Preparation of New Doxorubicin Hydrochloride Liposomes

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Abstract [**Objectives**] To prepare protopanaxadiol type doxorubicin hydrochloride liposomes by replacing cholesterol with protopanaxadiol, a derivative of ginsenoside, which has a similar structure with cholesterol, to reduce the adverse reaction of adriamycin (doxorubicin) and improve the shortcomings of ordinary doxorubicin hydrochloride. [**Methods**] Liposomes were prepared by thin film dispersion-ammonium sulfate gradient method, and the optimal formulation was screened by Box-Behnken experiment with particle size and encapsulation efficiency as the evaluation indicator through single factor experiment, and the drug release *in vitro* was verified. [**Results**] The average particle size of the liposomes was (149.21 ± 1.2) nm, the polydispersity index (PDI) was (0.22 ± 0.02), and the potential was − (15.22 ± 1.57) mV. The liposomes were spherical and uniform in size; the encapsulation efficiency and drug loading of the new doxorubicin hydrochloride liposomes were (89.71 ± 4.4)% and (7.28 ± 0.8)%, respectively. [**Conclusions**] The new doxorubicin hydrochloride liposomes was successfully prepared by a film dispersion-ammonium sulfate gradient method, the internal circulation of the doxorubicin hydrochloride liposomes was prolonged, and the new material has good stability. This study is expected to lay a foundation for the successful preparation of new doxorubicin hydrochloride liposomes *in vitro* and *in vivo*.

Key words Tumor, Doxorubicin hydrochloride liposomes, Targeted therapy, Protopanaxadiol, Clinical requirement

1 Introduction

From the perspective of clinical treatment, chemotherapy is still the main treatment for various tumors[1]. Anthracycline[2] drugs, taxanes, platinum drugs and other drugs are commonly used in chemotherapy regimens. Through the specific and targeted delivery of these drugs, their anti-cancer ability can be improved, while reducing harmful side effects. The use of doxorubicin hydrochloride, which belongs to the anthracycline class and has a broad spectrum of antitumor activity, has been a first-line clinical drug for many years[3], but its use has been limited due to its potential for significant cardiotoxicity during and several years after treatment[4]. Therefore, doxorubicin hydrochloride is the first liposome-encapsulated anticancer drug[5] to obtain clinical approval for the treatment of malignant tumors (including solid tumors, transplantable leukemia and lymphoma) by improving the traditional adriamycin (doxorubicin) formulation. However, it still has the shortcomings of insufficient circulation time *in vivo* and easy to be cleared[6]. But the doxorubicin hydrochloride liposomes undoubtedly provides a new idea for the further application of doxorubicin hydrochloride, and doxorubicin liposome is further improved on this basis, there are also a series of improved doxorubicin hydrochloride liposomes, but there are some problems[7]. Therefore, doxorubicin hydrochloride formulations still need to be improved. Protopanaxadiol (PPD) is a main component of ginsenoside[8] and the main metabolite of ginsenoside, which has the pharmacological effects of anti-tumor[9], anti-oxidation, anti-inflammatory and immunomodulation[10]. Studies have shown that ginsenoside has a similar structure with cholesterol[11], and can replace cholesterol as a membrane material to form liposomes with phospholipids to improve the adverse reactions of cholesterol. In this study, protopanaxadiol liposomes were used to load doxorubicin hydrochloride, which can reduce the adverse reactions of doxorubicin hydrochloride, prolong the circulation of liposomes *in vivo*, and have better killing effect on tumors. The traditional Chinese medicine component PPD in the film material can play a synergistic anti-tumor effect with doxorubicin hydrochloride, solves the problem that a single drug can not achieve a satisfactory therapeutic effect, and improves the tumor treatment effect.

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2 Materials and methods

2.1 Instruments and equipment Electronic balance (Shanghai Sunny Hengping Instrument Co., Ltd.); laser particle size analyzer (Dandong Better Size Instrument Co., Ltd.); ultrasonic cell disintegrator (Ningbo SCIENTZ Biotechnology Co., Ltd.); high-frequency CNC ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.); rotary evaporator (Zhengzhou Ketai Laboratory Equipment Co., Ltd.).

2.2 Experimental materials Doxorubicin hydrochloride (Shanghai Macklin Biochemical Technology Co., Ltd.); protopanaxadiol reference substance (Shandong Boyuan Pharmaceutical Co., Ltd.); phospholipid (AVT (Shanghai) Pharmaceutical Technology Co., Ltd.); cholesterol (Shanghai Macklin Biochemical Technology Co., Ltd.); methanol (Beyotime Biotechnology Co., Ltd.).

2.3 Establishment of doxorubicin determination method

2.3.1 Establishment of maximum absorption wavelength. Accurately weighed a proper amount of doxorubicin standard substance, prepared doxorubicin solutions with different concentrations using methanol and a gradient dilution method to obtain doxorubicin standard substance solutions, and scanned doxorubicin standard substance solutions within a wavelength range of 200 to 600 nm using methanol as a blank. The doxorubicin standard substance

solutions were not connected with a solvent peak, so the absorption wavelength was finally determined to be 480 nm.

2.3.2 Preparation of solutions. Preparation of ammonium sulfate solution: weighed solid ammonium sulfate with molar mass and dissolved it in clean water to prepare a solution with a concentration of 200 mmol; preparation of normal saline: weighed 9 g of sodium chloride and added 1.0 L of water.

2.3.3 Establishment of standard curve. Precisely pipetted doxorubicin hydrochloride solutions with different concentrations, measured the absorbance with an ultraviolet spectrophotometer, plotted a standard curve with the concentration (μg/mL) as the abscissa and the absorbance as the ordinate, and calculated the regression equation.

2.3.4 Specificity, precision, and stability. First, 3.0 mL of doxorubicin solution, 3.0 mL of new doxorubicin liposome solution and 3.0 mL of blank liposome solution were pipetted to investigate the specificity. Precisely pipetted 2.5, 3.0 and 3.5 mL of doxorubicin hydrochloride reference solution, continuously injected the sample for 6 times, and calculated the *RSD* according to the absorbance to test the precision. Precisely pipetted 2.0 mL of the test solution, and measure the particle size at 0, 24, 28 and 72 h after preparation to investigate the stability.

2.4 Preparation of doxorubicin liposomes

2.4.1 Preparation of liposomes. The liposomes were prepared by a film dispersion method and an ammonium sulfate gradient method. Weighed phospholipid and the PPD of the prescription amount, dissolved with a proper amount of methanol, ultrasonically dissolved, pipetted a certain amount of solution by a pipette gun and added into a round bottom flask, and evaporated by a rotary evaporator until a layer of film is formed on the bottle wall. Then, added the preheated ammonium sulfate solution with the concentration of 200 mmol, rotated and hydrated at 60 ℃ for 30 min. After hydration, the sample was broken by ultrasonic instrument at 200 W for 2 min, and then put into a dialysis bag for rotary dialysis with normal saline for 4 – 6 h. After dialysis, added doxorubicin hydrochloride standard solution, and bashed the sample in 60 ℃ water for 10 min to carry out drug loading, and then passed through a 800 nm polyester acid membrane, to determine the liposome particle size.

2.4.2 Single factor experiment. Liposomes were prepared according to the ammonium sulfate gradient drug loading method, keeping other conditions unchanged, only changing the drug loading time, the investigation time was 10 – 30 min, the encapsulation efficiency and particle size at each time were determined, and the ideal drug loading time was 20 min by comprehensive comparison. Then, the effects of drug-to-lipid ratio, ratio of water and oil and drug loading temperature on the particle size and encapsulation efficiency of liposomes were investigated, and the factors with greater influence were screened for the further experiment.

2.5 Characterization of new doxorubicin liposomes

2.5.1 Morphology of liposomes. The prepared liposome was diluted properly, dropped on the copper mesh for electron microscope, stained with 2% phosphotungstic acid, placed at room temperature until the sample dried naturally, and then placed under the transmission electron microscope to observe the morphology of the liposome and take photos.

2.5.2 Particle size, polydispersity index (PDI), potential. The particle size, potential and PDI of the prepared liposome were measured by laser particle size analyzer/Zeta potential analyzer.

2.5.3 Encapsulation efficiency and drug loading. The liposomes were screened through a 0.8 μm microporous membrane, collected the filtrate, and recorded the volume of the collected filtrate. Took 2.0 mL of liposome filtrate, added appropriate amount of methanol, vortex centrifuged, demulsified with ultrasound for 30 min, passed through 0.22 μm microporous membrane, measured with ultraviolet spectrophotometry, and recorded as the total amount of drug. Took another 2.0 mL of liposome filtrate was put into an ultrafiltration tube (M = 100 KD), centrifuged at 3 000 rpm for 30 min, and added the lower filtrate with appropriate amount of methanol. After passing through the membrane, directly measured by an ultraviolet spectrophotometer and recorded as the amount of free drug. Calculated encapsulation efficiency (*EE%*) and drug loading (*DL%*) of liposomes:

$$EE\% = (\text{Total drug content} - \text{Free drug content})/\text{Total drug content} \times 100\%$$

$$DL\% = (\text{Total drug content} - \text{Free drug content})/[(\text{Total drug content} - \text{Free drug content}) + \text{PLGA mass}] \times 100\%$$

2.6 Response surface optimization experiment In the single factor experiment, the drug-to-lipid ratio (A), the ratio of water and oil (B), and the ratio of phospholipid to PPD (C) were selected as the research factors, and encapsulation efficiency and particle size were used as the evaluation levels, and two levels of three factors were used. According to the orthogonal design table, the optimum prescription was selected.

2.7 *In vitro* release test (IVRT) The Liposomes were prepared according to the optimal prescription of drug-to-lipid ratio of 1∶10, ratio of water and oil of 2.5∶1, and the ratio of PPD to phospholipid of 1∶3. PBS buffer containing 1% Tween-80 with pH of 7.4 was prepared as the release medium, and samples were taken at 0, 1, 2, 4, 8, 16, 32 and 48 h, respectively, and the cumulative release concentration was calculated.

3 Results and analysis

3.1 Determination of doxorubicin content The maximum absorption peak of doxorubicin hydrochloride was determined at 480 nm by full scanning with an ultraviolet spectrophotometer, so the standard solutions of doxorubicin hydrochloride with different concentrations were determined at 480 nm, and the standard curve was plotted (Fig. 1), for the subsequent determination of liposome drug loading and encapsulation efficiency.

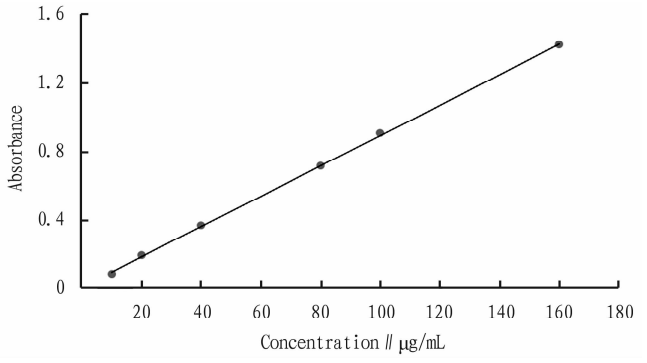


Fig. 1 Standard curve of doxorubicin hydrochloride solution

3.2 Preparation and characterization of new doxorubicin liposomes

3.2.1 Drug loading time. Liposomes were prepared using the ammonium sulfate gradient drug loading method, keeping other conditions unchanged, only changing the drug loading time, the investigation time was 10 – 30 min, and the encapsulation efficiency and particle size at each time were determined. By comprehensive comparison, the ideal drug loading time was 20 min (Fig. 2).

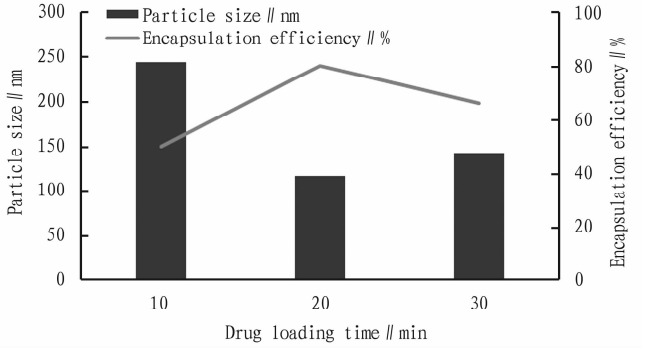


Fig. 2 Effect of drug loading time on particle size and encapsulation efficiency (n =3)

3.2.2 Drug-to-lipid ratio. Liposomes were prepared using the ammonium sulfate gradient drug loading method, keeping other conditions unchanged, only changing the ratio of drug to phospholipid, the drug-to-lipid ratio was 1 ∶ 5, 1 ∶ 10, 1 ∶ 20. The encapsulation efficiency and particle size under different drug-to-lipid ratios were measured, and the ideal drug-to-lipid ratio was 1 ∶ 20 (Fig. 3).

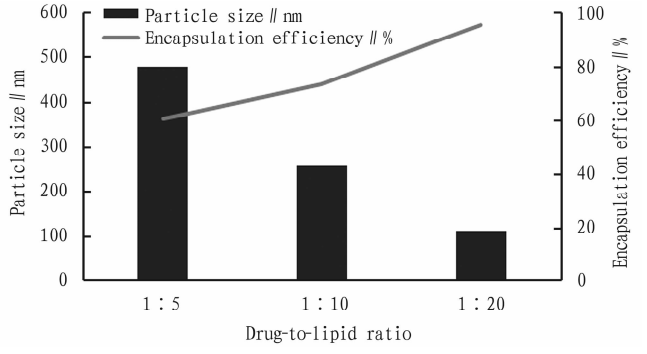


Fig. 3 Effect of drug-to-lipid ratio on liposome particle size and encapsulation efficiency (n =3)

3.2.3 Ratio of water and oil. Liposomes were prepared using the ammonium sulfate gradient loading method, keeping other conditions unchanged, only changing the ratio of water and oil, and the ratio of water and oil was 1 ∶ 2, 1 ∶ 2.5 and 1 ∶ 3. The encapsulation efficiency and particle size of the water-oil phase liposomes at different ratios were measured, and the ideal ratio of water and oil was 1 ∶ 2.5 (Fig. 4).

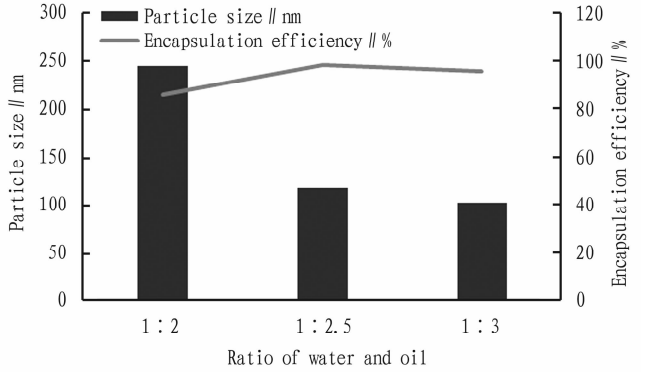


Fig. 4 Effect of ratio of water and oil on liposome particle size and encapsulation efficiency (n =3)

3.2.4 Drug loading temperature. Liposomes were prepared using the ammonium sulfate gradient drug loading method, keeping other conditions unchanged, only changing the drug loading temperature, the investigation time was 50, 60 and 70 ℃, the encapsulation efficiency and particle size at each temperature were measured, and the ideal drug loading temperature was 60 ℃ (Fig. 5).

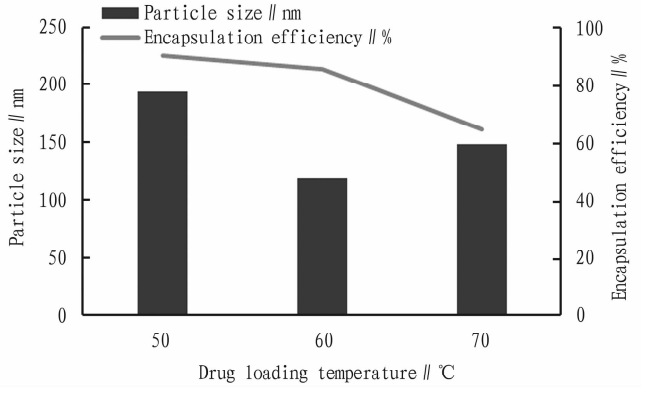


Fig. 5 Effect of drug loading temperature on particle size and encapsulation efficiency (n =3)

3.2.5 Phospholipid ratio. Liposomes were prepared using the ammonium sulfate gradient loading method, keeping other conditions unchanged, only changing the ratio of phospholipid to PPD, investigating the ratio of phospholipid to PPD as 1 ∶ 2, 1 ∶ 3, 1 ∶ 4, and determining the encapsulation efficiency and particle size of liposomes under different phospholipid ratios to PPD. The ideal phospholipid ratio of 1 ∶ 3 was obtained by comprehensive comparison (Fig. 6).

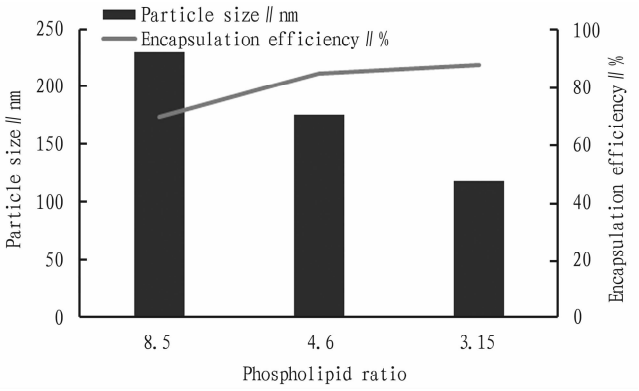


Fig. 6 Effect of phospholipid ratio on liposome particle size and encapsulation efficiency ($n=3$)

3.2.6 Observation of particle size and morphology of biomimetic doxorubicin liposomes. The particle size of the prepared novel doxorubicin liposomes was determined by laser particle size analyzer after dilution, and it was found that the particle size of the conventional doxorubicin liposomes was 118.52 nm, and the particle size of the new doxorubicin liposomes was 149.21 nm. The morphology of the prepared doxorubicin liposome was observed by transmission electron microscopy. The liposome was dropped on a 400 nm copper mesh and negatively stained with 2% phosphotungstic acid solution. After the sample was dried, it was observed under transmission electron microscopy. The prepared liposome was stable in morphology and had no adhesion (Fig. 7).

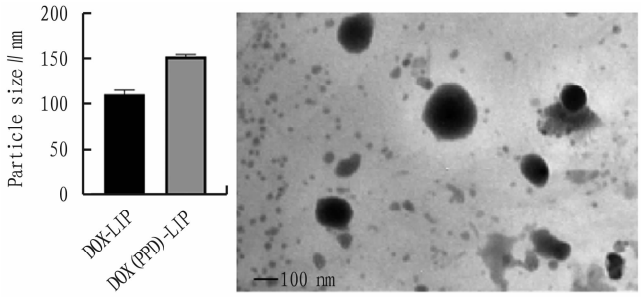


Fig. 7 Particle size and TEM observation of the new protopanaxadiol doxorubicin hydrochloride liposomes

3.3 Response surface optimization experiment

3.3.1 Box-Behnken experiment design. Based on the results of single factor investigation, three factors were selected as the major factors affecting the liposomes: drug-to-lipid ratio (0.05% −0.2%, X_1), water-oil ratio (0.3% −0.5%, X_2) and drug-lipid ratio (0.25% −0.3%, X_3) were used as factor levels, and a 3-factor 3-level experiment was designed using Design Expert 11, to optimize with particle size (Y_1) and encapsulation efficiency (Y_2) as response values. The experimental design results are shown in Table 1.

Table 1 Box-Behnken experiment design results

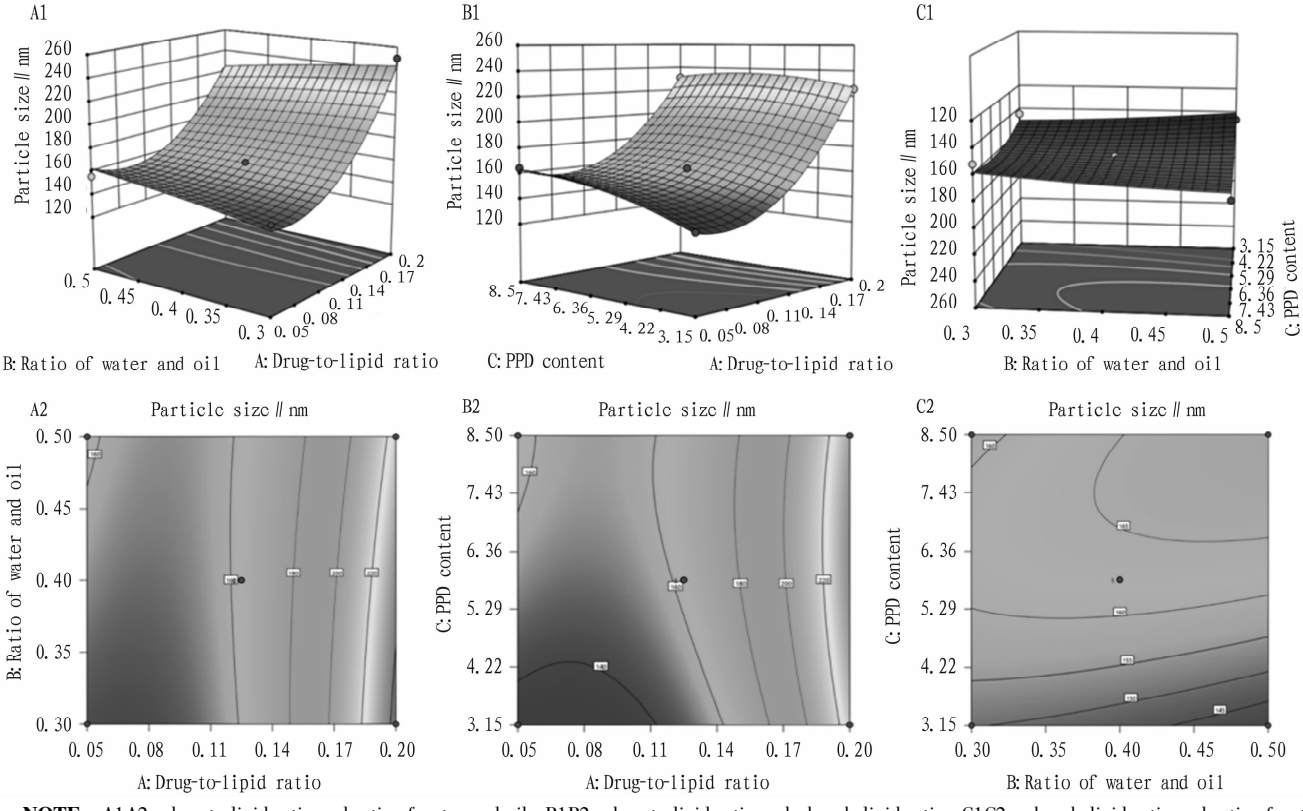
Experiment No.	Independent variable			Dependent variable	
	Drug-to-lipid ratio (X_1)	Water-oil ratio (X_2)	Drug-lipid ratio (X_3)	Particle size (Y_1) // nm	Encapsulation efficiency (Y_2) // %
1	0.05	0.3	0.3	147.80	86.75
2	0.20	0.3	0.3	250.68	76.51
3	0.05	0.5	0.3	155.88	85.55
4	0.20	0.5	0.3	220.46	75.25
5	0.05	0.4	0.25	132.51	88.21
6	0.20	0.4	0.25	225.64	78.21
7	0.05	0.4	0.5	164.03	82.51
8	0.20	0.4	0.5	230.95	72.51
9	0.125	0.3	0.25	144.65	88.89
10	0.125	0.5	0.25	146.60	89.12
11	0.125	0.3	0.5	152.24	82.51
12	0.125	0.5	0.5	174.34	83.21
13	0.125	0.4	0.3	162.31	89.21
14	0.125	0.4	0.3	163.25	88.18
15	0.125	0.4	0.3	162.21	89.31
16	0.125	0.4	0.3	163.22	88.61
17	0.125	0.4	0.375	162.24	88.68

3.3.2 Data processing. The quadratic polynomial regression equation between each factor and particle size (Y_1) and encapsulation efficiency (Y_2) was obtained by quadratic polynomial regression fitting of the experimental data in Table 1 through Design Expert 11 software: $Y_1 = 162.65 + 40.94A + 0.238\,7B + 9.02C - 9.57AB - 6.55AC + 5.04BC + 32.44A^2 - 1.38B^2 - 6.81C^2$, $R^2 = 0.985\,9$, $C.V.\% = 3.6 < 10\%$, indicating that the reliability and accuracy of the experiment are high. $Y_2 = 88.8 - 5.07A - 0.191\,2B - 2.96C - 0.015AB + 0.1175BC - 6.68A^2 - 1.11B^2 - 1.76C^2$, $R^2 = 0.995\,1$, $C.V.\% = 0.699\,6 < 10\%$, indicating that the reliability and accuracy of the experiment are high. According to the results of software analysis, the optimal parameters for preparing the biomimetic new doxorubicin liposomes were obtained as follows: $X_1 = 0.125$, $X_2 = 0.4$, $X_3 = 0.3$. A liposome with a particle size of 132.51 nm and an encapsulation efficiency of 89.31% was obtained theoretically (Table 2 and Figs. 8 −9).

Table 2 Regression coefficient and variance significance results of particle size model and encapsulation efficiency model

Source of variation	Particle size model						Encapsulation efficiency model					
	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value		Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value	
Model	19 240.41	9	2 137.82	54.42	0.000 1	Significant	236.74	9	26.3	38.52	<0.0001	Significant
A	13 407.85	1	13 407.85	341.3	0.000 1		225.78	1	225.78	330.62	<0.000 1	
B	0.456	1	0.456	0.012	0.917 2		4.79	1	4.79	7.01	0.033	
C	650.88	1	650.88	16.57	0.004 7		2.79	1	2.79	4.08	0.083 2	
AB	366.72	1	366.72	9.33	0.018 4		0.008 4	1	0.008 4	0.012 3	0.914 8	
AC	171.74	1	171.74	4.37	0.074 9		0.144 7	1	0.144 7	0.211 9	0.659 2	
BC	101.51	1	101.51	2.58	0.152 0		2.06	1	2.06	3.02	0.126 1	
A^2	4 431.51	1	4 431.51	112.80	0.000 1		14.76	1	14.76	21.62	0.002 3	
B^2	8.05	1	8.05	0.205	0.664 4		0.000 2	1	0.000 2	0.000 3	0.987 1	
C^2	195.01	1	195.01	4.96	0.061 2		0.288 5	1	0.288 5	0.422 4	0.536 5	
Residual error	275.00	7	39.29				4.78	7	0.682 9			
Lack of fit	273.83	3	91.28	0.979 798 8	0.085 959 883	Not significant	0.531 5	3	0.177 2	0.166 8	0.913 5	Not significant
Pure error	1.16	4	0.291				4.25	4	1.06			
Total variation	19 515.41	16					241.52	16				

NOTE $P<0.05$ means significant; $P>0.05$ means not significant.



NOTE A1A2: drug-to-lipid ratio and ratio of water and oil; B1B2: drug-to-lipid ratio and phospholipid ratio; C1C2: phospholipid ratio and ratio of water and oil. The same below.

Fig. 8 Response surface and contours affecting particle size

3.3.3 Process verification. Three batches of liposomes were prepared according to the optimal prescription, and their encapsulation efficiency and particle size were determined respectively. The results are shown in Table 3. It can be seen from Table 3 that the measured value of the experiment is close to the predicted value of the model, indicating that the model has good predictability and can be used to optimize the formulation of liposomes.

Table 3 Liposome verification experiment

Experiment No.	Particle size // nm	Encapsulation efficiency // %
1	141.80	89.59
2	137.23	90.09
3	135.79	88.90
$\bar{x} \pm SD$	138.27 ± 3.14	89.53 ± 0.59
RSD // %	2.27	0.67

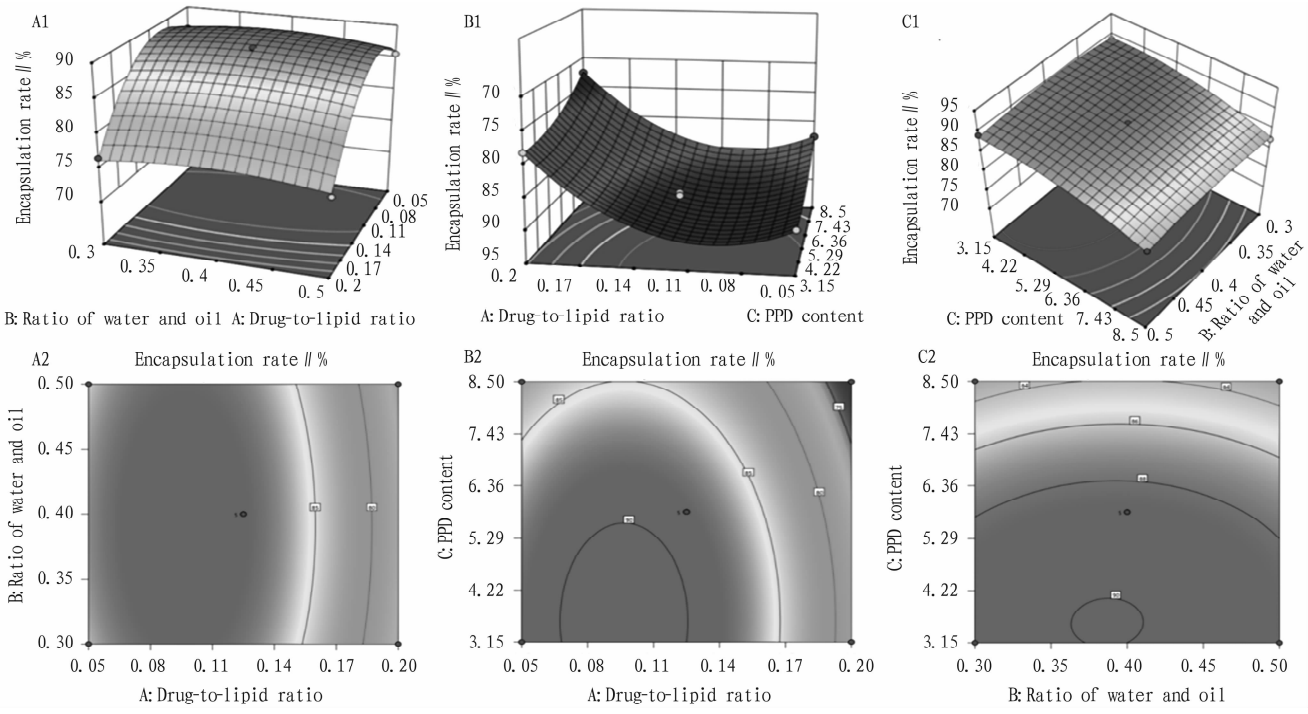


Fig. 9 Response surface and contours affecting encapsulation efficiency

3.4 *In vitro* release test The drug concentration was determined at different time points by *in vitro* release within 48 h, and the *in vitro* release curve was drawn (Fig. 10). The results showed that the doxorubicin hydrochloride liposomes modified by PPD could prolong the drug release time.

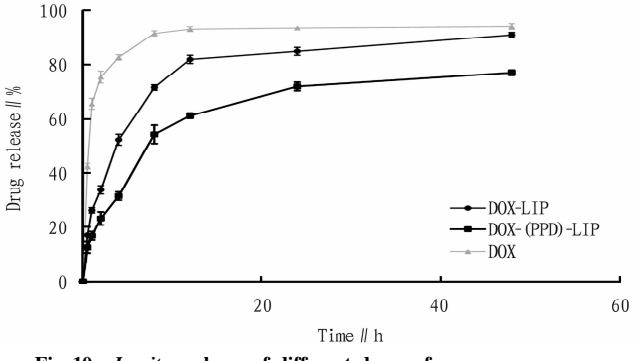


Fig. 10 *In vitro* release of different dosage form

4 Conclusions

In this study, liposomes were prepared by film dispersion method and ammonium sulfate gradient method, and the formulation of liposomes was optimized by Box-Behnken response surface design. The prepared liposomes had appropriate particle size, uniform distribution, spherical shape and good stability. This study is expected to lay a foundation for the successful preparation of new doxorubicin hydrochloride liposomes *in vitro* and *in vivo*.

References

[1] ROUBAUD MJ, PREVOT J, LECLERE JC, *et al.* Management of neonatal retro-auricular embryonal rhabdomyosarcoma-Case report[J]. International Journal of Surgery Case Reports, 2020, 75: 322 - 326.

[2] MARTINS-TEIXEIRA MB, CARVALHO I. Antitumour anthracyclines: Progress and perspectives[J]. ChemMedChem, 2020, 15(11): 933 - 948.

[3] VOCI S, GAGLIARDI A, AMBROSIO N, *et al.* Gliadin nanoparticles containing doxorubicin hydrochloride: Characterization and cytotoxicity [J]. Pharmaceutics, 2023, 15(1): 180.

[4] QI Y, CHEN J, DUAN J, *et al.* Major vault protein attenuates cardiomyocyte injury in doxorubicin-induced cardiomyopathy through activating AKT[J]. BMC Cardiovascular Disorders, 2022, 22(1): 77.

[5] RIGACCI L, ANNIBALI O, KOVALCHUK S, *et al.* Nonpeghylated liposomal doxorubicin combination regimen (R-COMP) for the treatment of lymphoma patients with advanced age or cardiac comorbidity[J]. Hematological Oncology, 2020, 38(4): 478 - 486.

[6] ZHU YX, JIA HR, DUAN QY, *et al.* Photosensitizer-doped and plasma membrane-responsive liposomes for nuclear drug delivery and multidrug resistance reversal[J]. ACS Applied Materials & Interfaces, 2020, 12 (33): 36882 - 36894.

[7] LI XR, CHENG XH, ZHANG GN, *et al.* Cardiac safety analysis of first-line chemotherapy drug pegylated liposomal doxorubicin in ovarian cancer [J]. Journal of Ovarian Research, 2022, 15(1): 96.

[8] LI C, ZHAN Y, ZHANG R, *et al.* 20(S)- Protopanaxadiol suppresses hepatic stellate cell activation via WIF1 demethylation-mediated inactivation of the Wnt/β-catenin pathway[J]. Journal of Ginseng Research, 2023, 47(4): 515 - 523.

[9] YANG L, ZHANG XY, LI K, *et al.* Protopanaxadiol inhibits epithelial-mesenchymal transition of hepatocellular carcinoma by targeting STAT3 pathway[J]. Cell Death & Disease, 2019, 10(9): 630.

[10] ALI M Y, ZAIB S, JANNAT S, *et al.* Inhibition of aldose reductase by ginsenoside derivatives via a specific structure activity relationship with kinetics mechanism and molecular docking study[J]. Molecules (Basel, Switzerland), 2022, 27(7): 2134.

[11] XIA J, ZHANG S, ZHANG R, *et al.* Targeting therapy and tumor microenvironment remodeling of triple-negative breast cancer by ginsenoside Rg3 based liposomes[J]. Journal of Nanobiotechnology, 2022, 20(1): 414.