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Research Article

Preparation and Characterization of Stable pH-Sensitive Vesicles Composed of α-Tocopherol Hemisuccinate

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Abstract. The current study aims to develop a stable pH-sensitive drug delivery system. First, cleavable polyethylene glycol-α-tocopherol hemisuccinate (PEG-THS) was synthesized. Conventional pH-sensitive vesicles composed of the Tris salt of α-tocopherol hemisuccinate (THST) were then prepared using the detergent removal technique. The vesicles had a mean particle size of (163.8±5.5) nm and a zeta potential of −74.5±6.4 mV. The THST vesicles were then modified using PEG-THS or uncleavable PEG-cholesterol (PEG-CHOL) (THST/PEG-lipids, 100:6 molar ratio). The mean vesicle particle size and absolute zeta potential decreased with increasing PEG-THS proportion. When the pH was decreased, the vesicle particle size and calcein release rate increased. The THST vesicles were initially Ca^{2+}-unstable but exhibited significantly improved stability after modification with PEG-THS, especially at PEG-lipid ratios above 6%. Incubation in an acid serum increased the calcein release rate of conventional THST vesicles to 45±1.98% at 10 min. However, the release rate of the PEG-CHOL vesicles remained low. The calcein release rate of PEG-THS vesicles was between those of conventional and PEG-CHOL-V. Therefore, PEG-THS can protect vesicles in serum and reconstitute their pH sensitivity in acidic conditions. Cleavable PEG-THS can be used in stable pH-sensitive preparations without loss of pH sensitivity. Free calcein and conventional vesicles eliminated from the plasma soon after injection, as well as the half-life ($t_{1/2}$) and area under the curve of PEG-THS-V encapsulating calcein, were dramatically increased. This phenomenon indicates that the use of PEG-lipid derivatives has gained a favorably long circulation effect in mice.

KEY WORDS: cleavage; long circulation; PEG-α-tocopherol hemisuccinate; pH-sensitive; vesicles.

INTRODUCTION

Cancer, inflammation, and other histopathological abnormalities often occur with acidification. Thus, pH-sensitive preparations such as pH-sensitive liposomes (PSLs) were initially proposed for drug delivery (1–3). However, the most severe tumor acidification occurs far from tumor capillaries and beyond the range of liposomes. In addition, the pH of tumor interspaces rarely drops below 6.5, making the integration or diffusion of liposomes within such a narrow pH range very difficult (4). Nevertheless, the pH in endosomes and lysosomes can reach values below 5.0 (2,5). Therefore, the destabilization of PSL depends on the postendocytic process.

Amphiphilic molecules spontaneously aggregate into organized bilayer molecular structures in solution. Vesicles are formed when these bilayers bend and the two ends close. PSLs are classified as pH-sensitive vesicles (PSV). Several related research on PSL have been conducted, but relatively few studies on PSV are available (6–10). Phospholipids, such as dioleoylphosphatidylethanolamine (DOPE), are used to construct PSLs and usually contain a pH-sensitive group (11,12). However, DOPE is quite expensive and not suitable for application in extensive research. In addition, like conventional liposomes, PSLs and PSVs are very unstable in plasma and fail to effectively deliver their contents to target tissues or cells (13). Therefore, improving the stability of PSLs has become a key issue.

Polyethylene glycol (PEG) lipids are used to modify PSLs to increase their surface hydrophilicity and steric hindrance and extend their circulation time *in vivo* (14–16). The chemical bonds between conventional PEG-lipids [such as PEG-distearoylphosphatidylethanolamine, PEG-cholesterol (PEG-CHOL), and so on] are usually amide or ether bonds, which have high chemical stability and thus are difficult to degrade. When PSLs modified by uncleavable PEG-lipids reach the target site, the

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ABBREVIATIONS: Calcein-S, Calcein solution; THST Vesicles, Tris (hydroxymethyl) aminomethane salt of alpha-tocopherol hemisuccinate vesicles; Calcein-TV, Calcein-tris (hydroxymethyl) aminomethane salt of alpha-tocopherol hemisuccinate vesicles; PEG-THS, Poly(ethylene glycol)-alpha-tocopherol hemisuccinate; PEG-CHOL, Poly(ethylene glycol)-cholesterol; PEG-THS-V, Poly (ethylene glycol)-alpha-tocopherol hemisuccinate vesicles; PEG-CHOL-V, Poly(ethylene glycol)-cholesterol vesicles.

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PEG chains are still attached to the liposome surface, so that the PSLs lose their responsiveness to the decline in pH (4,13,14,17,18). Therefore, the feasibility of the *in vivo* application of PSLs depends on their plasma stability and the preservation of their pH sensitivity when they reach the target sites.

The present study used the Tris salt of α-tocopherol hemisuccinate (THST) to prepare PSVs. THST offers a number of advantages, such as biocompatible characteristics and low cost, and eliminates the use of organic solvents in the vesicle preparation process. PEG-α-tocopherol hemisuccinate (PEG-THS), a cleavable PEG-lipid linked by ester bonds, was synthesized and used to modify the PSVs (see Fig. 1). The results indicate that unilamellar vesicles composed of THST underwent membrane fusion or aggregation at low pH. PEG-THS can increase the stability of vesicles and reconstruct the pH sensitivity of PSVs under certain conditions.

EXPERIMENTAL METHODS

Materials and Method

Chemicals

Methoxypolyethylene glycol (PEG, molecular weight of 2,000) was obtained from Fluka Co. (USA). THS was purchased from Xinchang Pharmaceutical Factory (Xinchang, China). Tris and calcein were purchased from Bodi Chemical Engineering Co. (Tianjin, China). Fetal bovine serum (FBS) was obtained from 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). The anion exchange resin was obtained from China National Medicines Corporation Ltd (Shenyang, China). All other chemicals were analytical reagent grade and used without further purification.

Structural Analysis

IFS-55 Fourier transform infrared spectrometer (KBr disk) was used to record the IR spectra of PEG-THS. ^{1}H-NMR and ^{13}C-NMR were performed on a Bruker ARX-300 using $CDCl_3$ as a solvent.

Animals

Kunming mice (male, 8 weeks old, weighing 18–22 g) were purchased from Shenyang Pharmaceutical University experimental animal center (Shenyang, China). All experiments were carried out in accordance with the guidelines of the local Animal Welfare Committee.

Synthesis of PEG-THS

THS (531 mg, 1 mmol) was mixed with PEG (600 mg, 0.6 mmol) in 20 mL dichloromethane at 0°C with gentle stirring, followed by the addition of 4-dimethylaminopyridine (40 mg, 0.32 mmol). After 10 min, *N*,*N*′-dicyclohexylcarbodiimide (DCC, 206 mg, 1 mmol) was added. The mixture was left to react for 4 h at room temperature. When the reaction was complete, the mixture was pump-filtered to obtain the crude product solution, which was successively extracted with 2 M HCl, saturated sodium bicarbonate solution, and distilled water. The organic layer was evaporated to dryness, and 20 mL diethyl ether was added to the residue. The precipitated white crystals were filtered and recrystallized three times from dehydrated alcohol and dried in vacuo over P_2O_5. The chemical structure was confirmed *via* TLC, IR, ^{1}H NMR, and ^{13}C-NMR. TLC: R_f=0.62 ($CHCl_3$/MeOH/H_2O=45:9:1). IR γ/cm^{-1}: 1758 and 1738 (ester, C=O); ^{1}H-NMR ($CDCl_3$, δ ppm): ^{1}H-NMR ($CDCl_3$, δ ppm): 0.85 (m, 12H, CH_3-phytyl), 1.0–1.3 (m, 18H, CH_2-phytyl), 3.38 (s, 3H, H-c), 3.64 (m, 176H, H-b), 4.27 (t, 2H, H-a); and ^{13}C-NMR($CDCl_3$): δ171.6 (C-4′), 170.4 (C-1′), 70.1 (C-a), 68.5 (C-b), 58.5 (C-c), 38.9, 36.9, 36.75, 32.2, 32.1, 30.6 (C-1″, 3″, 5″, 7″, 9″,11″, CH_2-phytyl).

Synthesis of Tris Salt of α-Tocopherol Hemisuccinate

THST was prepared following a previously reported procedure, with some modifications (19). Appropriate amounts of Tris (10 mmol) and THS (10 mmol) were separately dissolved

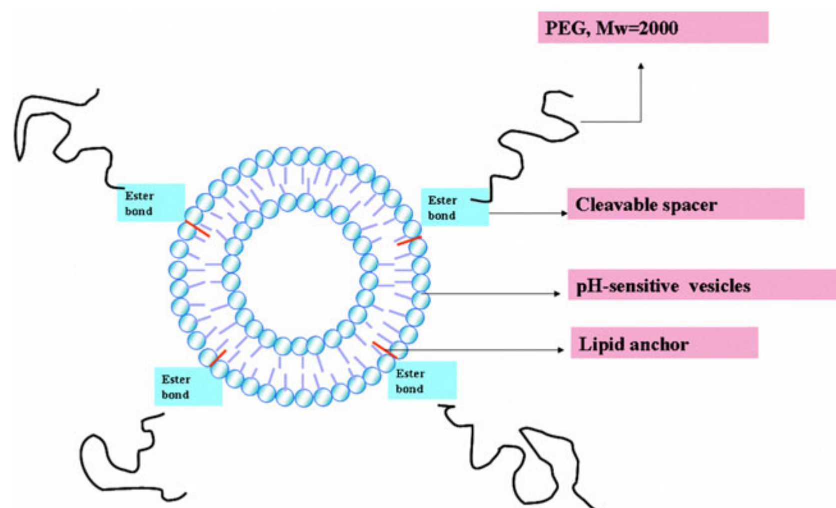

Fig. 1. pH-sensitive vesicles composed of α-tocophero hemisuccinate modified with cleavable PEG-lipids derivatives (PEG-THS)

in appropriate amounts of water and 150 mL alcohol at 57°C. The two solutions were then mixed and stirred with a magnetic stirrer for 2 h. The resulting solution was dried on a rotary evaporator (BE-52-Rotary Evaporator, Shanghai, China) under vacuum in a water bath at 57°C. The residue was recrystallized three times from ethyl acetate. Residual ethyl acetate was removed by heating overnight at 50°C under vacuum.

Preparation of pH-Sensitive THST Vesicles

Vesicles were prepared according to the detergent removal method (20), with some modifications. THST and sodium deoxycholate (5:1 molar ratio) without or with PEG-THS (2%, 4%, 6%, and 8% molar ratios) or with PEG-CHOL (6% molar ratio) were dissolved in 10 mM Tris–HCl buffer (1.54 M NaCl with or without 50 mM calcein, pH 7.40) and processed in a water bath sonicator until no precipitate remained in the solution. Sodium deoxycholate was removed by passing the vesicle suspension through stirred ultrafiltration cells (10 kDa cutoff) (Amicon stirred cell 8010, Millipore Co., USA). The vesicle residue on the ultrafiltration membrane was resuspended in 10 mM Tris–HCl buffer solution, and the resulting vesicles were extruded through 0.45 and 0.22 μm microporous membranes. Non-entrapped calcein was removed by passing the vesicle suspension through the anion exchange resin (21). All fluorescence measurements were performed on a Perkin Elmer LS-5B fluorospectrophotometer (Wellesley, MA, USA). The excitation and emission wavelengths were set at 490 and 520 nm, respectively.

Characterization of THST Vesicles

The structure of a small, single lamellar suspension was examined under a transmission electron microscope (TEM, H-100, Hitachi, Japan). The samples were negatively stained with 0.3% phosphotungstic acid and observed through TEM. The particle size and zeta potential of the pH-sensitive THST and PEGylated vesicles were measured using dynamic laser light scattering (NICOMP™ 380 Submicron Particle Sizer, Particle Sizing Systems, Santa Barbara, CA, USA) and a zeta potential analyzer (delta 440sx Zeta potential analyzer, Beckman Coulter, USA), respectively. The samples were diluted with the aqueous phase of the formulation for the measurements, which were all conducted in triplicate at 25°C.

Verification of the pH Sensitivity of the THST Vesicles

pH-Induced THST Vesicle Aggregation

A 3-mL sample of the vesicles was diluted with the appropriate sodium acetate buffer (10 mM) at various pH levels (4.4, 5.4, 6.4, and 7.4). The resulting vesicle suspensions were incubated at 37°C for 10 min. Aliquots of the samples were withdrawn and the mean particle diameters were determined *via* dynamic laser light scattering using the Nicomp 380ZLS particle sizer.

Calcein Release from PSVs Triggered by Low pH

Calcein-loaded vesicles (0.05 mL) were added to 1 mL 10 mM sodium acetate buffer or 20% FBS (pH 4.4, 5.4, 6.4, and 7.4) in disposable cuvettes. The mixtures were then vortex-mixed at 37°C. After 10 min, 0.1 mL of the samples was diluted to 5 mL and the pH was adjusted to 7.4. Calcein fluorescence was measured through a fluorospectrophotometer (HITACHI 650–60, Hitachi, Japan) before and after the addition of 10% Triton X-100 (22). The percentage of calcein release was calculated using the following equation:

$$\%\ Calcein\ release = ((F_t - F_0)/(F_{100\%} - F_0)) \times 100\% \quad (1)$$

where F_0 is the fluorescence intensity of calcein in the buffer (pH 7.4), F_t is the fluorescence intensity after incubation in the acid buffer, and F_{100} is the fluorescence intensity after the addition of Triton X-100.

Verification of the Stability of PEG-THS-V

The stability of the PEG-THS-modified vesicles was verified by measuring the changes in the particle size after the addition of different concentrations of $CaCl_2$. Different proportions (1%, 2%, 4%, 6%, 8%, molar ratio) of PEG-THS-V were mixed with different concentrations of $CaCl_2$ solution (1 and 10 mM). The resulting suspensions were incubated at 37 °C. At various time points (0, 1, 4, 8, 24 h), aliquots of the samples were withdrawn and the mean particle diameters were determined *via* dynamic laser light scattering.

PEG-THS Reconstruction of the Acid Sensitivity of PSVs (22)

Calcein-TV, PEG-THS-V (0.1 mL) or PEG-CHOL-V (0.1 mL) were mixed with 0.9 mL 75% FBS (pH 5.4) in a disposable cuvette at 37°C. At different time points (0, 0.1667, 0.5, 1, 2, 4, 8 and 24 h), the serum pH was adjusted to 7.4, and calcein fluorescence was measured at 490 and 520 nm through the fluorospectrophotometer before and after the addition of 10% Triton X-100, which represents the 100% calcein leakage from the vesicles. The percentage of calcein release was calculated using Formula (1).

Pharmacokinetics of Calcein-Loaded Vesicles *In Vivo*

Male KM mice were randomly divided into three groups for the pharmacokinetic studies on calcein-S, as well as conventional α-tocopherol derivatives (THST) and PEGylated vesicles. A dosage of 0.015 mL/kg calcein-labeled vesicles or calcein-S was injected *via* the tail vein. At 0.0167, 0.083, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 12.0 h following the IV injection, blood samples were collected *via* eye puncture. Plasma was obtained by centrifuging blood samples at 5,000×*g* for 10 min. The plasmas were kept at −20°C until analysis.

The concentration of calcein in plasma was assayed using a spectrofluorometric method. Briefly, 4.8 mL PBS (pH 7.4, containing 10% Triton X-100) was added to 20 μL plasma

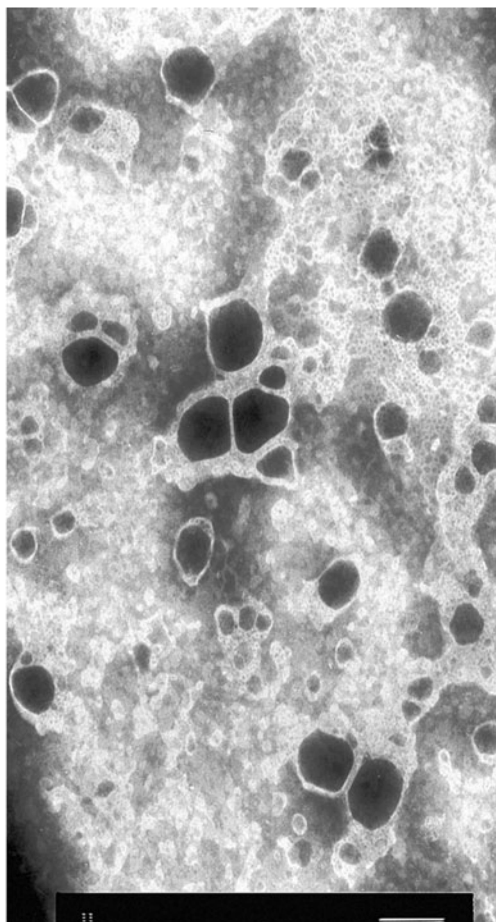

Fig. 2. The TEM photograph of THST vesicles, the *bar* represents 100 nm

samples. The mixture was assayed using a spectrofluorometer at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Statistical Analysis

Statistical comparisons were performed *via* the Student's *t* test for two groups and one-way ANOVA for multiple groups. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Morphology of the THST Vesicles

Vesicles prepared using the detergent removal method were investigated *via* electron microscopy. Figure 2 shows that the vesicles were spherical, and most were single-room. These results confirm that THST can form complete bilayer-structured vesicles in a buffer solution.

Tris salt was chosen to bond with THS to introduce additional hydroxyl groups and increase the hydrophilicity of THS (see Fig. 3 for the structures of Tris and THS). Otherwise, the large proportion of lipophilic fragments in the surfactant would lead to relatively poor stability of the vesicles (23). Aside from functioning as a drug delivery material, THS shows strong growth inhibition of a variety of tumor cells without affecting normal cells (1,24,25). Further exploration of the synergistic effect of THS with anticancer drugs is recommended.

The methods of vesicle preparation are basically the same as those for liposomes. In the present study, two methods, namely direct hydration and detergent removal, were investigated. The vesicles prepared *via* direct hydration must be passed through a microfluidizer or probe sonicator to decrease the particle size, thereby possibly reducing the entrapment efficiency. On the other hand, the detergent removal method allows the formation of smaller vesicles with high entrapment efficiency but without ultrasound or high-pressure homogenization. In addition, the detergent removal method is a very moderate approach and helps avoid the introduction of organic solvent residues.

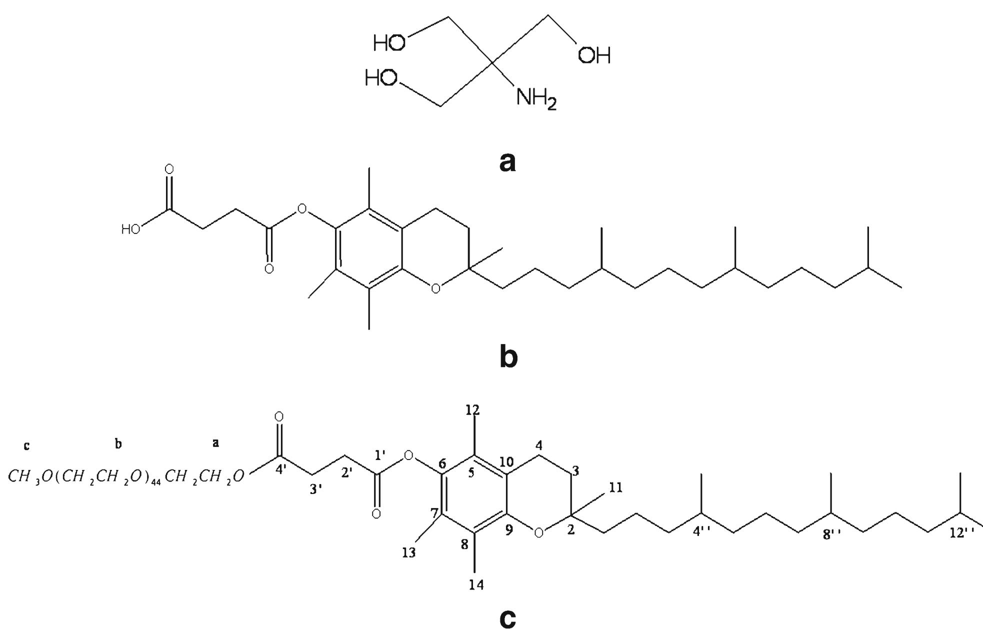

Fig. 3. Molecular structures of Tris **a**, THS **b** and PEG-THS **c**

Particle Size Distribution and Zeta Potential of THST Vesicles

The mean Gaussian particle size of the THST vesicles without PEGylation was 163.8±5.5 nm. The Nicomp analysis reveals two major particle size distributions, namely, 65.3±7.2 nm and 226.0±9.4 nm. Table I shows that the mean Gaussian particle size decreased with increasing proportion of PEG-lipid derivatives. This finding is consistent with those of previous studies (22,26). The small particle sizes allow a more compact packing of molecular layers, which reduces lateral repulsion and further enhances the stability of the vesicles.

The mean zeta potentials of the conventional THST and PEG-modified vesicles were determined using a zeta potential analyzer. As shown in Table I, increasing the PEG-lipid molar ratio reduces the absolute value of the zeta potential of the vesicles. PEG-lipid derivatives are neutral. When the vesicles are PEG-modified, they overlap and cover the vesicle surface, forming a compact conformation cloud (mushroom cloud), which then shields the negative charge carried by the vesicle itself (Fig. 4).

According to the DLVO theory, a strong electrostatic repulsion exists between particles when the particle system has a negative charge. PEGylation decreases the zeta potential, thereby reducing the electrostatic repulsion. However, this modification will not affect the stability of the vesicles. Given that the PEG chain exhibits good hydrophilicity and flexibility, it can produce a noticeable steric hindrance to increase the repulsion between particles (27,28). According to previous studies, when the absolute value of the zeta potential of a particle is close to zero, its capacity to avoid the phagocytic reticuloendothelial system is at its strongest (29,30).

Verification of the pH Sensitivity of the Vesicles

Effects of the Buffer pH on the THST Vesicle Particle Size

The changes in the particle size of the vesicles at different pH buffers are shown in Fig. 5. When the THST vesicles were incubated in pH 6.4 and 5.4 buffer solutions for 10 min, the particle size of the #2 peak increased by 98 and 173 nm, and that of the #1 peak increased by 57 and 33 nm, respectively, compared with those obtained after incubation at pH 7.40. When the vesicles were incubated in the pH 4.4 buffer, the mixed solution immediately produced visible precipitation. However, the particle size could not be determined, indicating that when the pH is reduced to a certain extent, the vesicles become very unstable. Extending the incubation times of the vesicles in the pH 6.4 and 5.4 buffer solutions also produced visible precipitation, indicating that the increase in the particle size in acidic solutions is time-dependent. When the incubation time at pH 7.4 was increased to 10 min or longer, no observable change in the particle size was detected.

Table I. Changes in the Mean Particle Size and Zeta Potentials of the Prepared Vesicles with the Increase of PEG-THS Concentration

Proportion of PEG-THS (%)	Mean particle size (nm)	Coefficient variation (CV)	Zeta potential (mv)
0	163.8±5.5	0.428±0.013	−74.5±6.4
2	145.3±4.8	0.405±0.026	−45.3±4.6
4	121.1±3.4	0.352±0.020	−31.4±3.3
6	98.2±3.8	0.385±0.015	−10.0±1.5
8	101.2±2.5	0.396±0.023	−1.3±0.3

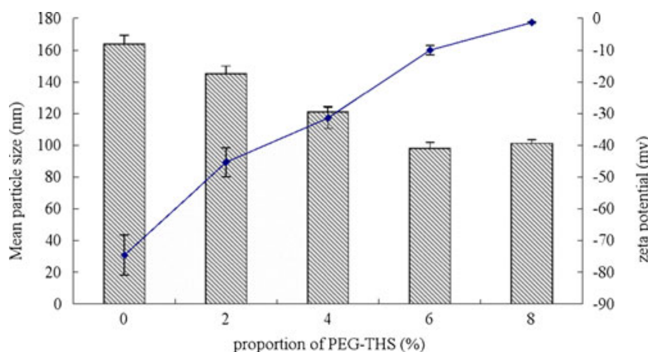

Fig. 4. Changes in the mean particle size and zeta potentials of the prepared vesicles with the increase of PEG-THS concentration

The particle size of liquid particles such as liposomes, vesicles, and emulsions often present an uneven (or multi-waveform) distribution. Nicomp analysis aids in understanding the actual particle distribution process. In the current study, the changes in the Nicomp and Gaussian diameters were used as comprehensive indicator values of vesicle stability. The results show that the Nicomp diameter is more suitable in characterizing the stability of the vesicles. As shown in Fig. 5, the amplification of the #1 peak at pH 6.4 was slightly larger than at pH 5.4, possibly because of the greater aggregate stability of small particles compared with that of large particles. The changes in the larger #2 peak may provide a better explanation for the stability of the vesicles. However, in the Nicomp analysis, the particle sizes of the primary and second peaks changed along with their proportion. Therefore, finding an appropriate comparison method for Nicomp is necessary.

Calcein Release of the THST Vesicles at Low-pH Buffer Solutions With or Without a Serum

Figure 6 shows that as the pH decreased, the calcein release rate of the PSVs increased with or without a serum. However, the presence of a serum slightly decreased the acid sensitivity of the vesicle. When the pH value reached 4.4, the

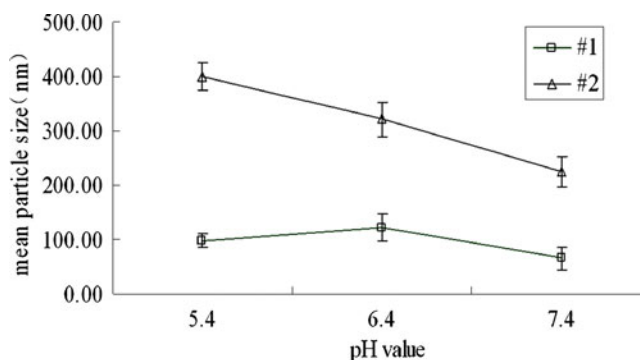

Fig. 5. pH-dependent particle size increase of THST vesicles incubated in sodium acetate buffer of various pH values for 10 min (#*1* small particle size peak; #*2* large particle size peak)

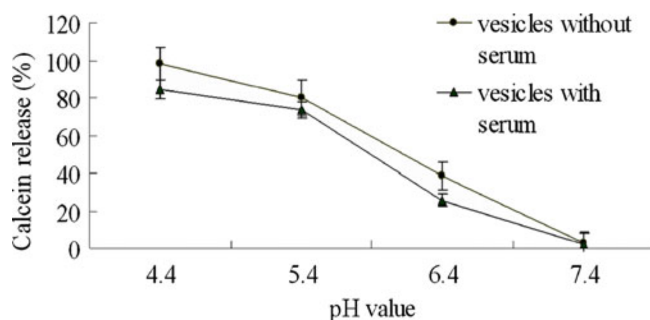

Fig. 6. Calcein release of THST vesicles incubated at sodium acetate buffer of various pH values with or without serum (37°C, 10 min)

percentage of calcein release was nearly 100%, indicating the pH sensitivity of the vesicles.

The pH sensitivity of the THST vesicles may be attributed to the low pK_a value of their carboxylic acid groups. The computer-generated stereogeometries of THS reveal that THS has hydrophobic portions adjacent to its hydrophilic moiety and flexible tails (31). When the pH value is neutral, the carboxyl ions of the fatty acids provide an effective electrostatic repulsion that keeps the vesicles in a stable layer phase. When the pH value decreases, carboxyl protonation of the fatty acids leads to the formation of a hexagonal phase (non-layer phase structure), and the stability of the vesicles decreases, causing them to aggregate, fuse, and release their contents.

The particle size of the THST vesicles increased with the decrease in pH, indicating the occurrence of vesicle aggregation or fusion. The calcein release results are consistent with the change in the particle size; that is, the pH decrease led to the destabilization of the vesicles and the release of the drug contents. The acid sensitivity of the vesicles decreased in the presence of 20% FBS, which is consistent with the conclusion of Shi (32). However, the reason for the decrease in the pH sensitivity remains to be determined.

PSLs are usually less stable in serum or plasma (4). The same can be said of THST vesicles. This instability is generally considered to be associated with the lipid composition of liposomes. In the present study, the vesicles were incubated in 20% FBS, which resulted in a significantly increased particle size (data not shown). The complement-mediated lysis,

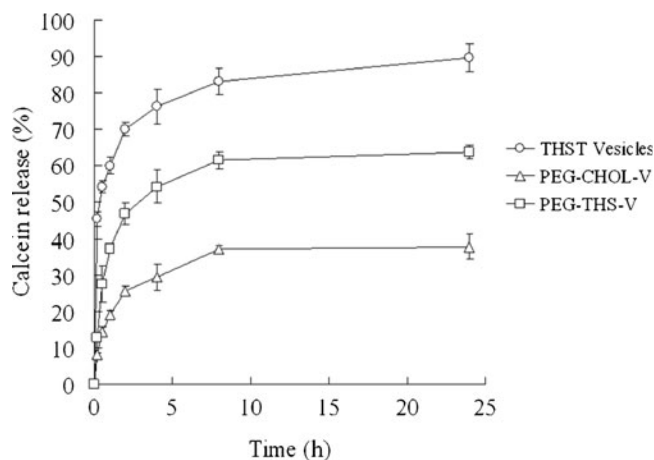

Fig. 8. PEG cleavage from vesicles modified with 6% molar ratio of PEG-lipid, when incubated for different time at 37°C in 75% FBS

destabilization by high-density lipoproteins, and bridging behavior of electrolytes such as calcium ions in plasma may be the reasons for the destabilization of the vesicles (33).

Verification of the Stability of PEG-THS-V

As shown in Fig. 7, when the molar ratios of PEG-THS were less than or equal to 4%, the mean particle size increased with the increase in incubation time and Ca^{2+} concentrations. However, when the PEG-THS ratio increased to 6%, the mean THST vesicle particle size was almost unchanged even when the incubation time was extended to 24 h and the Ca^{2+} concentration was increased to 10 mM.

Ca^{2+}-induced membrane fusion is widely used in the study of liposomes (13). In the current study, a $CaCl_2$ solution was used to determine the stability of the THST vesicles. The –COO– groups on the surface of conventional vesicles interact with Ca^{2+} in the plasma and induce vesicle fusion and aggregation. When the vesicles were modified with PEG-THS, the negative groups on the surface were shielded, resulting in increased vesicle stability. The Ca^{2+} stability of conventional and PEGylated vesicles was also investigated. Different concentrations of the $CaCl_2$ solution induced different changes

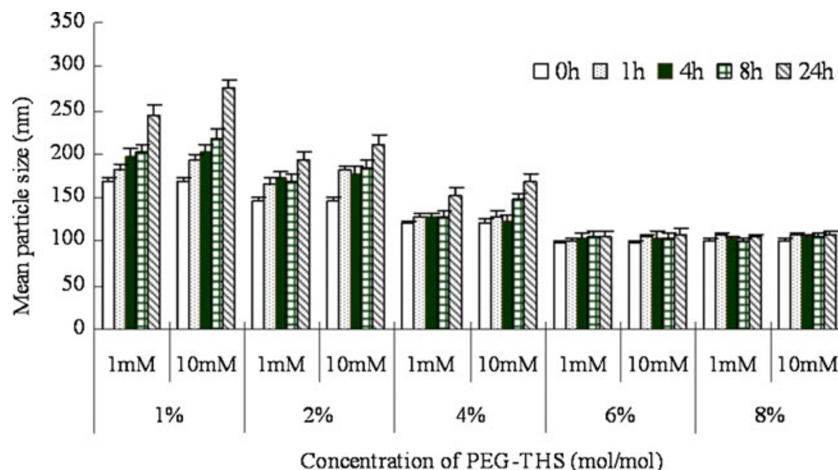

Fig. 7. Changes in the particle sizes of vesicles modified with PEG-THS incubated in different concentrations of $CaCl_2$ for different time

Table II. Analytical Recovery and Precision of Calcein in Plasma of Mice (n=5)

Conc. (μg·mL^{-1})	Recovery (%)	Intra-day RSD (%)	Inter-day RSD (%)
50.0	97.4±5.2	5.20	6.30
200	92.3±5.7	2.21	7.01
600	98.6±3.2	3.83	4.00

Table III. Pharmacokinetic Parameters of Calcein *In Vivo* Following i.v. Different Calcein Treatment Groups (n=3)

Parameter	Calcein-S	Calcein-TV	PEG-THS-V
$t_{1/2}$ (h)	0.38±0.05	0.43±0.04	2.80±0.61$^{\#\#}$
V_{ss} (mL/g)	5.54±2.91	1.74±0.32**	2.29±0.02
MRT (h)	0.50±0.03	0.86±0.17**	1.87±0.05$^{\#\#}$
AUC (μg·h/mL)	32.15±8.37	53.04±6.62*	146.44±4.42$^{\#\#}$
Cl (mL/min/g)	6.11±1.94	3.45±0.43**	1.23±0.04$^{\#\#}$

*P<0.1; **P<0.05, calcein-S group and calcein-TV
$^{\#\#}$ P<0.05; calcein-TV and PEG-THS-V

in the conventional vesicles. When the Ca^{2+} concentration was 10 mM, the vesicle solution immediately became turbid, indicating visible precipitation. When the concentrations were 5 and 2 mM, the vesicle solution became turbid after 5 and 10 min, respectively. When the concentration was 1 mM, the mean particle size of the vesicles increased in a time-dependent manner even though no visible change was observed (data not shown). Therefore, the THST vesicles are Ca^{2+}-unstable. As demonstrated in Fig. 7, the Ca^{2+} stability of the vesicles significantly improved after modification by PEG-THS, especially when the PEG-lipid ratio was above 6%.

The Debye–Hückel method was used to calculate the PEG layer thickness on the surface of the vesicles formed from the tris(hydroxymethyl) aminomethane salt of cholesteryl hemisuccinate (CHST). When PEG-cholesteryl hemisuccinate-modified CHST vesicles were exposed to Ca^{2+} solutions at 0%, 3%, and 7% molar ratios, the PEG layer thickness was 0.48, 2.58, and 2.82, respectively. This result indicated that the thickness of the protective layer increased with the increase in the PEG ratio. The vesicles became less sensitive to the ionic strength of the medium because of the repulsion between PEG layers (33).

Therefore, the role of PEG is to prevent the vesicles from coming into close contact with one another and prevent the bridging action of calcium ions (*via* electrostatic interaction). When the PEG concentration was low (<4%), PEG could not completely prevent this electrostatic bridging; thus, the presence of calcium could still increase the particle size of the vesicles (Fig. 7). Previous studies have shown that when the molar ratio in the prescription is less than or equal to 7%, PEG-lipid derivatives can produce a very good, sustained circulating effect (34). Thus, the present study used vesicles containing 6% PEG-THS for the subsequent experiments.

Reconstruction of the pH Sensitivity of THST Vesicles by PEG-THS

As shown in Fig. 8, the calcein release rate of conventional THST vesicles already reached 45%±1.98% at 0.1667 h and more than 80% at 8 h. These results suggest that the stability of the THST vesicles in an acid serum is very poor. When the vesicles were modified with PEG-lipid derivatives, their stability significantly increased. By contrast, the calcein release by PEG-CHOL-V was always at low levels and had not reached 40% after 24 h, indicating that although the stability of vesicles can significantly be enhanced, the pH sensitivity of the vesicles at low pH is reduced. For the PEG-THS-V, the calcein release rate was above 60% at 24 h, which is between the release rates of the aforementioned two vesicles. When the PEG-THS-V was incubated in a low-pH buffer solution, only a negligible amount of calcein was released (data not shown). Aside from effectively playing a protective role, PEG-THS could also reconstitute the pH sensitivity of PSVs in an acid serum. Notably, the calcein release by PEG-THS-V was higher than those by PEG-CHOL-V (P< 0.05). In our previous study, the chemical bonds between PEG and the lipids were proven to be ester bonds, implying that the PEGs can be gradually cleaved by esterases in plasma and tissues (22). The bond between PEG and THS is a carboxylic ester bond, whereas that between PEG and CHOL is an ether bond. Ether bonds are usually more stable than ester bonds, which is consistent with the current results.

Although the ability of PEG-THS to increase vesicle stability may be weaker than that of PEG-CHOL, PEG-THS may still be an acceptable material for vesicle preparation.

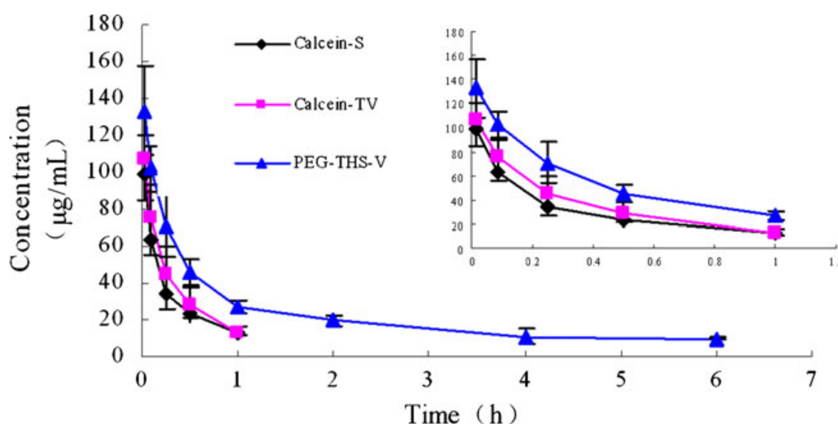

Fig. 9. Mean plasma concentration-time profiles of calcein after i.v. administration of calcein solution and different calcein THST vesicles (n=3)

Given that blood circulation in humans only takes about 40 s to complete one cycle (35), PEGylated vesicles have enough time to reach the target sites as long as they remain stable within that brief period. On the other hand, a very high PEG-lipid stability is not conducive to the reconstruction of the pH sensitivity of vesicles.

Plasma Concentration and Pharmacokinetic Parameters

In vivo methodological investigation (Table II) shows that the spectrofluorometric method used for determination of calcein plasma concentration was stable and reliable. As shown in Fig. 9, the free calcein and conventional vesicles were quickly eliminated from the plasma after injection. Calcein could not be detected 1 h after injection. PEG-THS-V significantly changed the general blood distribution of the conventional vesicles and solution.

The pharmacokinetic parameters were calculated by a non-compartment model, as shown in Table III. The results showed that the THST vesicles could not increase the $t_{1/2}$ of calcein ($P>$ 0.1). The $t_{1/2}$ of PEG-THS-V encapsulating calcein were 7.34 and 6.51 times longer than that of the un-PEGylated vesicles and solution, respectively. On the other hand, the AUC of the PEGylated vesicles were 4.55 and 2.76 times greater than that of the conventional vesicles and free calcein ($P<0.05$), respectively.

A comparison of the pharmacokinetic parameters and plasma concentration-time curve shows that almost no difference can be observed in the plasma distribution between conventional THST vesicles and the solution group. This result is consistent with that of calcium ions and plasma stability experiments *in vitro*. That is, ordinary THST vesicles were very unstable and were likely to rapidly release the encapsulated fluorescent material in the plasma, as well as a physiological concentration of calcium ion solution. The $t_{1/2}$ and AUC of the PEG-THS-V increased significantly in the mice. This result indicates that the use of PEG-lipid derivatives has gained a favorably long circulation effect.

α-Tocopherol could be used as a film-forming material that acts as an antioxidant in liposomes. In the present study, THST was used as pH-sensitive materials to construct pH-sensitive vesicles, which are expected to retain their advantages in liposome applications. PEG-THS could extend the circulation time of THST vesicles *in vivo*, indicating that these PEG-lipid derivatives could be applied to different membrane vesicles or liposomes.

CONCLUSIONS

PSVs composed of THST were prepared in the present study. The vesicles exhibited a very favorable pH response, with significant changes in the particle size and favorable release profiles of contents under low pH conditions. After modification by cleavable PEG-THS, the stability of the vesicles in high concentrations of electrolyte solutions increased. Meanwhile, the pH sensitivity of the vesicles was reconstructed using PEG-THS in an acid serum. Therefore, cleavable PEG-THS can efficiently protect vesicles such as conventional PEG-lipids and reconstruct the acid sensitivity of vesicles in low-pH tissues. The results of the current study could serve as references for the improvement of the stability of conventional pH-sensitive preparations.

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