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RESEARCH PAPER

The Bifunctional Liposomes Constructed by Poly(2-ethyl-oxazoline) -cholesteryl Methyl Carbonate: an Effectual Approach to Enhance Liposomal Circulation Time, pH-Sensitivity and Endosomal Escape

Huan Xu • Wei Zhang • Yan Li • Fei F. Ye • Peng P. Yin • Xiu Yu • Mei N. Hu • Yuan S. Fu • Che Wang • De J. Shang

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ABSTRACT

Purpose A novel bifunctional liposome with long-circulating and pH-sensitive properties was constructed using poly(2-ethyl-oxazoline)-cholesteryl methyl carbonate (PEtOz-CHMC) in this study.

Methods PEtOz-CHMC was synthesized and characterized by TLC, IR and ^{1}H-NMR. The obtained PEtOz lipid was inserted into liposomes by the post-insertion method. Through a series of experiments, such as drug release, tumor cell uptake, cytotoxicity, calcium-induced aggregation, pharmacokinetic experiments, etc., the pH-sensitive and long-circulating properties of PEtOzylated liposomes was identified.

Results PEtOz-CHMC modified liposomes (PEtOz-L) showed increased calcein release at low pH. Flow cytometric analysis results showed that the fusion and cellular uptake of PEtOz-L could be promoted significantly at pH 6.4 compared with those at pH 7.4. Confocal laser scanning microscope observations revealed that PEtOz-L could respond to low endosomal pH and directly released the fluorescent tracer into the cytoplasm. MTT assays in HeLa cells demonstrated that doxorubicin hydrochloride (DOX) loaded PEtOz-L exhibited stronger anti-tumor activity in a medium at pH 6.4 than in a medium pH 7.4. PEtOz-L remained stable when these liposomes were incubated in calcium chloride solution. The cumulative calcein release rate of PEtOz-L was significantly lower than that of CL when the liposomes were dialysed in PBS. The pharmacokinetic experiments of liposomes in rats showed that $t_{1/2}$ and AUC of PEtOz-L were 4.13 times and 4.71 times higher than those of CL.

Conclusions PEtOzylated liposomes exhibits excellent long-circulating and pH-sensitive properties. Our results suggest that PEtOz is a promising biomaterial for the modification of liposome in drug delivery.

KEY WORDS bifunctional · liposomes · long-circulating · pH-sensitive · poly(2-ethyl-oxazoline)

INTRODUCTION

An ideal nano drug delivery system (DDS) should have the following properties: (i) sufficiently long circulation time in the body; (ii) capable of specifically targeting the pathological site; (iii) release of encapsulated drugs in response to local irritation in the pathological site; and (iv) capable of increasing intracellular drug delivery (1). In recent years, the use of liposomes as carriers of new effective biological anti-cancer drugs, such as genes or proteins, has received increasing attention. When pH-sensitive liposomes (PSLs) are ingested by cancer cells, because the pH in endosomes can reach values below 5.5, the encapsulated genes or proteins drug should be released into the cytosol after this drug enters the endosome; otherwise, this drug becomes digested and metabolised by lysosomes and loses its anticancer activity. This process is called "endosomal escape" (2,3). Based on this process, pH-sensitive liposomes have aroused increasing research interest.

Similar to conventional liposomes (CL), PSLs are highly unstable in the plasma and cannot effectively transport the inclusion to the target sites. Therefore, the key issue in this study is to improve the stability of PSLs. The authors aimed to discover a polymer that could increase the stability of PSLs, avoid phagocytosis by mononuclear phagocyte system (MPS) and destabilise liposomes at low pH. In the present study, the hydrophilic, supple and pH-sensitive poly(2-ethyl-oxazoline)

H. Xu (✉) • W. Zhang • Y. Li • F. F. Ye • P. P. Yin • X. Yu • M. N. Hu • C. Wang
Department of Pharmacy, School of Chemistry and Chemical Engineering Liaoning Normal University, Dalian 116029, People's Republic of China
e-mail: huan.xu1@163.com

Y. S. Fu
Department of Anatomy, College of Basic Medical Science Dalian Medical University, Dalian 116044, People's Republic of China

D. J. Shang
Liaoning Provincial Key Laboratory of Biotechnology and Drug Discovery Liaoning Normal University, Dalian 116081, People's Republic of China

(PEtOz) was used to construct a bifunctional liposome that possesses both long-circulating and pH-sensitive properties.

PEtOz is a macromolecule and long-chain polymer with many advantages such as low toxicity, good hydrophilicity, flexibility and good biocompatibility (4–6). PEtOz has also been approved by U.S. Food and Drug Administration. PEtOz has broad application prospects in DDS. For instance, PEtOz can be used to modify the protein or enzyme conjugation product to increase its stability *in vitro* and *in vivo* (7,8). Zalipsky *et al.* found that PEtOz4420-DSPE can extend the circulation time of liposomes *in vivo* (9). The half-life of liposomes modified with the degree of polymerization of 50 PEtOz is more than 15 h in rats *in vivo*, and this long-circulating property is similar to that of the PEGylated liposomes (10).

PEtOz is also used to construct pH-sensitive micelles (11–13). Thus, PEtOz-lipid derivatives can provide liposomes with pH sensitivity, which is similar to some acid-sensitive polymer materials such as poly(acrylic acid) derivatives.

In this study, cholesteryl chloroformate (CHM) was chosen as the lipid anchor to couple with PEtOz. Cholesterol derivatives have many advantages such as extensive sources, stable physical and chemical properties and low cost compared with phospholipids (PLs). Although cholesterol cannot form a membrane structure itself, it can act as an important part of liposomes and stabilise the liposome structure. The lipids derivatization of the polymer could be formed by connecting PEtOz with cholesterol derivatives, and this lipid anchor could be inserted into the lipid bilayer of liposomes.

In summary, PEtOz was used to modify liposomes to obtain long-circulating and pH-sensitive bifunctional liposomes. The principles of our study are presented as follows (Fig. 1): (i) given the hydrophilicity and flexibility of PEtOz, the PEtOz liposomes (PEtOz-L) can avoid recognition by the opsonins in the plasma and engulfment by MPS to develop long circulation time; (ii) PEtOz-L can accumulate in tumour tissues via the enhanced permeability and retention (EPR) effect; (iii) the liposomes enter the tumour cells via the endocytic pathway; and (iv) at low endosomal pH, PEtOz induces liposome fusion with the endosomal membrane or destabilisation of endosomes and the liposomes release the drug directly into the cytoplasm.

MATERIALS AND METHODS

Materials

Acetonitrile and 2-ethyl-2-oxazoline (EtOz) were purchased from Sigma-Aldrich (USA). 4-(Dimethylamino) pyridine (DMAP) was obtained from Shanghai Medpep Co. Ltd (Shanghai, China). Cholesterol was provided by Shanghai Advanced Vehicle Technology Co. Ltd (Shanghai, China). CHM and methyl *p*-toluenesulfonate (MeOTS) were purchased from Shanghai Aladdin Reagent Co., Ltd. (Shanghai, China). Soybean phosphatidylcholine (S75, S100) was provided by LIPOID (Germany). Doxorubicin hydrochloride (DOX) was provided by Beijing Huafeng United Technology Co., Ltd. (Beijing, China). Calcein was obtained from Bodi Reagent Factory (Tianjin, China). Sephadex G-50 was provided by Biosharp (Japan). Dimethyl sulfoxide (DMSO), other reagents and solvents (AR grade) were purchased from Tianjin Kemiou Chemical Reagent Co. Ltd. (Tianjin, China).

The cell culture reagents were purchased from Gibco Corporation (USA). Lyso-Tracker Red, 4′,6-diamidino-2-phenylindole (DAPI), immunol Staining Fix Solution and Antifade Mounting Medium were obtained from Beyotime Biotechnology Company (Nantong, China).

Cell Culture

HeLa cells were obtained from the cell bank of Chinese Academy of Sciences. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v:v) Fetal Bovine Serum (FBS) in an incubator (Thermo Scientific, USA) at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity.

Animals

Male Wistar rats weighing 220±20 g were purchased from the experimental animal centre of Dalian Medical University. The rats were provided free access to water and rat chow. All of the experiments were performed in accordance with the guidelines of the local Animal Welfare Committee.

Synthesis of PEtOz-OH

Monohydroxyl poly(2-ethyl-2-oxazoline) (PEtOz-OH) (molecular weight 2 kDa) was synthesised by the cationic ring-opening polymerisation of 2-ethyl-2-oxazoline (EtOz), and MeOTS was used as an initiator according to a previously described procedure (Fig. 2a) with slight modifications (14). A solution of EtOz (20 mmol) and MeOTS (1 mmol) in acetonitrile (20 mL) was added to the pre-dried reaction flask and stirred at reflux (100°C) for 24 h in nitrogen atmosphere. The mixture was cooled to room temperature and then added to 10 mL of 0.1 M methanolic KOH. The reaction was maintained for 12 h; when the reaction was terminated, hydroxyl groups were introduced at the end of the PEtOz chain. The crude product flowed through the silica gels and precipitated in cooled diethyl ether. The product was vacuum-dried for 24 h to yield a white powdery product, PEtOz-OH.

The chemical structure of PEtOz-OH was confirmed by thin layer chromatography (TLC), IR (TENSOR27 infrared spectrometer, Bruker, Germany) and ^{1}H-NMR (ARX-300

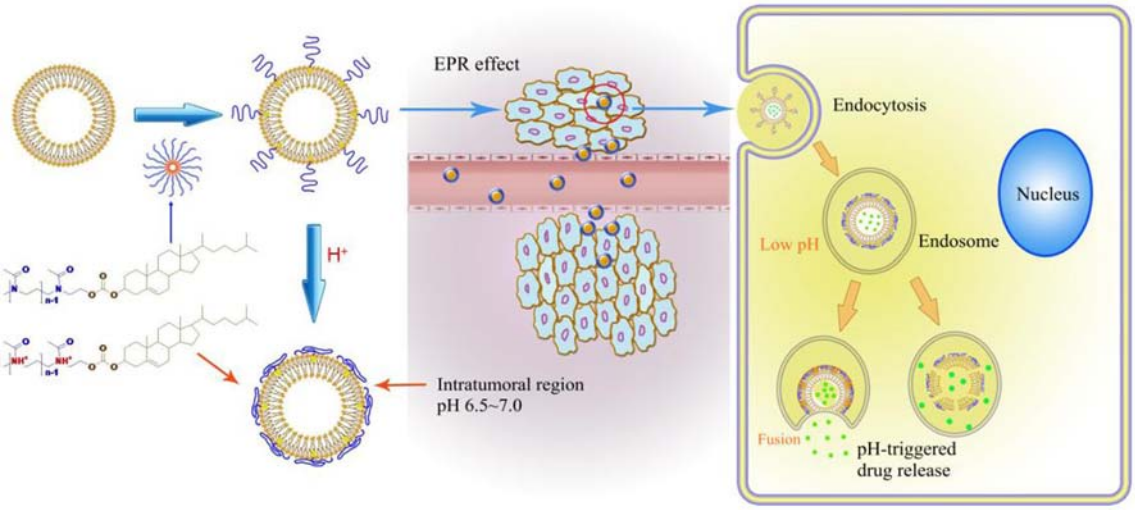

Fig. 1 Schematic representation of a bifunctional PEtOzylated liposomes and the strategy for the drug release mechanism.

spectrometry, Bruker, Germany). TLC: R_f=0.43 (chloroform: methanol: triethylamine=50:10:1, v:v). IR γ/cm^{-1}: 3458 (hydroxyl, −OH), 1628 (carbonyl, C=O) and 1576 (amide, −NH). ^{1}H NMR ($CDCl_3$, δ ppm): 1.12 (*t*, 3H, H-5), 2.3 (*q*, 2H, H-4), 2.5 (*s*, 3H, H-6), 3.45 (*t*, 2H, H-2, 3) and 3.79 (*t*, 2H, H-1). The molecular weight and molecular weight distribution were recorded by gel permeation chromatography (GPC; PL-GPC-220, Beijing West Broad Technology Co. Ltd, China). GPC (Mobile Phase: N,N-Dimethylformamide; Internal standard: polystyrene): Mn=2,276, Mw=3,271, PDI=1.320.

Synthesis of PEtOz-CHMC

The synthesis of PEtOz-CHMC is shown in Fig. 2b. In nitrogen atmosphere, a solution of CHM (1.2 mmol) in 20 mL of dichloromethane was added dropwise to PEtOz-OH (0.8 mmol); triethylamine (0.4 mmol) and DMAP (0.4 mmol) were then added. The mixture was reacted for 24 h at room temperature. After the reaction was completed, the mixture was washed with distilled water, 1 M HCl and saturated sodium chloride to remove the by-products. The organic layer was dried in vacuum with anhydrous magnesium sulphate. The crude product was purified by silica gel column. The conditions for column chromatography were as follows: silica gel column was packed with 200- to 300-mesh silica gel mixed with petroleum ether; dichloromethane: methanol (20:1, v:v) was used as eluent; sample volume was 1 mL.

The chemical structure of PEtOz-CHMC was confirmed by TLC, IR and ^{1}H NMR. TLC: R_f=0.50 (chloroform: methanol: triethylamine=50:10:1, v:v). IRγ/cm^{-1} (Fig. 3a): 1732 (ester carbonyl, C=O), 1628 (carbonyl, C=O) and 1580 (amide, −NH). ^{1}H NMR ($CDCl_3$, δ ppm) (Fig. 3b): 0.68 (*s*, 3H, H-4), 0.861 (*d*, 3H, H-1), 0.87 (*d*, 3H, H-2), 0.923 (*d*, 3H, H-3), 1.009 (*s*, 3H, H-5), 2.50 (*s*, 3H, H-9), 3.52 (*d*, 2H, H-7, 8) and 5.356 (*s*, 1H, H-6).

Preparation of CL and PEtOz-L

Blank CLs were prepared by the film dispersion method (15). S75 and cholesterol (molar ratio of 3:2) were dissolved in chloroform. After the chloroform was removed in an evaporator at 37°C, a thin lipid film was formed and then further dried in vacuum overnight to remove any traces of the remaining solvents. The lipid film was hydrated in 0.01 M PBS at 37°C.

Calcein liposomes were prepared by the reverse-phase evaporation method, which is used to prepare liposomes with a large internal aqueous space. In brief, the ether solution containing S75 and cholesterol (molar ratio of 3:2) was added to 60 mM calcein aqueous solution (3:1, v/v). The remaining procedure was the same as described by Szoka *et al.* Unencapsulated calcein was removed by gel filtration in a Sephadex G-50 column (16).

The coarse dispersed liposomes were sonicated to clarity in an ultrasonic cell disruptor (JY92-2D probe sonicator, Ningbo, China) to reduce the mean diameter of the particles. The resulting liposomes were extruded in polycarbonate membranes with gradually decreasing pore sizes (0.45 and 0.22 μm).

The ammonium sulphate gradient exchange method was used to load DOX in the liposomes (17,18). S100 and cholesterol (molar ratio of 3:2) were dissolved in chloroform. After the organic solvent evaporated, the resulting lipid film was hydrated in 250 mM ammonium sulphate solution. The liposomal solution was treated according to the aforementioned operation to obtain small unilamellar liposomes. The free ammonium sulphate was removed by dialysis using dialysis tubing (MWCO 8,000–12,000, Spectrum, USA). The

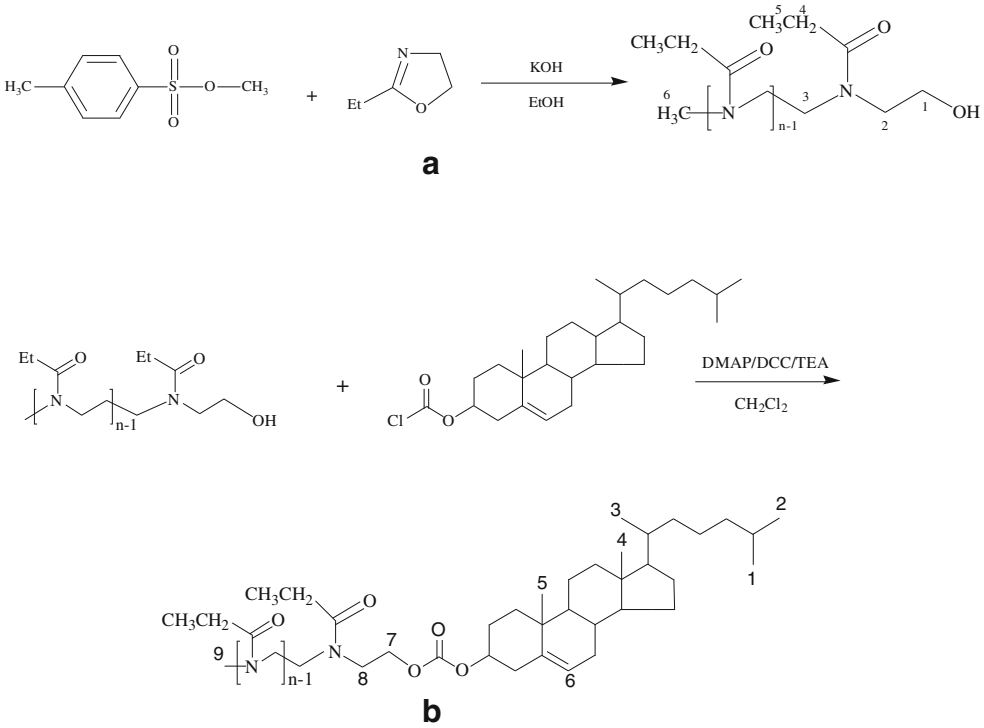

Fig. 2 The schematic of the synthesis of PEtOz-OH (**a**) and PEtOz-CHMC (**b**).

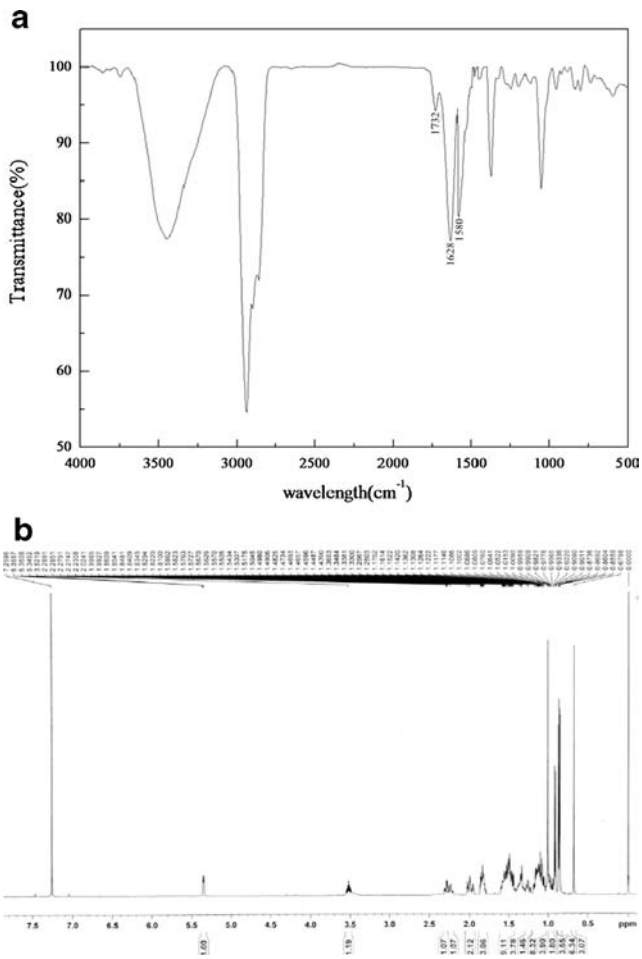

Fig. 3 The IR spectra (**a**) and ^{1}H NMR spectrum (**b**) of PEtOz-CHMC.

liposome suspension and DOX were mixed at 1:10 (w/w, drug/lipid); the mixture was incubated at room temperature for 3 h. After incubation, unencapsulated DOX was removed by gel filtration in a Sephadex G-50 column.

PEtOz-CHMC micelles were prepared by dissolving copolymers in 5 mL of chloroform. After the chloroform was removed in an evaporator, the conjugate film was hydrated in 5 mL of 0.01 M PBS at 37°C.

Using the post-insertion method (19), we efficiently modified liposomal surfaces with PEtOz. In brief, all of the aforementioned types of CL suspensions with PEtOz-CHMC micelles (lipids: PEtOz-CHMC=20:1, molar ratio) were mixed at room temperature for 1 h to obtain the PEtOzylated liposomes.

Characterization of Liposomes

The diameters and zeta potentials of the liposomes were measured using a Nicomp380 ZLS dynamic light scattering instrument (Submicron Particle Sizer, Particle Sizing Systems, Santa Barbara, CA, USA). Data were obtained as an average of more than three measurements of different samples.

Encapsulation efficiency (EE) of calcein and DOX in liposomes was determined according to the mini-column centrifugation method using a Sephadex G-50 column. Calcein concentration was analysed using a model F-7000 fluorescence spectrophotometer (Hitachi Inc., Japan) at excitation and emission wavelengths of 490 and 512 nm, respectively. The DOX concentration was quantified using a model 752N

UV–vis spectrophotometer (Shanghai Precision & Scientific Instrument Co. Ltd, China) at a wavelength of 480 nm.

Determination of PL Concentration

The PL concentration was detected according to Stewart's protocol (20). We added 50 μL of liposome suspension in 2 mL of ferrothiocyanate reagent. After mixing and centrifugation, the upper layer was removed and the PL concentration was assessed using a 752N UV–vis spectrophotometer at a wavelength of 485 nm.

Verification of the pH Sensitivity of PEtOz-L

Change in Morphology of Liposomes Under Different pH

The liposome sample (0.1 mL) was diluted tenfold by PBS at different pHs (5.4, 6.4 and 7.4). A drop of the sample dispersion was placed in a 100-mesh copper grid. A drop of 2% phosphotungstic acid solution (pH 7.4) was added to the grid and then dried for 12 h in a desiccator. Liposome morphology was observed by transmission electron microscopy (TEM; JEM-2000EX, JEOL, Japan).

Calcein Release from Liposomes Triggered by Different pH

The pH sensitivity of PEtOz-L was determined by the calcein-dequenching assay. Calcein-loaded liposome suspensions were added to PBS at different pHs (5.0, 5.4, 6.4 and 7.4) in a disposable cuvette. At 1, 5, 10, 20, 30 and 60 min, the fluorescence intensity (FI) of the sample was determined by fluorescence spectrophotometry. Triton X-100 was added to disrupt the remaining liposomal bilayers, thereby causing 100% calcein leakage from the liposomes. The percentage of calcein release was calculated based on the following equation:

$$\%\text{Calcein Release} = \left[\left(C_{pH}-C_0\right)/\left(C_{total}-C_0\right)\right] \times 100\% \quad (1)$$

where C_0 is the concentration of calcein in the buffer (pH 7.4), C_{pH} is the concentration of calcein following incubation at pH of the acidic buffer and C_{100} is the concentration of calcein after Triton X-100 was added.

Flow Cytometric Analysis

The cellular uptake of calcein-loaded liposomes was studied by flow cytometry. HeLa cells (2×10^5 cells/well) were cultured for 24 h in 35 mm glass bottom dishes. After the culture medium was removed, the cells were incubated in 500 μL of serum-free DMEM. Calcein-PEtOz-L and calcein-CL (final PL concentration was 50 μmol/L) were incubated with the cells at 37°C. After 1 or 4 h, the cells were washed twice with ice-cold PBS, removed by gently scraping and centrifuged at 1,000 × *g* for 5 min. The FI was analysed on a BD FACSCanto (Beckman Coulter, USA). A total of 10,000 events were recorded for each sample and the single cells were gated.

Confocal Laser Scanning Microscope (CLSM) Analysis

HeLa cells (1×10^5 cells/well) were grown to 30% to 40% confluence on 22 mm coverslips in six-well cell culture plates for 24 h and incubated in serum-free DMEM for 10 min. Calcein-PEtOz-L and calcein-CL (final PL concentration was 50 μmol/L) were incubated with the cells for 1 or 4 h at 37. After incubation, the cells were washed thrice with ice-cold PBS. Then, 2 mL of Lyso-Tracker Red (50 nM) diluted with DMEM was added to each well. After 1 h, the cells were carefully washed twice with ice-cold PBS. Subsequently, 2 mL of immunol Staining Fix Solution was added to each well to fix the cells. The coverslips were finally mounted on glass slides by using a vectashield medium with DAPI for microscopic observations. The location of intracellular fluorescence was validated using a CLSM (ECLIPSE-Ti, Nikon, Japan).

Cytotoxicity of Liposomes

The cytotoxicity of DOX-liposomes was evaluated by determining the cell viability using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HeLa cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated in 100 μL of pH 7.4 and 6.5 media for 24 h. Approximatley 5 μL of DOX-PEtOz-L and DOX-CL were added to the cells and incubated for another 24 h. At the given time intervals, 10 μL of MTT (5 mg/mL in pH 7.4 PBS) was added and incubated for 4 h in an incubator. DMSO (150 μL) was added to replace the medium to solubilise the formazan crystals. The intensity of UV absorbance was determined using a microplate reader (Thermo Electron Co., USA) at 492 nm. Each point was performed in triplicate. Cell viability was calculated by the following equation:

$$\text{Cell viability} = \text{OD}_{\text{liposomes}}/\text{OD}_{\text{control}} \times 100\% \quad (2)$$

where $\text{OD}_{\text{liposomes}}$ is the absorbance intensity of the cells treated with DOX-liposomes and $\text{OD}_{\text{control}}$ is the absorbance intensity of the cells incubated with the culture medium.

Evaluation of Liposome Stability

Calcium-Induced Aggregation of Liposomes

We diluted 200 μL of liposomes in 3 mM calcium chloride solution to obtain a final volume of 5 mL and incubated the resulting solution at 37°C. The absorbance of the sample was

determined at 450 nm by using UV–vis spectrophotometer at different times. The change rate of turbidity (CRT) was calculated by the following equation:

$$CRT\% = (A_T - A_0)/A_T \times 100\% \quad (3)$$

where A_T is the absorbance of the liposomes at different times and A_0 is the absorbance of liposomes mixed with PBS.

Release of Calcein Liposomes *In Vitro*

Calcein-loaded liposome suspensions or calcein solution (1 mL) were placed in dialysis bags (10,000 Da molecular weight cutoff). The dialysis bags were incubated under sink conditions of PBS at 37°C. The samples (3 mL) were withdrawn at a fixed time and fresh buffer was supplemented. Calcein release from liposomes was determined by fluorescence spectrophotometry as described above.

Pharmacokinetics of Calcein-Loaded Liposomes *In Vivo*

The pharmacokinetic behaviour of liposomes was evaluated in rats. The animals were injected with 25 μmol PL/kg via the tail vein. At different time points, 500 μL of blood samples was collected via eye puncture. Plasma was obtained by subjecting whole blood samples to centrifugation at 2,000 × *g* for 5 min. The plasma was diluted to 5 mL by PBS containing 1% Triton X-100. The calcein concentration in plasma was measured by fluorescence spectrophotometry as described above.

Statistical Analysis

Statistical comparisons were performed by Student's *t*-test for two groups, and one-way ANOVA for multiple groups. $P<0.05$ indicated a statistical significance.

RESULTS

Characterization of Liposomes

As shown in Table I, the mean particle size of the prepared liposomes was <150 nm. PEtOz modification increases the particle size of liposomes by approximately 25 nm. The absolute value of zeta potential of PEtOz-L decreased compared with that of CL but similar to that of PEGylated liposomes.

Table II shows that the zeta potential of CL almost did not change when pH decreased; by contrast, the zeta potential of PEtOz-L reduced significantly. Thus, some variations could occur in the membrane of PEtOzylated liposomes in low pH environments.

The EEs of calcein-loaded liposome and DOX-loaded liposome were 10.9±0.8% and 97.8±1.2%, respectively.

Verification of the pH Sensitivity of Liposomes

Liposome Morphology Under Different pH

As seen in Fig. 4, PEtOz-L showed good morphology in pH 7.4 buffer solution, and the average sizes of liposomes were smaller than 100 nm. PEtOz-L began to accumulate and aggregate and the mean particle size of liposomes increased and was much larger than 100 nm when pH was decreased to 6.4. When pH was decreased to 5.4, the membrane of PEtOz-L underwent phase transition and exhibited an irregular liposome shape. By contrast, the morphology of CL did not change at different pH levels.

Calcein Release of Liposomes at Different pH Buffer Solutions

Figure 5 shows that no significant difference was found between acidic buffer solutions (pH 5.0, 5.4 and 6.4) and pH 7.4 buffer solutions in terms of the calcein release of CL. The calcein release ratio reached only about 15% after 60 min. The release of calcein from PEtOz-L at pH 7.4 buffer solution was similar to that of CL and the release ratio was only 12.1± 1.3% after incubation for 60 min. After pH was decreased, the release ratio of calcein increased gradually. The release ratio of calcein increased to 55.3±2.8% immediately after incubation with the buffer solution at pH 5 for 1 min. After the time was extended to 60 min, the release ratio reached 82.6± 5.3%. These results suggested that low pH induced the destabilisation of PEtOz-L. Moreover, lower pH corresponded to a higher content of released calcein.

Flow Cytometric Analysis

Figure 6 shows that no significant difference was observed for the cellular uptake of CL at pH 7.4 and 6.4. When PEtOz-L was incubated in pH 6.4 buffer solution for 1 h or 4 h, the cellular uptake increased significantly compared with those obtained after incubation at pH 7.4. The cellular uptake of PEtOz-L was significantly higher at low pH than that of CL. With the extension of incubation time, the cellular uptake of two kinds of liposomes increased. At pH 7.4, the cellular uptake of PEtOz-L was higher than that of CL.

Endosomal Escape

Figure 7 shows that PEtOz-L was mainly distributed throughout the cytoplasm. PEtOz-L could enter the cell via endocytosis and the low pH environment of the endosome induced the liposomal membrane to fuse with the endosomal

Table I Mean Particle Sizes and Zeta Potentials of the Prepared Liposomes ($n=3$)

Liposomes	Particle size/nm	Zeta potential/mV
CL	95.5 ± 6.8	−26.75 ± 3.7
PEtOz-L	121.7 ± 11.4	−19.28 ± 3.2
Calcein-CL	120.3 ± 8.5	–
Calcein-PEtOz-L	134.8 ± 9.2	–
DOX-CL	98.2 ± 7.2	–
DOX-PEtOz-L	124.6 ± 8.7	–

membrane, and then release most of the drug to the cytoplasm directly. Thus, PEtOz-L successfully underwent "endosomal escape". By contrast, CL mainly gathered in the lysosome.

In Vitro Cytotoxicity of Liposomes

The cytotoxicity results of DOX liposomes in a different culture environment are shown in Fig. 8 DOX loaded CL showed a weaker cytotoxic effect compared with PEtOz-L at different pH ($P<0.01$). DOX loaded PEtOz-L exhibited stronger antitumor activity in a medium at pH 6.5 than in a medium pH 7.4 in different DOX concentration ($P<0.01$). PEtOz-L demonstrated higher toxicity compared with CL at acidic conditions.

Verification of the Liposome Stability

Calcium-Induced Aggregation of Liposomes

As shown in Fig. 9, when CL was incubated in 3 mM $CaCl_2$ solution, the turbidity increased with the extension of incubation time. When the time was extended to 24 h, the CRT increased to 50.1 ± 3.3%. By contrast, the CRT of PEtOz-L was significantly smaller than that of CL and when incubated for 24 h, the CRT was only 16.4 ± 1.2%. The images show that the turbidity of CL changed significantly and even exhibited precipitation, whereas the property and appearance of PEtOz-L almost no change. Thus, PEtOz exhibited favourable anti-Ca^{2+}-induced liposome aggregation and could maintain liposome stability.

Release Characteristics of Liposomes *In Vitro*

The cumulative release amount of calcein solution reached 100.4 ± 4.5% at 6 h. CL was highly unstable in buffer solution:

Table II Change of Zeta Potentials of Liposomes Incubated in Buffer of Various pH Values ($n=3$)

	Zeta potential of CL (mv)	Zeta potential of PEtOz-L (mv)
pH 7.4	−26.75 ± 3.7	−19.28 ± 3.2
pH 5.4	−23.72 ± 4.5	−10.68 ± 2.1

the calcein release from CL was quick, and the cumulative release amount reached 51.8 ± 3.2% after dialysis for 10 h (Fig. 10). With the extension of incubation time, the cumulative release rate of calcein increased to 62.5 ± 3.3% after dialysis for 24 h. However, the cumulative release amount of calcein from PEtOz-L was always lower than that of CL and the cumulative release amount was only 16.9 ± 1.2% after 24 h.

Pharmacokinetics of Calcein-Loaded Liposomes *In Vivo*

As shown in Fig. 11, CL was quickly eliminated from plasma after injection and calcein could not be detected 1 h after injection. PEtOz significantly changed the general blood distribution of liposomes. The circulation time of PEtOz-L in rats was significantly longer than that of CL.

The pharmacokinetic parameters were calculated by a non-compartment model. The values of $t_{1/2}$ and AUC of PEtOz-L encapsulating calcein were 4.13 and 4.17 times higher than those of CL, respectively ($P<0.01$).

DISCUSSION

Particle size is one of the basic properties of the liquid microparticle formulation, which can affect the stability of the preparation *in vitro* to a certain extent. If the particle size is too large, the particle will be prone to precipitation. Thus, controlling the particle size of the liposomes to increase its physical stability is crucial. A larger diameter can also reduce the uptake efficiency of liposomes during cellular uptake experiments *in vitro* (21). In addition, the particle diameter of liquid particles determines the behaviour of the formulations *in vivo*. When the particle size exceeds 200 nm, the particles are easily phagocytised by MPS (22). Given the increase of the pore size of endothelial cells or structural defects in the tumour site, the microparticle carrier can produce the EPR effect, which is also dependent on a smaller particle diameter (23). The particle size of the liposomes in this study was strictly controlled to below 150 nm, possessed good physical stability and did not affect the cellular uptake experiments and pharmacokinetics *in vivo*.

The direct incorporation method was applied to prepare the long-circulating liposomes, and the polymer was distributed on the liposomal surface and the liposomal interior. Thus, the proportion of the polymer to modify liposomes was not accurate. Given that the inner water cavity of liposomes was occupied, the EE of water-soluble drug decreased. Even the possible interaction between the drug and polymer materials in the liposomal interior could lead to the instability of liposomes (24). With the studies on the liposomes modified by multi-functional polymer materials, researchers focus on the post-insertion method. Compared with the direct

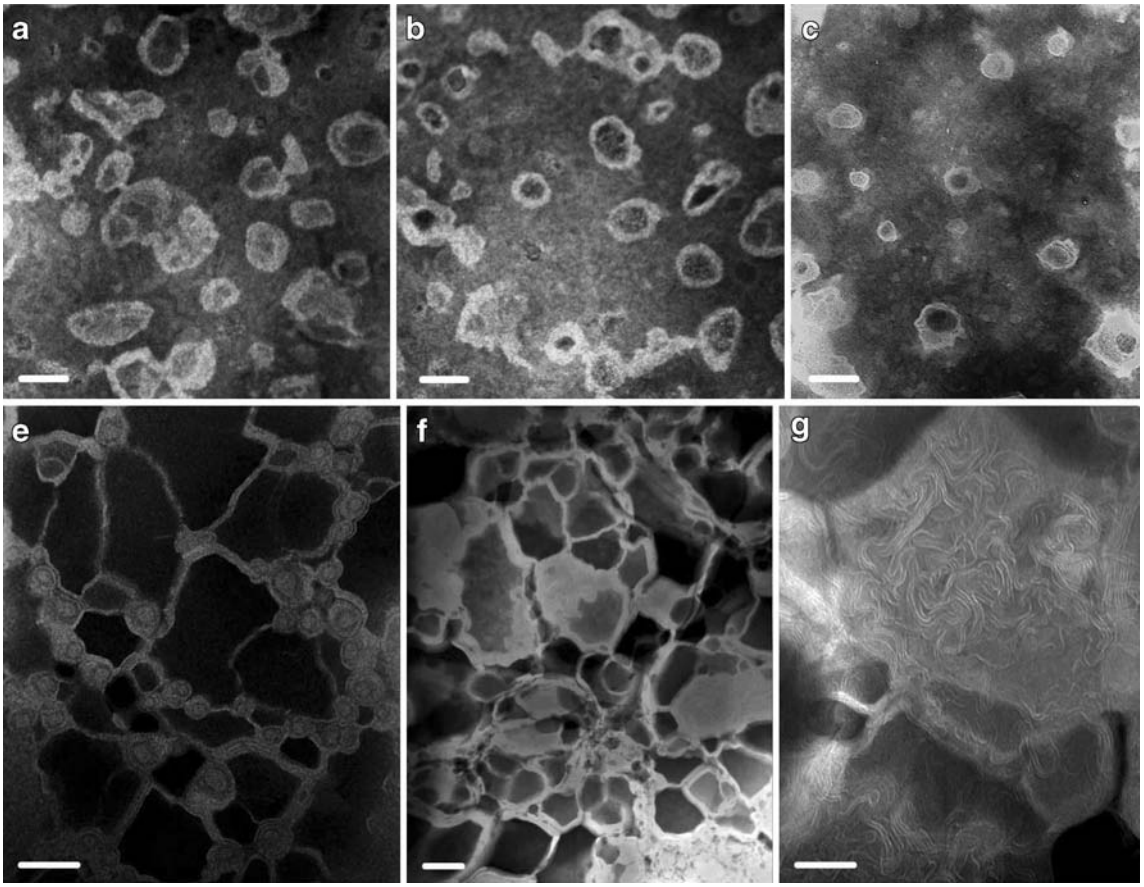

Fig. 4 Change in morphology of liposomes under different pH. (**a**, **b**, **c**) represent conventional liposomes incubated in buffer of pH 7.4, 6.4 and 5.4, respectively; (**e**, **f**, **g**) represent PEtOzylated incubated in buffer of pH 7.4, 6.4 and 5.4, respectively. Bars represent 100 nm.

incorporation method, the post-insertion method ensure the hydrophilic polymer more efficiently and accurately insert to the surface of the liposomes. Yoshinoa *et al.* found that when PEG-lipids are incubated with CL over the phase transition temperature of PL for 15 min, the insertion ratio of PEG is more than 90% (19). In this study, another advantage of the post-insertion method was that the EE of PEtOz-L and CL were uniform because these liposomes were from the same batch. Because the incubation temperature is low and time is short, the EE of PEtOzylated liposomes prepared by "post insertion loading" was similar with CL. During analyses of cellular uptake of calcein liposomes and cytotoxicity of DOX liposomes, the amount of drug was equal in the two kinds of liposomes, which was convenient in comparing the nature of the liposomes before and after modification. The particle size of PEtOz-L prepared by the post-insertion method was slightly larger than that by the direct incorporation method. Based on the aforementioned reasons, the post-insertion method was chosen for liposome preparation.

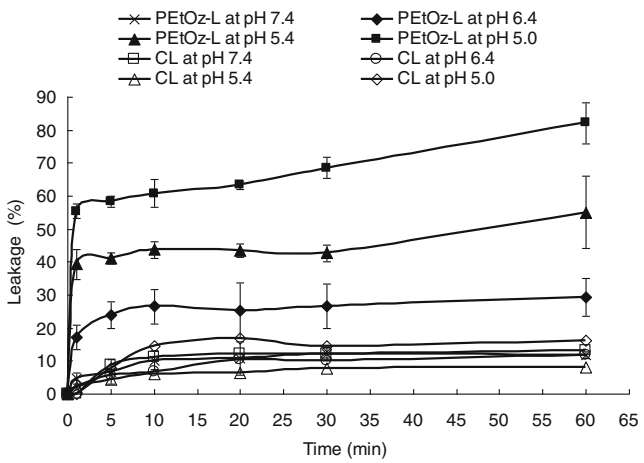

Fig. 5 Time course of pH-dependent release of calcein entrapped in conventional liposomes and PEtOzylated liposomes at 37°C. ($n = 3$). Results are presented as mean ± SD.

Calcein, as a water-soluble fluorescent probe, has been widely used to evaluate the release characteristics of nanoparticles (25–27). When the concentration of calcein aqueous solution is 60 mM or more, calcein undergoes polymerisation and fluorescence self-quenching, thus no FI exists for this concentration of calcein liposomes under fluorescence spectrophotometry. However, the reduced concentration can readily detect FI after calcein is released into the medium under low pH conditions. According to the change in FI, the

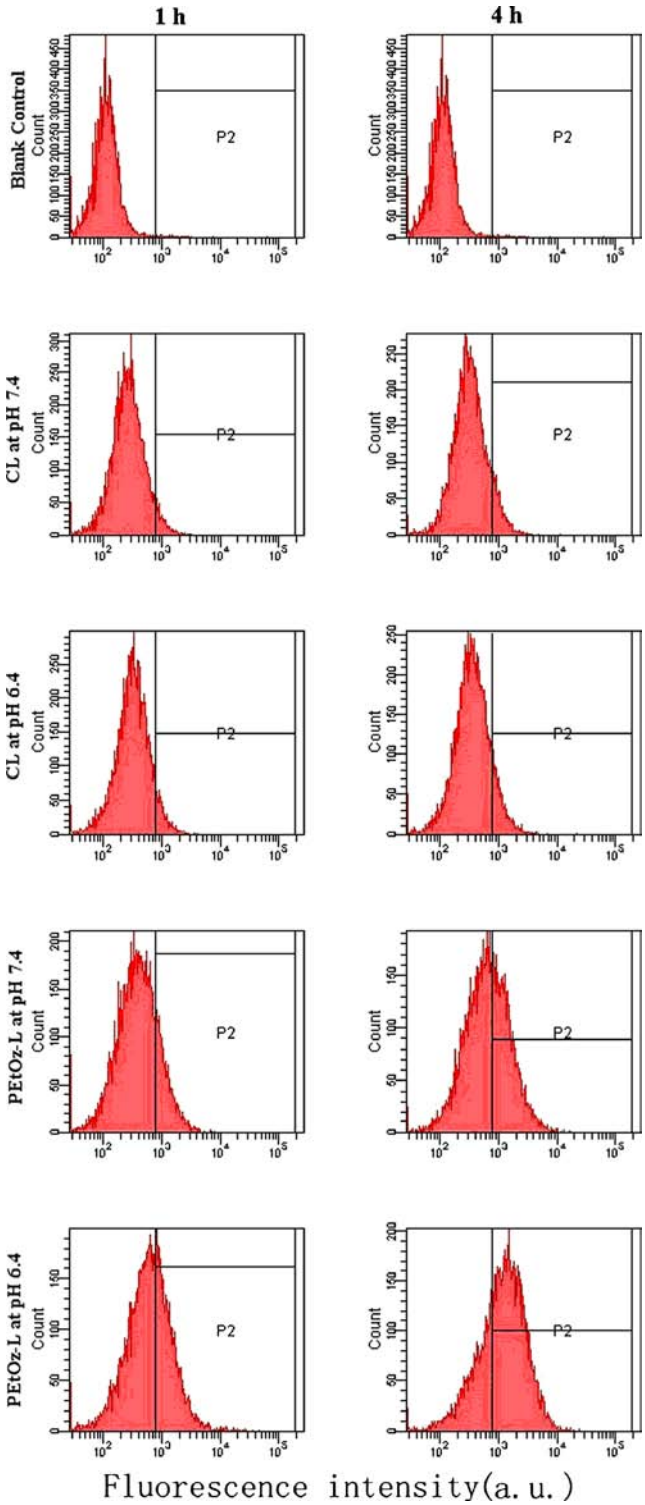

Fig. 6 Flow cytometric measurement of calcein uptake by HeLa cell after incubated with no treatment or different liposomes for 1 h and 4 h at pH 6.4 or 7.4.

pH sensitivity of liposomes can be described. If the other model drugs are chosen, the released drug must be isolated from liposomes before the assay. In this study, the use of calcein eliminated the tedious and time-consuming separation process and obtained more accurate test results. However, the FI of calcein solution is highly susceptive to many factors, such as pH, ionic strength and plasma protein, which leads to the fluctuation of experimental data. Some researchers incubated calcein liposomes with a large number of different pH buffers for a period time, and then the pH was readjusted to 7.4 by an appropriate buffer (25,28). The authors attempted to adopt this method to determine the release ratio of calcein liposomes. We found that such a dilution process demands a high EE of liposomes. However, obtaining a high EE of calcein liposomes by the conventional preparation method is very difficult. Secondly, the influence of pH on the FI of calcein is obvious, and a slight deviation in pH of solution results in the obtained data to be far from the true results. Some researchers adopted the calibrated FI method to solve this problem (29). The calibrated FI value was obtained by contrasting the FI of different pH calcein solutions *vs* that of pH 7.4. With the extension of time and the measurement frequency, the FI of calcein decreased, so the researcher measured the FI of a specific concentration and the pH of calcein per minute to obtain the calibrated curve. In this study, a similar method was adopted and the determined process was simple, convenient and resulted in more accurate test data.

The results of *in vitro* cellular uptake and flow cytometric analyses showed that the uptake of PEtOz-L was significantly higher than that of CL under low pH conditions. PEtOzylation helped liposomes to respond to the decreased pH and promoted liposomes to fuse with the cell membrane. The uptake rate of PEtOz-L was higher than that of unmodified liposomes at pH 7.4, which was opposite to the conclusion that PEGylation would decrease the cellular uptake of liposomes. Although PEtOz-L negatively charged, the absolute value of zeta potential of PEtOz-L is lower than that of CL. Therefore, the electrostatic repulsion between the PEtOz-L and cells is smaller than that of CL and cells and the uptake rate of PEtOz-L was higher than that of unmodified liposomes at pH 7.4.

The pH sensitivity of PEtOz has attracted significant research attention and pH-sensitive micelles composed of PEtOz-lipid derivatives are increasingly being developed (13,14,30). The "N" in the amide bond of PEtOz chain can be protonated under low pH, which induces configuration and flip-flop of the block copolymer and exposure of the lipophilic group, resulting in leakage of the encapsulated drug. PEtOz possesses polyelectrolyte properties, which can provide liposomes with pH sensitivity. In this study, PEtOz-lipid derivatives were used to modify liposomes and the pH sensitivity of liposomes was investigated. The TEM results, release of calcein liposomes, fusion with membrane of erythrocyte and a series of cell tests under different pH conditions showed that PEtOz-L could aggregate or even exhibit conformational changes at low pH. PEtOz-L possesses good pH sensitivity and has a function in

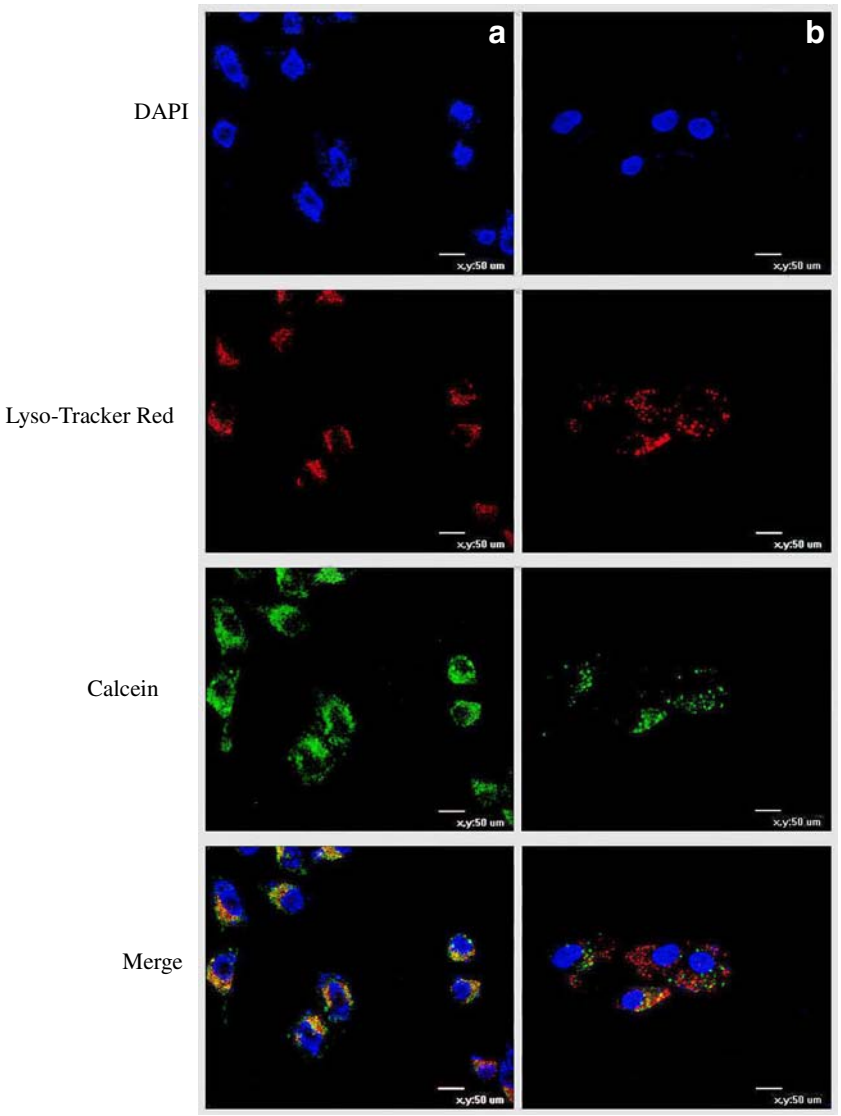

Fig. 7 The intracellular uptake and distribution of liposomes by confocal laser scanning microscopy. HeLa cells were incubated with PEtOzylated liposomes (**a**) and conventional liposomes (**b**) at 37°C for 4 h. In the CLSM images, green represents calcein, blue represents DAPI and red represents Lyso-Tracker Red.

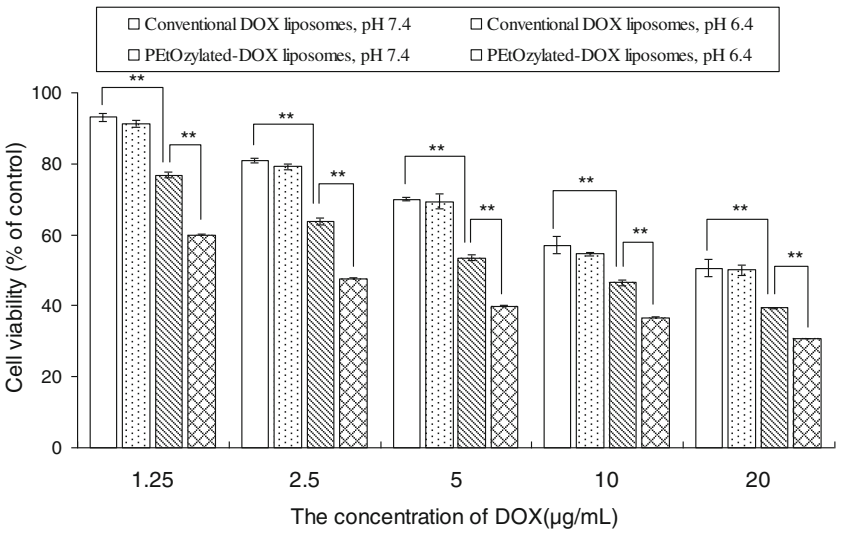

Fig. 8 Viability of HeLa cells was determined by MTT assay when cells were incubated with liposomes encapsulated different concentration of DOX at various pH for 24 h ($n = 3$). Results are presented as mean ± SD. $^{**}P < 0.01$.

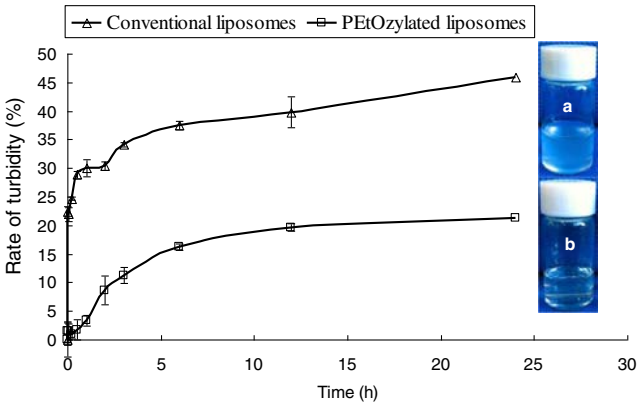

Fig. 9 Changes in the turbidity of liposomes when incubated in 3 mM $CaCl_2$ for different time. The images in Figure show the appearance of conventional liposomes (**a**) and PEtOzylated liposomes (**b**) incubated with $CaCl_2$ solution for 24 h ($n = 3$). Results are presented as mean ± SD.

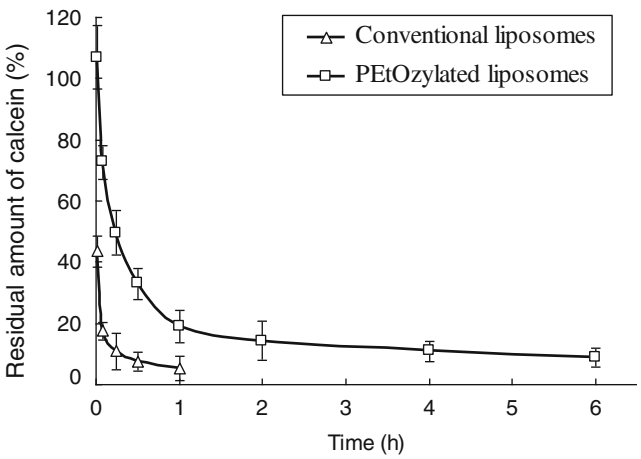

Fig. 11 Blood clearance of calccein in rats after injection of calcein liposomes ($n = 3$). Results are presented as mean ± SD.

"endosomal escape". Thus, PEtOzylate could be used to overcome the "PEG dilemma".

PEtOz-CHMC aqueous solution is a weak alkaline, which is related with the weak basic groups in the PEtOz structure. We have determined the titration curve of PEtOz-CHMC block copolymer aqueous solution and the curve exhibited a sudden change at pH 6.5, indicating that PEtOz-CHMC could be protonated in slightly acidic conditions (data not shown). Both the length of PEtOz and the type of lipids anchored affect the pKa of block copolymer, so the response to pH must be different for PSLs constructed by PEtOz with different molecular weights or different kinds of lipid anchors. We are attempting to carry out a related study. The presumable pH-sensitive mechanism of PEtOz-modified liposomes involves the following. PEtOz contains a protonated basic group NR^{3+}, which can form hydrogen bonds with the hydrogen donor under acidic conditions. The hydrophilic, stretched long chain of PEtOz changes to a dense and hydrophobic spherical structure. Decreased pH induces PEtOz to exhibit this conformational change and adsorb tightly on the surface of the liposomal membrane when PEtOz is on the surface of liposomes (Fig. 1). The lipid bilayer of the liposomes rearranges to accommodate the adsorptive PEtOz chain. Given the damage in the PL bilayer or fusion between membranes, the drug is quickly released.

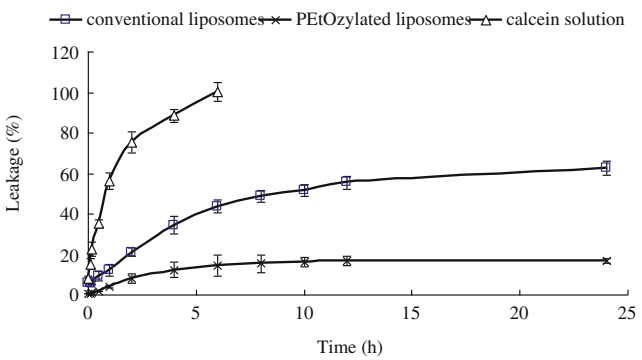

Fig. 10 Calcein accumulated release when solution liposomes incubated at PBS (pH 7.4) for different time ($n = 3$). Results are presented as mean ± SD.

Many factors in plasma such as high-density lipoprotein, serum albumin and metal ions are prone to combine with CL and are easily phagocytized by MPS. Metal ions, such as calcium, can induce the aggregation and fusion of CL (31,32). PEG can form a steric barrier layer on the surface of liposomes and improve their hydrophilicity. Thus, PEGylation can prevent the recognition of opsonins and prolong the blood circulation time of liposomes. PEGylation has been extensively applied to the modification of liposomes. Calcium ions can combine with the negatively charged surface of liposomes and induce liposome aggregation (21,22,33). Some researchers adopted the calcium-induced liposome fusion test to verify whether PEGylation can help liposomes resist the metal ion and increase stability (32). In this study, a similar test method was conducted to investigate whether or not PEtOz-L exhibits good resistance to calcium ion and prove PEtOz can increase liposome stability. To give more prominence to the contribution of PEtOz, we selected the unsaturated and negatively charged PL S75 to prepare the liposomes. The results (Figs. 9, 10) show that PEtOz significantly increased the stability of CL.

In this study, calcein was chosen as the fluorescent label to investigate the pharmacokinetics of liposomes in rats. Compared with radiolabelling, fluorescence labelling method does not contain radioactive contaminants and is easy to operate. As shown in Fig. 10, PEtOzylation could greatly improve the circulation time of liposomes *in vivo*. After injection at 6 h, this stability became less obvious (but was still significantly greater than that of CL). The chemical bond between PEtOz and CHMC is a carbonate bond, which is slightly sensitive to esterase in rat plasma or tissue. The hydrolysis of ester bond leading to the long-circulating effect of PEtOz-L is slightly weaker than that of PEGylation as reported in previous

studies (34). Although liposomes were placed at 4°C for 6 months, PEtOz-L still maintained its good liposomal appearance, whereas CL produced precipitates. Thus, PEtOz could provide liposomes with good stability.

CONCLUSION

Stability is one of the prerequisites of the application of nanoformulations and pH sensitivity can ensure that liposomes are released from the endosome. In this study, PEtOz greatly enhanced the stability of liposomes *in vitro* and *in vivo*. Liposomes were also provided with pH sensitivity by PEtOz. Thus, a long-circulating and pH-sensitive bifunctional liposome was successfully built. Our results can serve as reference to improve the stability of traditional PSLs. Given that PEtOz is temperature sensitive, future studies should investigate the optimal molecular weight and proportion of PEtOz to develop liposomes with more unique characteristics.

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REFERENCES

1. Torchilin V. Multifunctional and stimuli-sensitive pharmaceutical nanocarriers. Eur J Pharm Biopharm. 2009;71:431–44.
2. Hatakeyama H, Akita H, Harashima H. A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma. Adv Drug Deliv Rev. 2011;63:152–60.
3. Hatakeyama H, Akita H, Ito E, Hayashi Y, Oishi M, Nagasaki Y, *et al.* Systemic delivery of siRNA to tumors using a lipid nanoparticle containing a tumor-specific cleavable PEG-lipid. Biomaterials. 2011;32:4306–16.
4. Wiesbrock F, Hoogenboom R, Abeln CH, Schubert US. Single-mode microwave ovens as new reaction devices: accelerating the living polymerization of 2-ethyl-2-oxazoline. Macromol Rapid Commun. 2004;25:1895–9.
5. Wang X, Li X, Li Y, Zhou Y, Fan C, Li W, *et al.* Synthesis, characterization and biocompatibility of poly(2-ethyl-2-oxazoline)-poly(D, L-lactide)-poly(2-ethyl-2-oxazoline) hudrogels. Acta Biomater. 2011;7:4149–59.
6. Wang CH, Wang WT, Hsiue GH. Development of polyion complex micelles for encapsulating and delivering amphotericin B. Biomaterials. 2009;30:3352–8.
7. Miyamoto M, Naka K, Shiozaki M, Chujo Y, Saegusa T. Preparation and enzymatic activity of poly[(N-acy1imino)ethylene-modified catalase. Macromolecules. 1990;23:3201–5.
8. Mero A, Pasut G, Via LD, Fijten MW, Schubert US, Hoogenboom R, *et al.* Synthesis and characterization of poly(2-ethyl 2-oxazoline)-conjugates with proteins and drugs: suitable alternatives to PEG-conjugates? J Control Release. 2008;125:87–95.
9. Zalipsky S, Hansen CB, Oaks JM, Allen TM. Evaluation of blood clearance rates and biodistribution of poly(2-oxazoline)-grafted liposomes. J Pharm Sci. 1996;85:133–7.
10. Woodle MC, Engbers CM, Zalipsky S. New amphipatic polymer-lipid conjugates forming long-circulating reticuloendothelial system-evading liposomes. Bioconjug Chem. 1994;5:493–6.
11. Hsiue G, Chiang HZ, Wang CH, Juang TM. Nonviral gene carriers based on diblock copolymers of poly(2-ethyl-2-oxazoline) and linear polyethylenimine. Bioconjug Chem. 2006;17:781–6.
12. Lee SC, Lee HJ. pH-Controlled, polymer-mediated assembly of polymer micelle nanoparticles. Langmuir. 2007;23:488–95.
13. Hsiue GH, Wang CH, Lo CL, Wang CH, Li JP, Yang JL. Environmental-sensitive micelles based on poly(2-ethyl-2-oxazoline)-b-poly(l-lactide) diblock copolymer for application in drug delivery. Int J Pharm. 2006;317:69–75.
14. Wang CH, Hsiue GH. New amphiphilic poly(2-ethyl-2-oxazoline)/poly(l-lactide) triblock copolymers. Biomacromolecules. 2003;4:1487–90.
15. Bangham JA, Lea EJA. The interaction of detergents with bilayer lipid membranes. Biochim Biophys Acta. 1978;511:388–96.
16. Szoka Jr F, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Natl Acad Sci U S A. 1978;75:4194–8.
17. Han HD, Lee A, Hwang T, Song CK, Seong H, Hyun J. Enhanced circulation time and antitumor activity of doxorubicin by comblike polymer-incorporated liposomes. J Control Release. 2007;120:161–8.
18. Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta. 1993;1151:201–15.
19. Yoshinoa K, Taguchi K, Mochizuki M, Nozawa S, Kasukawa H, Kono K. Novel analytical method to evaluate the surface condition of polyethylene glycol-modified liposomes. Colloid Surf A. 2012;397:73–9.
20. Anabousi S, Laue M, Lehr CM, Bakowsky U, Ehrhardt C. Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy. Eur J Pharm Biopharm. 2005;60:295–303.
21. Basile L, Passirani C, Huynhc NT, Béjaud J, Benoit JP, Puglisi G, *et al.* Serum-stable, long-circulating paclitaxel-loaded colloidal carriers decorated with a new amphiphilic PEG derivative. Int J Pharm. 2012;426:231–8.
22. Kim JY, Kim JK, Park JS, Byun Y, Kim CK. The use of PEGylated liposomes to prolong circulation lifetimes of tissue plasminogen activator. Biomaterials. 2009;30:5751–6.
23. Couvreur P, Puisieux F. Nano-and microparticles for the delivery of polypeptides and proteins. Adv Drug Deliv Rev. 1993;10:141–62.
24. Nakamura K, Yamashita K, Itoh YK, Yoshino K, Nozawa S, Kasukawa H. Comparative studies of polyethylene glycol-modified liposomes prepared using different PEG-modification methods. Biochim Biophys Acta. 2012;1818:2801–7.
25. Shi G, Guo W, Stephenson SM, Lee RJ. Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic/anionic lipid combinations. J Control Release. 2002;80:309–19.
26. Asokan A, Cho MJ. Cytosolic delivery of macromolecules 4. Head group-dependent membrane permeabilization by pH-sensitive detergents. J Control Release. 2005;106:146–53.
27. Di ML, Marianecci C, Cinque B, Nazzarri M, Cimini AM, Cristiano L, *et al.* pH-sensitive non-phospholipid vesicle and macrophage-like cells: binding, uptake and endocytotic pathway. Biochim Biophys Acta. 2008;1778:2749–56.
28. Simões S, Slepushkin V, Düzgünes N, Pedroso de Lima MC. On the mechanisms of internalization and intracellular delivery mediated by pH-sensitive liposomes. Biochim Biophys Acta. 2001;1515:23–37.

29. Garg A, Kokkoli E. pH-sensitive PEGylated liposomes functionalized with a Fibronectin-Mimetic peptide show enhanced intracellular delivery to colon cancer cells. Curr Pharm Biotechnol. 2011;12:1135–43.
30. Seow WY, Xue JM, Yang YY. Targeted and intracellular delivery of paclitaxel using multi-functional polymeric micelles. Biomaterials. 2007;28:1730–40.
31. Holland JW, Hui C, Cullis PR, Madden TD. Poly(ethylene glycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. Biochemistry. 1996;35:2618–24.
32. Piperoudi S, Fatouros D, Ioannou PV, Frederik P, Antimisiaris SG. Incorporation of PEG-lipids in arsonoliposomes results in formation of highly stable arsenic-containing vesicles. Chem Phys Lipids. 2006;139:96–106.
33. Dadashzadeh S, Vali AM, Rezaie M. The effect of PEG coating on in vitro cytotoxicity and in vivo disposition of topotecan loaded liposomes in rats. Int J Pharm. 2008;353:251–9.
34. Xu H, Deng YH, Chen DW, Hong WW, Lu Y, Dong XH. Esterase-catalyzed dePEGylation of pH-sensitive vesicles modified with cleavable PEG-lipid derivatives. J Control Release. 2008;130:238–45.