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RESEARCH ARTICLE

Application of liposome encapsulation technique to improve anti-carcinoma effect of resveratrol

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Abstract

Aim: The promising anti-tumor effect of resveratrol (RES) has aroused much interest in recent years, but its clinical application was seriously hindered due to its poor solubility in water. The aim of this study was to improve the water solubility of RES by liposome encapsulation technique for effective tumor treatment.

Methods: This study develops two liposomal formulations to solubilize RES by reverse-phase evaporation method with or without poly(ethylene glycol-2000)-grafted distearolyl phosphatidylethanolamine (DSPE-PEG_{2000}). The effect of different formulation factors on the encapsulation efficiency (EE) and the particle sizes were investigated. These factors included the mass ratio of drug to soybean phosphatidylcholine (drug/SPC), the mass ratio of cholesterol to soybean phosphatidylcholine (chol/SPC), the volume ratio of water phase/organic phase and the microfluidization process. The drug release studies were performed in various media, simulating the desired application conditions. The cytotoxicity study was carried out by MTT assay on HeLa and Hep G2 cell lines.

Results: The RES EE of 95% was obtained when using drug/SPC (1:40 mass ratio), Chol/SPC (1:10 mass ratio), water phase/oil phase (1:2 volume ratio), microfluidization process (entrance pressure 6 kpa, two times of cycle time). The addition of DSPE-PEG_{2000} into the formulation showed little effect on the formation and properties of RES liposome. The release of RES was pH-independent. RES liposomes and PEG-modified liposomes performed significant inhibition effects on both cells growth due to the solubilized RES.

Conclusion: RES can be effectively loaded into liposomes and its anti-cancer effect was evidently improved by the application of liposome encapsulation technique.

Keywords: Resveratrol, liposome, reverse-phase evaporation method, cytotoxicity, anti-tumor effect

Introduction

Resveratrol (RES, trans-3,4′,5-trihydroxystilbene) is a naturally occurring polyphenol, which has been found in some fruits, vegetables and traditional oriental medicinal plants, such as grapes, nuts and polygonum roots. Early in the end of last century, the potential protective effect of RES on cardiovascular health has been discovered, which can prevent human cardiovascular diseases by inhibiting platelet aggregation[1,2], promoting vasorelaxation[3,4], suppressing atherosclerosis[5], decreasing lipid peroxidation[6] and improving serum cholesterol and triglyceride concentrations[7,8]. Moreover, RES has inhibition effect on cyclooxygenase activity *in vivo*, which can reduce the pro-inflammatory molecules production and perform the anti-inflammatory properties[9–11]. Besides, the immunity effect[12,13], protective property against brain damage[14] and the anti-aging effect of RES were also explored extensively[15,16]. With advents of these aspects, RES is always applied as one kind of basis ingredients in the formulation of health and care products.

In the last decades, the anti-tumor activity of RES has been noticed. Though the anti-tumor mechanism was investigated, it was explained complexly and diversely. In brief, the inhibition effect on the enzymatic activity of cyclooxygenase was considered as a major reason for RES to reduce the probability of cancers' growth[10,17,18]. The promising anti-tumor effect of RES has aroused much interest in recent years. Unfortunately, most studies were still restricted on *in vitro* cellular level[19] due to

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the poor water solubility of RES. RES can be well dissolved in various common organic solvents, such as ether, ethanol, methanol, dimethyl sulfoxide (DMSO), but barely dissolved in water. In order to achieve effective pharmacological doses of RES, a considerable amount of organic solvent is usually required in cell line tests, which is not suitable and acceptable for *in vivo* drug delivery[20,21]. Therefore, the clinical application of RES has been seriously hindered.

Liposomes are spherical lipid vesicles with bilayer structures of natural or synthetic amphiphilic lipid molecules[22]. The hydrophobic drug could be loaded into the hydrophobic domain formed by lipid bilayer membrane. As a result, liposomal encapsulation technique displays strong solubilization effect for hydrophobic drugs. On the other hand, the passive targeting to tumor and the potential to alleviate drug toxicity of liposomes were also beneficial for clinical application[23]. Early in 1995, the Doxil® (doxorubicin liposomes) was first approved for clinical use[24]. Furthermore, a lot of researches have recently been concentrated on long circulating liposomes or stealth liposomes, whose surfaces are modified with hydrophilic segments such as polyethylene glycol (PEG) to prolong circulation half-life and consequently enhance therapeutic effect[22,25]. The possible mechanism is that the modified PEG on lipid bilayer could form a protective layer over the liposome surface and prevent liposome from recognition by opsonins and the following clearance.

Our work was based on these concepts and aimed to load RES into conventional liposomes (RLs) or PEG-modified liposomes (PRLs) to improve the solubility of RES in an aqueous solution. The effect of various factors on drug loading into liposomes was investigated including the mass ratio of drug to phospholipids (drug/SPC), the mass ratio of cholesterol to phospholipids (Chol/SPC), the microfluidization process and different species of phospholipids. To overcome their instability on shelf, the RES-loaded liposomes were lyophilized with proper cryoprotectants. The drug release studies were investigated *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 *in vitro* cytotoxicity was carried out by MTT assay on human Hep G2 hepatocellular carcinoma cells and human HeLa cervical carcinoma cells.

Materials and methods

Chemicals

RES (purity 98%, Batch No. 038K5202) was purchased from Sigma-Aldrich Co. Ltd (America). 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 distearolyl phosphatidylethanolamine (DSPE-PEG_{2000}) was purchased from lipoid (GmbH Germany). Acetonitrile for HPLC analysis was obtained from Merck (Darmstadt, Germany). All other materials were analytical grade.

Preparation of RES liposomes

The RES liposomes (RLs) were prepared by reverse-phase evaporation method. SPC, Chol and RES were dissolved in ether and then mixed with phosphate buffer solutions (PBS, pH 7.4). The final mass ratio of drug to phospholipids, the mass ratio of SPC to Chol and the amount of ether and PBS were determined on the basis of optimal encapsulation of RES by liposomes. The two-phase mixture was subjected to sonication in a water bath for 30 min at room temperature until it became w/o emulsion. The solvent was then evaporated in a rotavapor at a temperature of 40°C in water bath for 20 min to form the vesicles, which were then sonicated with a probe sonicator. The pulse function was on 1 s and off 2 s for 5 min. The PEG-modified liposomes (PRLs) were also prepared by the method mentioned above except that 5 mol% DSPE-PEG_{2000} lipoid was additionally applied with SPC. Unentrapped RES was removed by dialysis for 7 h. The sonicated liposome samples were placed into dialysis bags (COMW = 7400) and then immersed in 500 mL PBS (pH 7.4). Every 1 or 2 h, all dialysis media were abandoned and replaced by new fresh PBS. The liposome suspension was handled by the microfluidization and the optimal entrance pressure and cycle times have been investigated. When one preparation factor was investigated, the level of other factors was fixed[26].

To prepare freeze-dried liposome, 2.5% trehalose and 2.5% lactose were used as cryoprotective agent. Then the vials with 10 mL of liposome suspension were frozen by positioning the samples on the shelf at –80°C for 10 h, and then the frozen samples were dried at –50°C for 24 h at 10 Pa. After this period, a second drying step at room temperature was kept for 12 h.

RES liposomes characterization

The freeze-dried samples were rehydrated by deionized water at room temperature. The encapsulation efficiency (EE) and drug loading content (LC) were calculated by the following equations:

$$EE\% = \left(\frac{\text{amount of drug encapsulated}}{\text{amount of used drug}} \right) \times 100\%$$

$$EE\% = \left(\frac{\text{amount of drug encapsulated}}{\text{total amount of drug encapsulated liposome}} \right) \times 100\%$$

The RES concentration was determined by the reversed-phase high performance liquid chromatography (HPLC).

The chromatographic system was made up of a Agilent 1100 system with 20 μL loop and a reversed-phase column (Kromasil ODS, 4.6 × 250 mm, 5 μm particle size) equipped with a guard column (C18, 4.6 × 10 mm). The mobile phase was made up of water, acetonitrile and glacial acetic acid (64.8:35:0.2, volume ratio). The detection wavelength was 303 nm and the flow rate was 1.0 mL/min[27]. All liposome samples were deemusified by methanol before analysis.

The particle size and potential were determined by Zetasizer-3000 HS (Malvern Instruments Ltd., UK). Liposomal samples were diluted with distilled deionized water to 3 mg/mL. The scattering angle was kept at 90°C, 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.

The transmission electronmicroscopy (TEM) images were detected by 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) to 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.

Drug release from liposomes

The liposomal RES release *in vitro* was studied by using dialysis method. 1 mL of rehydrated suspension of lyophilized liposomes (RES 1 mg/mL) was poured into the dialysis bag (COMW: 12000) and dialyzed against 25 mL PBS of different pHs (37°C, 100 rpm)[28]. The pH selection was considered to simulate the application environments, including the normal physiological fluid (pH 7.4), tumor tissues (pH 6.5) and endosomal compartments (pH 5.5). At the predetermined time, 4 mL of receptor phase was removed and replaced with fresh phosphate buffer solution of equal volume during 24 h. The released RES was quantified with UV spectrophotometry at 303 nm. All release tests were run in triplicate and the release experiments were all conducted under sink conditions.

Cell culture

Human Hep G2 hepatocellular carcinoma cells (TCHu 72, Shanghai Cell Bank of Science Academy of China, Shanghai, PRC) and Human HeLa cervical carcinoma cells (TCHu187, Shanghai Cell Bank of Science Academy of China, Shanghai, PRC) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) and incubated at 37°C in the humidified 5% CO_2.

MTT assay for the *in vitro* cytotoxicity

The MTT assay was performed according to Kang et al.[29] With minor modification, 5×10^3 cells (HeLa, Hep G2) were seeded in each well in a 96-well plate. Twenty-four hours later, PBS (pH 7.4) containing series concentration of various drug formulations, including free RES, PRL and RL were added. The cells incubated in medium without any drug or liposomes were used as controls. After 24 or 48 h incubation, 31.5 μL MTT (5 mg/mL) were added, and the plates were incubated for an additional 4 h. After culture medium was removed, the formazan crystals were dissolved in 100 μL DMSO, and the absorbance value was read on a microplate reader (Mlutiskan MK3, Turmo Corp.) at the dual wavelengths of 570 nm.

Results

Optimization of formulation and preparation procedure

Effect of drug/SPC

The effect of drug/SPC mass ratio on EE and LC was studied. As RES was fixed at 10 mg in the formulation, four groups of liposome samples were prepared with drug/SPC mass ratio at 1:20, 1:30, 1:40 and 1:50. The results show that the EE changes along with the weight ratio of drug to SPC (Figure 1). Before lyophilization, when decreasing the drug/SPC mass ratio from 1:20 to 1:40, the EE increased from 79 to 95% while the LC decreased from 3.19 to 2.11%. At 1:50 of drug/SPC ratio, the EE didn't increase anymore but the LC further decreased to 1.73%. After lyophilization, the EE% was still increased as decreasing the weight ratio of drug/SPC until drug/SPC ratio reached 1:40. It was also found that lyophilization procedure would result in the slight decrease of EE and this influence degree was proportionally related to the drug/SPC mass ratio. Considering in a comprehensive way, we finally selected that 1:40 was proper drug/SPC mass ratio to encapsulate the drug by liposome.

Effect of Chol/SPC

In order to determine the optimal amount of chol added in liposomes, the effect of chol/SPC on EE was investigated.

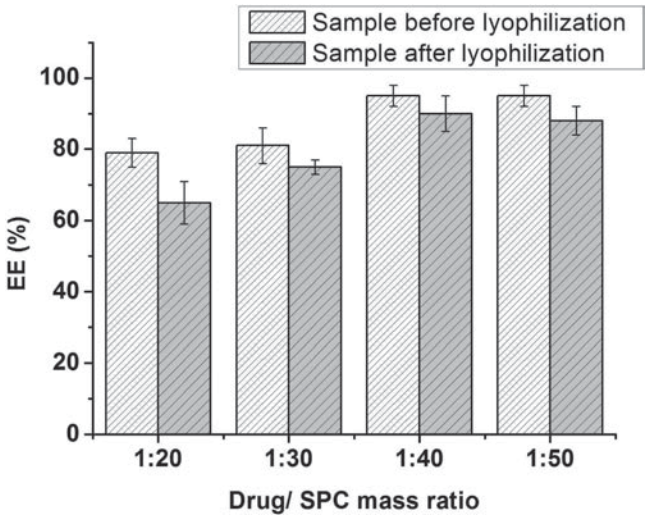

Figure 1. The encapsulation efficiency (EE) of resveratrol (RES) liposomes prepared with different drug/soybean phosphatidylcholine (SPC) mass ratio before lyophilization and after rehydration. The mean was calculated from three samples of different batches and the error bar represents the standard deviation from the mean (mean ± SD, $n = 3$).

The amount of SPC was fixed at 400 mg, while the added Chol changed to obtain Chol/SPC mass ratio of 1:5, 1:7.5 and 1:10. Before lyophilization, the EE increased from 79 to 80 and 89% when Chol/SPC decreased from 1:5 to 1:7.5 and 1:10 (Figure 2). Therefore, we could find that the EE was significantly influenced by the amount of cholesterol. After lyophilization, this trend of increased EE with decreased chol amount was still obvious. But compared with that before lyophilization, the EE of all samples decreased at various Chol/SPC mass ratios, and the EE differences between the samples before lyophilization and after lyophilization were obviously reduced as Chol/SPC mass ratio decreased. When Chol/SPC mass ratio was 1:10, the EE was 89% before lyophilization and 85% after lyophilization. Apparently the EE of both samples were at a high level. However, when the mass ratio of Chol/SPC was further reduced to 1:15, the EE didn't increase any more. Furthermore, considering the positive effect of chol on stabilizing the bilayer structure of liposome, a certain amount of Chol is always necessary for the formation of liposome. Therefore, Chol/SPC 1:10 mass ratio was finally applied in our study.

Effect of water phase/organic phase

The EE of RES in liposome was almost not influenced by the water phase/organic phase volume ratio in this study (The data were not given). However, the appropriate volume of water phase or oil-phase was necessary. The excessive water would make it quite difficult to homogenize oil-phase to obtain the liposomes with narrow particle size distribution, while inadequate amount of water phase would bring about the aggregation of phospholipids and hinder lipid bilayer formation. On the other hand, the increase of the organic phase would make a contribution of forming more stable w/o emulsion in the process of sonication with a water bath, but aggravate the difficulty to evaporate organic solvent completely. As a result, when 10 mg of RES were loaded into liposomes, 10 mL of PBS and 20 mL of ether were applied and the favorable volume ratio of water phase/organic phase was fixed at 1:2.

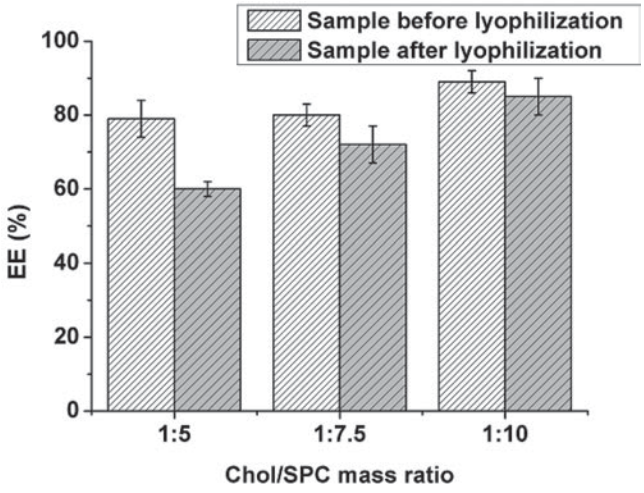

Figure 2. The encapsulation efficiency (EE) of soybean phosphatidylcholine (SPC) liposomes prepared with different drug/soybean phosphatidylcholine (SPC) mass ratio before lyophilization and after rehydration. The mean was calculated from three samples of different batches and the error bar represents the standard deviation from the mean (mean ± SD, $n = 3$).

Effect of DSPE-PEG$_{2000}$

The previous studies have suggested that 1–5 mol% DSPE-PEG$_{2000}$ is commonly added in phospholipid to prolong circulation time and 5 mol% DSPE-PEG$_{2000}$ is the sufficient and optimal concentration[30]. Therefore 5 mol% DSPE-PEG$_{2000}$ lipoid was used in our study to prepare PRL. The effects of drug/SPC and Chol/SPC on EE of PRL were also investigated in this study, and the results were almost similar with the conventional RES liposomes (data not shown). As a result, the same drug/SPC mass ratio, Chol/SPC mass ratio and water and ether volume were applied in the formulation of the PRL.

Effect of microfluidization

In order to make the particle size smaller and more homogenous, the RL solution was handled by the microfluidization process. The cycle time and the entrance pressure were the most important parameters in this process. As shown in Figure 3, the EE decreased as the cycle times increased. And when the cycle times were fixed, the EE decreased as the entrance pressure increased. Although the EE on the condition of entrance pressure 3 kpa was the highest, the particle sizes were not reduced ideally unless the cycle times were increased to four (Figure 4). Based on these two figures, we chose the entrance pressure 6 kpa and two times cycle. On this condition, the EE of RL was 83% and the particle size was around 105 nm. It has been suggested that small particle size with homogeneous distribution usually cannot be achieved at low pressure with limited cycle times. However, the excessive pressure and cycle times could result in the structure break of the lipid

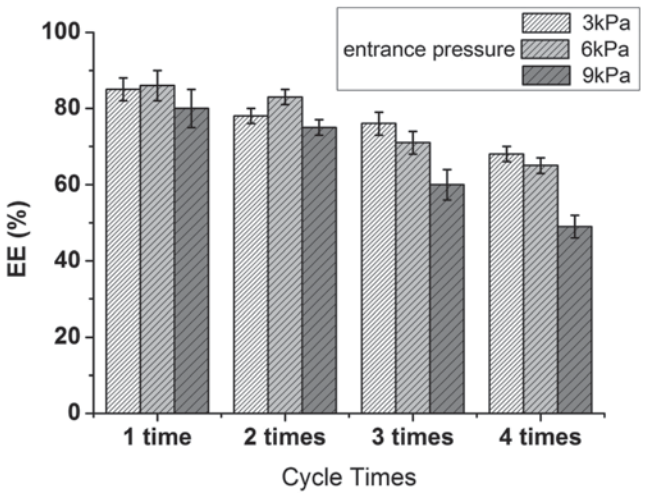

Figure 3. The influence of entrance pressure and cycle time on the encapsulation efficiency (EE)% of resveratrol (RES). The mean was calculated from three samples of different batches and the error bar represents the standard deviation from the mean (mean ± SD, $n = 3$).

bilayer so as to reduce the EE evidently[31,32]. Furthermore, heat produced in the microfluidization process should be taken into consideration. When the temperature exceeded the phospholipid phase-transition temperature, the permeability of the membrane of the liposome will increase, resulting in the leaking out of the drug and the low EE. Therefore ice pack was applied all the time during the process[33].

Characterization of liposomes

Considering all these factors discussed above, an optimized formulation has been determined as follows. The RL were prepared with the formulation of RES 10 mg, SPC 400 mg, Chol 40 mg, PBS (pH 7.4) 10 mL and ether 20 mL (drug/SPC mass ratio of 1:40, the Chol/SPC mass ratio of 1:10 and the water phase/organic phase of 1:2). For PRL, DSPE-PEG_{2000} lipoid 44.5 mg (5 mol% of SPC) was additionally applied with SPC. All the samples were prepared according to this optimal formulation and then characterized.

The morphology of liposomes

The vacant liposomes and the RES-loaded liposomes were observed by TEM. As shown in Figure 5, all samples of the liposomes were well identified. The vacant liposomes were nearly perfect sphere-shaped, having a large internal aqueous space and existing in disperse

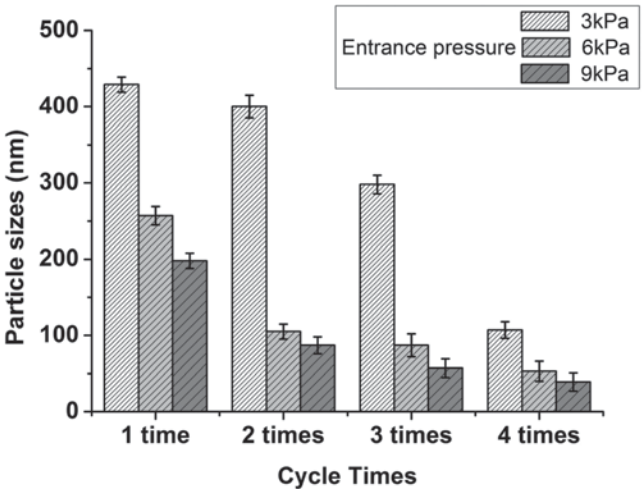

Figure 4. The influence of entrance pressure and cycle time on the particle sizes of liposomes. The mean was calculated from three samples of different batches and the error bar represents the standard deviation from the mean (mean ± SD, $n = 3$).

(Figure 5A). When RES was loaded into liposomes, the particle density increased so that could not be transmitted by light (Figure 5B–E).

The particle sizes and ζ-potential of liposomes

The particle size and ζ-potential were determined by Malvern Zetasizer-3000 HS. The particle sizes of RLs were at 120 ± 5 nm (PDI, 0.320 ± 0.187), and PRLs at 100 ± 8 nm (PDI, 0.291 ± 0.179). The mean was calculated from three samples of different batches (mean ± SD, $n = 3$). Obviously, incorporation of DSPE-PEG_{2000} into the membrane brought about the light decrease of particle sizes. According to the literature, the extensive hydration around the large ethylene oxide head group of the DSPE-PEG_{2000} enhanced the lateral repulsive properties[34]. For the purpose to reduce the repulsion effect, the curvature of the liposome surface increased, which thereby reduced the particle sizes. Moreover, the ζ-potential of RLs and PRL were −38.4 ± 6 and −39.2 ± 5 mV, respectively. The negative charge property of RLs and PRLs indicates that the liposomal formulation were stable since the electrical repulsion would help to avoid liposomes aggregation.

Release behavior of rehydrated RES liposomes *in vitro*

PBS with different pH (5.5, 6.5 and 7.4) was used as drug release media in our study, which imitated the various pH environments (physiological fluids, tumors and endosomes), respectively[33]. The cumulative drug release of RES liposomes were 59.9, 57.8 and 65.3% at pH7.4, 6.5 and 5.5 in 48 h, respectively, while only 48.1, 49.8 and 51.2% for PRL at the corresponding pH in 72 h probably due to the retardant of hydrophilic DSPE-PEG_{2000} for drug diffusion (Figure 6). RES was a hydrophobic compound with negligible solubility in water solution. Considering the nonionic and water-insoluble properties of RES, we may make a conclusion from the data shown in Figure 6 that the release of RES from the liposome was pH-independent. Though the data of drug *in vitro* release may not be predictive for *in vivo* environment, the relative lower level of drug release rate of PRLs at pH7.4 was promising to achieve the long circulating property.

MTT assay

We examined the toxicity of PRL and RL in HeLa cells and Hep G2 cells. The results (Figure 7) indicated that in comparison with free RES, either PRL or RL apparently caused inhibition effect on cell growth at 24 h and 48 h

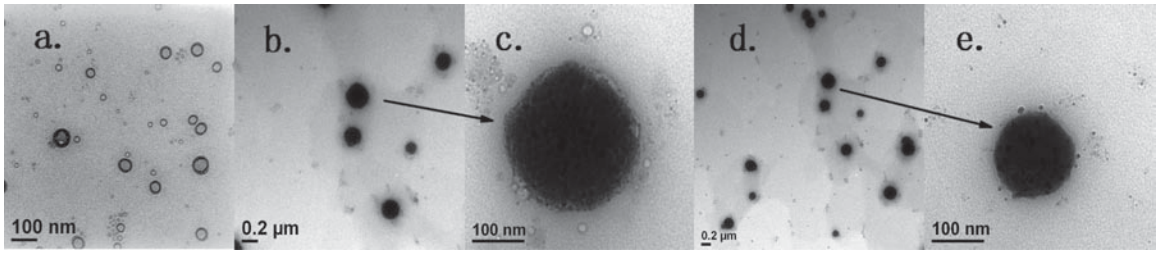

Figure 5. Images demonstrated the structure of (A) the vacant liposomes, (B) RLs, and (D) PRLs. (C) Illustrates the TEM image of one particle magnified from (B). (E) Illustrates the TEM image of one particle magnified from (D).

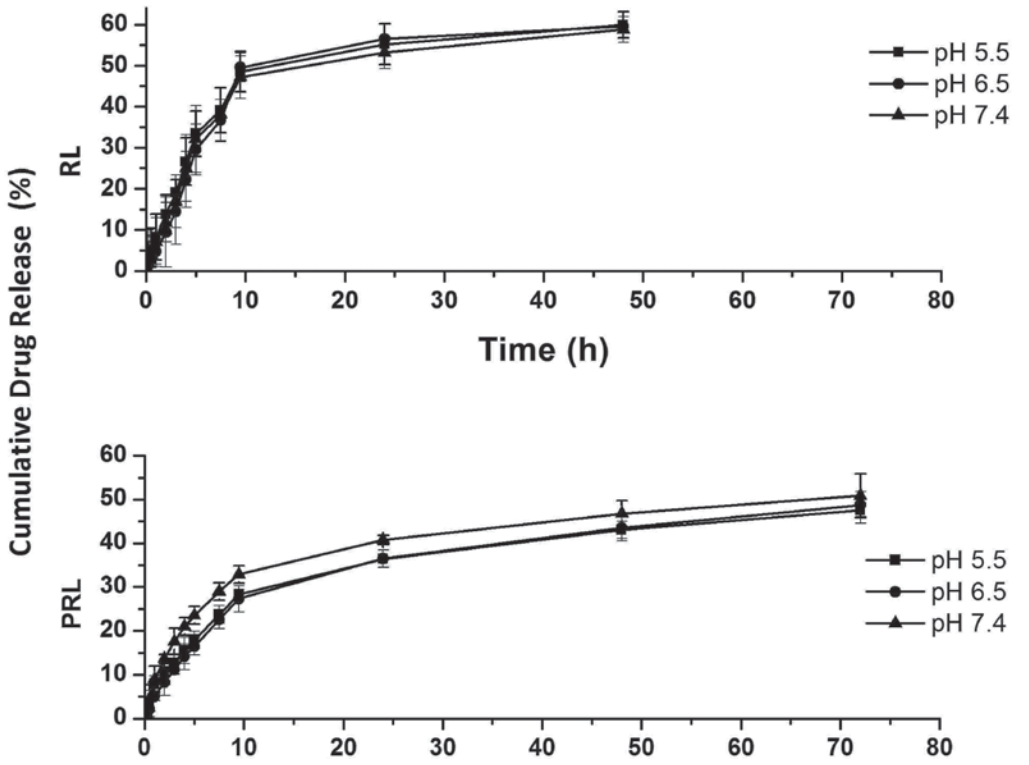

Figure 6. The RES release of PRL and RL in phosphate buffer solutions at pH 7.4, 6.5 and 5.5. The mean wais calculated from three samples of different batches and the error bar represents the standard deviation from the mean (mean ± SD, $n=3$).

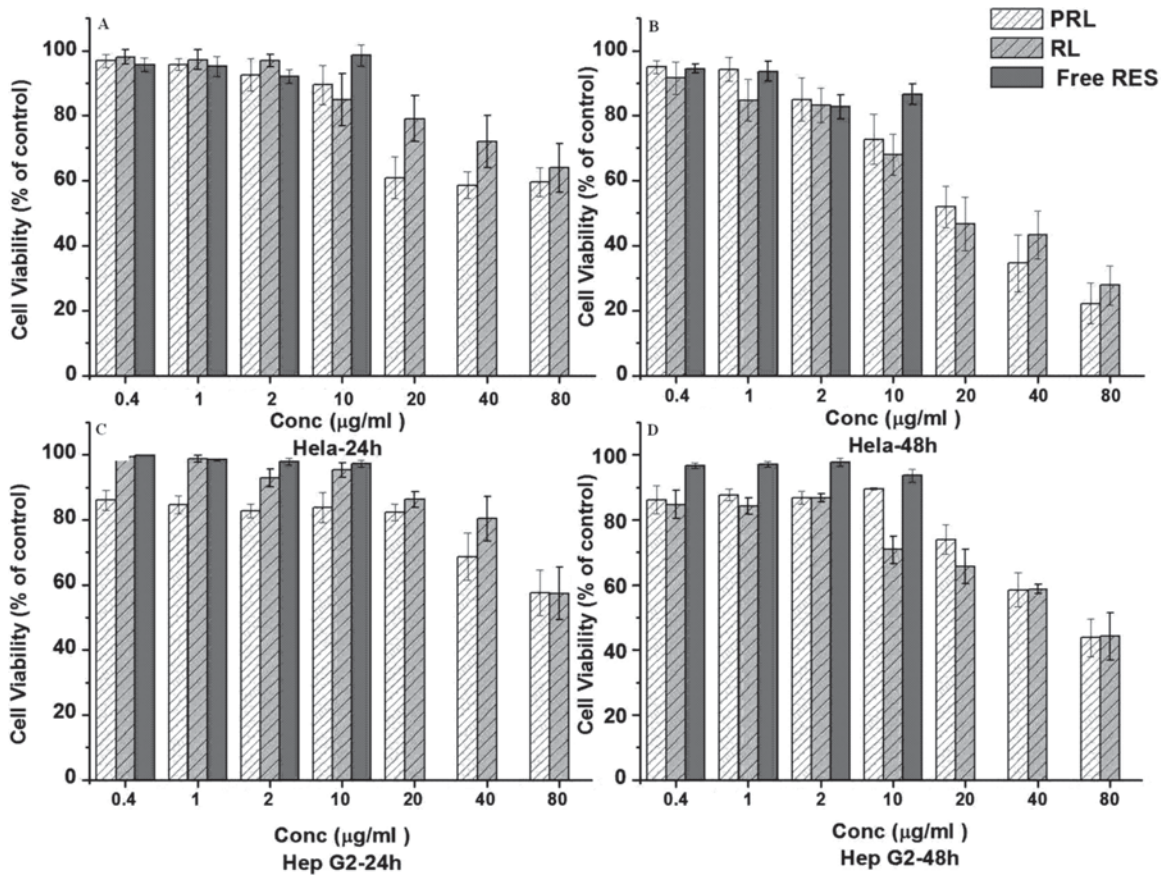

Figure 7. MTT assay of RES and RES liposomes on HeLa cells and Hep G2 cells. The mean was calculated from three samples of different batches and the error bar represents the standard deviation from the mean (mean ± SD, $n=3$).

treatment. This phenomenon may be attributed to the increase of RES concentration in liposome solution. The maximum concentration of RES in water is only 50 μg/mL. When this RES solution was treated with HeLa cell and Hep G2 cells, the final RES concentration in each well was 10 μg/mL which could not cause effective inhibition on both tumor cells. As shown in Figure 7A–D, once RES was loaded into RL or PRL, the cell viability of both HeLa cells and Hep G2 cells decreased noticeably as the concentration of RES increased from 0.4 to 80 μg/mL. The PEG-modified liposomal RES caused HeLa cell death of about 40.5 and 77.8%, and caused Hep G2 cell death about 42.4 and 56.23% in the concentration of 80 μg/mL at 24 h and 48 h treatment, respectively. Liposomal RES displayed slightly less cytotoxicity than PEG-modified liposomal RES, causing HeLa cell death about 36.1 and 72.3%, and caused Hep G2 cell death about 42.6 and 55.7% at 24 h and 48 h, respectively. As a control, the vacant RLs and PRLs did not affect the cell growth.

Discussion

RES was a water-insoluble drug and could easily dissolve in organic solvents. Due to the strong capability of dissolving drugs and the low boiling point to be readily evaporated, ether was used as the oil-phase in our study. According to the results shown above, it was found that the amount of SPC and Chol was important to influence the encapsulating capacity of liposomes. The drug loading capacity was influenced by the amount of phospholipid. Indeed, EE was improved as the amount of phospholipid increase (Figure 1), whereas excessive amount of lipid would induce the decrease of LC. On the other hand, cholesterol added in liposome could enhance the hydrophobic and rigidity of the membrane, thereby reducing the leakage or permeability of encapsulating drugs. However, excessive cholesterol would give rise to the disruption of regular linear structure of the liposomal membrane and the decrease of internal aqueous volume. As a result, the excess cholesterol in lipid phase led to the low EE[35]. In short, the amount of phospholipid and chol should be optimized for liposome formulation. Therefore, 1:40 drug/SPC mass ratio and 1:10 chol/SPC mass ratio were determined as the most appropriate condition in our study.

Recently, lyophilization technique has been widely applied for facilitating the storage of liposomes and was tested in our study as well. The process of lyophilization was generally harmful to the liposome integrity and induced marked loss in the EE. The data shown in Figures 1 and 2 confirmed that after lyophilization the EE was somehow decreased as compared with the samples before lyophilization. Fortunately, this adverse influence of lyophilization was minimized as the drug/SPC mass ratio and Chol/SPC mass ratio decreased. At 1:40 drug/SPC mass ratio and 1:10 Chol/SPC mass ratio, the resultant liposome achieved high EE% which dropped only a little during lyophilization procedure.

Incorporation of DSPE-PEG_{2000} into the membrane phase almost brought about no remarkable changes in the physicochemical properties comparing with RLs. Some previous researches improved that the incorporation of DSPE-PEG_{2000}, a highly water-soluble polymer, into liposomes reduced the liposome aggregation in some degree and improved the physical stability of liposomes[36–38]. On the other hand, in tumor tissues, partial tumor vessels could cause extravasation of small liposomes (i.e., <400 nm[38–42]). The past studies confirmed that 100 nm liposomes extravasate much easier than 200 or 400 nm liposomes[43,44]. Furthermore, small liposomes can diffuse better throughout tumor tissue so as to prevent limited perivascular accumulation. In our study, the particle sizes of vacant liposomes, RLs and PRLs were all at 100 nm approximately. The data measured by Tatesizer-3000 HS instrument indicated that the inclusion of DSPE-PEG_{2000} resulted in the slight decrease of mean particle sizes from 120 to 100 nm when compared with RLs. Therefore, both RLs and PRLs were promising to extravasate from tumor vessels and worth being investigated in our future work.

The major obstacle of clinical application of RES is its poor solubility in aqueous solutions. Loading drug into liposomes strikingly increased the RES concentration in aqueous solutions via solubilization effect and consequently performed remarkable cytotoxicity on HeLa cells and Hep G2 cells. The Figure 7A shows that when HeLa cells were treated with RLs for 24 h, the significant decrease of cell viability appears from drug concentration of 10 to 80 μg/mL. When HeLa cells were treated with RLs for 48 h, this trend was more notable. Moreover, as shown in Figure 7A and B, as the HeLa cells were treated with PRLs for 24 h at a relative high concentration (20, 40, 80 μg/mL), the inhibition effect had almost no meaningful differences. But when it was treated for 48 h, the cell viability extremely reduced. When Hep G2 cells were treated with PRLs and RLs, the cell viability were also decreased as the liposomal concentration increased. And at the high concentrations (20, 40 and 80 μg/mL), this trend was particularly apparent. Furthermore, the intensity of inhibiting tumor growth of both kinds of liposomes on HeLa cells was stronger than Hep G2 cells.

Since the liposome drug loading technique has been extensively developed, some research studies have been carried out on encapsulating RES into liposomes. Narayanan et al. prepared liposomes encapsulating of curcumin and RES in combination, and further animal studies confirmed that these liposomes could enhance chemopreventive efficacy in prostate cancer in PTEN knockout mice[45]. Kristl et al. loaded RES into liposomes and provided evidences of effective liposome-mediated uptake of RES for improvement of the cell-stress response[46]. Though the evaluation of liposomal RES on cell lines has proliferated, efforts in liposome formulation and preparation processes in detail is lacking. Our

work concerned the development of liposome preparation and series factors on preparation procedures, and explored an optimal formulation which was crucial for the liposome technique application. Further pharmacodynamics and pharmacokinetics study *in vivo* based on these two kinds of liposomes will be investigated and reported in our future paper.

Conclusions

RES was successfully encapsulated into liposomes by reverse-phase evaporation method. The factors of drug/SPC mass ratio, Chol/SPC mass ratio, microfluidization process and DSPE-PEG_{2000} used in phospholipid were optimized to achieve nano-sized liposomes with high EE. Furthermore, the drug release behaviors and cytotoxicity *in vitro* were studied, which revealed that both RES liposomes and RES PEG-modified liposomes inhibited the growths of HeLa cells and Hep G2 cells due to the increased drug concentration in water.

Declaration of interest

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