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pH-Sensitive mPEG-Hz-Cholesterol Conjugates as a Liposome Delivery System

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ABSTRACT: Hydrazone (Hz)-based pH-sensitive methoxy(polyethylene glycol)-cholesterol conjugates (mPEG-Hz-Chol), were synthesized and used to fabricate liposomes. The structures of the mPEG2000-Hz-Chol conjugate were confirmed by FT-IR and ^{1}H-NMR; they were stable at pH 7.4 but sensitive to mild acid conditions (pH 5.5). Plain liposomes were also prepared with S100PC/Chol, and the pH-insensitive liposomes with S100PC/Chol/mPEG2000-Chol for comparison; all the liposomes were similar in diameter ~200 nm. *In vitro*, the pH-sensitive liposomes released more of the model drug, paclitaxel (PTX), than the plain liposomes. The pH-sensitive liposomes were less toxic than the plain liposomes and exhibited higher cellular uptake of PTX compared with pH-insensitive liposomes by human breast cancer cells (MCF-7). The pH-sensitive mPEG2000-Hz-Chol liposomes are now being investigated as a potential new liposome drug delivery system.

KEY WORDS: mPEG2000-Hz-Chol, pH-sensitive liposomes, paclitaxel, cytotoxicity, cellular uptake.

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INTRODUCTION

Liposomes as drug carrier can improve the drug treatment by reducing the adverse effects. Many liposome studies have been made to develop a wide variety of amphiphilic associated conjugates containing a poly(ethylene glycol) (PEG) segment as a modifier, such as PEG bonded to dimyristoyl phosphatidylethanolamine (DMPE–PEG) [1] or to distearoyl phosphatidylethanolamine/methoxy (DSPE–PEG) [2]. PEG enhances blood circulation; however, when the drug carrier is intended for intracellular penetration PEG often prevents normal interaction of the carried drug with cells [3].

To solve this problem, several approaches have been tried, such as using pH-sensitive liposomes. The concept of pH-sensitive liposomes as drug carriers is based on the fact that many pathological sites, including tumors, demonstrate acidic properties. For example, Kale and Torchilin [4] developed cell-penetrating active PEGylated TATp-modified pH-sensitive liposomes with a pH-sensitive protective coating of PEG-Hz-PE. At $pH < 6$, the PEG coating is detached and the internalization of the nanocarriers into target cells is enhanced. Similarly, Shin et al. [3] developed pH-sensitive PEG-PE-modified liposomes with acid-labile vinyl ethers with the PEG coating, which was detached at lower pHs.

Paclitaxel (PTX) is a drug that is effective against a broad spectrum of cancers, especially carcinomas of the breast, ovary, and lung [5,6]. However, due to its low aqueous solubility, it is formulated with polyethoxylated castor oil and ethanol (Cremophor®EL). Unfortunately, many patients are hypersensitive to Cremophor®EL: therefore, another delivery formulation is needed for effective delivery [7,8].

Phosphatidylethanolamine (PE), which is used for liposome preparation, is very expensive and difficult to obtain in large quantities. Therefore, it is impractical and unsuitable for therapeutic and clinical applications. Cholesterol (Chol) may be a better candidate as it confers lipid bilayer cohesion, thereby increasing the half-life of liposomes *in vivo*. Also mPEGylated cholesterol molecules are easy to synthesize in large amounts [9].

Consequently, with this in mind, we studied the physicochemical characteristics of a mPEG-Hz-Chol conjugate with a hydrazone bond. Based on these results, an *in vitro* pH-dependent degradation and the *in vitro* release of PTX from mPEG2000-Hz-Chol-modified liposomes were explored.

MATERIALS AND METHODS

Soybean phosphatidylcholine (S100PC) was purchased from Dongshang Co. Ltd. (Shanghai, China). Cholesterol (Chol) and succinic anhydride were obtained from China Medicine Shanghai Chemical Reagent Corporation (Shanghai, China). Dicyclohexyl carboimide (DCC), 4-(dimethyl amino) pyridine (DMAP), mPEG2000, and ether 3-(4,5-dimethyltiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (MO, USA). PTX was supplied by Taihua Natural Plant Pharmaceutical Co. Ltd. (China). Sephadex G-25 were obtained from Sigma Chemical Co. (MO, USA). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate or DiI was purchased from Beyotime Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade.

MCF-7 human breast cancer cell line was obtained from the New Drug Screening Center of China Pharmaceutical University. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

Synthesis of Cholesterylhemisuccinate

Cholesterol (2.0 g, 5 mmol) and succinic anhydride (1.5 g, 15 mmol) were dissolved in CH_2Cl_2 (40 mL) and DMAP (0.1 g, 0.8 mmol) was added as the catalyst. An excess of succinic anhydride was used to ensure that the hydroxy terminals were completely reacted while the mixture was stirred at 65°C for 12 h. The unreacted succinic anhydride was removed by filtration and the CH_2Cl_2 was removed by rotary evaporation. The residue was washed with copious amounts of water and dried overnight. A white powdery product was obtained with 59% yield. The IR spectra were recorded on a Bruker Fourier transform infrared (FT-IR) Tensor 27 spectrophotometer (KBr disk): 3469(νOH), 2946(νCH_3), 2895, 2866, 1731(ester $\nu C=O$), 1709(carboxylic acid $\nu C=O$), 1467, 1285, and 1178 (νC–O).

Synthesis of Hydrazide-Activated Cholesterylhemisuccinate

Cholesterylhemisuccinate (CHEMS) 0.5 g, 1 mmol, with 0.5 mL of $SOCl_2$, was stirred at 43°C for 3 h. The solvent was removed and the

residue was dissolved in CH_2Cl_2. Hydrazine hydrate (0.3 mL) was added at 0°C and the mixture stirred for 3 h. The liquids were removed and the residue was dissolved in CH_2Cl_2. After purification on an activated silica gel column a white powdery product was obtained with 61% yield. IR (KBr, cm^{-1}): 3416(νNH_2), 3306(νNH), 3249(νNH_2), 2948, 2898, 2866, 1730(ester $\nu C=O$), 1665(amide $\nu C=O$), 1633(δNH_2), 1591(βNH), 1189(νC–O), and 996.

Synthesis of activated mPEG

A solution of mPEG2000 (2 g, 1 mmol) in dichloromethane was added to p-acetylbenzic acid (1.5 g, 10 mmol), DCC (2.05 g, 10 mmol), and DMAP (0.3 g, 2.5 mmol) and stirred for 24 h. The filtrate was concentrated, dissolved in isopropanol (20 mL), and cooled at 0°C for 2 h. The crystals that formed were collected and washed with isopropanol and diethyl ether. A light yellow powder was obtained in 80% yield. IR (KBr, cm^{-1}): 3500–3400(νOH), 3409(νOH), 2892, 1734($\nu C=O$), 1650($\nu C=C$), 1465(δCH_2), 1351, 1276, and 838.

Synthesis of mPEG2000-Hz-Chol Conjugate

Activated mPEG2000 (0.12 g, 0.06 mmol) were reacted with an excess of hydrazine activated cholesterol in 2 mL of chloroform at 25°C. After stirring overnight, the chloroform was evaporated in a rotary evaporator. The excess activated mPEG2000 was separated from mPEG2000-Hz-Chol conjugates by gel filtration chromatography using Sephadex G-25. The mPEG2000-Hz-Chol conjugate fractions were kept at −60°C overnight and then freeze dried. The mPEG2000-Hz-Chol conjugate was recovered as a white powder with 53% yield. IR (KBr, cm^{-1}): 3430(νOH), 3224(νNH), 2939, 2846, 1724(ester C=O), 1647(amide $\nu C=O$, $\nu C=N$), 1599($\nu C=C$), 1576, 1503($\nu C=C$), and 910–665(γ=CH). ^{1}H-NMR was performed on a 300 MHz Bruker NMR spectrometer using $CDCl_3$ as a solvent: 4(m, 43H, $-CH_3$, $-CH_2$, and –CH (cholesterol), 2.58–2.80(d, 4H, $OCOCH_2CH_2COOH$, succinyl group), δ2.9–4.6(m, $-CH_2$, $-OCH_3$ (mPEG), δ7.4–8.4(m, $-C_6H_4$, δ4.62–4.65 (d,1H, C_3–H, Chol), and δ5.37 (s, 1H, C_6–H, Chol).

In Vitro pH-dependent Degradation of the mPEG2000-Hz-Chol Conjugate

The mPEG2000-Hz-Chol conjugate was divided into equal volumes and incubated at 37°C at pH values 7.4, 6.5, 6.0, and 5.5, respectively.

The mPEG2000-Hz-Chol conjugate was analyzed at different intervals using reverse-phase high performance liquid chromatography (RP-HPLC; HP1100, Agilent, USA) [10] with a mixture of methanol and water (95 : 5, v/v) as the mobile phase. A Diamohsile column C18 ($4.6 \times 250\,mm^2$, Dikma, Beijing, China) was used at 25°C with a 1.0 dmL/min flow rate with a 300 nm UV detector, HP1100, Agilent, USA. The injected volume of the sample was 20 μL. As a control, a pH-insensitive conjugate mPEG2000-Chol was prepared and analyzed.

mPEG-Hz-Chol and mPEG-Chol-modified pH-insensitive Liposomes

The plain liposomes were composed of S100PC/Chol (90 : 10), the pH-insensitive liposomes were composed of S100PC/Chol/mPEG-Chol (90 : 10 : 3) and the S100PC/Chol/mPEG2000-Hz-Chol (90 : 10 : 3), were prepared by the thin-film hydration. The hydrophobic excipients, PTX, cholesterol, and lipids, were dissolved in chloroform and transferred into a conical flask and the solvent evaporated under reduced pressure. The dry lipid formed was hydrated with phosphate buffer saline (PBS; pH 7.4) and the liposomal suspension was filtered through 0.2 μm polycarbonate filters and stored at 4°C.

The particle sizes and zeta potentials of the liposomes were measured using a photon correlation spectrometer light scattering apparatus zeta potential/particle sizer 3000HS (Malvern Instruments, UK) and analyzed by the Zetasizer 3000H (Malvern software).

The entrapment efficiency (EE) and the drug loading were determined from three liposome samples containing PTX. An aliquot of liposome was treated with 50 times volume of methanol to disrupt the liposome and amount of PTX was determined by RP-HPLC (HP1100, Agilent, USA). The mobile phase was a mixture of methanol and water (75 : 25, v/v) with the Diamohsile column was used as described above. The EE and drug loading were calculated from the following equation:

$$\text{EE}\ (\%) = \frac{\text{Amount of PTX in liposome}}{\text{Amount of total PTX}} \times 100. \quad (1)$$

$$\text{Drug loading}\ (\%) = \frac{\text{Amount of PTX in liposome}}{\text{Amount of freeze} - \text{Dried liposome}} \times 100. \quad (2)$$

In Vitro Release

To assay the *in vitro* release characteristics of PTX from plain liposomes, mPEG2000-Chol-modified pH-insensitive liposomes, and mPEG2000-Hz-Chol-modified pH-insensitive liposomes, dialysis was used at room temperature with plain liposomes as the control. An aliquot of each liposomes (0.1 mL) was put into a dialysis tube and was tightly sealed. The tube was then immersed in 200 mL of PBS (pH 7.4) containing 0.1% (v/v) Tween 80 [11,12]. Samples (0.5 mL) were taken at predetermined times from the stirred medium for 24 h, with concomitant replacement with the same volume of fresh medium. The concentration of PTX was determined by HPLC after diluting with acetonitrile without further treatment.

In Vitro Cytotoxicity

The cytotoxicity of PTX with plain and mPEG2000-Hz-Chol-modified pH-sensitive liposomes against MCF-7 breast tumor cells was determined by using the MTT assay, with Taxol® as the control. Flat-bottomed, 96-well tissue-culture plates were seeded with 2.0×104 cells per well and incubated at 37°C in a 5% CO_2 incubator for 24 h. The liposomes were diluted with culture media to make specific concentrations of PTX and added to the wells (200 μL each in triplicate). Control wells were treated with equivalent volumes of PTX-free media. After 24 h (or 72 h), the supernatant was removed and MTT (0.5 mg/mL) in PBS (pH 7.4) and culture medium (100 μL each) was added to each well and incubated for 4 h. Each well was washed with 200 mL of PBS after which was added 200 μL of dimethyl sulfoxide (DMSO) to dissolve the MTT formazan crystals. Plates were shaken for 15 min and the absorbance was read at 560 nm using a microplate reader. The IC_{50} values of PTX were calculated from concentration effect curves, considering the control well as 100% [13].

Cellular Uptake

A mixture of S100PC/Chol (90 : 10), DiI, and either mPEG2000-Hz-Chol (pH-sensitive) or mPEG2000-Chol (pH-insensitive) at a molar ratio 10 : 0.3 : 0.3 was evaporated under reduced pressure. The dry lipid obtained was hydrated with 10 mL of PBS (pH 7.4). The liposomal suspension was filtered through 0.2 μm polycarbonate filters and stored at 4°C until use.

For qualitative analysis, MCF-7 human breast cancer cells were seeded onto coverslips in six-well plates in 1 mL of DMEM containing 10% FBS and then incubated for 48 h. Then the plates were washed twice with PBS. The DiI-labeled pH-sensitive liposomes (mPEG2000-Hz-Chol, pH-insensitive liposomes, and mPEG2000-Chol-modified liposomes) were preincubated for 5 h at pH 5.5 and pH 7.4. The different DiI-labeled liposome samples were added and incubated at 37°C for 2 h. Then the medium was removed and the plates washed with serum-free medium three times. The cells were viewed with an Olympus IX51 microscope and the images analyzed using DP controller software [14].

To quantify the level of PTX in the cells, MCF-7 cells were seeded in a 24-well plate at a seeding density of 1×106 cells per well in 1 mL of growth medium [15]. After 24 h, medium was changed with 1 mL of each culture media containing pH-sensitive liposomes or pH-insensitive liposomes with 100 ng/mL of PTX after both the liposomes were preincubated for 5 h at pH 5.5 and pH 7.4. Then different liposome samples were added and incubated at 37°C for 2 h. Then the cells were washed with cold PBS twice and the remaining cells were destroyed with 0.1 mL of 10% Triton and acetonitrile was added to precipitate the protein. The drug content in the supernatant after centrifugation was measured by RP-HPLC. Cellular uptake efficiency was calculated using the following formula:

$$\text{Cellular uptake efficiency (\%)} = \frac{\text{Amount of PTX in cells after incubation}}{\text{Amount of PTX in liposomes added to cells}} \times 100. \quad (3)$$

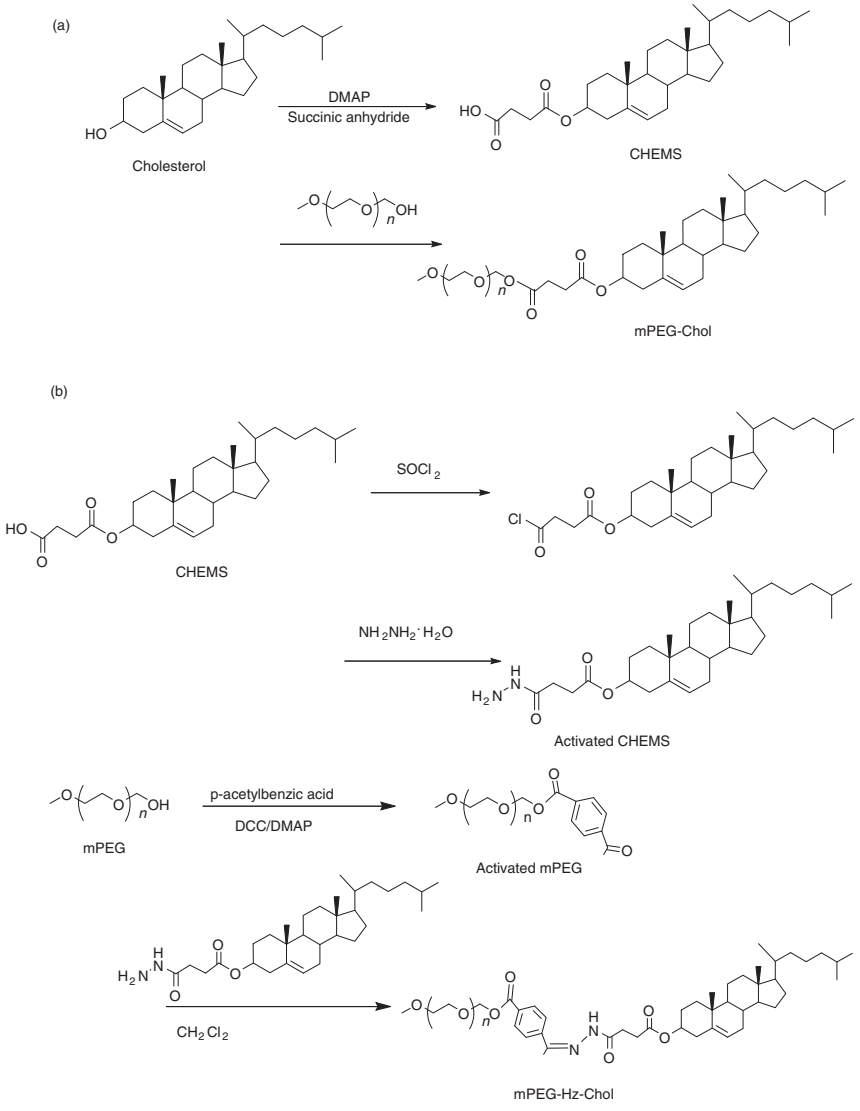
Statistical Analysis

All data were expressed in the form of the mean ± standard deviation. For comparison of mean values between the formulations, the Student's *t*-test was used. In all cases, $p < 0.05$ was accepted as denoting a statistical difference.

RESULTS

The synthesis routes for the mPEG2000-Chol and mPEG2000-Hz-Chol conjugates were shown in Scheme 1. The mPEG2000-Hz-Chol conjugates were prepared in three steps as it is difficult for directly react the three position hydroxyl on cholesterol with the long chain

PEGs. Therefore, CHEMS was mixed with excess of $SOCl_2$ to form the acyl chloride which was then reacted with hydrazine hydrate to acyl hydrazides. In the next step, activated mPEG was reacted with the Chol acyl hydrazide to form mPEG-Hz-Chol. The structures were verified by



Scheme 1. Reaction scheme for the synthesis of mPEG2000-Chol (a) and mPEG2000-Hz-Chol (b).

IR and ^{1}H-NMR spectroscopic methods. The carbonyl stretching vibration of ester linkage was located at 1730–1735 cm^{-1}, which indicated the formation of ester by the reaction between the hydroxyl group in mPEG2000 and the carboxylic group of CHEMS.

The degradation kinetics of mPEG2000-Hz-Chol at pH 7.4, 6.5, 6.0, and 5.5 in buffer solutions at 37°C, determined by HPLC, indicated that the degradation kinetics of mPEG2000-Hz-Chol was pH-dependent degradation (Table 1). The mPEG2000-Hz-Chol degraded at pH 5.5 with a 6.7 h half-life, while at physiological pH the half-life was 40.9 h. The pH-sensitive test indicated that the mPEG-Hz-Chol was stable in neutral solution (pH 7.4) while sensitive to mild acidic environments (pH 5.0–6.0). This PEGylated cholesterol synthesis (Scheme 1) can be easily carried out on an industrial scale.

Characteristics of pH-sensitive Liposomes

The transmission electron microscopy (TEM) images of both the plain and modified liposomes, shown in Figure 1, are discrete round structures

Table 1. pH-Sensitive hydrolytic kinetics of mPEG2000-Hz-Chol conjugate incubated in different pH buffer solutions at 37°C.

Conjugate	pH	Half-life (h)
mPEG2000-Hz-Chol	7.4	40.9 ± 8.7
	6.5	18.8 ± 1.6
	6.0	12.3 ± 2.4
	5.5	6.7 ± 1.8

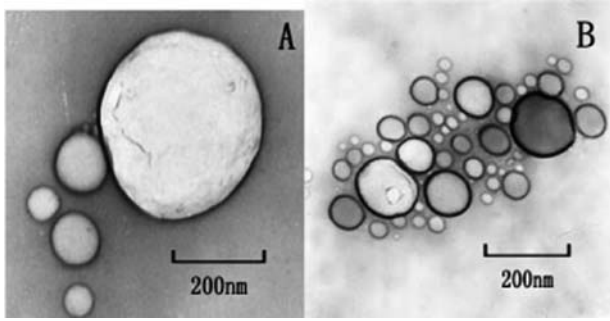


Figure 1. The TEM images of the liposome systems: (A) plain liposome; (B) mPEG2000-Hz-Chol-modified pH-sensitive liposome.

Table 2. The physicochemical parameters of pH-sensitive liposomes and plain liposomes.

Parameters	Plain liposomes	pH-sensitive liposomes
EE (%)	89.7 ± 1.4	92.2 ± 2.4
Drug loading (%)	2.43 ± 0.06	2.54 ± 0.08
Particle size (nm)	116.4 ± 4.5	132.6 ± 3.4
Zeta potential (mV)	−20.9 ± 1.6	−19.1 ± 2.1

that ranged in size from 100 to 200 nm with no significant differences. The EE of the pH-sensitive liposomes was slightly higher than the plain liposomes (Table 2); however, both were >90%. The encapsulation of PTX did not affect the particle size of the liposomes and the drug loading and zeta potential of the pH-sensitive liposomes were only slightly higher than the plain liposomes. Since small particles (<200 nm) are known to increase the accumulation of drug in the tumor *via* enhanced permeability and retention (EPR) effect [16,17], in this article the particle sizes of pH-sensitive liposomes were prepared within the 200 nm range.

In Vitro Release

To determine the pH-sensitivity of the modified liposomes, plain liposomes (PTX-loading 2.18%), mPEG2000-Chol-modified pH-insensitive liposomes (PTX-loading 2.36%), and mPEG2000-Hz-Chol-modified pH-sensitive liposomes (PTX-loading 2.54%) were prepared. The *in vitro* release behavior of the mPEG2000-Chol and mPEG2000-Hz-Chol liposomes is shown in Figure 2(a) and (b). The drug release was mainly by diffusion. The cumulative PTX release from the plain liposomes was 40.3 ± 2.6% for 24 h at pH 7.4, while from the mPEG2000-Chol and mPEG2000-Hz-Chol-modified liposomes, it was 18.6 ± 2.3% and 19.8 ± 4.5%, respectively (Figure 2(a)). Due to the steric hindrance of the PEG chain, the liposomes became more stable and, therefore, release from the inner part of the liposomes was more difficult. In pH 5.5 PBS (Figure 4(b)), the cumulative releases of PTX from the plain liposome and the mPEG2000-Chol-modified liposomes were 48.6 ± 5.0% and 20.4 ± 1.9% in 24 h, respectively, while in mPEG2000-Hz-Chol-modified liposomes the release was 65.8 ± 5.4%. For different values of pH, there were no releasing differences in the plain liposomes and mPEG2000-Chol-modified liposomes, while there were significant differences in mPEG2000-Hz-Chol-modified liposomes. The difference

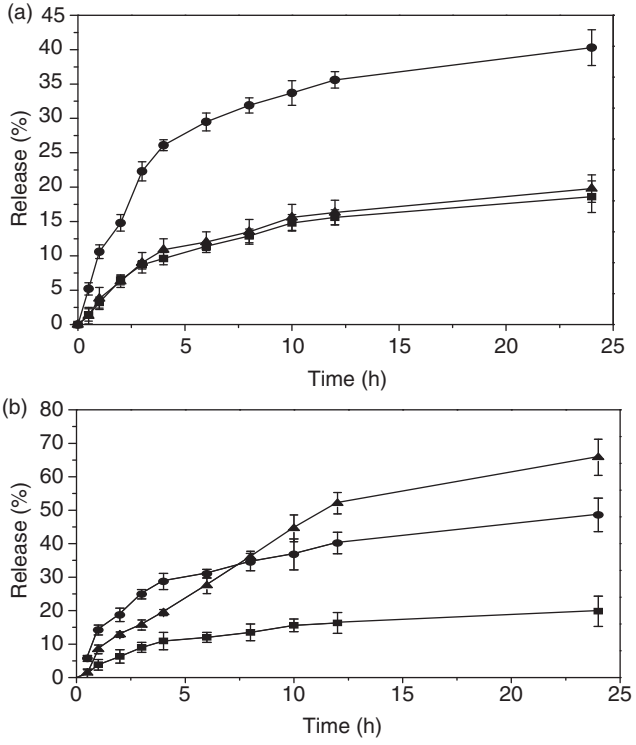


Figure 2. Release of PTX from the liposomes. (a) Release of PTX from plain liposome (PTX-loading 2.18%) (●), mPEG2000-Chol-modified pH-insensitive liposomes (PTX-loading 2.36%) (■), and mPEG2000-Hz-Chol-modified pH-sensitive liposome (PTX-loading 2.54%) (▲) into medium containing 0.1% (v/v) Tween 80 at pH 7.4. (b) Release of PTX from plain liposome (●), mPEG2000-Chol-modified pH-insensitive liposome (■), and mPEG2000-Hz-Chol-modified pH-sensitive liposomes (▲) into medium containing 0.1% (v/v) Tween 80 at pH 5.5 (mean ± SD $n = 3$).

was expected as mPEG could degrade and detach from liposomes under acidic condition, which leads to rapid drug release [18,19].

In Vitro Cytotoxicity

The cytotoxicity of plain and pH-sensitive liposomes containing PTX was evaluated by MTT against MCF-7 breast cancer cells, with Taxol® as the control. The IC_{50} values of Taxol®, plain liposomes (PTX-loading 2.28%), and pH-sensitive liposomes (PTX-loading 2.37%) at two different incubation times are shown in Table 3. Significantly higher IC_{50} values were observed for both liposomal formulations and after

Table 3. Cytotoxicity of PTX in Taxol®, plain liposome (PTX-loading 2.28%), and pH-sensitive liposome (PTX-loading 2.37%) against MCF-7 breast cancer cells (mean ± SD. n = 3).

	IC_{50} (nM)	
Formulation	24 h	72 h
Taxol®	153.87 ± 8.81	83.37 ± 8.98
Plain liposomes	183.33 ± 14.68	98.53 ± 14.65
pH-sensitive liposomes	287.83 ± 27.67	126.27 ± 12.21

24 h, compared to the Taxol® control. The pH-sensitive liposomes were less toxic than the plain liposomes after 24 h, as the drug release by the modified liposomes was slower than the plain liposomes (Figure 2(a)). However, after 72 h, the toxicities of all the liposomal formulations were similar to Taxol®, which is in accordance with the results of the release study, probably due to the slower release of PTX from the liposomes.

Cellular Uptake

The cellular uptake of the pH-sensitive liposomes was qualitatively evaluated by using DiI-labeled liposomes and quantitatively using PTX-loaded liposomes. There were no significant differences at pH 7.4 of the cellular uptake by the fluorescent DiI-labeled pH-sensitive liposomes, as shown in Figure 3. However, the fluorescence intensities were obviously enhanced while the pH-insensitive liposomes at pH 5.5 were not. Therefore, the cellular uptake of the pH-sensitive liposomes at pH 5.5 was greater than at pH 7.4. The pH-insensitive liposomes, as the controls, showed no significant differences at either pH 5.5 or 7.4.

The cellular uptake of the pH-sensitive liposomes with PTX, was quantitatively evaluated and the cellular uptake efficiency calculated. As shown in Figure 4(a), PTX in pH-sensitive liposomes (PTX-loading 2.37%) showed higher uptake at pH 5.5 than the pH-insensitive liposomes (PTX-loading 2.28%). The cellular uptake efficiency of PTX in pH-sensitive liposomes was 23.8% at pH 5.5, which was about 1.8 times greater than that at pH 7.4.

Moreover, no significant difference was observed between the two liposome types at pH 7.4. A possible mechanism for the uptake could involve a shielding/deshielding effect of the PEG corona from the liposomal surface. The PEG chain can be detached from mPEG2000-Hz-Chol under acidic conditions (pH 5.5), while the PEG on mPEG2000-Chol cannot. It is interesting to note that the PTX cellular uptake efficiencies

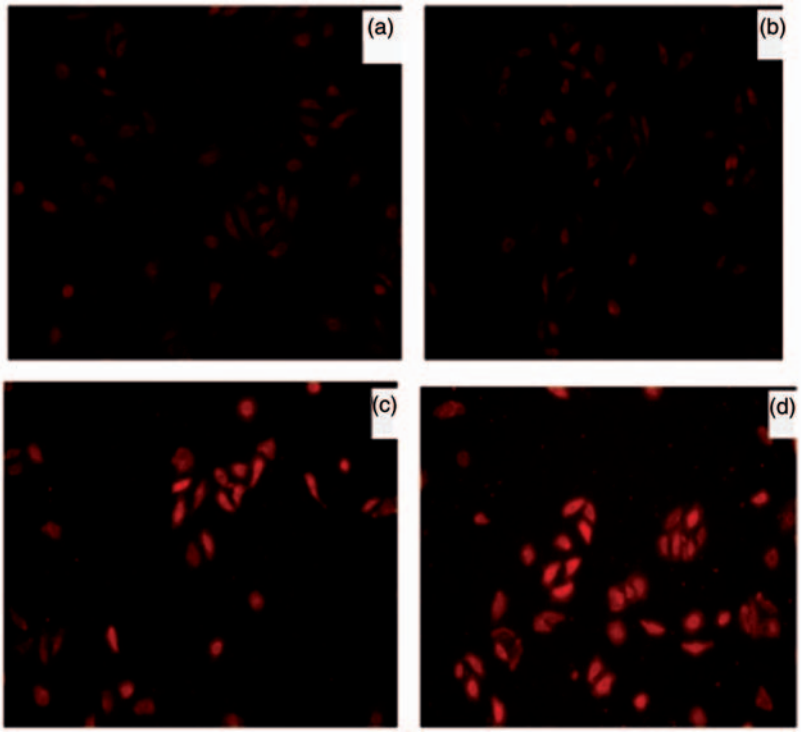


Figure 3. Fluorescence images after the MCF-7 breast cancer cells were incubated for 2 h with the DiI-labeled pH-sensitive and pH-insensitive liposomes: (a) pH 7.4 pH-insensitive liposome; (b) pH 7.4 pH-sensitive liposome; (c) pH 5.5 pH-insensitive liposome; and (d) pH 5.5 pH-sensitive liposome.

in pH-sensitive liposomes and pH-insensitive liposomes were significantly different when the pH was reduced to 5.5 (Figure 4(b)).

CONCLUSION

Novel mPEG-Hz-Chol conjugates were synthesized and characterized by FT-IR and ^{1}H-NMR. The pH-sensitive tests indicated that the mPEG-Hz-Chol structures were stable in neutral solutions (pH 7.4), but highly sensitive to mild acidic environments (pH 5.0–6.0). The physicochemical characterization of the liposomes, such as, drug loading, size, zeta potential, morphology, and EEs showed good reproducibility. The *in vitro* release by the mPEG2000-Hz-Chol-modified pH-sensitive liposomes showed greater accumulative release at pH 5.5 than at 7.4. The cellular uptake indicated that the uptake of

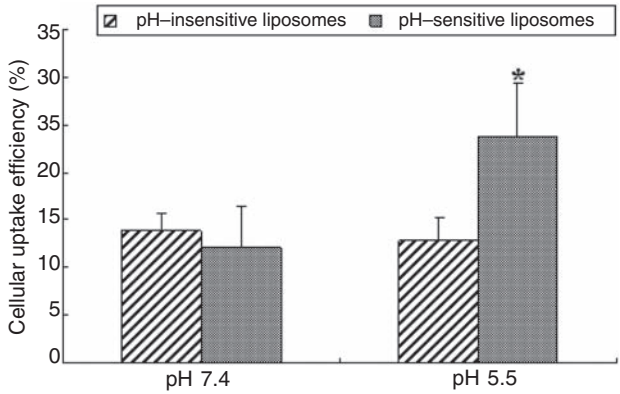


Figure 4. The cellular uptake of PTX-loaded pH-sensitive liposomes (PTX, 2.37%) and pH-insensitive liposome (PTX, 2.28%) by MCF-7 breast cancer cells incubated for 2 h compared with pH-insensitive liposomes at pH 5.5 (mean ± SD $n = 3$). $^*p < 0.05$.

mPEG2000-Hz-Chol-modified pH-sensitive liposomes at pH 5.5 was greater than at pH 7.4, while the pH-insensitive liposomes, as the control, showed no significant differences at pH 5.5 and pH 7.4, in both the qualitative and quantitative evaluations. The cytotoxicity of the PTX-loaded mPEG2000-Hz-Chol-modified pH-sensitive liposomes with MCF-7 breast cancer cells showed lower toxic effects than the plain liposomes after 24 h, while there were no significant differences after 72 h. In conclusion, these results indicate that mPEG-Hz-Chol conjugates could be useful as pH-sensitive liposomes for controlled drug delivery. Further *in vivo* research of mPEG-Hz-Chol-modified pH-sensitive liposomes is currently being carried out.

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