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Layer-by-layer assembly of chitosan stabilized multilayered liposomes for paclitaxel delivery

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ABSTRACT

Paclitaxel (PTX) loaded multilayered liposomes were prepared using layer-by-layer assembly in an effort to improve the stabilization of the liposomal compositions for PTX delivery. Stearyl amine was used to provide positive charge to the PTX-liposomes, and subsequently coated with anionic polyacrylic acid (PAA) followed by cationic chitosan. Various process variables were optimized and the optimum formulation was found to have particle size of 215 ± 17 nm, zeta potential of +27.9 ± 3.4 mV and encapsulation efficiency of 70.93 ± 2.39%. The lyophilized chitosan–PAA–PTX–liposomes formulation was stable in simulated gastrointestinal fluids and at different environmental conditions (4 °C and 25 °C). In vitro drug release experiments demonstrated that chitosan–PAA–PTX-liposomes formulation exhibited obvious sustained release behaviors compared to PTX-liposomes. Furthermore, chitosan–PAA–PTX-liposomes formulation revealed enhanced PTX induced cytotoxicity in human cervical cancer cell culture experiments compared to PTX-liposomes. In conclusion, the approach presented herein will provide a promising solution for PTX delivery.

1. Introduction

Paclitaxel (PTX), as one of the most widely used and effective antineoplastic agents, has a powerful antitumor effect against a wide spectrum of cancers, including ovarian and breast cancers, metastatic breast cancer, colon cancer, small and non-small cell lung cancer, and neck cancer (Mo et al., 2011; Xu et al., 2012). Due to its low water solubility (approximately <2 μg/ml) (Liggins, Hunter, & Burt, 1997), PTX has to be formulated in a 50/50 (v/v) mixture of Cremophor EL/dehydrated ethanol as commercial Taxol®, which is administered by intravenous injection (Tao, Xu, Chen, Bai, & Liu, 2012). Unfortunately, the use of Cremophor EL can induce severe side effects like hypersensitivity, neurotoxicity, and nephrotoxicity (Tao et al., 2012). Therefore, in recent years, considerable attempts have been made to find less toxic and better tolerated delivery systems for paclitaxel to substitute Taxol®. Among these delivery systems, liposome is regarded as one of the most promising drug carrier due to its biocompatibility in nature and decreased toxic effects of the drugs.

Very often, the phospholipids present at the surface of the liposomes are considered to be responsible for the instability in the biological media and during storage (Sulkowski, Pentak, Nowak, & Sulkowska, 2005). Therefore, surface modification has been conducted to stabilize the liposomes. Recently, polyelectrolytes coated liposomes by layer-by-layer assembly has been implemented for their stabilization (Ciobanu et al., 2007). Layer-by-layer assembly is a simple and versatile method to alternately expose a substrate to polycations and polyanions that are adsorbed as nanolayers on the liposome surface so as to protect the labile surface phospholipids from the outer physiological environments. A variety of polyelectrolytes, such as polyethylene glycol-derivatized nanotubes (Angelini et al., 2007), polypeptides (Ciobanu et al., 2007; Fujimoto, Toyoda, & Fukui, 2007), deoxyribonucleic acid (Fukui & Fujimoto, 2009) and polyallyl amine hydrochloride (Jain, Patil, Swarnakar, & Agrawal, 2012), have been applied to modify the liposomes to improve their stability, sustained release and oral bioavailability of encapsulated drugs. Chitosan, well known as the naturally cationic (1-4)-2-amino-2-deoxy-<beta>-D-glucan produced in various grades including the medical grade, is being studied extensively owing to its biocompatibility, biochemical significance, and absence of toxicity. In particular, it is recognized as a suitable carrier for anticancer drugs (Busilacchi, Gigante, Mattioli-Belmonte, & Muzzarelli, 2013; Majedi et al., 2014; Muzzarelli, 2009, 2010). However, to the best of our knowledge,

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Table 1
Effect of process variables on the physicochemical properties of PTX-liposomes.

Fixed parameters	Variables		Size (nm)	PDI	EE (%)
Theoretical drug loading: 10 wt%; sonication time: 30 s; bath temperature: 35 °C	Phospholipid to cholesterol molar ratio	4:1	326 ± 25	0.385 ± 0.067	55.14 ± 06.33
		3:1	205 ± 21	0.346 ± 0.090	67.52 ± 03.35
		2:1	**92 ± 9**	**0.228 ± 0.035**	**82.76 ± 04.15**
		1:1	127 ± 12	0.376 ± 0.072	70.43 ± 07.46
		1:2	295 ± 19	0.413 ± 0.083	46.38 ± 09.62
Phospholipid to cholesterol molar ratio: 2:1; sonication time: 30 s; bath temperature: 35 °C	Theoretical drug loading (wt%)	5	109 ± 7	0.315 ± 0.041	81.45 ± 3.51
		10	**126 ± 11**	**0.267 ± 0.036**	**83.33 ± 2.85**
		15	189 ± 18	0.363 ± 0.079	62.87 ± 4.06
		20	225 ± 24	0.456 ± 0.082	40.62 ± 6.67
Phospholipid to cholesterol molar ratio: 2:1; theoretical drug loading: 10 wt%; bath temperature: 35 °C	Sonication time (s)	15	186 ± 9	0.457 ± 0.036	85.64 ± 2.15
		30	**112 ± 6**	**0.253 ± 0.030**	**82.45 ± 3.74**
		45	98 ± 5	0.376 ± 0.061	53.57 ± 10.58
		60	72 ± 3	0.389 ± 0.058	40.75 ± 5.19
Phospholipid to cholesterol molar ratio: 2:1; theoretical drug loading: 10 wt%; sonication time: 30 s	Bath temperature (°C)	25	205 ± 11	0.418 ± 0.032	55.43 ± 3.15
		35	**102 ± 6**	**0.233 ± 0.026**	**80.45 ± 4.46**
		45	152 ± 4	0.392 ± 0.057	42.75 ± 2.28

The optimized values are represented in bold.

layer-by-layer deposition of chitosan to modify the liposomal PTX formulation has not been reported.

In the current study, the polyelectrolyte stabilized multilayered liposomes were prepared by firstly coating anionic polymer, polyacrylic acid (PAA), on cationic liposomes and subsequently cationic polymer, chitosan. The processing variables were optimized in terms of their size, size distribution and encapsulation efficiency. Furthermore, the stabilities in simulated gastrointestinal fluids and during storage, the in vitro drug release, and the cytotoxicity of the polyelectrolyte stabilized multilayered liposomes were also investigated.

2. Materials and methods

2.1. Materials

Soya lecithin (92% phosphatidyl choline), cholesterol, paclitaxel, trypsin–EDTA, MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide), and Tween 80 were purchased from Sigma Aldrich, USA. Polyacrylic acid (PAA), stearyl amine, mannitol, chloroform and methanol were obtained from Sinopharm Chemical Reagent Company, China. Chitosan with molecular weight of 50 kDa and degree of deacetylation >90% was supplied by Haili Biochemical Product Company, China. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) were purchased from PAA, Austria. Tissue culture plates and 8-well culture slides were procured from Tarsons and BD Falcon, respectively. Ultra-pure water was used for all the experiments. All other reagents used were of analytical grade.

2.2. Preparation of PTX loaded liposomes

Paclitaxel loaded liposomes were prepared by thin film hydration technique according to the previous literature (Szoka and Papahadjopoulos, 1980). For all the PTX loaded liposomes, the stearyl amine used was 5 wt% of the total weight. In brief, soya lecithin (24.5 mg), cholesterol (11.5 mg), stearyl amine (2 mg) and paclitaxel (2 mg) were dissolved in 20 ml mixture of chloroform and methanol (9:1, v/v) in a round bottom flask and a thin film was prepared by rotary evaporation of the organic solvents under vacuum. The thin film was further dried in vacuum oven (100 mbar, 35 °C) for 4 h to completely remove the traces of organic solvents. The dry lipid film was then hydrated with PBS, pH 7.4, and vortexed until all components were dissolved. The mixture was then probe sonicated (Kesheng JY, China) for 3 cycles of several seconds at amplitude 80 with 1 min gap in ice bath to get smaller sized liposomes (Tsumoto, Matsuo, Tomita, & Yoshimura, 2009). Various process variables (as listed in Table 1), such as phospholipid to cholesterol ratio, drug loading and sonication time, were optimized to obtain liposomes with desired quality. The quality of the liposomes was evaluated by their size, polydispersity index (PDI), zeta potential and encapsulation efficiency (EE).

2.3. Characterization of PTX-liposomes

The size and size distribution of liposomes were measured in aliquots of liposomal suspension diluted in large volumes of distilled water by the dynamic light scattering method (Zeta PALS, Brookhaven Instruments, US) and the values reported were averaged from 6 measurements. The zeta potentials of the liposomes were measured based on electrophoretic mobility under an electric field (Zeta PALS, Brookhaven Instruments, US), taking an average of 20 measurements.

The drug encapsulation efficiency of the PTX loaded liposomes was determined as follows: the liposomes were diluted in large volumes of distilled water, centrifuged at 40,000 × *g* for 60 min at 4 °C, and then ultrafiltrated to remove the PTX loaded pellet. The PTX amount in the ultrafiltrate was regarded as unentrapped drug amount (W_1) which was measured by HPLC method, while the amount of drug initially taken for preparation of liposomes was taken as W_0. The EE of PTX-loaded liposomes could be calculated by the following equations:

$$\mathrm{EE} = \frac{W_0 - W_1}{W_0} \times 100$$

2.4. Preparation of polyelectrolyte coated PTX-liposomes

The optimized PTX-liposome formulation screened from Section 2.2 was coated with series of polyelectrolytes using layer-by-layer assembly, mediated by ionic interactions. In brief, 1 ml of the cationic PTX-liposome (charge imparted by stearyl amine) obtained from Section 2.2 was firstly coated by 500 μl of anionic PAA aqueous solution (0.1 wt%, molecular weight 3000 Da) under constant stirring at 2000 rpm for 1 h using magnetic stirrer. They were then subjected to centrifugation at 20,000 × *g* using high speed centrifuge followed by single washing with water. 1 ml of the single layer coated liposome (PAA–PTX-liposome) was then coated with 500 μl of cationic chitosan aqueous solution (0.1 wt%, molecular weight 50 kDa) under constant stirring at 2000 rpm for 1 h using

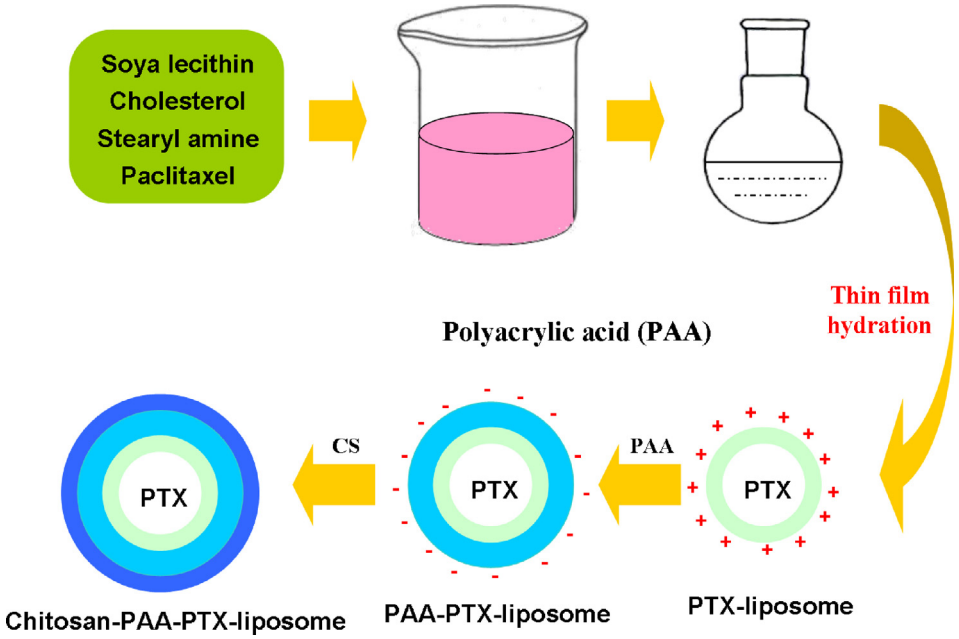

Fig. 1. Scheme of the preparation process of multilayered PTX loaded liposomes by layer-by-layer assembly.

magnetic stirrer. The dispersion was then centrifuged at 20,000 × *g* followed by single washing using water to get final formulation (chitosan–PAA–PTX-liposomes). All these formulations were analyzed for particle size, zeta potential and encapsulation efficiency. The schematic illustration of the preparation process of multilayered PTX loaded liposomes is shown in Fig. 1.

2.5. Morphology of polyelectrolyte coated PTX-liposomes

The shape and surface morphology of the developed formulations were observed using transmission electron microscopy (TEM, JEOL JEM-2100, Japan) with an acceleration voltage of 100 kV. Samples were negatively stained with 2% aqueous solution of uranyl acetate (Swarnakar, Jain, Dubey, Mishra, & Jain, 2007). The sample was dropped on the 400-mesh carbon coated grids for direct TEM imaging.

2.6. Stability studies

2.6.1. Stability in simulated gastrointestinal fluids

Freeze dried PTX-liposomes, PAA–PTX-liposomes and chitosan–PAA–PTX-liposomes were evaluated for their stability in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8). The SGF comprised of 0.2% NaCl, 0.7% pepsin, and HCl while the SIF consisted of 0.685 monobasic potassium phosphate, 1% NaOH and 1% pancreatin. Furthermore, sodium taurocholate (3 mM) was incorporated into the SIF to simulate the effect of bile salts (Vertzoni et al., 2004). 1 ml of the formulations was added into 9 ml of simulated gastrointestinal fluids, and then incubated for 2 h and 6 h in SGF and SIF, respectively. The parameters such as particle size, PDI, zeta potential and encapsulation efficiency were measured for each formulation.

2.6.2. Storage stability

Storage stability of all freeze dried formulations was tested at 4 °C and at 25 °C for six months. The parameters such as particle size, zeta potential and encapsulation efficiency were evaluated for each formulation after reconstitution.

2.7. In vitro drug release studies

The in vitro release profiles of PTX from chitosan–PAA–PTX-liposomes were investigated by dialysis membrane method. Phosphate buffer (pH 7.4) containing 0.1% Tween 80 (to solubilize the released drug) was used as release medium (Kalaria, Sharma, Beniwal, & Kumar, 2009). The formulation (1 ml) was introduced into a dialysis membrane bag, subsequently suspended in 15 ml of release medium at 37 °C. The release medium was stirred at a speed of 100 rpm. At predetermined time intervals, samples (0.5 ml) were withdrawn and replaced with an equal volume of fresh medium. The whole release study was carried out up to 24 h (Jain, Jain, Prasad, Jain, & Vyas, 2009; Jain et al., 2012). Finally, samples were analyzed using HPLC as described below and % cumulative drug release was calculated. For comparison, PTX release from PTX-liposomes and PAA–PTX-liposomes placed in a dialysis bag was conducted under the same conditions. Moreover, the mean dissolution time (MDT) is calculated from the following equation (Barakat, Elbagory, & Almurshedi, 2009):

$$\mathrm{MDT} = \frac{\sum_{i=1}^{n} t_{mid} \times \Delta M}{\sum_{i=1}^{n} \Delta M}$$

where i is the sample numbers, n is the number of dissolution times, t is the time at the midpoint between times t_i and t_{i-1}, and ΔM is the additional amount of drug dissolved between times t_i and t_{i-1}.

2.8. HPLC analysis of PTX

The concentration of PTX in different formulations was determined by the reverse phase HPLC (1100 HPLC system, Agilent, US) using a Symmetry column (Waters, Milliford, MA). The mobile phase consisted of acetonitrile–water (70:30, v/v) with a flow rate of 1 ml/min. A diode array detector was set at 227 nm and linked to Chem-Station software for data analysis. Each run was done in triplicate. The PTX concentrations in the samples were obtained using a calibration curve.

2.9. Cell culture

The human cervical cancer cell line (HeLa) was purchased from the ATCC (American Tissue Culture Collection, Manassas, VA) and cultured in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen), supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (PAA, Austria) in a humidified air with 5% CO_2 at 37 °C. The cells were harvested in 0.25% trypsin–EDTA solution (Sigma, USA) when 90% confluence in the cell culture medium was attained. The HeLa cells were then seeded at a density of 50,000 cells/well in a 96 well plate and incubated overnight to allow cell attachment for subsequent studies.

2.10. MTT assay

The cultured cells were washed by Hank's Buffered Salt (HBS) solution (PAA, Austria) for three times. After removing the HBS solution, cells were incubated with fresh DMEM (200 μl) containing pure PTX, PTX-liposomes and chitosan–PAA–PTX-liposomes at concentrations of 0.1, 1, 10 and 100 μg/ml for 24 h, respectively. Cell viability was then measured using MTT assay (Yang et al., 2007). Briefly, cells were washed with HBS solution and cultured again with 200 μl fresh DMEM containing 0.5 mg/ml MTT (Sigma, USA) for 3 h. The medium was then removed followed by dissolving MTT formazon in 200 μl DMSO. The optical density was measured at 550 nm using an EnSpire plate reader (PerkinElmer, US) (Wu, Liu, & Lee, 2006).

2.11. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine significance among groups followed by Tukey–Kramer multiple comparison tests. Statistical significance was established at $p < 0.05$.

3. Results and discussion

3.1. Preparation and optimization of PTX-liposomes

3.1.1. Effect of phospholipid to cholesterol molar ratio

The effect of the ratio of phospholipid to cholesterol on the formulation of PTX-liposomes was investigated, as listed in Table 1. Significant decrease in the particle size and PDI is observed for the formulations from 4:1 to 2:1, accompanying with increase in the encapsulation efficiency. This could be attributed to the assembling of cholesterol within the phospholipid molecules to provide rigidity to the resultant vesicular structures (Haidar, Hamdy, & Tabrizian, 2008), and these vesicular structures enable PTX molecules to pass in. However, it is also noted that further increase in the concentration of cholesterol in the formulations leads to undesirable quality of prepared liposomes. The particle size and PDI decrease with the increase in the cholesterol concentration in the liposomes from 2:1 to 1:2, which is probably ascribed to the destruction of the membrane vesicles at higher concentration. Furthermore, the notable reduction in encapsulation efficiency might originate from increased competence of cholesterol with PTX to incorporate within the vesicles and leaching of PTX molecules owing to destruction of membrane vesicles (Socaciu, Jessel, & Diehl, 2000). Therefore, ratio of phospholipids to cholesterol is selected as 2:1 (molar ratio) for further optimization.

3.1.2. Effect of drug loading

Table 1 illustrates the effect of drug loading on the quality attributes of the prepared liposomes. The particle size and PDI dramatically increase, whereas encapsulation efficiency simultaneously decreases with increase in the drug loading. Although the smaller particle size is observed in case of 5 wt% drug loading, its PDI and the encapsulation efficiency are inferior to those of 10 wt% drug loading. The higher drug loading leads to a drastic fall in the encapsulation efficiency, since liposomes are saturated. It is found that desired quality attributes of the liposomes could be achieved at 10 wt% drug loading, so it is selected to perform further studies.

3.1.3. Effect of sonication time

As evident from Table 1, sonication time has a significant impact on the quality attributes of PTX-liposomes. The particle size dramatically decreases, whereas the encapsulation efficiency simultaneously decreases with increase in the sonication time, indicating that drug-particle surface interactions do not play an important role in drug loading. The phenomenon could be also attributed to that the higher energy input results in abruption of vesicles via mechanical collision and release of entrapped drug. Although the highest encapsulation efficiency is observed in case of sonication time of 15 s, its PDI is much worse than that of sonication time of 30 s. Therefore, the sonication for 30 s at amplitude 80 with three cycles at 1 min interval is selected as optimized experimental time.

3.1.4. Effect of bath temperature

Bath temperature is also considered as an important parameter during preparation of the liposomes and its effect on the quality attributes of prepared formulations is shown in Table 1. In case of bath temperature at 25 °C, the molecular motion of cholesterol and phospholipid is too slow to form effective vesicular structures; while at 45 °C, the high temperature induces the oxidation of phospholipids and the destabilization of PTX. Therefore, 35 °C is selected as suitable bath temperature for optimization of the liposomes.

On the basis of the studies above, the optimized process variables are found to be phospholipid to cholesterol molar ratio of 2:1, drug loading of 10 wt%, sonication time of 30 s, and bath temperature of 35 °C, which are in accordance with the orthogonal array results (see supplementary data, Table S1 and S2). Furthermore, the optimized quality of the chitosan–PAA–PTX-liposome formulation possesses the particle size of 215 ± 17 nm, zeta potential of +27.9 ± 3.4 mV and encapsulation efficiency of 70.93 ± 2.39%. This optimized formulation is further utilized to perform the stability tests, in vitro release and cytotoxic assays.

3.2. Morphological features

Fig. 2 reveals the TEM images of PTX-liposomes, PAA–PTX-liposomes and chitosan–PAA–PTX-liposomes, which demonstrates the spherical structure of these formulations. Layer-by-layer assembly of polyelectrolyte on the PTX-liposomes does not alter the spherical shape. The average size of PTX-liposomes, PAA–PTX-liposomes and chitosan–PAA–PTX-liposomes is estimated to be approximately 100, 150, 200 nm, respectively, which agrees well with that obtained from dynamic light scattering analysis (Table 2). The increment in the size of PAA–PTX-liposomes and chitosan–PAA–PTX-liposomes is probably attributed to the polyelectrolyte coating on the surface of PTX-liposomes.

3.3. Stability studies

The stability studies of different PTX formulations in simulated gastrointestinal fluids are summarized in Table 2. PTX-liposomes and PAA–PTX-liposomes are found to be unstable in the simulated gastrointestinal fluids. The particle size, zeta potential and encapsulation efficiency of PTX-liposomes are significantly affected in both SGF and SIF. This could be attributed to that the bile salt monomers can permeate into lipid bilayers and thus lead to disruption of the vesicular structures (Haidar et al., 2008); the excess

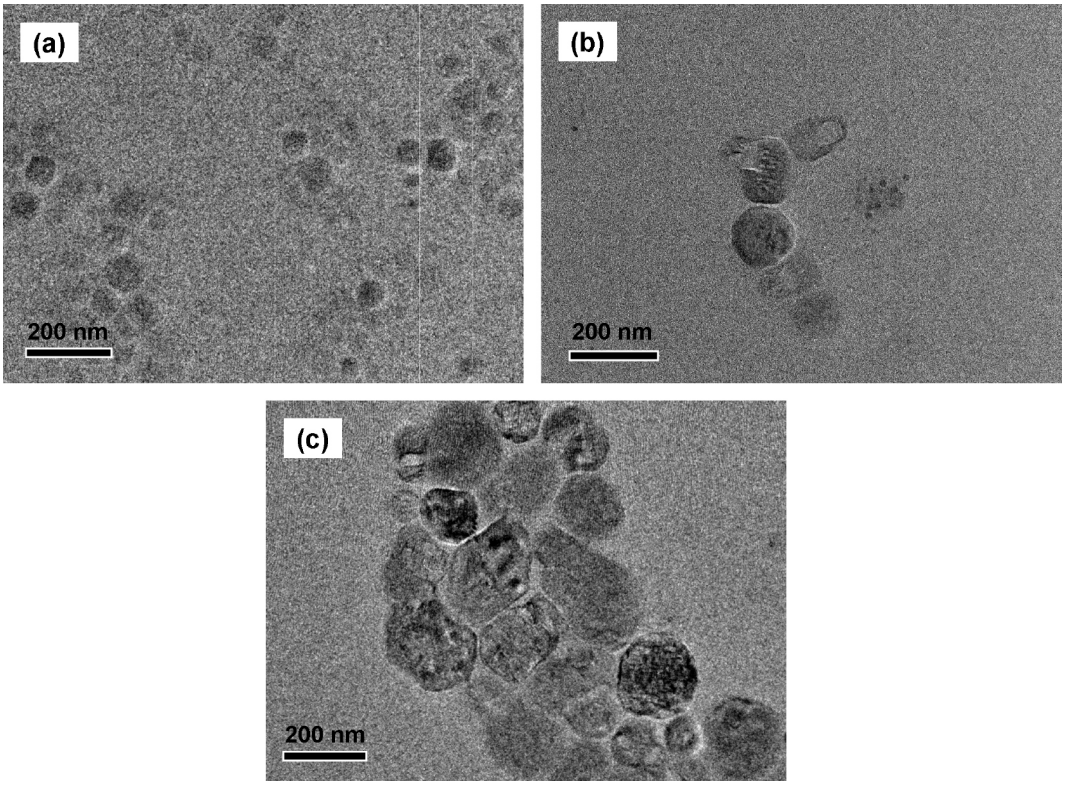

Fig. 2. TEM images of (a) PTX-liposome, (b) PAA–PTX-liposome and (c) chitosan–PAA–PTX-liposome.

hydrogen ions in the external environment can also diffuse in the inner aqueous phase of liposomes and catalyze the decomposition of them. The decomposition of the liposomes can be accounted for the reduced particle size and encapsulation efficiency. In case of PAA–PTX-liposomes, all the parameters are also significantly affected in both media, which could be ascribed to the aggregation of PAA–PTX-liposomes. This aggregation results from two aspects: for one, the electrostatic attraction is generated between the negative charges on the surface of liposomes and the positive charges in exterior environments; for another, as counter ions are freer to move within the continuous phase, there is a marked increase in entropy, which is the main driven force for aggregation (Tavares, Caroni, Dantas-Neto, Pereira, & Fonseca, 2012). Therefore, the particle size is drastically increased together with a sharp reduction in the encapsulation efficiency, indicating the destabilization of this system. In contrast, insignificant changes ($p > 0.05$) of chitosan–PAA–PTX-liposomes in terms of particle size, zeta potential and encapsulation efficiency are observed, suggesting their stability in both SGF and SIF. The improved stability is believed to be the presence of multiple coatings, which protect the labile surface of phospholipids from the outer physiological environments so as to preserve the original structure for efficient delivery of the drugs.

Table 3 represents the storage stability studies conducted for different PTX loaded formulations. After 6 months of storage at 4 °C and at room temperature, no significant changes are observed in the physical parameters, including the particle size, zeta potential and encapsulation efficiency, before and after storage, clearly revealing the stabilization of the formulations.

3.4. In vitro drug release studies

The in vitro drug release curves of PTX-liposomes, PAA–PTX-liposomes and chitosan–PAA–PTX-liposomes are shown in Fig. 3. As can be observed, PTX-liposomes demonstrate a relatively rapid drug release rate with 42% in 1 h and 69% in 4 h. In comparison, PAA–PTX-liposomes slow down the drug release rate with lag time of 3 h and 36% of drug in 4 h. Chitosan–PAA–PTX-liposomes exhibit more obvious retarded drug release rate, releasing only 31% of drug in 4 h. Furthermore, the MDT of PTX-liposome, PAA–PTX-liposome and chitosan–PAA–PTX-liposome is 2.80, 5.28 and 5.83 h,

Table 2
Stability studies of formulations in simulated gastrointestinal fluids.

Parameters	Size (nm)		Zeta potential (mV)		% Encapsulation efficiency	
	Initial	Final	Initial	Final	Initial	Final
PTX-liposomes						
SGF pH 1.2	108 ± 9	56 ± 7	+45.2 ± 3.8	+41.3 ± 2.5	82.85 ± 4.76	30.06 ± 3.08
SIF pH 6.8	108 ± 9	91 ± 11	+45.2 ± 3.8	+39.5 ± 3.9	82.85 ± 4.76	33.72 ± 3.60
PAA–PTX-liposomes						
SGF pH 1.2	152 ± 12	386 ± 35	−37.6 ± 2.6	+34.5 ± 3.4	77.18 ± 3.23	36.27 ± 4.36
SIF pH 6.8	152 ± 12	238 ± 16	−37.6 ± 2.6	+32.9 ± 4.1	77.18 ± 3.23	47.52 ± 5.87
Chitosan–PAA–PTX-liposomes						
SGF pH 1.2	215 ± 17	226 ± 14	+27.9 ± 3.4	+31.6 ± 2.7	70.93 ± 2.39	63.28 ± 4.16
SIF pH 6.8	215 ± 17	208 ± 19	+27.9 ± 3.4	+30.8 ± 3.1	70.93 ± 2.39	61.53 ± 3.35

Values are expressed as mean ± SD ($n = 6$).

Table 3
Storage stability studies of paclitaxel loaded formulations.

Parameters	Size (nm)		Zeta potential (mV)		% Encapsulation efficiency	
	Initial	Final	Initial	Final	Initial	Final
PTX-liposomes						
4 °C	108 ± 9	112 ± 8	+45.2 ± 3.8	+43.5 ± 2.6	82.85 ± 4.76	80.76 ± 4.28
25 °C	108 ± 9	105 ± 6	+45.2 ± 3.8	+44.2 ± 1.9	82.85 ± 4.76	80.12 ± 3.73
PAA–PTX-liposomes						
4 °C	152 ± 12	156 ± 10	−37.6 ± 2.6	−36.6 ± 2.4	77.18 ± 3.23	76.26 ± 3.62
25 °C	152 ± 12	159 ± 15	−37.6 ± 2.6	−35.9 ± 3.5	77.18 ± 3.23	74.87 ± 3.75
Chitosan–PAA–PTX-liposomes						
4 °C	215 ± 17	208 ± 11	+27.9 ± 3.4	+26.7 ± 2.6	70.93 ± 2.39	68.28 ± 2.16
25 °C	215 ± 17	226 ± 16	+27.9 ± 3.4	+25.8 ± 2.8	70.93 ± 2.39	67.56 ± 3.68

Values are expressed as mean ± SD ($n = 6$).

Table 4
Correlation coefficients of various dissolution models for different PTX loaded formulations.

Release model	PTX-liposome	PAA–PTX-liposome	Chitosan–PAA–PTX-liposome
Zero order	0.681	0.745	0.673
First order	0.827	0.714	0.649
Hixson–Crowell	0.910	0.780	0.709
Higuchi	0.849	0.914	0.929

respectively. A higher MDT value indicates greater drug retarding ability. The correlation coefficient values calculated from drug release kinetics based on various dissolution models of different PTX loaded formulations are summarized in Table 4. It could be observed that the drug release kinetics of PTX-liposomes corresponds well with Hixson–Crowell model, indicating diffusion and erosion as mechanism of drug release from liposomes (Rudra, Deepa, Ghosh, Ghosh, & Mukherjee, 2010). In contrast, the drug release profiles of PAA–PTX-liposomes and chitosan–PAA–PTX-liposomes are in good agreement with Higuchi type drug release kinetics, suggestive of diffusion from matrix systems as mechanism of drug release (Haidar et al., 2008). Similar results with PTX based particulate nanocarriers have been reported by previous literature (Pandita et al., 2009).

3.5. *In vitro cytotoxicity*

The cytotoxicity of blank chitosan nanoparticles, PTX-liposome, PAA–PTX-liposome and chitosan–PAA–PTX-liposome was evaluated by MTT method. PTX was used as positive control. MTT assay was tested for four different concentrations (0.1, 1, 10, 100 μg/ml) with the HeLa cells. Fig. 4 reflects the % cell viability of the HeLa cells treated with free PTX, PTX-liposome, PAA–PTX-liposome and chitosan–PAA–PTX-liposome at various concentrations. From Fig. 4, it can be observed that in the concentration range used for PTX formulations, the blank chitosan nanoparticles do not show significant cytotoxicity with more than 90% of HeLa cells survived. The % cell viability decreases proportionately as the concentration of formulations increases in all cases. At low concentrations (0.1 μg/ml), free PTX is more effective to suppress the cell growth than PTX-liposome, PAA–PTX-liposome and chitosan–PAA–PTX-liposome, which may be attributed to the relatively low amount of drug released from the carriers. At high concentrations (1, 10 and 100 μg/ml), chitosan–PAA–PTX-liposome shows decreased cell growth as compared to PTX-liposome and PAA–PTX-liposome, suggesting that incorporation of multiple coatings into liposome formulations improves the cytotoxicity of PTX against the cancer cells. Furthermore, the cell affinity of chitosan coated on the surface of liposome improves the cellular uptake of PTX, which is also responsible for the enhanced cytotoxicity.

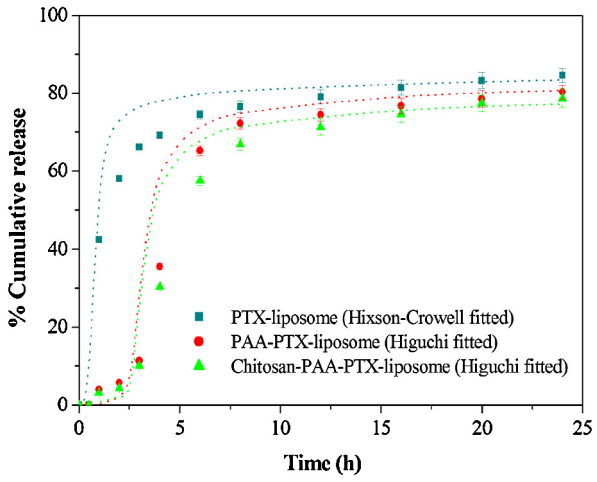

Fig. 3. In vitro drug release profiles of various PTX loaded formulations.

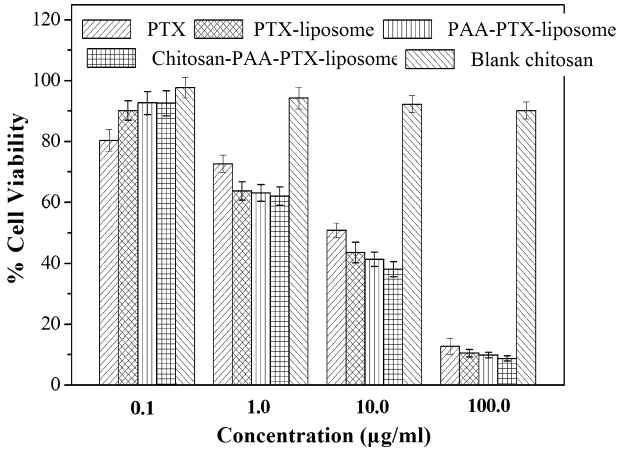

Fig. 4. Cell cytotoxicity of various formulations at different concentrations against HeLa cell line after 24 h incubation.

4. Conclusion

Layer-by-layer assemblies of anionic polyacrylic acid followed by cationic chitosan were successfully deposited on the surface of liposomes to improve their stabilization. Various

process variables were optimized and the optimum formulation of chitosan–PAA–PTX-liposome was found to have particle size of 215 ± 17 nm, zeta potential of +27.9 ± 3.4 mV and encapsulation efficiency of 70.93 ± 2.39%. The lyophilized chitosan–PAA–PTX-liposome formulation exhibited good stability in simulated gastrointestinal fluids, and remained stable in six-month storage (both 4 °C and room temperature) as well. In vitro release studies showed that the chitosan–PAA–PTX-liposome formulation sustained the drug release with lag time of about 3 h compared with PTX-liposome. The blank chitosan had almost no cytotoxicity, and the minimum viability was more than 90%. The chitosan–PAA–PTX-liposome formulation exhibited enhanced PTX induced cytotoxicity in human cervical cancer cells as compared to PTX, PTX-liposome and PAA–PTX-liposome, which was probably attributed to that the cell affinity of chitosan improved the cellular uptake of PTX. These results suggested that chitosan stabilized multilayered liposome would serve as a promising vehicle for PTX delivery.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.04.038.

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