International Journal of Pharmaceutics 361 (2008) 56–63

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Preparation and in vitro evaluation of liposomal chloroquine diphosphate loaded by a transmembrane pH-gradient method

Liyan Qiu*, Na Jing, Yi Jin

College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China

ARTICLE INFO

Article history:
Received 3 February 2008
Received in revised form 30 April 2008
Accepted 12 May 2008
Available online 16 May 2008

Keywords:
Chloroquine diphosphate
Liposomes
pH gradient
Lyophilization

ABSTRACT

This study developed an active loading method for encapsulating chloroquine diphosphate (CQ) into liposomes. The effects of different formulation factors on the encapsulation efficiency (EE) and the size of CQ liposomes were investigated. These factors included the internal phase of liposomes, the external phase of liposomes, the ratio of drug to soybean phosphatidylcholine (drug/SPC), the ratio of cholesterol to soybean phosphatidylcholine (Chol/SPC), and the incubation temperature and time. The EE (93%) was obtained when using drug/SPC (1:50 mass ratio), SPC/Chol (1:5 mass ratio) at 0.10 M citrate–sodium citrate buffer (pH 3.6). As 5 mol% methoxypoly(ethylene glycol)$_{2000}$ cholesteryl succinate (CHS-PEG$_{2000}$) or distearoyl phosphatidylethanolamine-poly (ethylene glycol)$_{2000}$ (DSPE-PEG$_{2000}$) was added, the size of particle was reduced and the EE was improved. Freeze-drying with 5% trehalose as a cryoprotectant was carried out to achieve long-term stability. The drug release studies were performed in vitro simulating the desired application conditions, such as physiological fluids (pH 7.4), tumor tissues (pH 6.5) and endosomal compartments (pH 5.5). The release of CQ from the liposomes prepared via remote loading showed the significant pH-sensitivity and retention properties, which favored the application of liposomal CQ at tumor tissues and endosomal compartments.

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1. Introduction

In recent years increasing attention is drawn to gene therapy in medicine, which refers to delivery of functional DNA into target cells via efficient vectors. To achieve transgene expression, vector/DNA complexes must escape from endosomes as soon as possible to avoid lysosomal enzyme degradation (Trentin et al., 2005). It has been observed that chloroquine (CQ) is further protonated to diproton CQ^{2+} after entering acidic endosome. Due to low bilayer permeability, CQ^{2+} would be captured in the endosome, which results in osmotic swelling and breakage of endosome. Therefore, as an extensively applied lysosomotropic agent, CQ favors the survival of vector/DNA complexes from the endosome and accelerates their release into the cytosol, which finally significantly improves the gene expression of many nonviral gene delivery systems in vitro (Ciftci and Levy, 2001; Keil et al., 2001). There are also other reports noting that CQ can interact with DNA and may increase the transfection efficiency by facilitating dissociation of DNA (Cheng et al., 2006). Furthermore, its effectiveness on the gene transfection in vivo has been confirmed by previous study (Zhang et al., 2003).

However, the CQ use is limited by its cytotoxic effect, which is induced by the higher effective concentration and the low bioavailability in gene transfection (Ciftci and Levy, 2001). A systemic administration of CQ aqueous solution would cause inclusion body retinopathy in patients chronically and even life-threatening toxicity (Crommelin et al., 1990). Liposome delivery system is considered to be an ideal choice to solve the above problems. It has been widely explored to improve the therapeutic index of chemotherapeutic agents during the therapy of cancers and infectious diseases by enhancing drug retention characteristics and altering biodistribution by the passive or active targeting (Adlakha-Hutcheon et al., 1999; Barenholz, 2001; Martins et al., 2007). Up to now, the application of liposomal CQ in gene therapy has not been reported, while recently our work has been focusing on the creation of liposomal CQ with enhanced drug retention and targeting characteristics to achieve improvement effect on gene transfection of anti-cancer DNA segments. And as the first part of this work, the objective of this paper is to expatiate the loading mechanisms of CQ into liposomes.

CQ liposomes with combination of distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidylglycerol (DPPG) can achieve a high EE by passive loading method (Peeters et al., 1989). In such system, DSPC is a neutral phospholipid and

* Corresponding author. Tel.: +86 571 8820 8434; fax: +86 571 8820 8434.
E-mail address: lyqiu@zju.edu.cn (L. Qiu).

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doi:10.1016/j.ijpharm.2008.05.010

DPPG is a negatively charged phospholipid. Because the positively charged CQ^+ (at pH 7.4) could easily interact with the negatively charged liposomes, the negatively charged phospholipid played a key role in passive loading procedures (Khan et al., 2005). However, if the CQ was encapsulated by neutral phospholipid (soybean phosphatidylcholine or egg phosphatidylcholine) alone, the EE was very low. So, the CQ encapsulation into liposomes employing passive loading method was depended on phospholipid composition and type.

The naturally occurring phospholipid has been industrialized, and it is more common in use and much cheaper than charged phospholipid. Therefore, this study aimed to develop CQ-loaded liposomes composed of neutral phospholipid with high EE. A transmembrane pH-gradient method was tried to encapsulate CQ considering that CQ is a kind of amino-containing drug which would be protonized under certain acid condition (Maurer-Spurej et al., 1999). The effect of various factors on liposome characterization was investigated including the internal phase of liposome, the external phase of liposome, the mass ratio of drug to phospholipids (drug/SPC), the mass ratio of cholesterol to phospholipids (Chol/SPC), the incubation temperature, incubation time and different phospholipid or cholesterol. To overcome the instability, the liposomes were lyophilized with proper cryoprotectant. The drug release studies were performed in vitro under the simulated application conditions as desired, such as physiological fluids (pH 7.4), tumor tissues (pH 6.5) and endosomal compartments (pH 5.5). The effect of liposomal CQ on the gene transfection in vivo and in vitro is under going and will be reported later.

2. Materials and methods

2.1. Chemicals

CQ (purity 99.6%, Batch no. 061014) was purchased from Kaiyang Biotechnology Pharmaceutical Co. Ltd. (Shanghai, China). Soybean phosphatidylcholine (SPC) was purchased from Taiwei Pharmaceutical Industry Co. Ltd. (Shanghai, China). Cholesterol (Chol) was purchased from Southern Chemical Reagent Co. Ltd. (Guangdong, China). Poly(ethylene glycol-2000)-grafted distearoyl phosphatidylethanolamine ($DSPE\text{-}PEG_{2000}$) and egg phosphatidylcholine (EPC) was purchased from Lipoid (GmbH Germany). Methoxypoly(ethyene glycol)$_{2000}$ cholesteryl succinate ($CHS\text{-}PEG_{2000}$) was a gift from Shenyang Pharmaceutical University. Sodium hexanesulfone was purchased from Yuwang Industrial Co. Ltd. (Shandong, China). Acetonitrile for HPLC analysis was obtained from Merck (Darmstadt, Germany). All other materials were analytical grade.

2.2. Methods

2.2.1. Preparation of CQ liposomes

Liposomal vesicles were prepared by a transmembrane pH-gradient method, which included two steps, namely the formation of blank liposome and drug loading. Blank liposomal vesicles were first prepared by reverse phase evaporation method as follows. An aqueous solution at low pH containing the appropriate buffer substance was added to diethyl ether containing SPC and Chol with various ratios. The two-phase mixture was subjected to sonication in a water bath for 10 min at room temperature until the mixture became w/o emulsion. The solvent was removed by a rotary evaporator at 40 °C in a water bath for 20 min to get multilamellar liposome vesicles, which were then sonicated with a probe sonicator. The pulse function was on 1 s and off 2 s for 5 min. CQ was dissolved with the liposome dispersion and the transmembrane pH gradient was implemented by adjusting the external pH of liposomes with the proper alkali to certain pH. Finally, the liposomes were incubated under an adequate condition (for 10 min at 40 °C). A series of liposomes containing trehalose as cryoprotective agent were also prepared according to the method stated above. When one preparation factor was investigated, the level of other factors was fixed.

2.2.2. Freeze-drying of liposome suspension

The vials with 10 ml of liposome suspension were frozen by positioning the samples on the shelf at −50 °C for 10 h and the frozen samples were dried at −50 °C for 24 h at 10 Pa. After this period, a second drying step was applied at room temperature for 12 h (Mohammed et al., 2006). The lyophilized liposomes were reconstructed with water at room temperature.

2.2.3. Determination of the buffer capacity of different buffer

The buffer capacity of internal phase of liposomes was measured by acid–base titration using a pH-meter. The buffer was titrated with 1 M NaOH (pH from 3.6 to 7.0) and the volumes of NaOH consumed were recorded. The buffer capacity was calculated by applying the Van Slyke equation:

$$\beta = \frac{\Delta C_b}{\Delta \mathrm{pH}}, \quad (1)$$

where C_b is base concentrations, ΔpH is the difference between final pH and initial pH, and β is the buffer capacity (Pertusatti and Prado, 2007).

2.2.4. Chloroquine liposomes characterization

Untrapped CQ was removed by eluting liposomes over a Sephadex G-50 column (1 cm × 23 cm) with PBS (NaCl 137 mM, KCl 2.7 mM, NaH_2PO_4 10 mM, KH_2PO_4 1.76 mM, pH 7.4). The EE and drug loading content (LC) was calculated using the follow equation:

$$\mathrm{EE\%} = \left(1 - \frac{\text{amount of drug unencapsulated}}{\text{amount of drug used}}\right) \times 100, \quad (2)$$

$$\mathrm{LC\%} = \frac{\text{amount of drug encapsulated}}{\text{amount of drug encapsulated} + \text{amount of lipid}} \times 100. \quad (3)$$

The CQ concentration was determined by reverse-phase liquid chromatography (HPLC). The chromatographic system consisted of a Agilent 1100 system with 20 μl loop and a reversed-phase column (Diamonsil ODS, 4.6 mm × 250 mm, 5 μm particle size) equipped with a guard column (C18, 4.6 mm × 10 mm). The mobile phase consisted of A and B (22:78, v/v). A was acetonitrile, while B was deionised water containing 50 mM kalium dihydrogen, 6.5 mM sodium hexanesulfonate and 7 mM triethylamine (adjusted to pH 3.0 with phosphoric acid). The detection wavelength was 330 nm and the flow rate was 1.0 ml/min (Bell et al., 2007).The particle size and ζ-potential was determined by Zetasizer-3000HS (Malvern Instruments Ltd., UK). Liposomal samples were diluted with distilled deionized water and the final lipid concentration was 3 mg/ml. The scattering angle was kept at 90°, the wavelength in vacuum was set as 658 nm and the temperature was set at 25 °C during whole experiments. All determination was repeated three times.

Transmission electron microscopy (TEM) images were obtained using a JEM 1230 operating at an acceleration voltage of 80 kV. Liposomal samples were diluted with PBS (NaOH 29 mM, KH_2PO_4 50 mM, pH 7.0) and the final lipid concentration was 5 mg/ml. The diluted sample was placed on a copper grid and most of the liquid was removed with filter paper. Finally, a drop of 3% aqueous solution of sodium phosphotungstate was added for negative staining.

2.2.5. Drug release from liposomes

The liposomal CQ release in vitro was studied using dialysis bag (cut-off molecular weight: 12 000). 1 ml of rehydrated suspension of lyophilized liposomes or unlyophilized liposome suspension (chloroquine diphosphate 1 mg/ml) was poured into the dialysis bag and dialyzed against 25 ml phosphate buffer solutions of different pHs (37 °C, 100 rpm). The pH selection depended on the application environments, including the physiological fluids (pH 7.4), tumor tissues (pH 6.5) and endosomal compartments (pH 5.5) (Fritze et al., 2006). At predetermined time, 4 ml of receptor phase was removed and replaced with fresh phosphate buffer solution of equal volume during 6 h. The released chloroquine was quantified with UV spectrophotometry (330 nm). All release tests were run in triplicate and the release experiments were all conducted under sink conditions. The differences of cumulative drug release at various pHs were evaluated for significance using one-way ANOVA and the difference at $P < 0.05$ was considered to be significant.

2.3. Stability studies

The lyophilized liposomes were stored at 4 °C and 25 °C for 3 months in the sealed situation, respectively. The pH of system, particle size and drug EE was determined each month.

3. Results and discussion

3.1. Effect of internal phase on the EE

In the case of pH-gradient method, CQ was added to pre-formed blank liposomes with an imposed pH gradient (acidic internal phase). CQ possesses two basic ionization sites as weak base and the pK_a values were 8.10 and 9.94, respectively (Ottiger and Wunderli-Allenspach, 1997; Verbeeck et al., 2005). It was further protonated to diproton CQ^{2+} in a depletion of the internal proton pool once exposed to acidic intravesicular environment. Due to low bilayer permeability, CQ^{2+} would be captured inside of the liposomes. The protonation, however, induced the increase of interior pH if the interior aqueous was not adequately buffered (Fahr et al., 2005; Fritze et al., 2006). At equilibrium, the ratio of CQ concentration inside the liposomes to that outside the liposome must equal the ratio square of proton concentrations in the two compartments (Cullis et al., 1997). If the acidic interior was highly buffered, the higher concentration of interior H^+ should induce the higher drug concentration. Therefore, the pH gradient and internal buffering capacity is very important for drug loading. The buffering capacity is related to the composition and concentration of intravesicular buffer. The relative factors were examined as follows.

CQ was loaded with several kinds of citrate buffer (pH 3.6, 0.10 M) as the internal aqueous phase. With β (buffering capacity) of $C_6H_8O_7$–$C_6H_5Na_3O_7$ buffer (0.0765) > $C_6H_8O_7$–Na_2HPO_4 buffer (0.0619) > $C_6H_8O_7$–Na OH buffer (0.0474), the EE was 93% > 90% > 81% (Table 1), which indicated that the buffer system composed of weak base could achieve stronger buffering capacities than that composed of strong base and consequently induced the higher EE. In addition, increasing the internal buffering capacity could improve the retention characteristics of drugs which was encapsulated by pH-gradient technique (Lee et al., 1998).

Liposomes were prepared with $C_6H_8O_7$–$C_6H_5Na_3O_7$ buffer (0.10 M) at different pH-values (3.0, 3.6, 4.0 and 5.0) as internal aqueous phase. As presented in Table 1, the lower pH-value resulted in the higher EE. When the pH of the intravesicular medium was reduced, the pH gradient was increased and the more proton was provided by the internal aqueous phase (Madden et al., 1990). According to the earlier study, for the weak bases with one amino function, the concentration of the weak base inside liposomes compared to outside concentration exhibited a positive correlation with the ratio of internal proton concentration to the outside concentration. A pH gradient of 3 units (ΔpH = (external pH − internal pH) = 3) was predicted to produce a 1000-fold higher internal concentration of weak base than the external concentration. The effect of pH gradient on the CQ with two amino functions was more significant than the weak base with one amino function (Cullis et al., 1997), so increasing the pH gradient (ΔpH) could significantly improve the EE of liposomal CQ. But a further decrease of pH exacerbated lipid stability due to hydrolysis. Considering the stability of lipid and retention characteristic, pH 3.6 was chosen.

Table 1
The effect of internal phase on the EE before lyophilization and after rehydration

Internal aqueous phase	EE (%)	
	Before lyophilization	After rehydration
$C_6H_8O_7$–Na_2HPO_4 (pH 3.6, 0.10 M)	90 ± 2	96 ± 3
$C_6H_8O_7$–Na OH (pH 3.6, 0.10 M)	81 ± 4	79 ± 5
$C_6H_8O_7$–$C_6H_5Na_3O_7$ (pH 3.6, 0.10 M)	93 ± 3	97 ± 3
$C_6H_8O_7$–$C_6H_5Na_3O_7$ (pH 3.0, 0.10 M)	95 ± 2	91 ± 4
$C_6H_8O_7$–$C_6H_5Na_3O_7$ (pH 4.0, 0.10 M)	85 ± 3	89 ± 1
$C_6H_8O_7$–$C_6H_5Na_3O_7$ (pH 5.0, 0.10 M)	69 ± 5	74 ± 5
$C_6H_8O_7$–$C_6H_5Na_3O_7$ (pH 3.6, 0.05 M)	65 ± 4	61 ± 3
$C_6H_8O_7$–$C_6H_5Na_3O_7$ (pH 3.6, 0.20 M)	99 ± 1	87 ± 4

The mean is calculated from three samples of different batches (mean ± S.D., $n = 3$).

$C_6H_8O_7$–$C_6H_5Na_3O_7$ (pH 3.6) buffers of various concentrations (0.05, 0.10 and 0.20 M) as internal aqueous phase were used to prepare the liposome, respectively. The EE was listed in the following order: 99.2% (0.20 M) > 93.6% (0.10 M) > 65.7% (0.05 M) (Table 1) because improving the internal concentration of buffer ions strengthened its buffering capacity. Citrate buffer is used widely in injection formulations as pharmaceutically acceptable excipient. However, care must be taken with regards to the total amount and concentration of citrate in vivo, as citrate is able to chelate plasma calcium. Furthermore, isosmotic formulations are preferable and the use of higher citrate concentration should be restricted (Stensrud et al., 2000). The destructive effect of freeze-drying was significant on the liposome with high citrate–sodium citrate concentration, so 0.10 M was selected to avoid the leakage in the process of freeze-drying.

3.2. Effect of exterior phase on the EE

Different alkalis were used to adjust the external pH of liposomes to 7.0, including NaOH (1 M), $NaHCO_3$ (1 M) and Na_2HPO_4 (1 M). It has been confirmed that the final ion species and ion strength in the system affect the membrane ζ-potential, which is an important physicochemical parameter determining the interaction between the charged drug and membrane (Maurer-Spurej et al., 1999). For the weak basic drug, the negative potential or lower positive membrane potential favors its membrane–water partitioning due to charge interactions, which would increase the accumulated drug amount (Westman et al., 1982; Madden et al., 1990; Das et al., 2005).

As shown in Table 2, the EE was improved by using alkali with stronger basicity. NaOH, a strong base, was consumed the least at molar quantity, so the concentration of Na^+ was the lowest in the final determination system. Adjusting the external pH to the same level with weaker base $NaHCO_3$ (pK_a 7.62), more Na^+ was dissociated to the system. And Na_2HPO_4 (pK_a 7.20) containing two Na^+ would make the system have Na^+ at the highest concentration. Since the increase of positive-ion concentration would improve the ζ-potential and decrease the membrane–water partitioning of CQ, the EE of liposomes was lowest when Na_2HPO_4 was employed.

Table 2
The effect of exterior phase on the EE before lyophilization and after rehydration

Alkali	External pH	ζ-Potential (mV)	EE (%)	
			Before lyophilization	After rehydration
NaOH	7.0	1.5 ± 0.7	97.9 ± 3.1	74.5 ± 5.8
$NaHCO_3$	7.0	5.9 ± 1.3	95.2 ± 1.8	84.3 ± 3.4
Na_2HPO_4	7.0	10.7 ± 2.5	93.6 ± 2.7	97.5 ± 2.3
Na_2HPO_4	6.2	−20.5 ± 1.9	90.9 ± 3.0	61.5 ± 3.3
Na_2HPO_4	6.6	−15.7 ± 2.1	97.3 ± 1.8	78.5 ± 2.6
Na_2HPO_4	7.4	14.2 ± 3.2	85.2 ± 1.9	79.1 ± 4.8

The mean is calculated from three samples of different batches (mean ± S.D., n = 3).

However, in the process of freeze-drying, the destruction effect of strong alkali was great. The EE fell from 97.9 ± 3.1 to 74.5 ± 5.8% after lyophilization when NaOH was used to adjust external pH. Therefore, Na_2HPO_4 with moderate basic strength was suitable in this study.

The external pH of liposomes was regulated by Na_2HPO_4 (1 M) to pH 6.2, 6.6, 7.0 and 7.4, respectively. With improving the ΔpH unit from 2.6 (internal pH 3.6, external pH 6.2) to 3.0 (internal pH 3.6, external pH 6.6), the EE achieved the highest 97.3%. However, the EE reduced with the further increase of pH gradient (external pH 7.0 or 7.4) (Table 2). Besides the pH gradient, the membrane–water partitioning partition coefficient was also an important factor, which affected the remote drug loading. When the pH gradient and buffering capacity was sufficient, the membrane–water partitioning played the main role in the drug loading. In the experiment, because the weak base Na_2HPO_4 was used, the minute change of external pH would introduce massive positive ion Na^+, which would improve the ζ-potential greatly and decrease the EE by reducing the membrane–water partitioning of CQ (Van Balen et al., 2004). On the other side, the product exhibited most stable in the neutral condition in the process of lyophilization. Therefore, the external pH 7.0 was optimum to produce freeze-dried CQ liposomes.

3.3. Effect of drug/SPC on the EE

With decreasing the drug/SPC (mass ratio) from 1:10 to 1:50, the EE increased from 47.4 to 93.6% (Fig. 1), while the LC decreased from 3.9 to 1.7%, respectively. If the amount of lipid and internal buffering capacity was given, the encapsulation volume and drug loading capacity was limited. Because too high level of accumulated drug depleted the interior buffering capacity and collapsed the pH gradient, further drug uptake was inhibited (Otake et al., 2001). When the ratio was further decreased from 1:50 to 1:80, the EE had no remarkable change but the LC continued to decrease to 1.0%. During the lyophilization, the size of liposomes increased with increasing the amount of lipid, which was associated with the aggregation of liposomes induced by higher lipid concentration. With comprehensive consideration of the EE and LC, 1:50 was proper drug/SPC.

3.4. Effect of Chol/SPC on the EE

As shown in Fig. 2, the EE increased from 56.5 to 93.6% when reducing Chol/SPC from 1:3 to 1:10 and the EE of liposomes without Chol (only SPC) was 90.2%, which showed that the EE was not influenced obviously when the amount of cholesterol was further decreased. The presence of cholesterol in liposome could keep the membrane hydrophobic and rigid in order to reduce the leakage or permeability of encapsulating drugs. But the excessive cholesterol decreased internal aqueous volume and disrupted the regular linear structure of the liposomal membrane, so the formulation with excess cholesterol in lipid phase led to the lower EE (Glavas-Dodov et al., 2005). Therefore, Chol/SPC (1:5 mass ratio) was applied in our study.

3.5. Effect of temperature and time on the EE of liposomes

As presented in Fig. 3, the leakage of CQ from liposomes could be significantly reduced by maintaining the incubation temperature not over 40 °C, because the thermal mobility at a higher temperature (60 °C) may cause the leakage and rapid loss of the pH gradient (Chou et al., 2003). Drug uptake into the liposome reached a maximum after 10 min incubation, and could keep stable for up to 35 min at 40 °C. Thus, a period of 10 min for loading at 40 °C was proper. If the incubation time was further lengthened, the EE decreased. When the incubation was performed at room temperature, it would cost 20 min to reach the maximum drug loading.

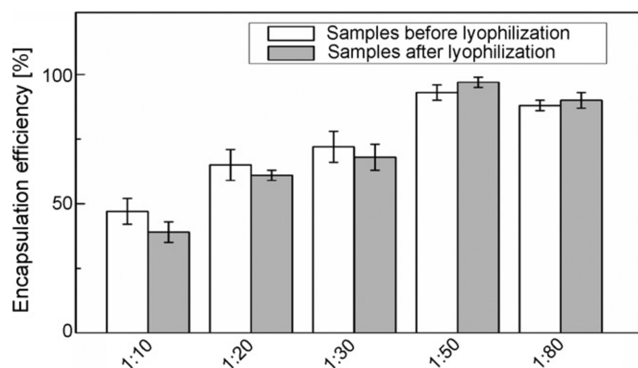

Fig. 1. The EE of CQ into liposomes prepared with different drug/SPC mass ratio before lyophilization and after rehydration. The mean is calculated from three samples of different batches and the error bar represents standard deviation from the mean (mean ± S.D., n = 3).

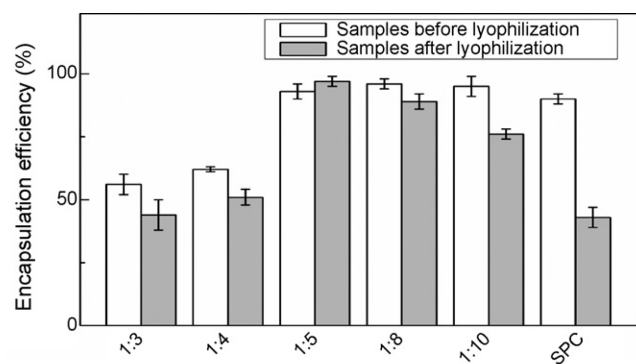

Fig. 2. The EE of CQ into liposomes with various Chol/SPC mass ratios before lyophilization and after rehydration. The mean is calculated from three samples of different batches and the error bar represents standard deviation from the mean (mean ± S.D., n = 3).

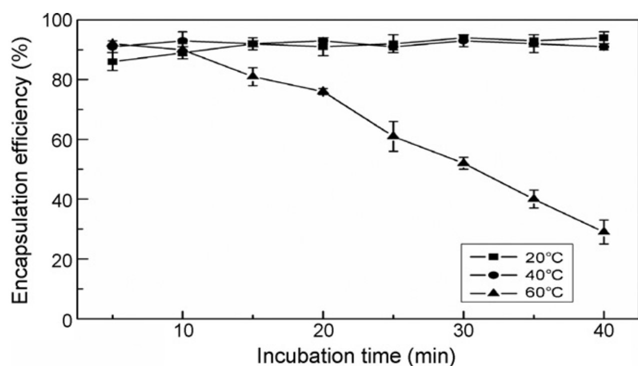

Fig. 3. The EE of CQ into liposomes incubated for different time at different temperature. The mean is calculated from three samples of different batches and the error bar represents standard deviation from the mean (mean ± S.D., $n = 3$).

A lot of study reported that the permeability of bilayer was bad at the temperature below phase transition temperature. Under this condition, the foreign substances were difficult to overcome the activation energy (Chou et al., 2003; Dos Santos et al., 2004). In our study, the uptake could reach the higher level at the lower temperature, which showed that the uptake was dependent on the physicochemical properties of drug. It was possible that CQ interacted with the head groups of the phospholipid, which caused the change in phase transition behavior of liposomes (Stensrud et al., 2000; Flaten et al., 2007).

3.6. Effect of phospholipid or cholesterol type on the EE and size of liposomes

As shown in Table 3, the composition of phospholipid and cholesterol somewhat influenced the characterization of CQ liposomes. Determination results revealed that the liposomes had the narrow particle size distribution and the smaller PDI before lyophilization. The inclusion of 5 mol% DSPE-PEG_{2000} resulted in the increase of EE (97.8%) and decrease of the size. The others had reported the higher EE of liposomes containing DSPE-PEG_{2000} as well (Johnsson et al., 1999; Yang et al., 2007). It could be explained by that DSPE contains a negatively charged phosphate group, which was favorable to the encapsulation of positively charged CQ^+. It was well known that the membrane–water partition coefficient of weak base was highly sensitive to the charge on the membrane and the use of negatively charged lipids in liposomes would increase the membrane–water partition coefficient of weak base (Ceh and Lasic, 1997). EPC was also used to prepare the liposome instead of SPC and there was no significant change in the EE and size of liposomes.

Table 3
The effect of phospholipid, cholesterol type and lyophilization on the EE and size before lyophilization and after rehydration ($n = 3$)

Series	Size, mean ± S.D. (nm)	PDI	EE (%)
A	110 ± 8	0.201 ± 0.209	93.6 ± 2.7
A_1	458 ± 15	0.564 ± 0.056	97.5 ± 2.3
A_2	279 ± 12	0.267 ± 0.117	81.8 ± 3.1
B	95 ± 10	0.197 ± 0.115	96.2 ± 3.0
B_1	432 ± 34	0.483 ± 0.153	84.5 ± 3.9
B_2	262 ± 21	0.260 ± 0.178	75.1 ± 7.0
C	87 ± 6	0.164 ± 0.137	97.8 ± 1.9
C_1	467 ± 29	0.436 ± 0.182	86.4 ± 5.5
C_2	256 ± 18	0.248 ± 0.091	85.6 ± 6.7

The mean is calculated from three samples of different batches (mean ± S.D., $n = 3$). (A) conventional liposomes; (B) liposomes composed of 5 mol% CHS-PEG_{2000}; (C) liposome composed of 5 mol% DSPE-PEG_{2000}; (1) freeze drying the liposome suspension directly; (2) freeze drying the diluted liposome suspension of lower lipid concentration.

3.7. Effect of lyophilization on the EE and size of liposomes

The process of lyophilization was harmful to the liposome integrity and induced a pronounced decrease in the EE. After screening, trehalose was chosen as a cryoprotectant to improve the stability of liposomes during freezing drying. The effect of lipid concentration on the lyophilization was examined. For series (1) samples, trehalose was directly added to the mother liposome suspension (5%, w/v) for lyophilization. For series (2), the liposome suspension was diluted with water (v:v = 1:1) to get the lower lipid concentration, then trehalose was added (5%, w/v).

The process of lyophilization made the great effect on the particle size of liposomes despite of trehalose as a cryoprotectant. Comparing with liposomes before lyophilization, series (1) showed a considerable size-increase after rehydration (Table 3), but the increased particle size did not induce the leakage of drug, which was contrary to previously reported results (Stensrud et al., 2000). The possible explanation for the phenomenon was that the absolute value of ζ-potential of the liposomes was low, which induced aggregation of vesicles in lyophilization, but no fusion of liposome membranes. The effect of lyophilization on the size of series (2) was less than series (1). Taking the liposome composed of 5 mol% DSPE-PEG_{2000} for example, comparing with liposomes before lyophilization (Fig. 4C), C_1 showed a serious aggregation after rehydration (Fig. 4C_1), however, C_2 displayed a good dispersity if the liposome suspension was diluted before lyophilization (Fig. 4C_2). It suggested that decreasing the lipid concentration could avoid serious aggregation during the lyophilization. Moreover, the PEGylation was effective in enhancing the physical stability of liposomes and improving the aggregation in some degree, because the PEGylation could prevent interaction among particles sterically during lyophilization (Armstrong et al., 2002).

3.8. Release behavior of reconstructed CQ liposomes in vitro

As well known, the obstacle to develop the clinical application of liposomal drug lies on its instability, which leads to drug leakage before it reaches disease target site. If drug retention characteristics of liposome is ensured, the therapeutic index could be significantly improved (Crommelin et al., 1990). Therefore, an ideal liposome delivery system should be stable enough during circulating in the physiological fluids but presents notable drug release at the action site (Dutta, 2007).

In our study, several factors were examined how to affect drug retention, such as lyophilization, phospholipid and cholesterol type, pH gradient, internal buffering capacity and the concentration of internal buffer.

The drug release study was carried out in phosphate buffer solutions of pH 7.4, 6.5 or 5.5, which imitated the various pH environments (physiological fluids, tumors and endosomes) from the point of application to the target. In general, all experimental groups displayed a strong pH-dependent drug release behavior (Fig. 5a–f). The release of CQ from liposomes could be related to the diffusion through the lipid bilayer acting as a membrane barrier for encapsulated drug, and the release was just in opposite direction compared with the initial remote loading mechanism. After the liposome dispersion was exposed to the lower pH, a fraction of CQ^{2+} in the liposome tended to distribute into the outside phase along the concentration difference to reach equilibrium. When the pH of receptor phase was 7.4, the CQ^{2+} would convert to neutral molecule CQ or CQ^+, which could reside in liposomal bilayers or convert to CQ^{2+} again in liposome chamber.

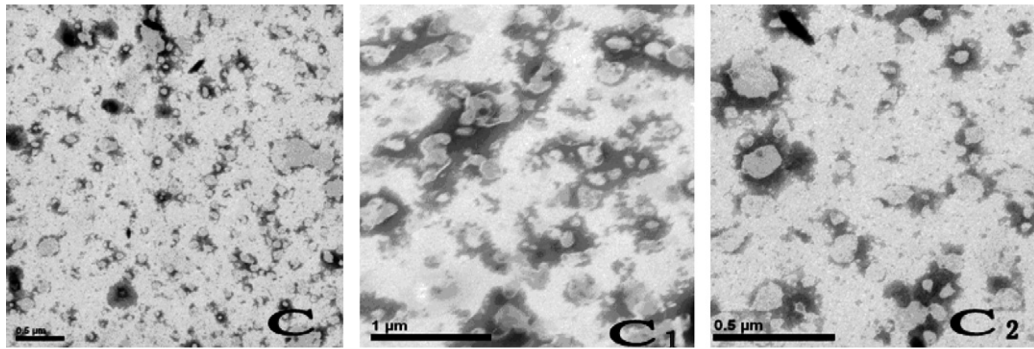

Fig. 4. TEM micrographs of the chloroquine liposomes composed of 5 mol% DSPE-PEG$_{2000}$ before lyophilization and after rehydration. (C) liposome before lyophilization and the bar represents 500 nm; (C$_1$) reconstructed liposomes which was lyophilizated according to series (1) and the bar represents 1000 nm; (C$_2$) reconstructed liposomes which was lyophilizated according to series (2) and the bar represents 500 nm.

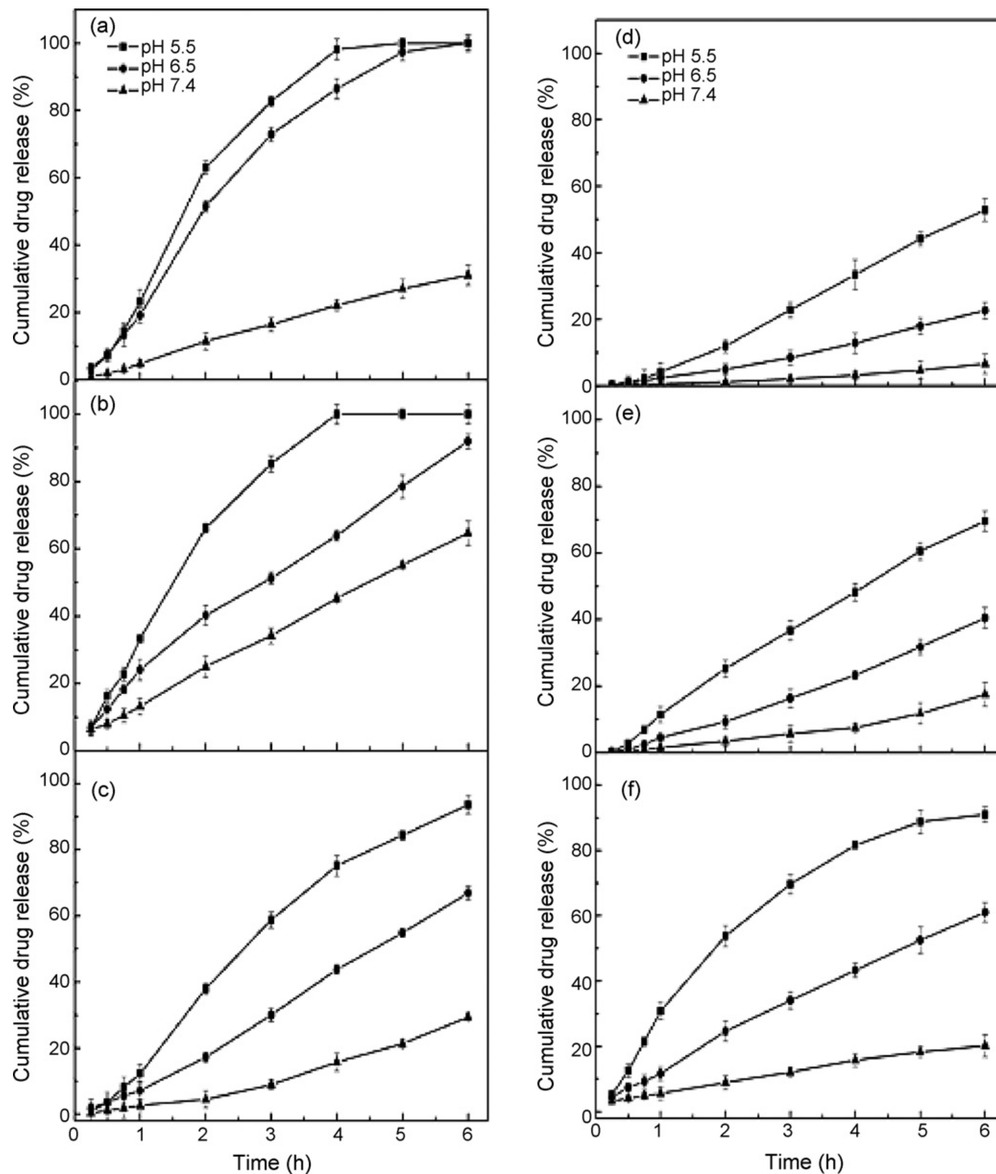

Fig. 5. The release of CQ from reconstructed liposomes in phosphate buffer solutions of pH 7.4, 6.5 or 5.5. (a) A$_2$ conventional liposomes with SPC and Chol; (b) B$_2$ liposomes composed of 5 mol% CHS-PEG$_{2000}$; (c) C$_2$ liposomes composed of 5 mol% DSPE-PEG$_{2000}$; (d–f) the release of CQ from unlyophilized conventional liposomes with different pH-value buffers as internal aqueous phase. (d) Conventional liposomes with buffer (pH 3.0) as internal aqueous phase. (e) Conventional liposomes with buffer (pH 3.6) as internal aqueous phase. (f) Conventional liposomes with buffer (pH 5.0) as internal aqueous phase. The mean is calculated from the cumulative drug release of three samples (from the same batch) and the error bar represents standard deviation from the mean (mean ± S.D., n = 3).

Thus the release of CQ^{2+} from the liposomes was quite slower at pH 7.4.

For the conventional liposomes, the cumulative drug release at physiological pH 7.4 was 30% within 6 h, while more than 90% of CQ was released from liposomes at the buffer pH 5.5 and 6.5 (Fig. 5a). The release of CQ in a physiological fluids environment may be slower than that in the tumor tissues and endosomes. The inclusion of 5 mol% CHS-PEG_{2000} slowed down the release of CQ from liposomes at pH 6.5, but accelerated the CQ release at pH 7.4 (Fig. 5b). The inclusion of 5 mol% DSPE-PEG_{2000}, however, could reduce the release of CQ from liposomes at pH 6.5 and 5.5 significantly (Fig. 5c). The possible explanation was that the membranes composed of negatively charged lipids (DSPE-PEG_{2000}) exhibited higher membrane–water partition coefficient for positively charged drug, which should lead to the high level of entrapped drug and improve retention properties (Maurer-Spurej et al., 1999). Therefore, on the viewpoint of drug retention, the modification of lipid or cholesterol seems not necessary.

For the same formulation, the rehydrated liposomes (Fig. 5a) showed faster release rate compared with the unlyophilized liposome suspension group (Fig. 5e). This result was also in accordance with the forementioned conclusion that the process of lyophilization was harmful to the liposome membrane integrity.

In addition, some factors which favored the drug loading were observed to enhance the drug retention such as the pH gradient and the internal buffering capacity. When raising the pH gradient (ΔpH) from 2.0 (Fig. 5f), 3.4 (Fig. 5e) to 4.0 (Fig. 5d), the drug retention was significantly enhanced ($p < 0.01$). Similarly, with β (buffering capacity) of internal buffer increasing ($C_6H_8O_7$–NaOH buffer < $C_6H_8O_7$–Na_2HPO_4 buffer < $C_6H_8O_7$–$C_6H_5Na_3O_7$ buffer), the drug retention was also significantly enhanced ($p < 0.05$, the figure was not shown). However, improving the concentration of internal buffer (from 0.1 to 0.2 M) did not enhance the drug retention, which could be explained by the higher buffer concentration inducing the higher osmotic pressure ($p > 0.05$, the figure was not shown).

3.9. Stability studies

The lyophilized liposomes were more stable at 4 °C than at 25 °C. The primary results showed that the EE had no significant change at 4 °C during 3 months, but the particle size increased slightly from original 279 to 302 nm ($n = 3$). At 25 °C, the entrapment efficiency reduced greatly (from 81.8 to 52.7%). To some extent, the lyophilization could avoid the hydrolysis of phospholipid, but the hydrolysis still occurred, which caused a significant decrease in the pH of system (from 7.4 to 6.7). In general, the low temperature was favorable to preserve lyophililized powder. The further stability study was still going on.

4. Conclusions

CQ was successfully encapsulated into liposomes by pH gradient method, which showed that it was an appropriate alternative technique to load chloroquine diphosphate into lipid vesicles. The preparation condition of internal phase, exterior phase, drug/SPC, Chol/SPC, incubation temperature and incubation time was optimized to achieve stable and nano-sized liposome with high EE. Furthermore, the pH-triggered release and improved retention of liposomal CQ offered perspectives for the improvement of cancer target treatment. The assistance effect of liposomal CQ on the gene delivery for cancer in vitro and in vivo would be reported in our future paper.

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