

PEG-Benzaldehyde-Hydrazone-Lipid Based PEG-Sheddable pH-Sensitive Liposomes: Abilities for Endosomal Escape and Long Circulation

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ABSTRACT

Purpose To fabricate an acid-cleavable PEG polymer for the development of PEG-cleavable pH-sensitive liposomes (CL-pPSL), and to investigate their ability for endosomal escape and long circulation.

Methods PEG-benzaldehyde-hydrazone-cholesterol hemisuccinate (PEG_B-Hz-CHEMS) containing hydrazone and ester bonds was synthesised and used to fabricate a dual pH-sensitive CL-pPSL. Non-cleavable PEGylated pH-sensitive liposome (pPSL) was used as a reference and gemcitabine as a model drug. The cell uptake and endosomal escape were investigated in pancreatic cancer Mia PaCa-2 cells and pharmacokinetics were studied in rats.

Results The CL-pPSL showed accelerated drug release at endosomal pH 5.0 compared to pPSL. Compared to pPSL, CL-pPSL released their fluorescent payload to cytosol more efficiently and showed a 1.4-fold increase in intracellular gemcitabine concentration and higher cytotoxicity. In rats, injection of gemcitabine loaded CL-pPSL resulted in a slightly smaller V_d (149 ± 27 ml/kg; 170 ± 30 ml/kg) and shorter terminal $T_{1/2}$ (5.4 ± 0.3 h; 5.8 ± 0.6 h) (both $p > 0.05$) but a significantly lower AUC ($p < 0.01$), than pPSL, due to the lower PEGylation degree (1.7 mol%) which means a ‘mushroom’ configuration of PEG. A five-time increase in the dose with CL-pPSL resulted in a 11-fold increase in AUC and a longer $T_{1/2}$ (8.2 ± 0.5 h).

Conclusion The PEG-detachment from the CL-pPSL enhanced endosome escape efficiency compared with pPSL, without significantly compromising their stealth abilities.

KEY WORDS dual pH-sensitive liposomes · endosome escape · hydrazone · PEG detachment · tumor

ABBREVIATIONS

AUC	The area under the concentration-time profile curve
CHEMS	Cholesterol hemisuccinate
CL-pPSL	Cleavable PEGylated pH-sensitive liposomes
DL	Drug loading
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
EE	Entrapment efficiency
Hz	Hydrazone
IC ₅₀	The drug concentration causing 50% inhibition
PSL	pH-sensitive liposomes
pPSL	PEGylated pH-sensitive liposomes
PEG	Poly(ethylene glycol)
TEM	Transmission electron microscopy

INTRODUCTION

Since the discovery by Bangham in early 1960's, liposomes have attracted unprecedented attention as drug delivery systems which resulted in numerous publications, however, this has resulted in few FDA approved clinical products (1). To date, 15 liposomal products have been approved for clinical use and nearly twice as many are in clinical trials (2). However still there is a room for improvement in these numbers by rational formulation design. Two critical factors needed attention in the development of liposomal anticancer drug delivery systems are their long circulation time in the blood stream which facilitates drug accumulation at tumors, and their ability to release cargos at the

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target cells (3). Surface modification of liposomes with hydrophilic polymers, such as poly(ethylene glycol) (PEG), poly(vinyl pyrrolidone) (PVP) (4), and Poloxamer 188 (5), have been demonstrated to extend liposome circulation times. The hydrophilic polymers sterically prevent adsorption of opsonins; thus, allowing liposomes bypass the recognition of the host mononuclear phagocyte system, a major clearance pathway for liposomes. This technique is known as ‘steric stabilization’ or ‘stealth coating’. To date, the gold standard for stealth coating is still PEG (6). However, from the first PEGylated liposome, Doxil (containing doxorubicin) approved in 1995, to the latest Onivyde (irinotecan liposome injection) for metastatic pancreatic adenocarcinoma in 2015, although the side effects in patients have been minimized, the improvement in efficacy is marginal (7). This could partially be attributed to the poor cellular uptake and slow intracellular drug release due to PEGylation, known as PEG dilemma (8).

The pH-sensitive liposomes (PSL) have been developed to refine the conventional liposomes to promote extra- and intracellular drug release to cancer cells (9). PSLs, conceptualized by Yatvin *et al.* in 1980 (10), are designed to maintain stability in physiological pH, but release the payloads at a threshold of low pH (10), such as the endosomal lumen (pH 5–5.5), i.e. ‘endosome escape’ allowing drug to be released into the cytosol. In contrast, conventional liposomes can be entrapped in endosomes and transported to lysosomes where drug may be degraded (11). Similar to the conventional liposomes, PEGylation is required to stabilise PSL *in vitro* and *in vivo*. However, PEGylation hinders the cellular uptake of liposomes by cancer cells (12), reduces the pH sensitivity of PSL and their ability of endosomal escape (13), compromising the efficacy.

To overcome the above ‘PEG dilemma’ (14), PEG-detachable liposomes are designed using various tumor specific features as stimuli, low pH (15), enzymes (16), and reduction (17). Low-pH triggered PEG-detachable liposomes are typically constructed by conjugating PEG to liposomes surface via a pH-sensitive bond (18). The bond cleavage at low pH initiates PEG shedding that facilitates endocytosis, followed by membrane fusion and endosomal escape (19). The chemical bonds used are important in determining the PEG detachment efficiency, as well as the stability at physiological pH, and thus the circulation half-life. Various pH-sensitive bonds have been investigated, such as hydrazone (Hz), ester and vinyl ether (15), and hydrazone (20) is commonly used due to its greater stability at pH 7.4. Chen *et al.*, reported PEG-detachable liposomes (non-pH sensitive bilayers) using mPEG-Hz-CHEMS based on ketone-derived hydrazone. The study revealed that the PEG-detachable liposomes might overcome another PEG dilemma, the accelerated blood clearance (ABC) phenomenon induced by repeated injection in animal models. However, an increased accumulation in liver and spleen was found with these novel liposomes compared with the conventional liposomes (21).

In this paper, we aimed to develop a PEG-cleavable pH-sensitive liposome system (CL-pPSL) and investigate the effects of PEG detachment on cellular uptake, endosome escape, and stealth property of the dual pH-sensitive liposomes. A novel PEG-lipid, PEG-benzaldehyde-hydrazone-cholesteryl hemisuccinate (PEG_B-Hz-CHEMS) was synthesized and subsequently used to fabricate CL-pPSL with backbone bilayers composed of CHEMS and 1, 2-distearoyl-sn-glycero-3-phosphocholine (DOPE). The proposed mechanism was that the cleavage of pH-labile bonds, the succinic ester bond of CHEMS, but mainly hydrazone (Hz), enables PEG detachment from CHEMS at extracellular or endosomal pH, which generates naked CHEMS/DOPE PSL. Gemcitabine, a first line chemotherapeutic agent for pancreatic cancer was chosen as a model drug. Gemcitabine is a prodrug which is activated in the cells after its phosphorylation which inhibit synthesis of DNA. Additionally, the cellular uptake of gemcitabine relies on active influx transporters, and may be limited by the low expression of human equilibrative nucleoside transporter-1 (hENT1) (22), one of the mechanisms associated with pancreatic cancer resistance (22–24). The PEGylation degree of CL-pPSL was determined by HPLC analysis of PEG_B-Hz-CHEMS. The cell uptake and endosome escape of CL-pPSL was investigated on Mia PaCa-2 pancreatic cancer cell line using confocal imaging, and cytotoxicity by MTT assay. Liposomal stealth property was investigated in Sprague Dawley rats.

MATERIALS AND METHODS

Materials

Phospholipids, 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesteryl hemisuccinate (CHEMS), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀) were purchased from Avanti Polar Lipids (Alabama, USA). Poly(ethylene glycol) methyl ether (mPEG2000), dicyclohexyl carbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), and Sephadex G-25 were obtained from Sigma Chemical Co. (MO, USA). HPLC grade acetonitrile and methanol were obtained from Merck. All other chemicals and solvents were reagent grade. Gemcitabine HCL (99.95% purity) was obtained from Selleckchem (Houston, USA). Calcein, Nile Red and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, for cytotoxicity studies) were from Sigma (Auckland, New Zealand). Gibco™ Dulbecco’s Modified Eagle’s Medium (DMEM) cell culture media was purchased from Thermofisher Scientific (Auckland, New Zealand).

Sprague-Dawley (SD) rats were obtained from the Vernon Jansen Unit (The University of Auckland) and used for the

pharmacokinetic study. All procedures were approved by the Committee on Animal Experiments of The University of Auckland (Ethics approval number 001228).

Synthesis of PEG_B-Hz-CHEMS

The synthesis of PEG_B-Hz-CHEMS was carried out in three steps.

- Step 1: CHEMS (1 mM) in 20 mL of dichloromethane was reacted with 0.5 mL of thionyl chloride at room temperature for 2 h. The solvent was evaporated under vacuum to leave a sticky, oily product, to which was then added benzene (50 mL). The benzene was removed under reduced pressure, and the residue was dissolved in dichloromethane (20 mL). The above solution was placed on ice bath and added with hydrazine hydrate (0.3 mL) with stirring for 1 h, and concentrated to dryness under reduced pressure. The residue was dissolved with dichloromethane, filtered and the filtrate was concentrated under reduced pressure to obtain CHEMS with a yield of 50%. The structure of final product was confirmed by ¹H NMR spectroscopy using a Bruker Avance-400 spectrometer.
- Step 2: A solution of mPEG₂₀₀₀ (1 mmol) in 20 mL of dichloromethane was added to 4-carboxybenzaldehyde (10 mmol), DCC (10 mmol), and DMAP (2.5 mmol) and stirred for 24 h. The resulting reaction mixture was filtered, and the solid product obtained was collected, washed with isopropanol and diethyl ether and dried under vacuum. A light-yellow powder was obtained with 80% yield. The final product, PEG-benzaldehyde (PEG_B) was confirmed by ¹H NMR spectroscopy.
- Step 3: PEG_B (0.06 mmol) was reacted with hydrazine activated CHEMS (0.09 mmol) in 2 mL of chloroform at 25°C in a tightly closed reaction vessel. After overnight stirring, the solvent was removed under reduced pressure, and the residue was purified by dissolving in milliQ water (pH adjusted to 7.4) and applied to a Sephadex G-25 column. The turbid fractions containing the component were pooled, and freeze dried overnight at -80°C. The structure and molecular weight of PEG_B-Hz-CHEMS were confirmed by ¹H NMR and mass spectrometry.

Characterization of PEG_B-Hz-CHEMS Micelles

Size and Morphology of Micelles

The size of polymeric micelles of PEG_B-Hz-CHEMS was determined by dynamic light scattering (DLS, ZS90, Malvern, U.K.).

The polymeric micelles diluted in 10 mM PBS, pH 7.4, was filtered through 0.45 μm pore size filters and the measurements were conducted in a 1.0 mL quartz cuvette, using a diode laser of 800 nm at 25°C, and the scattering angle was fixed at 90°. Morphology of the micelles was observed by Transmission Electron Microscopy (TEM, JSM5600LV, Japan).

Critical Micellar Concentration (CMC)

The CMC of the micelles was determined by a modified fluorescence technique using Nile Red as a fluorescent probe (25). To prepare micelles loaded with Nile Red, 30 mg PEG_B-Hz-CHEMS and 0.2 mg Nile Red were first dissolved in 2 mL methanol, followed by addition of 10 mL PBS, 0.01 M, pH 7.4 dropwise into the solution. After stirring for 8 h at 37°C, excess of Nile Red was removed from the polymeric micelles by filtration through 0.45 μm membrane. For CMC determination, Nile Red loaded micelles were diluted to a concentration of the polymer range from 2×10^{-7} mol L⁻¹ to 2×10^{-4} mol L⁻¹. Fluorescence measurements were taken at an excitation wavelength of 550 nm and the emission from 580 to 720 nm using Fluoro Max-4 Spectrofluorometer. Excitation and emission slit widths were maintained at 5.0 nm and spectra were accumulated with a scan speed of 200 nm/min. The CMC of the polymer was determined by plotting the fluorescence intensity against log polymer concentration (mol L⁻¹).

Preparation and Characterization of Dual pH-Sensitive Liposomes

Preparation of pH-Responsive Liposomes

Two kinds of liposomes were prepared by thin film hydration method. Conventional pPSL were prepared by DOPE, CHEMS, DSPC, cholesterol, and DSPE-PEG₂₀₀₀ at the molar ratio of 4:2:2:2:0.5. Our previous study (9) showed this pPSL membrane had superior pH-sensitivity using a non-PSL as control. CL-pPSL were prepared using the same lipids at the same ratios but by replacement of DSPE-PEG with PEG_B-Hz-CHEMS. All lipids apart from the PEG polymers were dissolved in chloroform, and then solvent was removed on rotary evaporator at 30°C under vacuum to form thin lipid films, which was kept under vacuum overnight. The resulting lipid films were hydrated with phosphate buffered saline (PBS, 0.1 M, pH 7.4) at 30°C. The hydrated suspension was subjected to 7-cycles of freeze and thaw before extrusion through a 100 nm membrane. The liposomes were then subjected to ultracentrifugation to obtain the pellets. PEG_B-Hz-CHEMS was coated on liposomes by post-insertion, i.e., incubation of polymer solution with the liposomes. Optimal incubation time and polymer concentrations (equivalent to 3, 5, and 10 mol% to lipids) were determined.

Size, Zeta Potential and Morphology of Liposomes

The selected liposome formulations with better IE of PEG_B-Hz-CHEMS were characterized. Hydrodynamic particle diameter (*Z*-average) and polydispersity (PDI) were determined by photon correlation spectroscopy (PCS) on a Zetasizer Nano ZS (Malvern Instruments, Germany) at a temperature of 22°C and a scattering angle of 173°. Liposome pellet was resuspended in 1 mL PBS, pH 7.4, 0.01 M and further diluted as required. Particle size and zeta potentials before and after PEGylation were compared to evaluate the surface modification of liposomes with PEG.

Determination of PEGylation Degree and PEG Configuration

The degree of PEGylation was determined by measuring the insertion efficiency PEG_B-Hz-CHEMS using an Agilent 1260 series HPLC system with a diode array detector (DAD) on a Luna C18, 5 μm, 4.6 mm × 250 mm column connected with a 4.6 mm × 30 mm pre-column of same type (Phenomenex®, New Zealand). The mobile phase consisted of methanol–acetonitrile–Milli Q water (5:45:50, *v/v*) and the flow rate was set at 1 ml/min. Dual UV wavelength was set at 300 nm for detection of PEG_B-Hz-CHEMS and at 254 nm for its degradation product PEG_B. The method was validated to be linear in the concentration range of 8 to 125 μg/mL ($r^2 = 0.9999$, $n = 3$).

The insertion efficiency (IE) of PEG_B-Hz-CHEMS into the liposomes was determined by analysing both supernatant and the pellet. In brief, the liposome suspensions were subjected to ultracentrifugation (186,000×*g*) for 2 h which was proved to have complete separation of unbound PEG_B-Hz-CHEMS from the liposomes. The supernatants were collected and diluted with 0.01 M PBS (pH 7.4) to the concentration levels within the calibration range and analysed by HPLC immediately. In parallel, the liposome pellet was destroyed with 10% triton X-100 solution and diluted with PBS before quantification of PEG_B-Hz-CHEMS. The IE (%) was calculated as percentage of the polymer inserted in liposomes to the total amount of polymer used in preparation.

The mean distances *D* between the grafting sites of the PEG-chains and *R_F* (Flory radius) will determine the conformation of the polymer on the liposome surface (26). Distance *D* can be calculated using Eq. 1

$$D = \left(\frac{A}{\text{Mole ratio of PEG attached}} \right)^{1/2} \quad (1)$$

A is the area per lipid molecule in the liposome, which is reported in literature as 50 Å (27).

R_F (Flory radius), can be determined using Eq. 2 (26).

$$R_F = aN^{3/5} \quad (2)$$

Where, *a* is monomer size; *N* is the degree of polymerization.

Gemcitabine Loading and Entrapment Efficiency Into Liposomes

Gemcitabine was loaded into the selected CL-pPSL and pPSL using small volume incubation (SVI) method (28). In brief, 20 μL of gemcitabine suspension (1.5 mg/ml), pH 7.4 was added to the liposomal pellet, vortexed for 3 min and incubated at 60°C for 3 h for drug loading. Free drug was removed by eluting the liposomal suspension through gel filtration column, Sephadex G-75 column (100 × 20 mm) using 20 mM PBS containing 0.15 M NaCl at pH 7.4. Collected liposomal fractions were dissolved using Triton X-100 and subjected to HPLC analysis. Drug concentration was measured to determine the entrapment efficiency (EE) and drug loading (DL) using the following equations.

$$EE (\%) = \frac{\text{mass of the drug liposomes}}{\text{mass of the drug used for loading}} \times 100 \quad (3)$$

$$DL (\%) = \frac{\text{mass of the drug in liposomes}}{\text{mass of drug-loaded liposomes}} \times 100 \quad (4)$$

pH-Responsive Drug Release

The pH-responsive drug release from PSL and CL-pPSL was compared using a dialysis method. Cellulose acetate dialysis bags (MWCO 12–14 kDa) containing 1 ml of the liposome suspension was placed in 50 ml release medium (PBS 50 mM, pH 7.4, 6.5 and 5.0, adjusted with NaCl to 320 mOsm) at 37°C with stirring throughout the experiment. At different time intervals, samples (0.1 ml each) were taken and analysed by HPLC to obtain the percentage of the drug released (28).

The *in vitro* drug release-time profiles were compared using the similarity factor (*f₂*) approach (28). The *f₂* value is a logarithmic transformation of the sum-squared error of the differences in % release between two formulations (*T_j* and *R_j*) through all the time points, as described in Eq. 5:

$$f_2 = 50 \log \left\{ \left[1 + \left(\frac{1}{m} \right) \sum_{j=1}^m w_j |R_j - T_j|^2 \right]^{-0.5} \times 100 \right\} \quad (5)$$

Where, *m* represents the total time points, and *w_j* is an optional weight factor.

An *f₂* value between 50 and 100 indicates the release profiles are similar.

Quantitative Determination of Cellular Drug Uptake Using HPLC

To determine the cellular uptake, MIA PaCa-2 pancreatic cancer cells were seeded in 6-well plates (2 × 10⁶ cells/well in 2 ml) and cultured in DMEM with 10% FBS for 24 h at

37°C, 5% CO₂ to form cell monolayers. The cells were further incubated with free drug, pPSL or CL-pPSL at 37°C with the final drug concentration fixed at 120 µM, which was shown to cause minimal cell death. After drug exposure for 4 h, the medium was aspirated and the plate was placed on ice and washed with ice-cold PBS three times before the cells were lysed with 80 µl methanol. The sample was dried and reconstituted with mobile phase and drug concentration was determined by a validated HPLC method (28). Intracellular drug concentration (C_i) was calculated based on the drug amount and cell size, measured by microscope. The ratio of C_i/C_e (extracellular drug concentration) was determined to evaluate the cellular uptake of each formulation.

Cytotoxicity to MIA PaCa-2

MTT assay was used to evaluate cytotoxicity of gemcitabine formulations on Mia PaCa-2 pancreatic cell line. Briefly, cells in medium were seeded into 96-well plate (800 cells in 100 µL/well) and cultured at 37°C, 5% CO₂ for 24 h. Various concentrations of free gemcitabine or drug loaded CL-pPSL and pPSL dispersed in PBS (pH 7.4) were separately added into the wells and cultured for 4 and 24 h. After drug exposure, cells were washed, 100 µL of fresh medium was added and cells were allowed to grow for 72 h. After this, MTT cell viability was measured with untreated cells in culture medium used as controls (100%). Blank liposomes were also tested for cytotoxicity. The drug concentration causing 50% inhibition of viable cell density (IC₅₀) with the 95% confident intervals was calculated using a non-linear fitting model in GraphPad Prism (GraphPad Software, USA).

Intra-Cellular Trafficking of CL-pPSL and pPSL

To investigate their intra-cellular trafficking or endosomal escape, liposomes were dual labelled with fluorescent dyes: Lipophilic Nile Red (0.2 µg/ml) was incorporated into the lipid membrane to probe the intracellular fate of liposomes whereas hydrophilic calcein at its self-quenched concentration (80 mM) was loaded into the core, which acts as an indicator of lipid vesicle leakage to study their endosome escape properties (29). Mia Paca-2 cells were seeded at 1 × 10⁵ cells/well in four-well chambered slides and cultured for 24 h. The dual-labelled liposomes were added to the plates with a total lipid concentration of 0.4 mmol/L.

After incubation at 37°C for 1 and 2 h respectively, the cells were washed three times with cold PBS (pH 7.4) and fixed using 4% paraformaldehyde for 10 min. Then, nucleus was stained by incubation with DAPI for 5 min, followed by positioning the coverslips with mounting agent. The cellular uptake and endosomal escape of various liposomes was observed using a confocal laser scanning microscope (Olympus Fluoview FV1000, Olympus Corporation, Japan) with

excitation wavelength of 366 nm for DAPI, 488 nm for calcein and 546 nm for Nile Red.

Pharmacokinetics in Rats

SD rats (195–205 g) were randomly divided into three groups, namely gemcitabine solution (*n* = 4), pPSL (*n* = 4) and CL-pPSL (*n* = 6). Each formulation (0.2 mg/ml) was injected via the tail vein at 1 mg/kg equivalent gemcitabine. Blood samples (100–200 µl) were collected from the tail vein at various time points. Furthermore, a high dose of 5 mg/kg was injected to test the dose dependence of pharmacokinetic parameters of CL-pPSL. Blood samples were treated with a 2-step protein precipitation method by acetonitrile and analysed using the method previously reported (30). Gemcitabine concentration was calculated using a freshly prepared external standard curve ranging from 0.1 to 10 µM. The assay was linear with *r*² = 0.9992. No interference was observed from plasma components and the recovery was > 94% at both low and high concentrations.

The gemcitabine pharmacokinetic profiles were fitted to a non-compartmental model using a Kinetica 5 program and the pharmacokinetic parameters such as the area under the concentration-time profile curve (AUC), mean residence time (MRT) and elimination half-life (T_{1/2}), were obtained.

Statistical Analysis

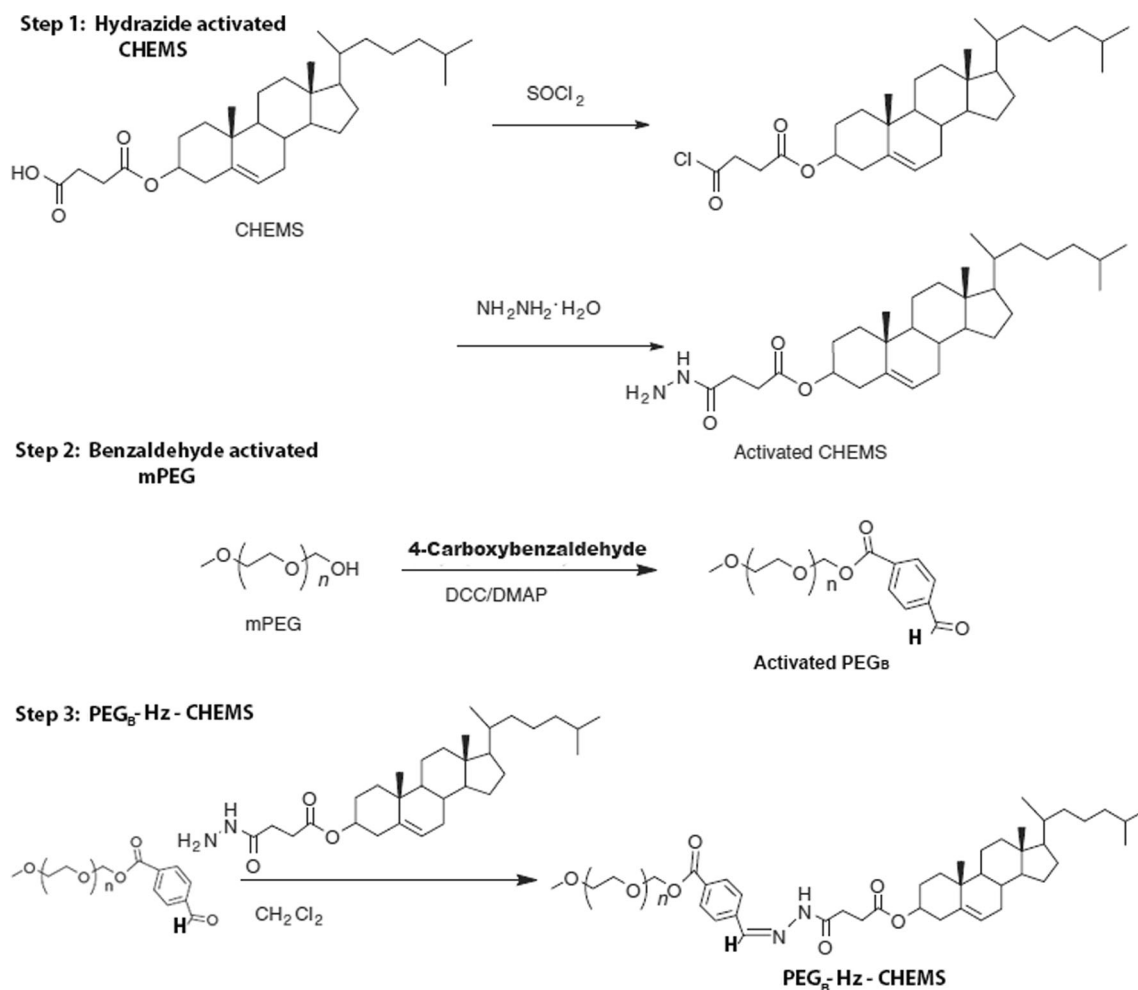
The level of significance for all statistical analysis was set at 0.05. Data was analysed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, U.S.A).

RESULTS AND DISCUSSION

Synthesis of PEG_B-Hz-CHEMS

As shown in Scheme 1, PEG_B-Hz-CHEMS was successfully synthesized via three steps: 1) Activated CHEMS was synthesized based on the literature (31) in which CHEMS was reacted with thionyl chloride to form the acyl chloride, which readily reacted with hydrazine hydrate to form hydrazine-activated CHEMS; 2) Benzaldehyde-derivatized PEG (PEG_B) was obtained by introducing 4-carboxybenzaldehyde into mPEG 2000 under catalysis of DCC and DMAP; and 3) PEG_B was then reacted with 1.5 M excess of hydrazine-activated CHEMS to form PEG_B-Hz-CHEMS. The yield of final product was 90%.

The structure of PEG_B-HZ-CHEMS was confirmed by ¹H NMR spectroscopy. As shown in Fig. 1a, the proton signals attributed to the (-OCH₂CH₂)_{*n*} cluster of PEG_B (peak a) at δ 3.09–4.00 ppm, the adjacent methyl cluster group (peak b) at δ



Scheme 1 Synthesis of PEG_B-Hz-CHEMS with three steps.

0.8–1.2 ppm and peaks at δ 7.9–8.0 ppm for protons of the aromatic ring of benzaldehyde demonstrated the successful conjugation of aldehyde group of PEG with hydrazide. Of particular significance was the disappearance of the peak representing the aldehyde group at chemical shift δ 10.50 ppm and the appearance of new peaks (c and c^1) for Ph-HC=N bond formation at δ 8.32 ppm and δ 8.60 ppm, indicating the successful formation of the hydrazone bond. Furthermore, mass spectrometry of the final compound using Q-Exactive Hybrid Quadrupole mass spectrometer showed an average molecular mass of 2600, indicating successful addition of CHEMS (MW: 501) to PEG_B (average MW: 2100; Fig. 1b).

CMC of PEG_B-Hz-CHEMS

As expected, PEG_B-Hz-CHEMS could self-assemble into micelles in aqueous medium due to its amphiphilic property. The average particle size of micelles was 30 nm (Fig. 2a). Cryo-TEM showed that the micelles appeared as discrete particles with spherical shape (Fig. 2b). The CMC value determined was 14.4 μ M (Fig. 2c), equivalent to 0.04 mg/mL.

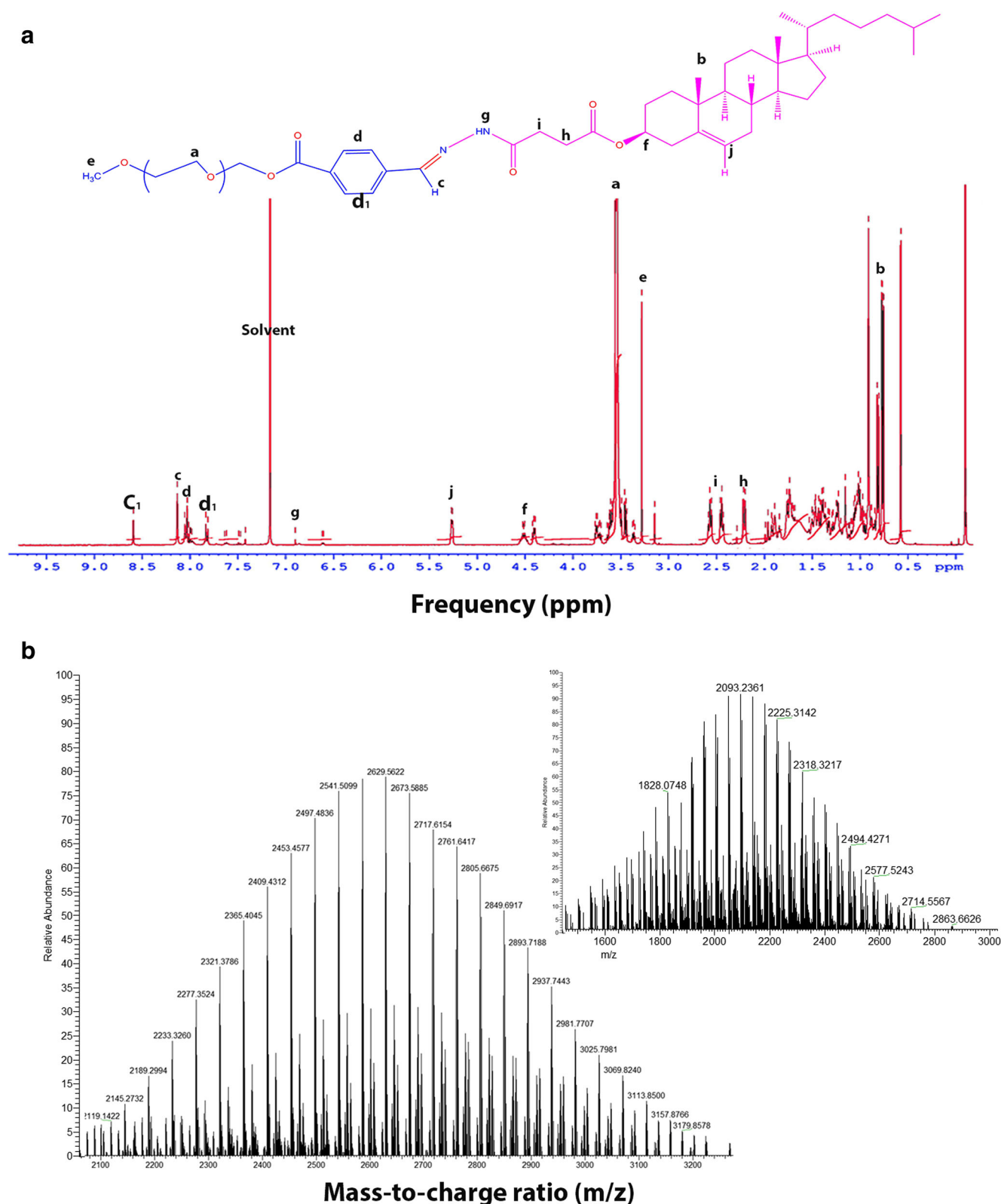
Characterization of Dual pH-Sensitive Liposomes

Size, Zeta Potential and Morphology

Blank liposomes before PEG modification showed an average size of 100.5 ± 1.4 nm for CL-pPSL (Fig. 3a), which increased to 124.2 ± 0.9 nm after incubation with 5% PEG_B-Hz-CHEMS (molar ratio to other lipids). PEG coordinates about three water molecules per monomeric unit resulting in a large hydrodynamic volume thus an increase particle diameter was observed post PEG modification. However, a further increase in polymer concentration to 10% (mol to lipids) during post-insertion resulted in reduction in pellet size, possibly because that excessive PEG caused micellization of lipids, as previously reported (32).

Along with the size change, the zeta potential of liposomes (-25.4 mV before PEGylation) was neutralized after PEGylation due to the shielding effect of PEG chains.

Similarly, the reference formulation, pPSL with 5% mol DSPE-PEG2000 had a particle size of 128.8 ± 1.3 nm and zeta potential of -8.0 ± 0.3 mV.



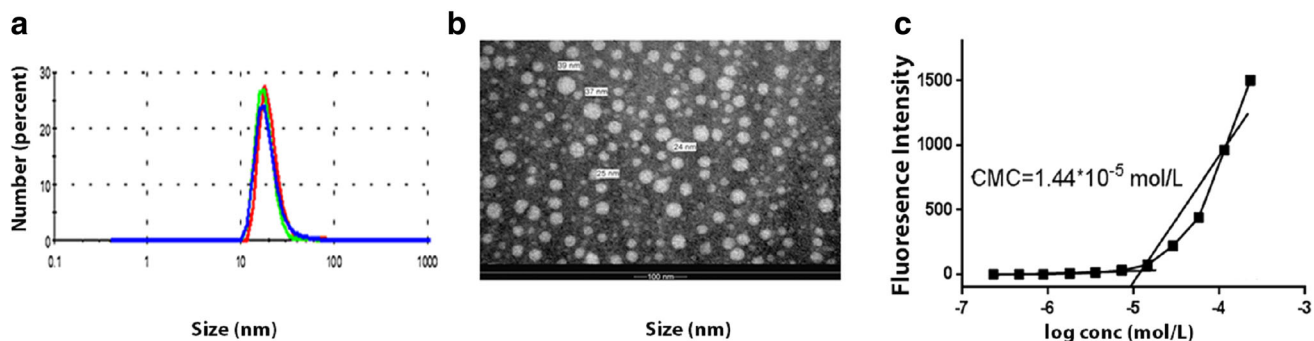


Fig. 2 Physicochemical characterization of PEG_B-Hz-CHEMS micelles: (a) Particle size by DLS, (b) Morphology by TEM; (c) CMC determination by a Nile Red method.

Quantification of Degree of PEGylation

Degree of PEGylation for CL-pPSL with 3, 5 and 10 mol% PEG_B-Hz-CHEMS to other lipids as shown in Fig. 3 was determined by HPLC analysis. Surprisingly, only <20% of the PEG was found to be inserted into liposomes after incubation for 8 h (Table I). An increase in the insertion time to 24 h resulted in an increased IE, but only to about 34%. Incubation with 10% of PEG_B-Hz-CHEMS destabilized the liposomes as seen from the liposomal pellet size. Therefore, the CL-pPSL with 5% PEG_B-Hz-CHEMS, which resulted in a PEGylation degree of 1.7% mol was selected for further studies.

The formation of micelles reduces IE as the thermodynamic barrier to insertion into the liposome bilayers is lower in the case of monomeric PEG-lipids compared to the micellar PEG-lipids as reported (33).

PEG Conformation on Surface of Liposomes

Once IE was determined the conformation of the PEG chains on the surface of liposomes depends on the Flory radius R_F and the distance D between the grafting sites. If $D < R_F$, PEG coils start to repel each other and extend outward from the liposome surface resulting in 'brush' conformation (34). When, $D > R_F$, individual polymer chains remain widely

separated and do not interact with each other resulting in 'mushroom' conformation.

With a molecular weight of 2000, the PEG₂₀₀₀ derivatives used in this synthesis was estimated to have a R_F of 3.7 nm (37 Å) (35). The distance 'D' in the selected CL-pPSL (PEGylation 1.7% mol) and pPSL (PEGylation 5% mol) was calculated to be 54 Å and 32 Å, respectively. Therefore $D > R_F$ for CL-pPSL but $D < R_F$ for pPSL which predicts 'mushroom' and 'brush' configurations on the liposomal surface, respectively.

Drug Loading and Entrapment Efficiency

With the SVI method, the optimized incubation time on the EE of gemcitabine was determined to be 3 h to give an EE of $37.0 \pm 1\%$, and DL 4% (w/w), similar to the previous report (28). The mechanism for drug loading was passive diffusion of the drug into liposomes, which is driven by a high concentration gradient established using a drug suspension. After drug loading size of liposomes was increased to about 145 nm for both liposomes.

pH-Sensitive Drug Release

Release of gemcitabine from CL-pPSL after 24 h was 41.9% at pH 7.4, 52.1% at pH 6.5 and increased to 95.5% at pH 5.0. The faster release profile at pH 5.0 demonstrated that the endosomal acidic environment could expedite the

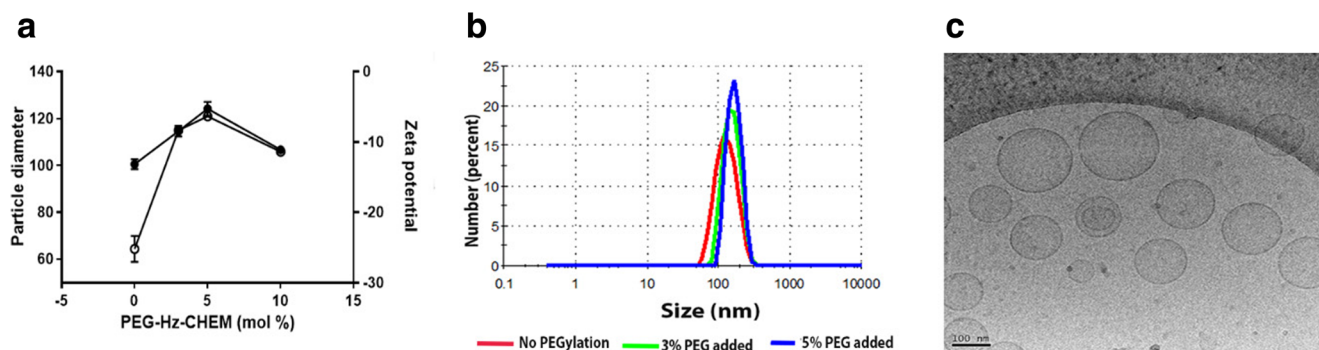


Fig. 3 (a) The influence of PEGylation with PEG_B-Hz-CHEMS on the particle size (closed symbols) and zeta potential (open symbols) (mean \pm S.D., $n = 3$). The concentrations of PEG_B-Hz-CHEMS represent the molar ratio to total lipids of the added PEG. (b) DLS showing shift in average particle size with PEGylation extent, (c) Cryo-TEM showing morphology of CL-pPSL with 1.7 mol% PEG_B-Hz-CHEMS.

Table I Insertion Efficiency (IE) of PEG_B-Hz-CHEMS Estimated by Analysing Pellet and Supernatant After Incubation at 4°C At Different Concentrations of Polymer (*n* = 3)

Polymer added (%)	Incubation time (hours)	PEG _B -Hz-CHEMS found in supernatant (%)	PEG _B -Hz-CHEMS found in pellet (%)	IE (%)	Mole ratio in liposomes %
3	8	81.1 ± 0.05	18.4 ± 0.04	18.4	0.6
	24	71.4 ± 0.08	34.3 ± 0.01	34.2	1.0
5	8	82.7 ± 0.03	17.9 ± 0.02	18.0	0.9
	24	68.4 ± 0.06	33.7 ± 0.07	33.6	1.7*

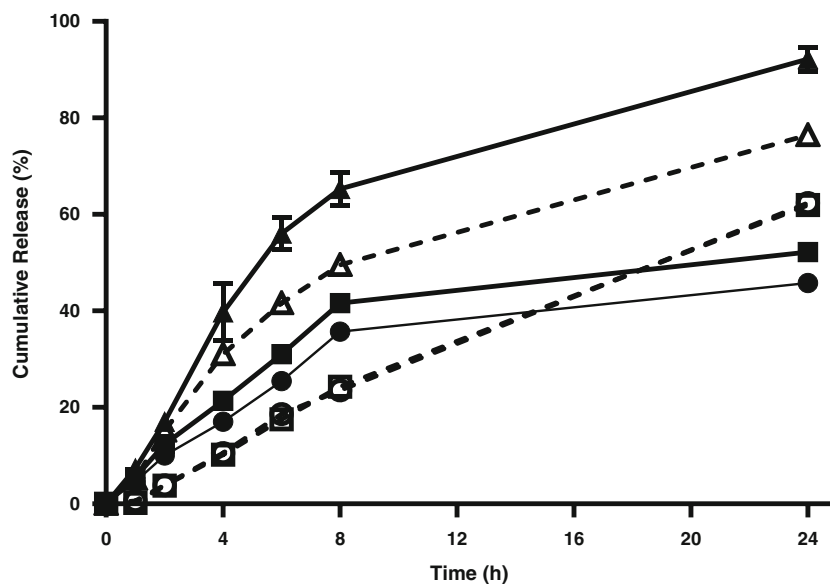
*Liposome selected for further studies. Incubation with 10% of PEG_B-Hz-CHEMS reduced liposomal pellet size

gemcitabine release from CL-pPSL. Similarly, pPSL also showed pH-responsive gemcitabine release, but to less extent than CL-pPSL with 47.4% at pH 7.4, 54.1% at pH 6.5 and 72.7% at pH 5.0 after 24 h.

Between CL-pPSL and pPSL, the overall release profiles (Fig. 4) are similar at pH 7.4 ($f_2 = 61.6$). Whereas, at pH 6.5 and 5.0, an f_2 value of 41.6 and 38.6 was obtained, which indicates that the release profiles from the two liposomes were not similar, with CL-pPSL producing faster release than pPSL. This can be due to the presence of the non-cleavable DSPE-PEG-2000 coating on the outer surface, which hindered the release of the drug. At pH 5.0, PEG detachment from CL-pPSL may have contributed to the rapid drug release.

Cellular Drug Uptake Determined by HPLC

The Mia PaCa-2 cells had an average diameter of $28.8 \pm 2.54 \mu\text{m}$ and based on that the total intracellular volume

Fig. 4 pH-sensitive drug release profiles of dual pH-sensitive liposomes (CL-pPSL, close symbols) and pPSL (open symbols), at pH 5.0 (triangles), pH 6.5 (diamonds) and pH 7.4 (circles). Data are mean ± S.D., *n* = 3.**Table II** Ratios of Intracellular and Extracellular Concentrations (C_i/C_e) of Gemcitabine in Mia PaCa-2 Cells after Incubation with Free Drug, pPSL, CL-pPSL for 4 h (Data are mean ± SD, *n* = 3 Experiments)

Formulation	C_i (μM)	C_e (μM)	C_i/C_e ($\times 10^{-2}$)
Free drug	2.41 ± 0.31	102.80 ± 0.46	2.34 ± 0.25
pPSL	4.31 ± 0.20	100.12 ± 0.20	4.30 ± 0.20
CL-pPSL	6.05 ± 0.27	100.22 ± 1.82	6.04 ± 0.15

was calculated to be $1.25 \pm 0.21 \mu\text{l}$ in each well. Following free drug exposure to cells for 4 h at 37°C, all C_i values were significantly lower than C_e (Table II), indicating gemcitabine had limitations in entering cells, or had been converted to its metabolites. However, C_i/C_e was 2.0 and 3.0-fold higher for pPSL ($P < 0.01$) and CL-pPSL ($P < 0.001$), respectively compared with the drug solution. Notably, cells treated with CL-pPSL showed a 2.3-fold higher intracellular drug concentration compared with those treated with pPSL ($P < 0.01$).

In general, cell uptake of both liposomes should be similar at neutral pH given their size and surface charge are similar. The higher intracellular concentrations found in CL-pPSL treated cells could be due to their more rapid endosomal escape than pPSL. Studies emphasize that lysis of lysosomes is only achieved by extraction using hypotonic solutions such as concentrated glucose (36). Otherwise endosomes-lysosomes stay intact to retain the contents. In this study methanol used as extraction solvent to lyse the cells, which is most commonly used solvent for extract intracellular drug, but endosome-lysosomes might not be completely lysed and therefore the entrapped drug was not measured by HPLC. Therefore, the rapid endosomal escape of CL-pPSL, rather than the cellular uptake, lead to their higher cytoplasmic concentration compared to pPSL.

Table III Cytotoxicity of CL-pPSL, pPSL and Free Gemcitabine Following 4 h or 24 h Exposure, and after Growth for 72 h of MIA PaCa-2 Cells, Measured with MTT Cell Viability Assay ($n = 3$)

Formulation	4 h		24 h	
	IC ₅₀ (μ M)	95% CI (μ M)	IC ₅₀ (μ M)	95% CI (μ M)
Free gemcitabine	0.570	0.404–0.787	0.045	0.024–0.078
pPSL	0.663	0.560–0.783	0.079	0.048–0.139
CL-pPSL	0.585	0.464–0.730	0.052	0.040–0.067

Cytotoxicity to MIA PaCa-2

Blank liposomes with lipid concentrations equivalent to the gemcitabine-loaded liposomes caused negligible toxicity in the MIA PaCa-2 cells over 24 h exposure (>85% cell viability). As shown in Table III, after 4 h exposure, no significant difference in cytotoxicity was observed between free drug, CL-pPSL and pPSL treated cells. Interestingly, with an increase in treatment periods as 24 h, CL-pPSL was significantly more cytotoxic ($P < 0.05$) than pPSL. This could attribute to the PEG-detachment, with the ester bonds but more effectively the hydrazone bond. Their ability to undergo accelerated destabilisation in endo-lysosomes facilitated rapid drug release into the cytoplasm.

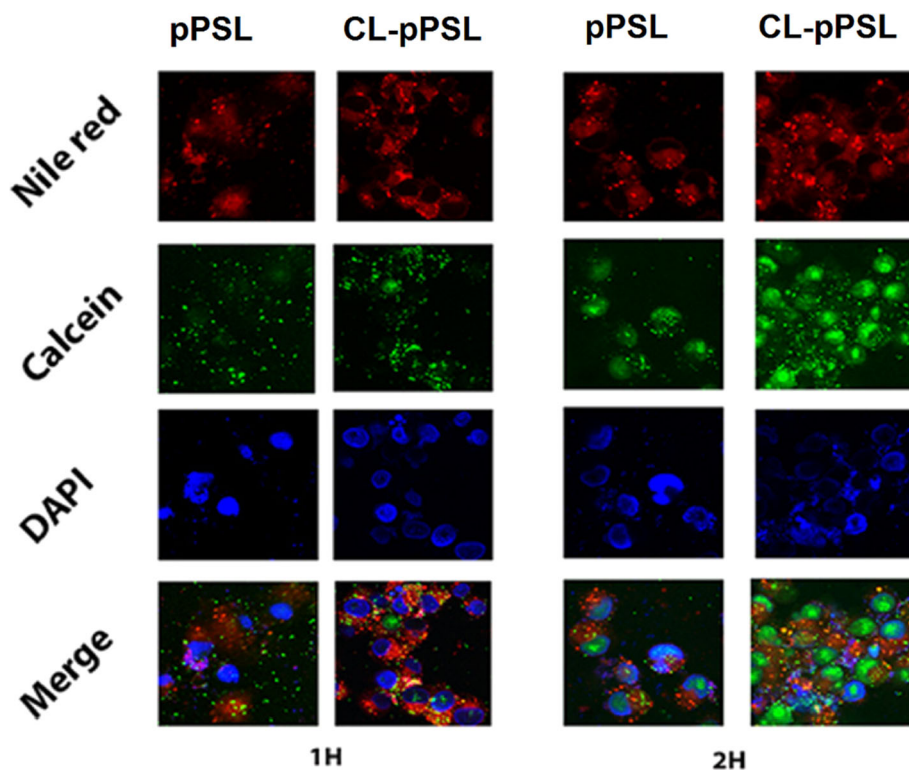
Gemcitabine is a nucleoside analogues and works by replacing one of the building blocks of nucleic acids during DNA replication. Therefore, in this study the cells were allowed to grow for 72 h after treatment before the MTT assay.

Intracellular Trafficking of Liposomes

As shown in Fig. 5, in the cells a strong red fluorescence intensity produced by the Nile Red in liposome membrane was observed for both liposomes, particularly CL-pPSL at 1 h, indicating that both the liposomes were internalized through endocytosis. It was found clathrin-mediated endocytosis accounted for about 50% (9). The Nile Red signal increased at 2 h with more red 'dots' presented.

The kinetics of endosome escape was shown with the second fluorescence marker, calcein (80 mM) which was loaded in the liposomal cores. The fluorescence of calcein is quenched at concentration of 80 mM (37) and thus liposomes are less visible if sequestered in endosomes but would produce strong fluorescence if content released from endosomes into the cytoplasm. Our previous study (9) showed that the fluorescence of calcein peaked at 10 mM. After 1 h treatment with CL-pPSL or pPSL, intracellular calcein fluorescence was observed but mostly as 'dots' (punctate), which indicates the sequestration of liposomes in endosomes. After 2 h, the calcein fluorescence in both cases enhanced, however the fluorescence in CL-pPSL treated cells was seen more homogeneously distributed in the cytosol (Fig. 5), providing evidence that CL-pPSL had more rapid endosome escape than pPSL. Interestingly, some strong calcein 'dots' with a larger size were collocated with the Nile Red 'dots' at 2 h, indicating endosomal escape was still in process, and possibly by fusion of liposomes with endosomes. Using a EPC/EPE/Chol (40:20:30 mol%) liposomes as an endosome membrane model, Vanic *et al.* (38)

Fig. 5 Confocal fluorescence microscopy images of Mia PaCa-2 cells excited with laser at 360 nm (blue), 488 nm (green) and red (546 nm) visualizing the cell uptake and endosome escape after 1 and 2 h incubation with CL-pPSL or pPSL both dual labelled with Nile Red and 80 mM of calcein.



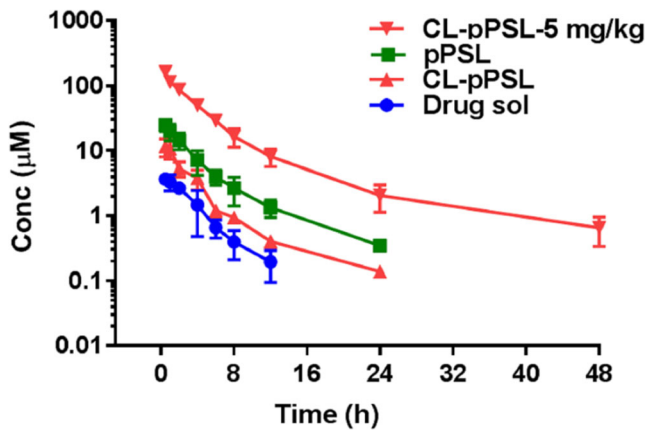


Fig. 6 (a) Pharmacokinetic profiles following *i.v.* injection of gemcitabine solution, pPSL and CL-pPSL ($n = 6$) in rats at 1 mg/kg equivalent gemcitabine and dose dependent pharmacokinetic profile of CL-pPSL. Data are mean \pm SD, $n = 4$ unless stated otherwise.

reported that half of the non PEGylated DOPE/CHEMS liposomes fused with endosome membrane at pH 5.5 within 30 min. Any PEGylation ($\geq 0.6\%$ mol) would reduce the fusion unless the pH was lower than 4.5.

Pharmacokinetics in Rats

Figure 6 presents the gemcitabine plasma concentration-time profiles in SD rats after *i.v.* administration of various gemcitabine formulations at a dose of 1 mg/kg. Compared with the gemcitabine solution, an approximate 4 to 6-fold increase in the $AUC_{0-\infty}$ along with 1.5 to 2-fold increases in $T_{1/2}$ and MRT were observed for both liposomes. Furthermore, the volume of distribution (V_d) and clearances (CL) were both significantly reduced (Table IV). Between the liposomal formulations, the CL-pPSL had 2-fold lower AUC and slightly shorter $T_{1/2}$ ($p > 0.05$) than pPSL, suggesting the PEGylation of CL-pPSL was not as efficient as for pPSL.

Differences in half-life and AUC between the two liposome systems could be explained by the difference in PEG density on their surface, which was predicted from the results of quantification of surface PEGylation. Even though an equivalent amount of PEG_B-Hz-CHEMS and PEG₂₀₀₀-DSPE was used

to prepare the liposomes (Table I), only 34% of the added polymer was found to be successfully inserted in CL-pPSL, resulting in differences in the PEG density and conformation. IE with PEG₂₀₀₀-DSPE to a pPSL similar to the one in this study was found to be nearly 100% (12). As determined earlier, PEG_B-Hz-CHEMS acquires a dense ‘mushroom’ conformation compared to pPSL, $D > R_F$, in ‘brush’ conformation which has a stronger stealth ability.

Further study with an increased dose of CL-pPSL to 5 mg/kg showed a 3-h longer $T_{1/2}$ and an 11-fold, rather than 5-fold, increase in AUC. This again supports the insufficient PEGylation which resulted in initial rapid entrapment of liposomes by liver or spleen. As the major clearance of liposomes is by these organs the increase in liposomes dose could lead to saturation of liver/spleen uptake (39), resulting in a longer half-life of these liposomes in the bloodstream. Overall, the pharmacokinetics study confirms that the pH-sensitive PEG-detachment from PEG_B-Hz-CHEMS in CL-pPSL may compromised their stealth abilities, but not significantly.

CONCLUSIONS

This study demonstrated that PEG-detachment using the new polymer PEG_B-Hz-CHEMS containing acid labile hydrazone and ester bonds, facilitated cellular uptake and particularly endosomal escape. Quantification of the extent of surface PEGylation indicated that that polymer adopted a mushroom conformation on the surface of liposomes, which later correlated with their plasma circulation half-lives. The gemcitabine loaded CL-pPSL demonstrated rapid pH-triggered drug release, and enhanced cellular uptake, and cytotoxicity, compared to the classical pPSL. The pharmacokinetics study suggested that stealth abilities of CL-pPSL were compromised to some extent, but it was likely due to the lower PEGylation degree, rather than PEG-detachment in the blood stream. In addition, this study also highlights the importance to quantify the PEGylation degree of liposomes as the insertion efficiency of some polymers such as PEG_B-Hz-CHEMS may be low.

Table IV Plasma Pharmacokinetic Parameters in SD Rats after a Single *i.v.* Administration of Drug Solution or Liposome Formulations with Dose of 1 mg/kg unless Stated Otherwise. Data are mean \pm SD, $n = 6$ for CL-pPSL and $n = 4$ for the other formulations

Formulation	AUC ($\mu\text{g/ml}\cdot\text{h}$)	V_d (ml/kg)	Clearance (ml/h/kg)	$T_{1/2}$ (h)	MRT (h)
Drug solution	17.0 \pm 1.7	742 \pm 4	202.3 \pm 18.4	3.2 \pm 0.4	3.5 \pm 0.2
pPSL	103.1 \pm 12.8	170 \pm 30	29.3 \pm 4.4	5.8 \pm 0.6	5.0 \pm 0.3
CL-pPSL	63.0 \pm 2.8**	149 \pm 27 ^{ns}	33.3 \pm 1.2*	5.4 \pm 0.3 ^{ns}	4.5 \pm 0.1 ^{ns}
CL-pPSL- 5 mg/kg	682.1 \pm 37.1	141 \pm 11	24.7 \pm 1.3	8.2 \pm 0.5	5.8 \pm 0.8

* $p < 0.05$; ** $p < 0.01$, and ns $p > 0.05$ compared with pPSL

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