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Preparation and Characterization of pH-Sensitive Vesicles Made of Cholesteryl Hemisuccinate

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pH-sensitive vesicles are designed to promote efficient release of entrapped agents in response to low pH. In this study, such vesicles were prepared from cholesteryl hemisuccinate (CHEMS) in Tris-HCl buffer. Vesicles prepared by this direct hydration method had a small particle size of 74.1 nm based on intensity of Gaussian and a size of 65.8 nm based on volume of Gaussian. The mean zeta potential was about –73mV, indicating that the vesicle system was stable. Entrapment efficiency for calcein by these vesicles was measured at 37 ± 2.36%, which is higher than that by phospholipid MLVs. These calcein loaded vesicles exhibited excellent stability at pH 7.4 and underwent rapid destabilization upon acidification as showed by calcein release. However these vesicles were unstable in Fetal Bovine Serum (FBS). Optimization of formulation might improve their stability in serum.

Keywords vesicle; CHEMS; pH-sensitive; calcein

INTRODUCTION

pH-sensitive liposomes have been developed to improve the efficiency of cytoplasmic delivery of various polar molecules and macromolecules. Many studies have shown the effectiveness of pH-sensitive liposomes as carriers for these molecules (Ishida, Kirchmeier, Moase, Zalipsky, & Allen, 2001; Lopes, Pilarkski, & Belch, 2000; Oliveira, Rosilio, & Lesieurs, 2000; Roux, Francis, & Winnik, 2002; Torchilin, Zhou, & Huang, 1993). This approach relies on the selective destabilization of liposomes following acidification by the surrounding medium. The initial rationale for the design of pH-sensitive liposomes was to exploit the acidic environment of tumors to trigger destabilization of liposomal membranes (Drummond, Zignani, & Leroux, 2000). However, while significant advances have been made in overcoming many of the barriers associated with liposomal drug delivery, a major problem has been the use of organic solvents for their preparations. To avoid the use of organic solvents, we adopted the direct hydration method to prepare a new type of pH-sensitive vesicles.

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Sterols and derivatives of sterols have been incorporated into phospholipid liposome membranes to improve the physical properties of lipid bilayers. For example, Ellens, Bentz, and Szoka (1984) discussed the effects of hydrogen ion on the stability of lipid vesicles composed of phosphatidylethanolamine and cholesteryl hemisuccinate. Cholesteryl hemisuccinate (CHEMS) is an acidic cholesterol ester formed by succinic acid and the β-hydroxyl group of cholesterol (Figure 1A) (Ismail & Pieter, 2000). It self-assembles into bilayers in alkaline and neutral aqueous media and it is commonly used with dioleoylphosphatidylethanolamine (DOPE) to form pH-sensitive fusogenic vesicles. We show here that CHEMS itself exhibited pH-sensitive polymorphism.

In this study, we showed that a pH-sensitive vesicle composed of tris(hydroxymethyl) aminomethane salt of cholesteryl hemisuccinate (CHST) offered many advantages over liposomes, such as high physical stability, low cost, and easy preparation. In addition, CHST can also be used to prepare suspensions of multilamellar or small unilamellar vesicles with or without the use of organic solvents. Our findings indicated that unilamellar vesicles composed of CHST underwent membrane fusion or aggregation. We also showed that trapped calcein was released upon acidification.

MATERIALS AND METHODS

Materials

CHEMS was synthesized in our laboratory. Tris and calcein were purchased from Bodi Chemical Engineering Co. (Tianjin, China). Fetal Bovine Serum(FBS)was provided by Haoyang Biological Product Co. (Tianjin, China). Triton X-100, acetic acid, sodium acetate, and ethyl acetate were obtained from Shenyang Chemical Reagent Factory (Shenyang, China). All other chemicals were analytical reagent grade and used without further purification.

Synthesis of Tris Salt of Cholesteryl Hemisuccinate

Tris salt of cholesteryl hemisuccinate was prepared by the reported procedure with modifications (Janoff, Kurtz, &

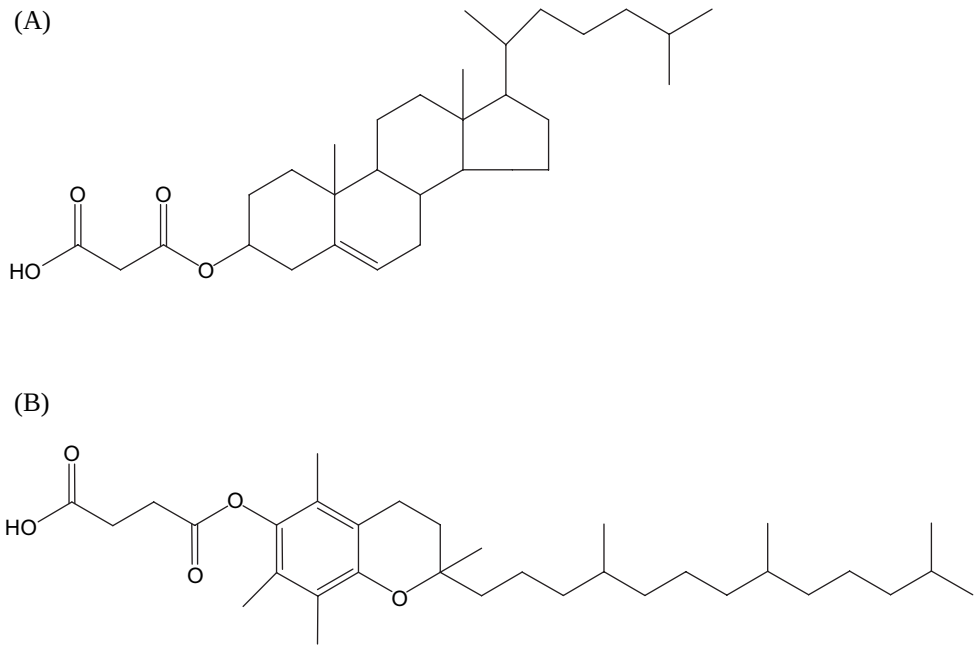

FIGURE 1. Molecular structures of CHEMS (A) and THS (B).

Jablonski, 1988). Appropriate amounts of Tris (0.01 mol) and CHEMS (0.01 mol) were separately dissolved in 400 and 150 mL alcohol at 57°C. The two solutions were then mixed and stirred with a magnetic stirrer (78HW-1 homeothermal magnetic stirrer, Hangzhou, China) for 2 hr. The resulting solution was dried on a rotary evaporator (BE-52-Rotary Evaporator, Shanghai, China) under vacuum in water bath at 57°C. The residue was recrystallized three times from ethyl acetate. Residual ethyl acetate was removed by heating at 50°C under vacuum over night. Product was analyzed by thin-layer chromatography (TLC) and differential scanning calorimetry (DSC).

Preparation of pH-Sensitive CHEMS Vesicles

Tris salt of cholesteryl hemisuccinate was dissolved in 10 mM Tris-HCl buffer (1.54 M NaCl with or without 80 mM calcein, pH 7.40) in a high speed dispersing apparatus (T18 ULTRA-TURRAX®, Guangzhou, China) for 2.5 min. To make single lamellar systems, the coarse dispersion vesicles were sonicated to clarity in a probe sonicator (JY92-2D probe sonicator, Ningbo, China) operating at 400 W (2 min) and 600 W (8 min) or passed through a microfluidizer (M-110 L Microfluidizer, Microfluidics, Newton MA) at 4.66 kpsi for 3 cycles, 9.32 kpsi for 5 cycles, 13.98 kpsi for 5 cycles, and 16.31 kpsi for 5 cycles. The resulting single lamella vesicles were extruded through polycarbonate membranes of gradually decreasing pore sizes (0.45 and 0.22 μm). Non-entrapped calcein was removed by passing the vesicle suspension through the stirred ultrafiltration cells (Amicon stirred cell 8010, Millipore Co.) and the residue of vesicles on the ultrafiltration membrane were collected. Calcein concentrations were determined by measurement of absorbance at 493.6 nm through a spectrophotometer (UV-260, Shimadzu Inc., Japan).

Morphology, Particle Size, and Zeta Potential of CHEMS Vesicles

The coarse dispersion vesicles were examined under a microscope to confirm that Tris salt of cholesteryl hemisuccinate spontaneously formed vesicles. The samples were directly dropped onto the slide and observed through oil immersion objective (DMBA 450 Motic Digital Microscope, China). The structure of a small single lamellar suspension that was formed through sonicator or Microfluidizer was examined under transmission electron microscope (TEM). The samples were negatively stained with 0.3% phosphotungstic acid and observed through TEM (H-100 TEM, Hitachi, Japan). The particle size and zeta potential of pH-sensitive CHEMS vesicles were measured by dynamic laser light scattering (NICOMP™ 380 Submicron Particle Sizer, Particle Sizing Systems, Santa Barbara, CA.) and zeta potential analyzer (delta 440 SX Zeta potential analyzer, Beckman Coulter Inc.), respectively. Samples were diluted with aqueous phase of the formulation for the measurements. All measurements were conducted at 25℃ in triplicates.

Entrapment Efficiency of Calcein in CHEMS Vesicles

Entrapment efficiency of calcein by CHEMS vesicles was assayed by ultrafiltration. The concentration of the calcein solution used for preparing vesicle dispersions was 80 mM.

First, the absorbance at 493.6 nm of calcein that was used to prepare calcein-containing vesicles was measured. After calcein-containing vesicle suspensions were ultrafiltrated by stirred ultrafiltration apparatus, the absorbance of ultrafiltrate was also determined. Entrapment efficiency of vesicles for calcein was estimated by the following equation.

$$\mathrm{EE}(\%) = (A_{\mathrm{tot}} - A_{\mathrm{u}}) / A_{\mathrm{tot}} \times 100\% \quad (1)$$

where A_{tot} is the total absorbance of calcein in vesicle suspensions and A_{u} is the absorbance of ultrafiltrate.

pH-Induced Vesicle Aggregations

Vesicles aggregations in response to reduced buffer pH were measured by increase in particle size (Shi, Guo, & Stephenson, 2002). A sample of 3 mL vesicles containing 100 nmol of lipid was diluted with 15 mL of sodium acetate buffer of various pHs (100 mM NaCl, 10 mM sodium acetate, pH 4.5, 5.5, and 6.5). The resulting vesicle suspensions were incubated at 37°C for 10 min or 1 hr. Then aliquots of the samples were withdrawn and the mean particle diameters were determined by dynamic laser light scattering with Nicomp 380ZLS particle sizer.

Calcium Induced CHEMS Vesicle Aggregations

The aggregations of vesicles induced by $CaCl_2$ were investigated by measuring the changes of particles sizes after addition of different concentrations of $CaCl_2$ (1, 2, 5, and 10 mM). Samples of 3 mL vesicles containing 100 nmol of lipid were diluted with 15 mL $CaCl_2$ solution of various concentrations. The resulting suspensions were incubated at 37°C. At various time points, changes of the clarity of vesicle suspensions were observed and aliquots of the samples were withdrawn and the mean particle diameters were determined by dynamic laser light scattering with a Nicomp 380ZLS particle sizer.

Calcein Release from Vesicles Triggered by Low pH

Vesicle calcein release was characterized by ultraviolet spectrometry as described (Sudimack, Guo, Tjarks, & Lee, 2002). Calcein-loaded vesicles (0.1 mL) containing 3.3 nmol of lipid were added to 1.4 mL Tris-HCl (pH 7.4, with or without serum) or sodium acetate buffer (100 mM NaCl, 10mM sodium acetate, pH 4.5, 5.5, and 6.5, with or without serum) in a disposable cuvette. After 10 min incubation at 37°C, vesicle suspensions were passed through the stirred ultrafiltration apparatus. The absorbance at 493.6 nm of ultrafiltrate was measured. The absorbance of the same amount of suspensions was also measured following the addition of 10% TritonX-100, which caused 100% calcein leakage from the vesicles. The percentage of calcein release was calculated based on the equation:

$$\%\text{Calcein Release} = ((A_{\mathrm{pH}} - A_0)/(A_{100\%} - A_0)) \times 100\% \quad (2)$$

where A_0 is the absorbance at pH 7.4, A_{pH} is the absorbance following incubation at acidic pHs, and A_{100} is the absorbance after the addition of TritonX-100.

Determination of Vesicle Stability in Fetal Bovine Serum

Vesicles (0.1 mL) entrapping 80 mM calcein and containing 3.3 nmol lipid were added to 1.4 mL 10% Fetal bovine serum (FBS). After 1 hr incubation at 37°C, calcein absorbance was measured and the percentage of calcein release was calculated as described above. In addition, vesicle particle size measurements were performed at various time points.

RESULTS AND DISCUSSION

Examination of CHEMS Vesicles by Microscope and Electron Microscope

Microscopic examination (Figure 2A) confirmed that CHEMS formed liposome-like vesicles on addition of Tris-HCl buffer through high speed dispersing apparatus. The fine structure of the vesicles (Figure 2B) was also investigated by electron microscopy. The electron micrograph also demonstrated the formation of liposome-like vesicles. A few multilamellar vesicles that appeared in the electron micrograph were most likely caused by the uneven dispersal of the probe sonicator.

Particle Size Distribution and Zeta Potential of Vesicles

The vesicle size distribution was measured by a particle sizing system. The particle distributions based on intensity% and volume% of the Gaussian or Nicomp diameter of CHEMS vesicles were shown in Figure 3. The CHEMS vesicles were filtered sequentially through polycarbonate membranes with 0.45 and 0.22 μm pore diameters.

As clearly shown in the particle size distribution based on intensity and volume of Gaussian, the mean particle size of CHEMS vesicles was 74.1 nm by intensity and 65.8 nm by volume. There was no significant difference between the size of intensity and the size of volume. However, in particle size distribution based on intensity and volume of Nicomp, there were two major particle size distributions. The smaller particle size distribution was about 35 nm and the larger was 85 nm by both intensity and volume. The percentage of the larger particle size was higher than that of the small particle size. The particle size distribution (PSD) is known to have a strong influence on the pharmacokinetics and pharmacodynamics of vesicles. Our CHEMS vesicles showed a narrow particle size distribution, which could be important for its biological applications.

In order to characterize the CHEMS vesicles in more detail, its zeta potential was measured. The mean zeta potential was about –73 mV. It showed that the vesicle system possessed negative charge in the experimental conditions, indicating a strong electrostatic repulsion between vesicles according to the DLVO theory. The presence of repulsive force on the surface

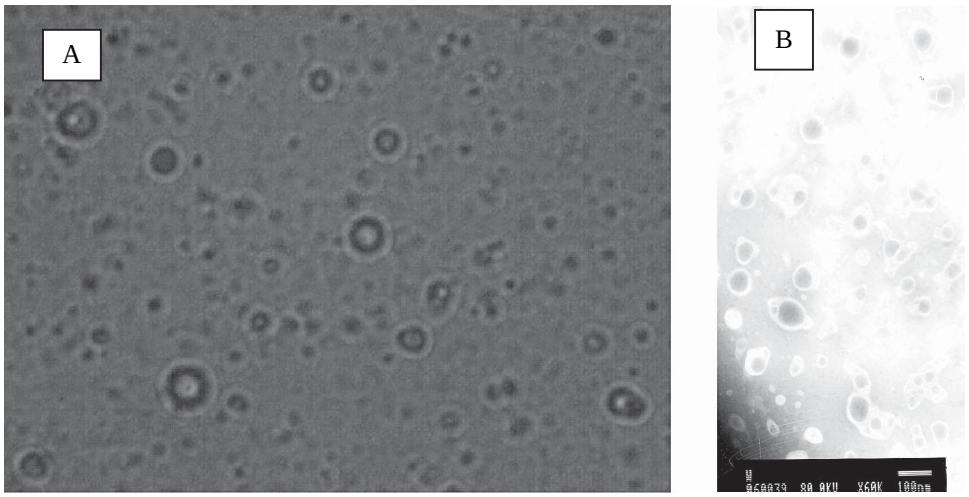

FIGURE 2. The microphotograph of CHEMS vesicles (A) and TEM photograph of vesicles (B), the bar represents 100 nm.

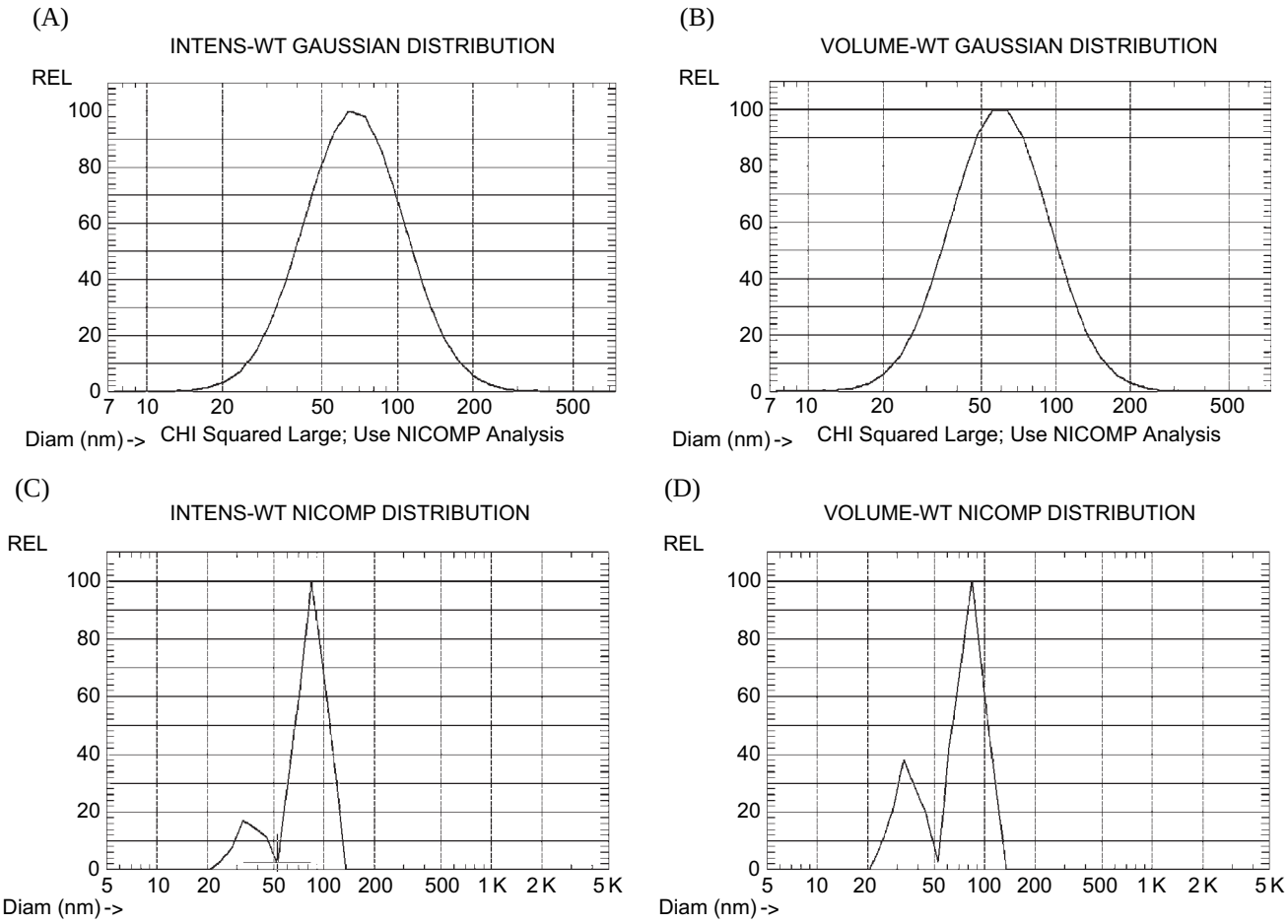

FIGURE 3. Average diameter distributions of CHEMS vesicles: (A) Intensity-WT gaussian distribution; (B) Volume-WT gaussian distribution; (C) Intensity-WT nicomp distribution; (D) Volume-WT nicomp distribution.

of vesicles suggested that CHEMS vesicles possessed better stability in vitro compared with conventional liposomes.

Entrapment Efficiency of Calcein by CHEMS Vesicles

Ultrafiltration was used to determine the entrapment efficiency in this study. Recovery rates of free drug were determined for all samples and they were around 99.2% of the amounts used in preparation. Entrapment efficiency of vesicles for calcein was determined at 37 ± 2.36%, which was generally higher than the reported entrapment efficiency of liposome. When calcein was added to the outside of "empty" liposomes, an encapsulation efficiency of about 15% was achieved after freeze-thawing or freeze-drying in the presence of mannitol (Huang & MacDonald, 2004). The highest calcein entrapment efficiency of the vesicles (niosomes) prepared with hydrated mixture of various non-ionic surfactants and cholesterol was only 6% with the largest particle size (Manosroi, Wongtrakul, Manosroi, Sakai, Sugawara, Yuasa, & Abe et al., 2003). One of the generally applicable approaches to improve entrapment efficiency is to prepare fewer but bigger liposomes instead of many small ones. This can be achieved by using dehydration-rehydration or freeze-thawing techniques. If the passive-loading protocols were applied, the liposome often entrapped a lower amount of hydrophilic substance (Pavelic, SB, & Schubert, 2001). Our CHEMS vesicles have high entrapment efficiencies as compared with phospholipid MLVs, suggesting that the CHEMS vesicle is likely a good carrier for water-solubility drug. We believe that the entrapment efficiencies of CHEMS vesicles will be increased further if the remote-loading protocols were applied.

Effects of Buffer pH on the Size of Vesicle Particles

The effects of pH on vesicle size were studied and the results are shown in Figure 4. As shown, incubation at pH 3.83, 4.45, and 5.48 for 10 min lead to 5-fold, 4-fold, and 2-fold increases in mean diameter as compared with incubation at pH 7.40, respectively. And incubation at these pHs for 1 hr showed 25-fold, 12-fold, and 3-fold in mean diameter as compared with pH 7.40. The larger increases in particle size with longer incubation time indicated that the increases in particle size in acidic buffers were time dependent. No change in particle sizes was observed when incubated in pH 7.40 buffer for 10 min and 1 hr. Moreover, even though no obvious changes in particle size based on intensity and volume of Gaussian were observed when these vesicles were kept in pH 6.44 buffers for 10 min and 1 hr as compared with incubation at pH 7.40, significant increases in particle size based on intensity and volume of Nicomp were observed (data not shown). The mean diameters of the vesicles were greatly increased at lower buffer pHs, indicating vesicle aggregation and/or membrane fusion. Jizomoto, Kanaoka, and Hirano (1994) had shown that α-tocopherol succinate (THS) (Figure 1B) can undergo self-assembly at neutral pH and the sizes of the resulting vesicles are sensitive to acidic pH. This acidic pH effect is attributed to the low pK_a of the THS carboxyl group. The stereogeometries

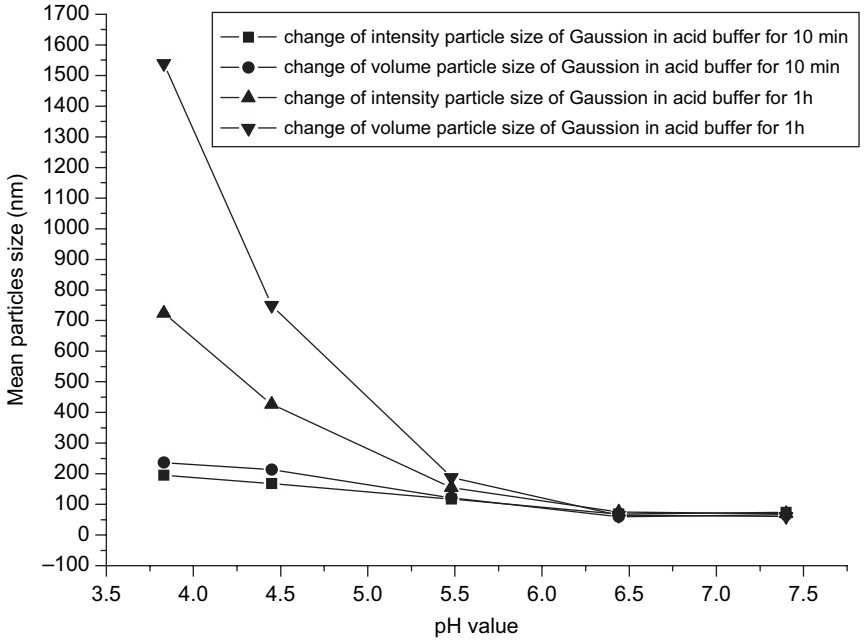

FIGURE 4. pH-dependent particle size increase of CHEMS vesicles incubated in sodium acetate buffer of various pH values for 10 min or 1 hr. change of intensity particle size of gaussian in acid buffer for 10 min (■), change of volume particle size of gaussian in acid buffer for 10 min (●), change of intensity particle size of gaussian in acid buffer for 1 hr (▲), change of volume particle size of gaussian in acid buffer for 1 hr (▼).

drawn by computer graphics revealed that there were similarities between THS and CHEMS. Both have hydrophobic portions adjacent to the hydrophilic moiety and both molecules have flexible tails. Therefore, the CHEMS vesicles could display similar sensitivity to acidic pH as THS vesicles.

Effects of Calcium on the Size of Vesicle Particles

Calcium-induced fusion of liposomes containing phosphatidylserine has been studied extensively as a model of membrane fusion (Holland, Hui, Cullis, & Madden, 1996). It has been proposed that Ca^{2+} acts either by neutralizing the charge repulsion between negatively charged liposomes, thereby promoting dehydration and bilayer contact through the formation of interbilayer Ca^{2+}-phosphatidylserine complexes, or by inducing formation of non-bilayer fusion intermediates. Regardless of the structures formed during or after fusion, the initial step must involve close apposition of bilayers so that membrane contact can occur. In order to determine the effects of Ca^{2+} on the structures of CHEMS vesicles, we carried out the calcium induced aggregation experiment. After addition of various concentrations of $CaCl_2$, the vesicle suspensions showed different patterns of changes. When 10 mM $CaCl_2$ was added, the suspension became turbid immediately. When 5 and 2 mM $CaCl_2$ were added, the suspension became turbid after 10 and 15 min, respectively. The Ca^{2+} concentration of 2 mM is close to the normal concentration of Ca^{2+} in human serum which is between 2.1–2.6 mM. When 1 mM $CaCl_2$ was added, we did not observe any change through naked eyes. However, when determined by dynamic laser light scattering at various time points, the particle size increased in a time dependent manner (Figure 5). The instability of CHEMS vesicles in the presence of $CaCl_2$ demonstrated that Ca^{2+} likely eliminated the charge repulsion between negatively charged vesicles.

Calcein Release from Vesicles Triggered by Low pH

Vesicles entrapping 80 mM calcein were evaluated for low pH-triggered calcein release. As illustrated in Figure 6, vesicles showed increased calcein release at pH 6.5, 5.5, and 4.5 with or without serum. The pH dependent calcein release profiles of CHEMS vesicles were consistent with the pH dependent profiles of aggregation, both indicating that CHEMS vesicles were sensitive to pH change around 6.5. However the presence of 20% serum rendered the calcein release of vesicles much less sensitive to the changes of pH. pH-sensitive vesicles are designed to undergo rapid destabilization in acidic environments. Even though some vesicles exhibited pH-sensitivity in the absence of serum, it might lose pH-sensitivity in the presence of serum (Shi et al., 2002). At pH 4.5, calcein release of CHEMS vesicles dropped from 87.17 to 61.83% in the presence of 20% FBS, agreeing with the report of Shi et al. (2002).

The Stability of Vesicles in Serum

Some pH-sensitive liposome preparations have been shown to be destabilized and release their contents in the presence of serum or plasma (Drummond et al., 2000). This serum-induced destabilization was exquisitely sensitive to the lipid composition of the liposome. To determine the stability of CHEMS vesicles in serum, we incubated the vesicles in the fetal bovine serum for various periods of time and measured particle size changes. As shown Figure 7, incubation in serum led to increases in mean diameter of CHEMS vesicles and the increases in particle size were time dependent. We also measured calcein release in serum. Calcein release from vesicles after 1 hr incubation was 43.21% in the presence of 20% serum. The results of calcein release in serum were consistent with the changes of the mean particle size of vesicles incubated

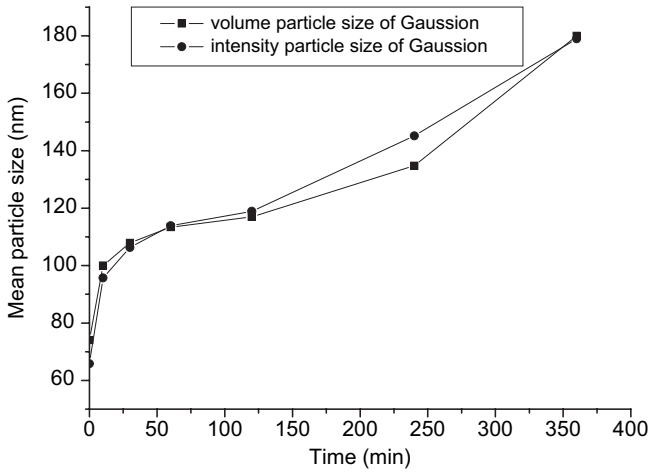

FIGURE 5. Changes of particle size of vesicles incubated in 1 mM $Cacl_2$, intensity particle size of gaussian (■) and volume particle size of gaussian (●).

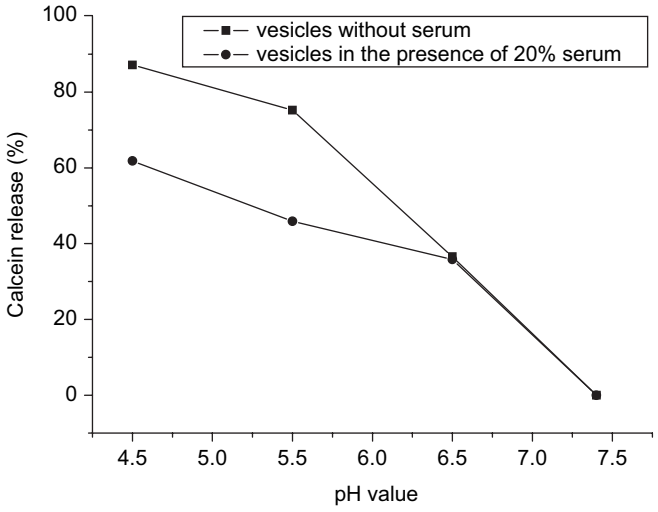

FIGURE 6. Calcein release of CHEMS vesicles incubated at sodium acetate buffer of various pH values with serum or without serum for 10 min. vesicles in the presence of 20% serum (●) and vesicles without serum (■).

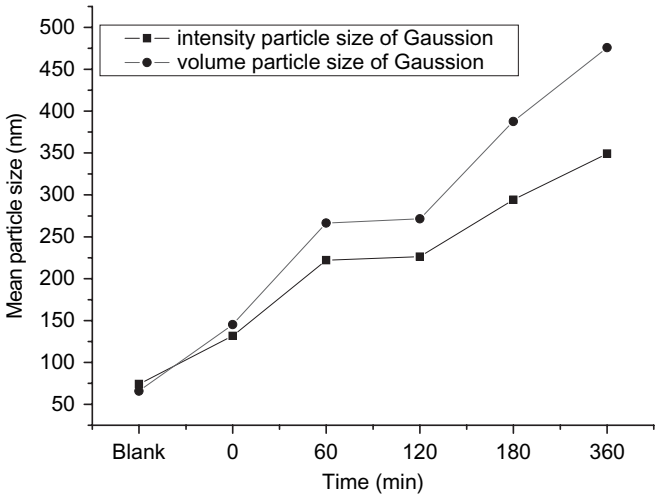

FIGURE 7. Increase in particle size of vesicles incubated in serum, intensity, particle size of gaussian (■) and volume particle size of gaussian (●).

with serum. The CHEMS vesicles were destabilized and released their contents in serum. As indicated by others (Drummond et al., 2000), several factors, including complement-mediated lysis and destabilization by HDL, could be involved in the destabilization of liposomes in serum. It is likely that these factors could affect the stability of CHEMS vesicles too. Moreover, some lipid compositions in serum could interact with the CHEMS vesicles and the possible adsorption of the serum proteins to the surface of the vesicles could result in the destabilization of the vesicles.

One way to increase the stability of vesicles in serum is to optimize the formulation. For example, PEG-lipid derivate had been incorporated into the formulation to prolong the circulation time of pH-sensitive liposomes or vesicles (Roux, Passirani, Scheffold, Benoit, & Leroux, 2004). But it also led to a significant decrease in liposome or vesicle pH-sensitivity (Kirpotin, Hong, Mullah, Papahadjopoulos, & Zalipsky, 1996). We are actively investigating for an optimal formulation to prolong the stability of CHEMS vesicles in serum.

CONCLUSIONS

The pH-sensitive vesicles composed of CHEMS were prepared and the vesicle suspensions showed significant changes in particle sizes and the release profiles of encapsulated marker under acidic pH conditions. The pH-sensitive properties of CHEMS vesicles could be used in the development of targeted drug delivery systems. The drug release from pH-sensitive CHEMS vesicles could be significantly increased if the vesicles reached tissues with a lower pH, like tumor. Moreover, CHEMS vesicles formed spontaneously with high entrapment efficiency and no organic solvent was used in the preparation.

In addition to its drug delivery potential as vesicles, CHEMS molecules showed anti-tumor properties similar to THS (Yamada, Arita, Kobuchi, Yamamoto, Yoshioka, Tamai, & Utsumi, 2003), which can induce apoptosis of cultured human promyelocytic leukemia cells (HL-60). Their results suggested that CHEMS induced apoptosis of HL-60 cells by modulating mitochondrial membrane functions, therefore activating the caspase cascade pathway via mitochondrial Cytochrome C release. When CHEMS vesicles were used to deliver anti-tumor drugs, itself might show some anti-tumor activity.

REFERENCES

Drummond, D. C., Zignani, M., & Leroux, J. C. (2000). Current status of pH-sensitive liposomes in drug delivery. *Prog. Lipid Res., 39,* 409–460. doi:10.1016/S0163-7827(00)00011-4.

Ellens, H., Bentz, J., & Szoka, F. C. (1984) pH-Induced destabilization of phosphatidylethanolamine-containing liposomes: Role of bilayer contact. *Biochemistry, 23,* 1532–1538. doi:10.1021/bi00302a029.

Holland, J. W., Hui, C., Cullis, P. R., & Madden, T. D. (1996). Poly(ethylene glycol)-Lipid Conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry, 35,* 2618–2624. doi:10.1021/bi952000v.

Huang, S. L., & MacDonald, R. C. (2004). Acoustically active liposomes for drug encapsulation and ultrasound-triggered release. *Biochim. Biophys. Acta, 1665,* 134–141. doi:10.1016/j.bbamem.2004.07.003.

Ishida, T., Kirchmeier, M. J., Moase, E. H., Zalipsky, S., & Allen, T. M. (2001). Targeted delivery and triggered release of liposomal doxorubicin enhances cytotoxicity against human B lymphoma cells. *Biochim. Biophys. Acta, 1515,* 144–158. doi:10.1016/S0005-2736(01)00409-6.

Ismail, M. H., & Pieter, R. C. (2000). Cholesteryl hemisuccinate exhibits pH sensitive polymorphic phase behavior. *Biochim. Biophys. Acta, 1463,* 107–114. doi:10.1016/S0005-2736(99)00186-8.

Janoff, A. S., Kurtz, C. L, & Jablonski, R. L. (1988). Characterization of cholesterol hemisuccinate and α-tocopherol hemisuccinate vesicles. *Biochim. Biophys. Acta, 941,* 165–175. doi:10.1016/0005-2736(88)90177-0.

Jizomoto, H., Kanaoka, E., & Hirano. K. (1994). pH-Sensitive liposomes composed of tocopherol hemisuccinate and of phosphatidylethanolamine including tocopherol hemisuccinate, *Biochim. Biophys. Acta, 1213,* 343–348. www.biophysj.org/cgi/content/full/79/3/1438

Kirpotin, D., Hong, K., Mullah, N., Papahadjopoulos, D., & Zalipsky, S. (1996). Liposomes with detachable polymer coating destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol). *Febs. Lett.,* 388, 115–118. doi:10.1016/0014-5793(96)00521-2.

Lopes de Menezes, D. E., Pilarski, L. M., & Belch, A. R. (2000). Selective targeting of immunoliposomal doxorubicin against human multiple myeloma in vitro and ex vivo. *Biochim. Biophys. Acta, 1466,* 205–220. doi:10.1016/S0005-2736(00)00203-0.

Manosroi, A., Wongtrakul, P., Manosroi, J., Sakai, H., Sugawara, F., Yuasa, M., & Abe, M. (2003). Characterization of vesicles prepared with various nonionic surfactants mixed with cholesterol. *Colloids and Surfaces B: Biointerfaces, 30,* 129–138. doi:10.1016/S0927-7765(03)00080-8.

Oliveira, M. C., Rosilio, V., & Lesieur, P. (2000). pH-Sensitive liposomes as a carrier for oligonucleotides: A physico-chemical study of the interaction between DOPE and a 15-mer oligonucleotide in excess water. *Biol. Chem., 87,* 127–137. www.wanfangdata.com.cn/qikan/periodical.articles/GWYY-HCY/gwyy2006/0607/060715.htm

Pavelic, Z., SB, N., & Schubert, R. (2001). Liposomal gels for vaginal drug delivery. *Int. J Pharm., 219,* 139–149. doi:10.1016/S0378-5173(01)00637-8.

Roux, E., Francis, M., & Winnik, F. O. M. (2002). Polymer based pH-sensitive carriers as a means to improve the cytoplasmic delivery of drugs. *Int. J Pharm., 242,* 25–36. doi:10.1016/S0378-5173(02)00183-7.

Roux, E., Passirani, C., Scheffold, S., Benoit, J. P., & Leroux, J. C. (2004). Serum-stable and long-circulating, PEGylated, pH-sensitive liposomes, *J Contr. Release, 94,* 447–451. doi:10.1016/j.jconrel.2003.10.024.

Shi, G. F., Guo, W. J., & Stephenson, S. M. (2002). Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic / anionic lipid combinations. *J Contr. Release, 80,* 309–319. doi:10.1016/S0168-3659(02)00017-2.

Sudimack, J. J., Guo, W. J., Tjarks, W. & Lee, R. J. (2002). A novel pH-sensitive liposome formulation, containing oleyl alcohol. *Biochim. Biophys. Acta, 1564,* 31–37. doi:10.1016/S0005-2736(02)00399-1.

Torchilin, V. P., Zhou, F., & Huang, L. (1993). pH-Sensitive liposomes. *J Lipos. Res., 3,* 201–255. www.jbc.org/cgi/reprint/272/4/2382.pdf

Yamada, K., Arita, K., Kobuchi, H., Yamamoto, S.J., Yoshioka, T., Tamai, H., & Utsumi, K. (2003). Cholesteryl-hemisuccinate-induced apoptosis of promyelocytic leukemia HL-60 cells through a cyclosporine A-insensitive mechanism. *Biochem. Pharmacol., 65,* 339–348. doi:10.1016/S0006-2952(02)01518-6.