, carbohydrate-binding proteins (lectins) of cell surface, which express on the plasmic membrane of many malignant cells, can be a good model of surface-modified liposomes. In this study, we investigated the in vitro characteristics of liposomes of which the surface was modified with a disaccharide molecule, sucrose or maltose. The disaccharide-modified lipids such as sucrose-modified lipid and maltose-modified lipid, in which the disaccharide was conjugated to the one end of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol)-2000 (DSPE-PEG2000), was synthesized. The disaccharide-modified liposomes were prepared by thin film-hydration method and then doxorubicin (DOX), an anticancer drug, was loaded to the prepared liposomes by the remote loading method with ammonium ion gradient. Flow cytometry and confocal microscopy analyses showed that the disaccharide-modified liposomes enhanced the intracellular uptake of liposomes into various cancer cell lines via lectin-mediated endocytosis. The disaccharide-modified liposomes in which DOX was loaded inside of liposomes exhibited higher cytotoxicity against various cancer cells than DOX-loaded SSL did. These results suggest that disaccharide-modified liposomes may be promising cancer targeting carriers which can enhance intracellular uptake and cytotoxicity of the drug-loaded liposomes via lectin-mediated endocytosis.

1. Introduction

Liposomes as drug carrying vehicles for chemotherapeutic agents including anticancer drugs have been investigated since 1960s. Many studies on cancer chemotherapy have developed various drug delivery systems that can release the drug in a controlled manner or enable drugs to be targeted for selective destruction of cancer cells (Arap et al., 1998; Harper et al., 1999). Among them, sterically stabilized liposomes (SSL) are known to be passively accumulated in cancer because the SSL retain their stability in bloodstream and hence prolong blood circulation time. It has been demonstrated that SSL avoid fast accumulation in the organs of the reticuloendothelial system (RES) and accumulate in cancer due to the effect of enhanced permeability and retention (EPR) (Gabizon et al., 1997; Han et al., 2007; Park, 2002). Recently, many studies have shown that modification of the surface of liposomes with hydrophilic moiety, such as polyethylene glycol (PEG) could increase the circulation time of liposomes in bloodstream. As an example, DOXIL® (Alza Co., USA) is well known to use PEG-modified liposomes as SSL (Yuan et al., 1994). However, SSL seem to be inefficient for intracellular delivery of anticancer drug due to lack of specific interaction between liposomal carriers and the target cells.

In previous literatures, the surface of liposomes has been modified in order to improve the selectivity of drug carrying vehicles to target cells by receptor-mediated endocytosis or electrostatic uptake. Monoclonal antibodies, small peptide fragments, folate, aptamer and small oligosaccharide were commonly used as cell-specific targeting molecules for the modification of liposomes. Among them, lectin as carbohydrate-binding protein may offer possibility as a good marker molecule for binding of glycoprotein on the surface of cancer cells (Lotan and Raz, 1988; Walker et al., 1985; Yao et al., 1998). Indeed, the cell glycocalyx, saccharide rich area on the cell surface, is an attractive model for increase of circulation time by surface modification of liposomes with saccharide molecules due to their properties which create hydrate barriers for protecting the cells. For example, monosialoganglioside (GM_1) has been used to increase the stability of liposomes in bloodstream as PEG has Allen and Hansen (1991).

In this study, we prepared surface modified doxorubicin-loaded liposomes with disaccharide such as sucrose or maltose. The effects

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of disaccharide molecules on the intracellular uptake and on physicochemical properties were investigated.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine(soy-hydrogenated) (HSPC), cholesterol (CHOL), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG-2000) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy (polyethylene glycol)-2000] (DSPE-cPEG-2000) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Maltose, sucrose, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and ammonium hydrogen carbonate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Doxorubicin (DOX) was purchased from Boryung Pharm. Co. (South Korea). For cell culture, Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM) and RPMI Medium 1640 (RPMI1640) were purchased from GIBCO BRL. (Burlington, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). All other materials were used without further purification.

2.2. Preparation of aminated-disaccharides

The aminated-maltose or -sucrose was synthesized by using similar methods described previously (Zhu et al., 2005). Maltose or sucrose (11 g, 32 mmol) was dissolved in 100 mL of water. Each solution was added to a flask containing ammonium hydrogen carbonate (15 g, 190 mmol) under stirring at room temperature. The termination of reaction was determined by thin layer chromatography by using chloroform, methanol and water (1:2:0.5, v/v/v) as the developing solvent. After 4 days of dialysis, final products were obtained by lyophilization. The product was directly used in next step without further purification.

2.3. Synthesis of DSPE-PEG2000-disaccharides

DSPE-cPEG-2000 (28.49 mg, 10 μmol) was dissolved in 3 mL of phosphate buffered saline (PBS) at pH 7.4 and then added to EDC (57.51 mg, 300 μmol) and NHS (34.52 mg, 300 μmol) for activation of carboxy group. The solution was stirred for 2 h before aminated-disaccharide (maltosyl amine or sucrosyl amine) (34 mg, 100 μmol) in 2 mL of PBS was added slowly. The reaction mixture was stirred for 20 h at room temperature. The excess EDC, NHS and aminated-disaccharide were removed by dialysis for 4 days at 4 °C by using cellulose dialysis tube (MWCO 1000, Spectrum, CA, USA). The chemical structure of DSPE-PEG2000-disaccharide was confirmed by ^{1}H NMR spectra. The products (yield: 89%) were lyophilized and then kept at −20 °C before further use. The chemical structure of DSPE-PEG2000-disaccharides was confirmed by ^{1}H NMR spectra and MALDI-TOF mass spectrometry (Voyage DE-STR). MALDI-TOF mass was performed by dissolving the sample and the matrix of 2,5-sinapinic acid in formic acid, acetonitrile and water (0.1:50:50, v/v/v). Mass spectra were typically accumulated from 200 laser shots. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.88 (CH_3, terminal of DSPE), 1.25 (CH_2, carbon chain of DSPE), 3.64 (CH_2, polyethylene oxide), 3.7–4.4 (OH, CH_2, disaccharide). MALDI-TOF MS Calcd for DSPE-PEG2000-disaccharides: 3170.9, found $[M+Na]^+$: 3194.0.

2.4. Preparation of liposomes

The disaccharide-modified liposomes used for loading of DOX were prepared by the remote loading method with ammonium sulfate gradient (Bolotin et al., 1994; Haran et al., 1993). The prepared liposomes and their lipid compositions were as follows: (1) PEG-liposomes; HSPC:CHOL:DSPE-mPEG-2000 = 9.57:3.19:3.19 mg/mL; (2) disaccharide-modified liposomes; HSPC:CHOL:DSPE-PEG2000-disaccharide = 9.57:3.19:3.19 mg/mL. Briefly, the lipids corresponding to the above each composition were dissolved in chloroform and subsequently the solvent was removed under reduced pressure at 50 °C, followed by evaporation with a rotary evaporator (Buchi Rotavapor R-200, Switzerland) under high vacuum. The resulting lipid film was hydrated with 250 mM ammonium sulfate solution by gentle mixing. The liposomal solution was extruded each 6 times through a polycarbonate filter (pore size: 100 and 200 nm, Whatman, USA) by using an extruder (Northern Lipids Inc., USA). The ammonium sulfate that was not included inside of the liposomes was removed by dialysis for 72 h at 4 °C with cellulose dialysis tube (MWCO 12,000, Viskase Co., IL, USA). The liposomal solution and 2 mg/mL of DOX solution were mixed and then incubated for 2 h at 60 °C. The mixture was dialyzed to remove the unloaded DOX. The disaccharide-modified liposomes loaded with DOX were stored at 4 °C until use. Concentration of DOX was determined by measuring the fluorescence intensity of DOX (excitation/emission: 490/585 nm).

2.5. Physicochemical characterization of liposomes

The morphology of liposomes was examined by transmission electron microscopy (TEM) (Tecnai G2, FEI Co., Eindhoven, Netherlands) by using 120 kV acceleration voltages. The average diameter, polydispersity and *Z*-potential of liposomes were measured by light scattering with a particle size analyzer (ELS-Z, Zeta potential and particle size analyzer, Otsuka, Japan).

2.6. Cell culture

To broadly cover the phenotype of cellular uptake exhibited by cancer cells in vitro, five human cancer cell lines such as HepG2, A375P, MCF-7, NCI-H358 and Hela were used for the intracellular uptake studies. All cell lines were grown in appropriate medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 2 mM nonessential amino acids. The cultures were sustained at 37 °C in a humidified incubator containing 5% CO_2.

2.7. Flow cytometry analysis for intracellular uptake of disaccharide-modified liposomes

Cancer cell lines were placed in a 24-well culture plate and cultured until 80% confluent at 37 °C in 5% CO_2. Cells were washed twice with PBS and then incubated with different formulation such as PEG-liposomes, sucrose-modified liposomes or maltose-modified liposomes in which DOX was loaded at concentration of 15 μg DOX/mL for 2 h at 37 °C in 5% CO_2 incubator in appropriate serum-free medium. Subsequently, each cancer cell line was washed with PBS and the cellular uptake of liposomes was analyzed flow cytometry (FACS) by using a Becton-Dickinson FACScan with CELLQuest software (Becton-Dickinson Immunocytometry System, Mountain View, CA).

2.8. Confocal microscopy analysis for intracellular uptake of DOX-loaded liposomes

Confocal microscopy was used to compare the intracellular uptake of DOX-loaded liposomes (excitation/emission: 480/540 nm). One × 10^4 cells/cm^2 of cancer cells were seeded into 8-well chambers (Lab-Tek®, Nalge Nunc International, Naperville, IL, USA) at 200 μL media/well and then cultured until 50% confluent at 37 °C in 5% CO_2. Cells were washed twice with PBS and

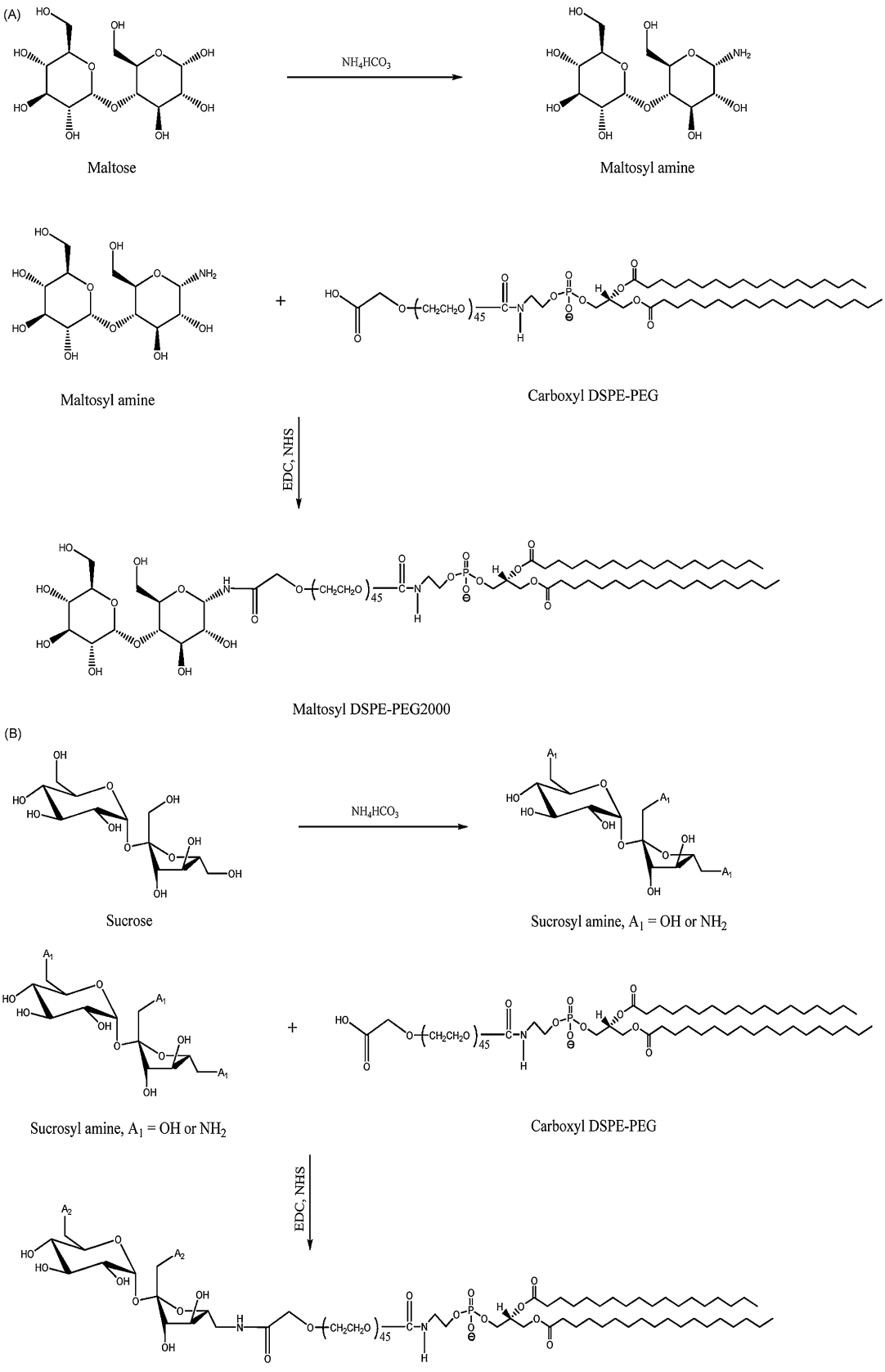

Fig. 1. Synthetic scheme for the preparation of DSPE-PEG2000-maltose (A) and DSPE-PEG2000-sucrose (B).

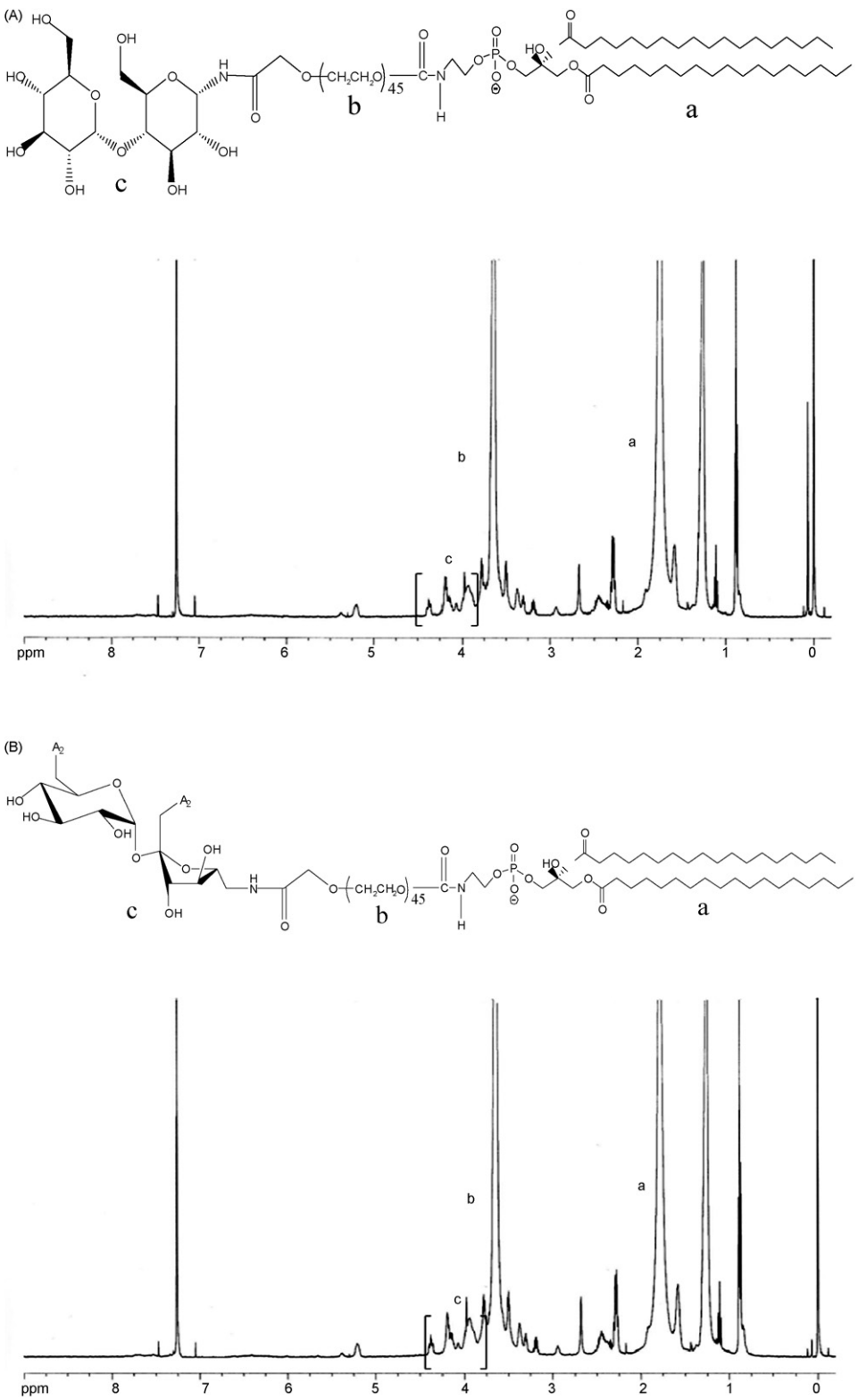

Fig. 2. ^{1}H NMR spectra of DSPE-PEG2000-maltose (A) and DSPE-PEG2000-sucrose (B). The broad peaks at 3.7–4.4 ppm of DSPE-PEG2000-sucrose and DSPE-PEG2000-maltose correspond to the conjugated sucrose and maltose, respectively.

they were incubated for 1 h at 37 °C with free DOX, DOX-loaded PEG-liposomes, DOX-loaded sucrose-modified liposomes and DOX-loaded maltose-modified liposomes, respectively. Concentration of DOX of the free DOX and DOX-loaded liposomes was adjusted to 6 μg DOX/mL by the dilution of them with serum-free medium. The cells were washed 3 times with PBS and fixed in 95% ethanol for 10 min. They were observed by confocal laser microscopy (LSM510, CarlZeis, Germany).

2.9. Cytotoxicity studies

The viability of cells treated with liposomes was determined by using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Mosmann, 1983). To evaluate cytotoxicity of disaccharide-modified liposomes containing DOX various cancer cells (5×10^3 cells/well) were cultured in 96-well flat-bottomed microtiter plates for 24 h at 37 °C. The medium was replaced with serum-free medium containing various concentrations of free DOX or DOX-loaded liposomes and subsequently cultured for 72 h at 37 °C. Twenty microlitres of 0.5 mg/mL MTT were added to each well and the cells were incubated for another 4 h at 37 °C in 5% CO_2. One hundred and ninety microlitres of medium in each well were removed and 150 μL of DMSO were added to each well for solubilization of blue formazan crystals. The viability of cells was measured with microplate reader (EL808, Biotek Inc., USA) at absorbance 590 nm.

2.10. Statistics

Values are presented as mean ± standard deviation (S.D.). Student's *t*-test was used to measure statistical significance between pairs of samples. Multiple comparisons were performed by using analysis of variance (ANOVA). A *p*-value below 0.05 was considered significant.

3. Results

3.1. Synthesis of DSPE-PEG2000-disaccharide

Synthesis of DSPE-PEG2000-dissachride was carried out as shown in Fig. 1A and B. DSPE-PEG2000-dissachride was synthesized by using EDC/NHS-coupled reactions and purified through dialysis in order to remove excess EDC/NHS and disaccharides for 4 days. The synthesis of DSPE-PEG2000-dissachride was investigated by analysis of ^{1}H NMR spectra of maltosyl DSPE-PEG2000 and sucrosyl DSPE-PEG2000 as shown in Fig. 2A and B. The synthesis of DSPE-PEG2000-disaccharide was confirmed by appearance of proton signals from the disaccharides at δ 3.7–4.4 ppm as broad peaks, which indicated a covalent conjugation of disaccharide to sucrosyl and maltosyl DSPE-PEG2000.

3.2. Characteristics of disaccharide-modified liposomes

As shown in Table 1, the mean particle diameter of PEG-liposomes, sucrose-modified liposomes and maltose-modified liposomes ranged from 97 nm to 113 nm. Zeta potential value of disaccharide-modified liposomes showed that the disaccharide-modified liposomes were more negative than PEG-liposomes because the hydroxyl group of disaccharide molecule in the disaccharide-modified liposomes could increase negative charge of liposomes. These results indicated that sucrose or maltose of liposomal surface could enhance absolute zeta potential value of the liposomes. DOX loading efficiency of the prepared liposomes was approximately 86–93%. Size and morphology of the sucrose-modified or maltose-modified liposomes were observed by TEM and the results were shown in Fig. 3A and B.

3.3. Intracellular uptakes of disaccharide-modified liposomes

Intracellular uptake of the liposomes was evaluated by using confocal microscopy observation and flow cytometry analysis. As shown in Fig. 4, confocal microscopy was used to compare the intracellular uptake of liposomes containing DOX (excitation/emission: 480/540 nm). Fig. 4A, E, I, M and Q showed strong red fluorescence in all kinds of cancer cells treated with free DOX for 1 h. Although there was no significant difference between PEG-liposome (B, F, J, N and R) and disaccharide-modified liposomes (sucrose: C, G, K, O and S; maltose: D, H, L, P and T), distribution of weak DOX fluorescence to cytoplasm and nuclei could be observed in the cells treated with disaccharide-modified liposomes.

To clarify the intracellular uptake of DOX-loaded liposomes, the fluorescence intensity of DOX against human cancer cells was evaluated by using flow cytometry. As shown in Fig. 5, the intracellular

Table 1
Physical properties of the various liposomal formulations.

Composition	Liposome size [nm]	Zeta potential [mV]	DOX loading efficiency [%]
PEG-liposomes (HSPC:CHOL:DSPE-mPEG-2000)	97.9 ± 0.9	−20.6 ± 1.7	89.8
Sucrose-modified liposomes (HSPC:CHOL:DSPE-PEG2000-sucrose)	110.7 ± 1.8	−38.6 ± 2.1	92.9
Maltose-modified liposomes (HSPC:CHOL:DSPE-PEG2000-maltose)	100.0 ± 2.1	−34.3 ± 4.7	86.7

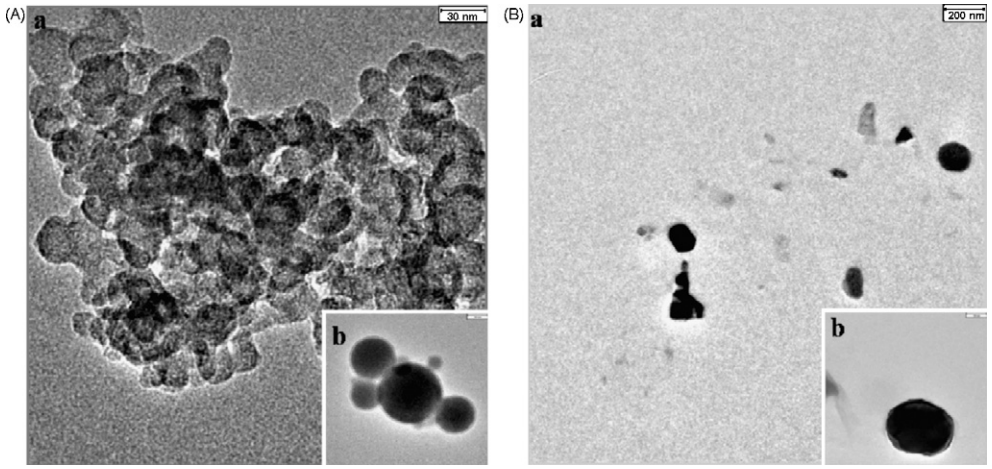

Fig. 3. TEM images of sucrose-modified liposomes (bar scale = a: 30 nm; b: 50 nm) (A) and maltose-modified liposomes (bar scale = a: 200 nm; b: 50 nm) (B) obtained by using TEM without staining. The images approximately represented the morphology of spherical liposome vesicles of 100 nm.

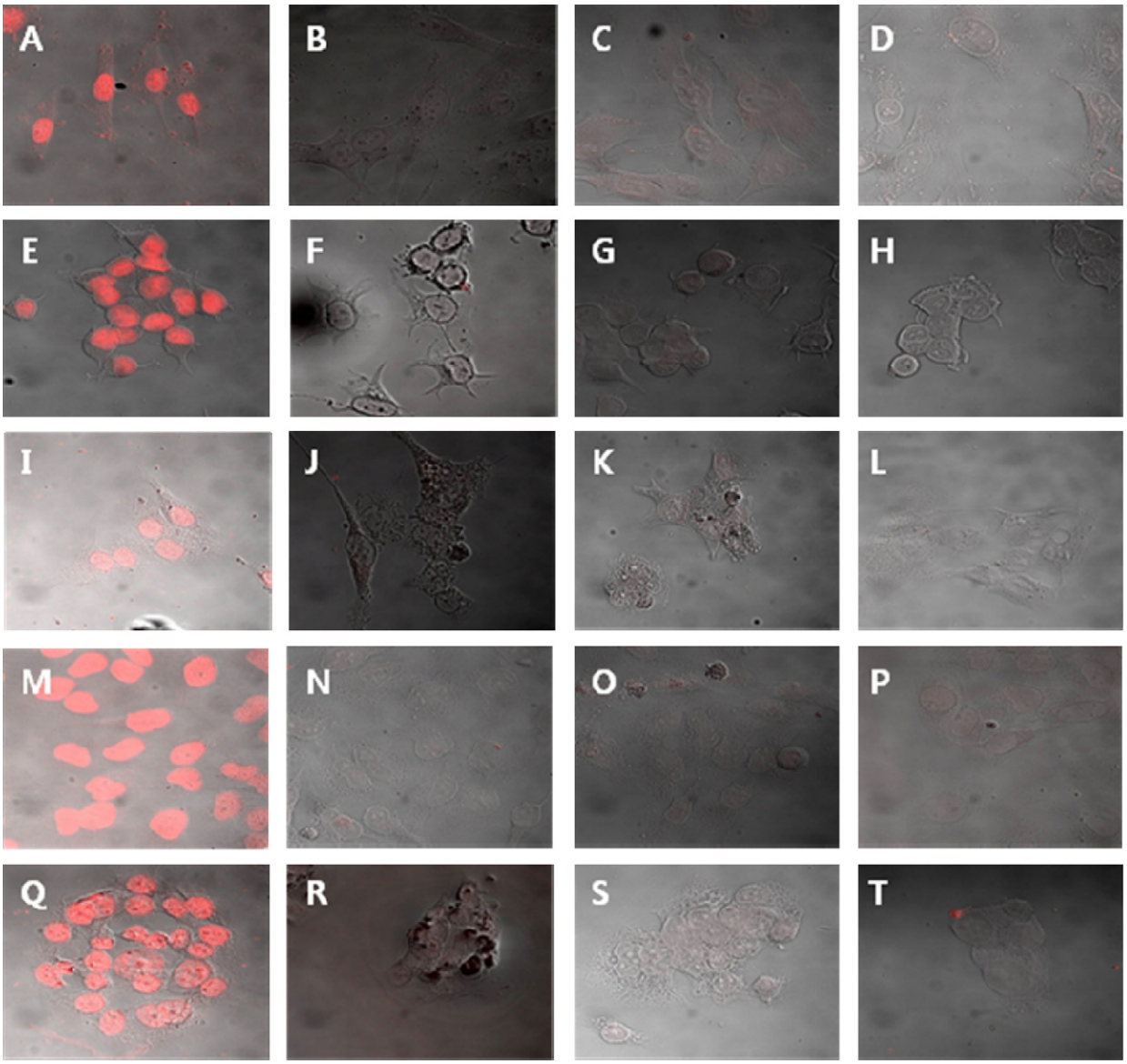

Fig. 4. Confocal laser microscopy analysis of A375P (A–D), MCF7 (E–H), HepG2 (I–L), Hela (M–P) and NCI-H358 (Q–T) cells treated with free DOX and DOX-loaded liposomes (excitation/emission: 480/540 nm); All cells were incubated with free DOX (A, E, I, M and Q), PEG-liposomes (B, F, J, N and R), sucrose-modified liposomes (C, G, K, O and S) and maltose-modified liposomes (D, H, L, P and T) at 6 μg/mL of DOX in serum-free medium for 1 h at 37 °C.

uptake of the disaccharide-modified liposomes containing DOX to the human cancer cells was higher than that of PEG-liposomes, indicating that carbohydrate molecules such as sucrose or maltose on liposomal surface could bind to lectins which are known as proteins or glycoproteins to bind carbohydrate molecules and also frequently expressed on the surface of cancer cells. Sucrose-modified liposome showed slightly higher intracellular uptake than maltose-modified liposome. As shown in Figs. 4 and 5, the free DOX displayed the highest level of DOX in the cancer cells.

3.4. Cell cytotoxicity test

Fig. 6 showed the cell survival curves of each cell line according to increase of the concentration of DOX solution and DOX-loaded liposomes. As shown in Fig. 6A (A375), Fig. 6B (NCI-H358) and Fig. 6C (Hela), viability of cells after exposure to disaccharide-modified liposomes was much lower than that of PEG-liposome. In the case of HepG2 and MCF7, there was no statistical difference in the viability of cells among liposomes (data not shown). The cytotoxicity of disaccharide-modified liposomes was more sensitive than PEG-liposomes but sucrose-modified and maltose-modified liposomes did not show remarkable difference. These results indicated that disaccharide-modified liposomes could target cancer cell lines efficiently as shown in Fig. 5 and also the disaccharide-modified liposomes exhibited higher intracellular uptake to the cancer cell lines than PEG-liposome did.

4. Discussion

In cancer chemotherapy, drug delivery system using PEG-modified liposomes has been developed in many clinical models. Many studies demonstrated that antitumor effects could be increased by enhanced permeation and retention (EPR) effect of the angiogenic blood vessel because PEG-modified liposomes prolonged the circulation times of anticancer drug-loaded liposomes in bloodstream (Fang et al., 2003; Papahadjopoulos et al., 1991; Klibanov et al., 1990). However, PEG-modified liposomes could interfere with ligand-mediated cellular uptake due to the presence

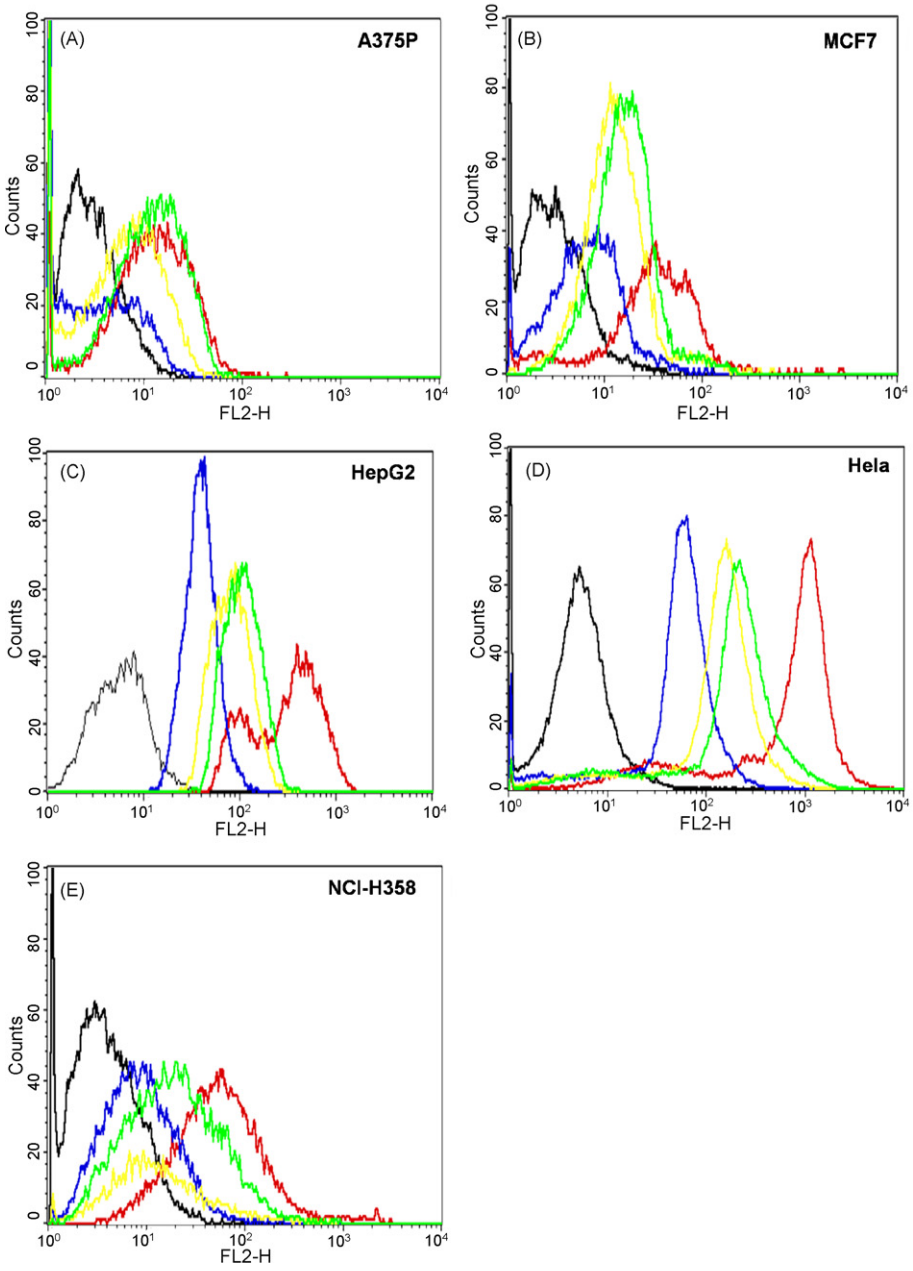

Fig. 5. Flow cytometry analysis of human cancer cell lines, A375P (A); MCF7 (B); HepG2 (C); Hela (D) and NCI-H358 (E), incubated with free DOX (red), sucrose-modified liposomes (green), maltose-modified liposomes (yellow) or PEG-liposomes (blue). Cells were incubated for 2 h at 37 °C in 5% CO_2 incubator. Intracellular uptake was determined by flow cytometer and evaluated by intensity of FL-2 (red fluorescence) at horizontal axis. The black peak indicated the back ground control of each cell line.

of steric hindrance of PEG-modified liposomes when target moiety is directly conjugated to the surface of liposomes (Lee and Low, 1995; Torchilin et al., 1992). Mono- and oligosaccharide-conjugated liposomes are an attractive model for malignant targeting because they could bind to the lectins which are found on many malignant cells (Monsigny et al., 1988, 1994; Yamazaki et al., 2000). The glycan as glycosylated cell surface proteins and cell membrane lipid are known to binding sites for lectin (Lehr, 2000). Thus, we synthesized disaccharides (sucrose and maltose) conjugated DSPE-PEG2000 via a carboxyl group of DSPE-cPEG-2000, and prepared disaccharides-modified liposomes as drug delivery system for glycoconjugates to sugar receptors (lectins) at the surface of cancer cells. Liposomes having DSPE-PEG2000-disaccharide or liposomes modified with PEG were prepared by using thin film-hydration and extrusion method. The particle diameter of disaccharide-modified liposomes was almost equal to that of PEG-liposomes. However, the zeta potential value of disaccharide-modified liposomes decreased due to the presence of abundant hydroxyl groups on the surface of them. In order to compare intracellular uptake of disaccharide-modified liposomes with PEG-modified liposomes, we investigated intracellular uptake of them by using confocal microscopy observation and flow cytometry analysis as shown in Figs. 4 and 5. As shown in Fig. 4, the fluorescence of the cells treated with free DOX was mainly visible in nuclear compartment, indicating the free DOX was internalized into nuclei of cancer cells after 1 h of incubation. In addition, significantly higher DOX level of cells treated with free DOX compared to the liposomal DOX indicates that the DOX uptake was by the mechanism of diffusion (Xiong et al., 2005). On the other hand, DOX fluorescence of the cells treated with the DOX-loaded disaccharide-modified liposomes distributed to both cytoplasm and nuclei although the fluorescence intensity was weak, while DOX fluorescence of the cells treated with the DOX-

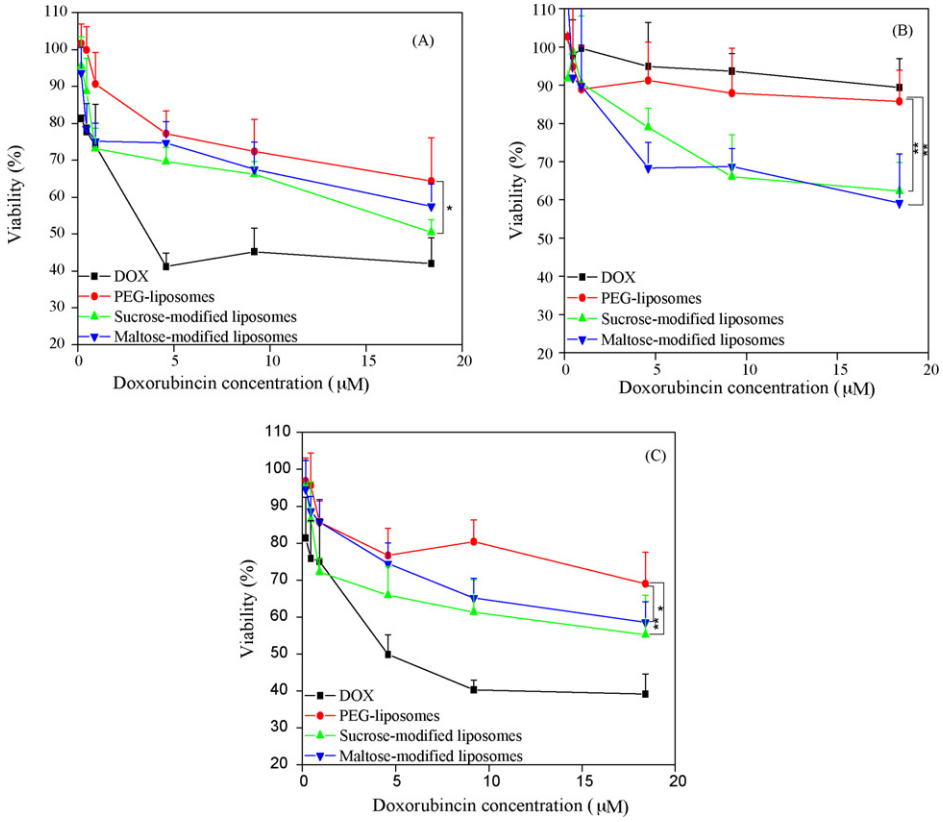

Fig. 6. Cell viability of A375P (A), NCI-H358 (B) and Hela (C) against DOX concentration. Cell viability was evaluated by using MTT assay after incubation of various cancer cells with DOX solution, PEG-liposomes, sucrose-modified liposomes, and maltose-modified liposomes for 3 days. Each value represents the mean ± S.D. ($n = 6$), $^{*}p < 0.05$ and $^{**}p < 0.01$.

loaded PEG-liposome was not discernible. The weak fluorescence of DOX-loaded liposomes was probably due to self-quenching by aggregated state of DOX (Lee and Low, 1995). The mechanism of action for cellular uptake of drug-loaded liposomes is explained by diffusion, endocytosis or membrane fusion (Düzgünes and Nir, 1999). DOX uptake by membrane fusion could hardly occur because the liposomes in this study does not have lipid component that can fuse with cell membrane. In addition, several studies suggested that liposomes without an internalizing ligand could not be endocytosed by cancer cells (Yuan et al., 1994; Huang et al., 1992; Sapra and Allen, 2002.). Therefore, the DOX uptake for PEG-liposome can be explained by diffusion and the insufficient release of DOX from DOX-loaded PEG-liposome in cell culture medium may be responsible for the indiscernible DOX fluorescence of cells. Although the intensity was weak, our observation that the distribution of DOX fluorescence to cytoplasm by disaccharide-modified liposomes also supported that endocytosis was involved in the DOX uptake by disaccharide-modified liposomes. After DOX-loaded liposomes entered the cells by endocytosis, DOX of DOX-loaded liposomes was released from the endocytotic vesicles and concentrated in the nucleus (Xiong et al., 2005). In contrast to confocal microscopy observation, cellular uptake efficiency observed by flow cytometry revealed a remarkable difference in DOX level in cancer cells treated with PEG-liposome and disaccharide-modified liposomes. As shown in Fig. 5, red fluorescence intensity of disaccharide-modified liposomes in cancer cells was much higher than that of PEG-liposomes, indicating that endocytosis was responsible for the improved cellular uptake of DOX for disaccharide-modified liposomes compared to PEG-liposomes. Cell–cell interaction was known to bind cell surface carbohydrate with cell surface lectin (Sharon and Lis, 1989). The results of flow cytometry analysis suggested that disaccharide-modified liposomes might enter the cells by lectin-mediated endocytosis because terminal monosaccharide residue bound to surface lectin of cancer cells. To investigate cell viability according to the concentration of DOX-loaded liposomes in cancer cell line, we performed cell cytotoxicity test against A375P, NCI-H358 and Hela by using MTT assay. As shown in Fig. 6, cell viability of disaccharide-modified liposomes was significantly lower than that of PEG-liposomes at 18.39 μM of DOX except that of maltose-modified liposomes against A375P.

In this study, we prepared liposomes containing disaccharide-conjugated DSPE-PEG2000 and evaluated intracellular uptake of liposomes having disaccharide molecules such as sucrose or maltose on the surface of liposomes. The results of flow cytometry analysis showed that disaccharide-modified liposomes could be highly internalized into the cancer cell when compared with PEG-liposome. In addition, cell viability of disaccharide-modified liposomes having DOX has been compared with that of PEG-liposomes. As shown in Fig. 6, cell viability after exposure of disaccharide-modified liposomes for 3 days decreased significantly when compared with the cell viability after exposure of PEG-liposomes. These results suggest that disaccharide-modified liposomes may enhance intracellular uptake of the sterically stabilized liposomes and therefore the therapeutic efficacy of DOX-loaded liposomes.

5. Conclusion

Disaccharide-modified liposomes were developed in order to increase intracellular uptake of anticancer drug to cancer cells. The saccharides are good models for tumor targeting molecules because many malignant cells express the lectin, sugar-binding protein. Intracellular uptake in vitro of disaccharide-modified liposomes was higher than that of sterically stabilized liposomes. More-

over, the disaccharide-modified liposomes had better cytotoxicity against cancer cells than sterically stabilized liposomes. Therefore, the liposomal carriers modified with disaccharide molecules offer possibility of efficient targeted drug delivery system in vivo.

References

Allen, T., Hansen, C., 1991. Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim. Biophys. Acta: Biomembr. 1068, 133–141.

Arap, W., Pasqualini, R., Ruoslahti, E., 1998. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279, 377.

Bolotin, E., Cohen, R., Bar, L., Emanuel, N., Ninio, S., Barenholz, Y., Lasic, D., 1994. Ammonium sulfate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandoliposomes. J. Liposome Res. 4, 455–479.

Düzgünes, N., Nir, S., 1999. Mechanisms and kinetics of liposome–cell interactions. Adv. Drug Deliv. Rev. 40, 3–18.

Fang, J., Sawa, T., Maeda, H., 2003. Factors and mechanism of "EPR" effect and the enhanced antitumor effects of macromolecular drugs including SMANCS. Adv. Exp. Med. Biol. 519, 29–50.

Gabizon, A., Goren, D., Horowitz, A.T., Tzemach, D., Lossos, A., Siegal, T., 1997. Long-circulating liposomes for drug delivery in cancer therapy: a review of biodistribution studies in tumor-bearing animals. Adv. Drug Deliv. Rev. 24, 337–344.

Han, H.D., Lee, A., Hwang, T., Song, C.K., Seong, H., Hyun, J., Shin, B.C., 2007. Enhanced circulation time and antitumor activity of doxorubicin by comblike polymer-incorporated liposomes. J. Control. Release 120, 161–168.

Haran, G., Cohen, R., Bar, L., Barenholz, Y., 1993. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim. Biophys. Acta: Biomembr. 1151, 201–215.

Harper, E., Dang, W., Lapidus, R., Garver, R., 1999. Enhanced Efficacy of a Novel Controlled Release Paclitaxel Formulation (PACLIMER Delivery System) for Local-Regional Therapy of Lung Cancer Tumor Nodules in Mice 1, vol. 5. AACR, pp. 4242–4248.

Huang, S., Lee, K., Hong, K., Friend, D., Papahadjopoulos, D., 1992. Microscopic localization of sterically-stabilized liposomes in colon carcinoma-bearing mice. Cancer Res. 52, 5135–5143.

Klibanov, A., Maruyama, K., Torchilin, V., Huang, L., 1990. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett. 268, 235–237.

Lee, R., Low, P., 1995. Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro. Biochim. Biophys. Acta: Biomembr. 1233, 134–144.

Lehr, C., 2000. Lectin-mediated drug delivery: the second generation of bioadhesives. J. Control. Release 65, 19–29.

Lotan, R., Raz, A., 1988. Lectins in cancer cells. Ann. N.Y. Acad. Sci. 551, 385–398.

Monsigny, M., Roche, A., Kieda, C., Midoux, P., Obrenovitch, A., 1988. Characterization and biological implications of membrane lectins in tumor, lymphoid and myeloid cells. Biochimie 70, 1633–1649.

Monsigny, M., Roche, A., Midoux, P., Mayer, R., 1994. Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. Adv. Drug Deliv. Rev. 14, 1–24.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Papahadjopoulos, D., Allen, T., Gabizon, A., Mayhew, E., Matthay, K., Huang, S., Lee, K., Woodle, M., Lasic, D., Redemann, C., 1991. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc. Natl. Acad. Sci. U.S.A. 88, 11460–11464.

Park, J.W., 2002. Liposome-based drug delivery in breast cancer treatment. Breast Cancer Res. 4, 95–99.

Sapra, P., Allen, T., 2002. Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs. Cancer Res. 62, 7190–7194.

Sharon, N., Lis, H., 1989. Lectins as cell recognition molecules. Science 246, 227–234.

Torchilin, V., Klibanov, A., Huang, L., O'Donnell, S., Nossiff, N., Khaw, B., 1992. Targeted accumulation of polyethylene glycol-coated immunoliposomes in infarcted rabbit myocardium. FASEB J. 6, 2716–2719.

Walker, R., Hawkins, R., Miller, W., 1985. Lectin binding and steroid receptors in human breast carcinomas. J. Pathol. 147, 103–106.

Xiong, X., Huang, Y., Lu, W., Zhang, X., Zhang, H., Nagai, T., Zhang, Q., 2005. Enhanced intracellular delivery and improved antitumor efficacy of doxorubicin by sterically stabilized liposomes modified with a synthetic RGD mimetic. J. Control. Release 107, 262–275.

Yamazaki, N., Kojima, S., Bovin, N., André, S., Gabius, S., Gabius, H., 2000. Endogenous lectins as targets for drug delivery. Adv. Drug Deliv. Rev. 43, 225–244.

Yao, Z., Zhang, M., Sakahara, H., Saga, T., Nakamoto, Y., Sato, N., Zhao, S., Arano, Y., Konishi, J., 1998. Imaging of intraperitoneal tumors with technetium-99m GSA. Ann. Nucl. Med. 12, 115–118.

Yuan, F., Leunig, M., Huang, S., Berk, D., Papahadjopoulos, D., Jain, R.K., 1994. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. Cancer Res. 54, 3352–3356.

Zhu, J., Yan, F., Guo, Z., Marchant, R., 2005. Surface modification of liposomes by saccharides: vesicle size and stability of lactosyl liposomes studied by photon correlation spectroscopy. J. Colloid Interface Sci. 289, 542–550.