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Optimization of preparation of DHAQ-loaded PEG-PLGA-PEG nonaparticles using central composite design

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Abstract Mitoxantrone (DHAQ)-loaded poly (ethylene glycol)-poly (lactic acid-co-glycolic acid) –poly (ethyleneglycol) (PELGE) nanoparticles (NP) were fabricated using an emulsification/solvent evaporation technique. A central composite design (CCD) was applied to evaluate the joint influence of three formulation variables: the amounts of polymer, concentration of the DHAQ, and the ratio of the organic phase (inner-phase) and the aqueous phase (outer-phase). In this study, we optimize the preparation technology on the basis of the single factor evaluation. The optimal conditions for the preparation of DHAQ-loaded nanoparticle were found to be: the concentration of PELGE was 9 mg/mL, the concentration of inner-phase of DHAQ was 27.5 mg/L, and the ratio of inner-phase/outer-phase was 8.5/1. The results showed that CCD is an ideal technique for formulation studies. The entrapment efficiency ratio (ER) was 90% and particle sizes are less than 500 nm. The nanoparticles, as examined by transmission electron microscopy (TEM), have a smooth and spherical surface. The DHAQ could be loaded into PELGE copolymers. In this study, the DHAQ nanoparticle-polymer delivery system was established by using PELGE polymers as carrier material.

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1. Introduction

It has been long recognized that the efficient use of drugs requires that they should be delivered selectively at the site of cancer cells, preferably at a controlled rate. This is especially true for potent drugs with strong side effects, such as the anticancer drugs. In addition to selectivity, such as beta-ray radioactive elements, requires that they should be protected from *in vivo* before reaching cancer cells. The particles of various sizes may allow their delivery to various parts of the body due to passive targeting of the carrier. Hence, cancer cells localized in such organs can be treated without adversely affecting the healthy cells in the body. The preparation of nanoparticles is a critical step for this targeting delivery system. Pharmaceutical formulators often face the challenge of finding the right combination of formulation variables that will produce a product with optimum properties. Optimization becomes even more important when the formulation is a controlled-release dosage form for this targeting delivery system, since many often inter-dependent factors can affect the release rate [1].

DHAQ is a very potent anticancer drug. However, the full therapeutic exploitation of DHAQ is limited by its toxicity in healthy tissues. Our aim was to investigate the possibility of a more selective delivery of DHAQ to tumor cells using PELGE nanoparticles as DHAQ carriers for intravenous administration. In this work we prepared PELGE nanoparticles by using the emulsion-solvent evaporation technique (w/o/w) with DHAQ as the model drug. A Central Composite Design has been used to elucidate the effect of

Table 1 Independent variables and their levels investigated in the central composite design

	Factor level in coded form				
Factor	$-\sqrt{3}$	-1	0	1	$\sqrt{3}$
X_1	5	7.11	10	12.89	15
X_2	0.05	0.166	0.325	0.484	0.6
X_3	6	6.85	8	9.15	10

*X_1 is the concentration of PELGE. X_2 is the concentration of DHAQ. X_3 is the ratio of inner-phase/outer-phase.

these independent variables on the optimized production of PELGE -NP.

The use of experimental designs is the most common method of simultaneously analyzing the influence of different factors on the properties of the system being studied [2, 3]. Most formulation studies involve variation of one factor at a time, keeping other factors constant. Such an empirical method is acceptable only when the factors are independent of one another. Factorial designs enable all factors to be varied simultaneously, thus allowing quantization of the effects caused by independent variables and interactions between them; thus, it is an ideal technique for formulation studies [4–6].

The simplest factorial design involves use of two levels of each variable, thus allowing estimation of linear relationships only. In situations where quadratic relationships exist, factorial designs with more than two levels can be used. However, in such studies, an increase in the number of factors markedly increases the number of experiments to be carried out. An alternative approach under these circumstances is to include extra center and star points in a two-level factorial design. This is then known as central composite design [7–9]. Table 1 shows the three independent variables investigated in this study and their levels.

2. Materials and methods

2.1. Materials and equipments

Beckman culture centrifugation (Allegra X-22R centrifuge), CS501 super constant water bath box (Shanghai Yue-xin scientific instrument manufactory) GBC UV cintra 10 e Spectrophotometer, transmission electron microscope (TEM, JEM 1200 FXII, Jeol Ltd, Tokyo, Japan), laser diffractometry (Mastersize/2000, Malvern), high-performance liquid chromatography (HPLC, Chiyoda-Ku, Tokyo, Japan); PELGE was synthesized by our own laboratory, Sodium citric acid, calcium chloride and other chemicals and reagents used were obtained commercially. All chemicals and reagents were analytical grade.

2.2. Synthesis of PELGE

Varied amount of lactide and glycolide crystals and specified amount of mPEG were accurately weighed and put into 25-ml glass ampoules. Catalyst stannous octoate was added at a concentration of 0.05% by weight of the feed and the tubes were evacuated. Then the tubes were heated in an oil bath at 150°C for 6 h. The coupling reaction of diblock copolymers was preformed with hexamethylene diisocyanate (HMDI) in toluene at 60 for 12 h, followed by refluxing for 6 h. The triblock copolymers were purified by methanol precipitation of polymer from methylene chloride using diethyl ether. The PELGEs having 70/30 or 80/20 or 50/50 molar ratio of lactic to glycolic acid moieties and 10% mPEG but different molecular weights were synthesized [10].

2.3. Preparation of nanoparticles using central composite design

Central Composite Design enables several independent variables to be investigated at the same time using a relatively small number of experiments. The independent variables in our studies were (1) PELGE concentration (2) DHAQ concentration (3) inner-phase/outer-phase ratio. For each factor, an experimental range was selected, based on the results of preliminary experiments and took the feasibility of preparation nanoparticles at the extreme values into consideration [11–13]. The layout of the design is shown in Table 2.

The nanoparticles loaded with DHAQ were fabricated using the double emulsion method with dextran70 as surfactant in the external aqueous phase. An aqueous solution of DHAQ was emulsified in 1 ml acetone/DCM, in which 10 mg of the copolymer had been dissolved, using probes sonication at 360 w for 30 s. This w/o emulsion was transferred to an

Table 2 Experimental design for three factors and experimental values of the objectives variables

No.	X_1 (mg/ml)	X_2 (mg)	X_3 (v/v)	SIZE (nm)	ER(%)
1	7.11	0.166	6.85	109.6	40
2	12.89	0.166	6.85	108.4	48.04
3	7.11	0.484	6.85	680.7	83.05
4	12.89	0.484	6.85	124.6	78.71
5	7.11	0.166	9.15	149.6	52.61
6	12.89	0.166	9.15	128.8	40
7	7.11	0.484	9.15	103.2	72.19
8	12.89	0.484	9.15	111.7	68.71
9	5	0.325	8	105.8	68.66
10	15	0.325	8	126.1	54.53
11	10	0.05	8	107	40
12	10	0.6	8	99.47	90.23
13	10	0.325	6	101	75.02
14	10	0.325	10	110.3	62.77
15	10	0.325	8	188.2	71.65

aqueous solution of dextran70 and the mixture was probe sonicated at 200 w for 30 s. The resulting w/o/w emulsion was gently stirred at room temperature until the evaporation of the organic phase was completed.

2.4. Determination of the DHAQ entrapment efficiency

The samples were centrifuged at 12,000×g for 10 min and the amount of DHAQ in the supernatants was measured by high-performance liquid chromatography (HPLC). The HPLC system was consisted of an SPD-10A variable UV-VIS detector, a Model FCV-12AH column-switching valve, and a set of Model LC-10AT liquid chromatograph including two pumps, a manometric module and a dynamic mixer from Shimadzu. The mobile phase consisted of methanol/0.2 Mol ammonium acetate buffer (48/52) solution. A Shimpack ODS column (150 × 4.6 mm, 5 μm) was eluted with the mobile phase at a flow rate of 1.0 mL/min. The eluate was monitored by measuring the absorption at 599 nm when the temperature of column was at 30°C. The Class VP V5.0 software was employed for the date analysis. The loading efficiency was calculated by the following equation: loading efficiency (wt%) = [(amount of remaining drug in nanoparticles)/(initial feeding amount of drug)] × 100%.

2.5. Characteristics of nanoparticles size

The morphological examination of nanoparticles was performed using a transmission electron microscope following negative staining with sodium phosphotungstate solution (0.2%, w/v). The samples were diluted with distilled water and measured at room temperature with a scattering angle of 90° and the size distribution of nanoparticles was determined by laser diffractometry.

2.6. Analysis of data

Based on the observed data, polynomial equations were generated to establish the correlation between the independent variables (i.e., concentration of PELGE, concentration of DHAQ, the ratio of inner-phase/outer-phase) and each of the dependent variables (i.e., the diameter, entrapment efficiency ratio) [4]. Table 1 shows the three independent variables investigated in this study and their corresponding levels, and Table 2 listed the 15 combinations of the different levels of the three variables used. All the preparations were obtained in triplicate and the dependent variables were determined two times in each one.

3. Results and discussion

3.1. Factors affecting nanoparticles size

The size of the particles is a very important parameter, because it is one of the factors that control the kinetics of drug release. The formation of the nanoparticles is affected by a number of factors.

3.1.1. The concentration of the polymers

This study demonstrates that the concentration of the vector materials affects the precipitation rate of nanoparticles in the nanoparticles formation process. When the concentration of PELGE or PEG-PLGA (PELGA) is between 10∼30 mg/mL, the nanoparticle size will increase with the increase of the concentration of the polymers. The concentration of polymers in the organic phase increases, and this increase of concentration of the polymers will result in their coagulation. At the same time, with the increase of the concentration of the polymers in the organic phase, the viscosity of the organic phase increases. This high viscosity slows down the rapid dispersion of the polymers. Therefore, it is impossible to produce small equal-sized particles. The polymers will coagulate to form relatively large particles, which result in the increase of the size of the nanoparticles.

3.1.2. The volume ratio of organic phase to water phase

The organic/aqueous phase ratio played a predominant role. The formation of nanoparticles depended on the rate of diffusion of the organic solvent into the aqueous phase, thus influenced the precipitation of polymers. When o/w ratio (v/v) decreases, the diameter of the nanoparticle will decrease. The organic phase disperses to form small particles under emulsifier. If the concentration of the particles in the dispersion medium of water is very low, the viscosity of the solution will be very low and the emulsion will be evenly distributed. Therefore, the chance for particles to get coagulated will be low and relatively stable dispersion system can be obtained. But when the o/w ratio (v/v) decreases to a certain degree, there are no such changes on the particle size.

3.1.3. The organic dissolvent

The physical and chemical properties of the organic dissolvent in the organic phase can greatly affect the emulsification and sphere-formation property [14–16]. The selected initial solvent used to dissolve the copolymer had a slight effect on the size of the nanoparticles, but had an evident effect on the drug loading. Dichloromethane has certain solubility in water, after the primary emulsion formation, dichloromethane gradually dissolves and disperses into

water through the organic-water interface and volatilizes through the water-air interface. In these processes, organic drops gradually solidify to form microspheres. Because the solubility of dichloromethane in water is very low, the solidification is very slow and therefore, it is very easy to form particles with good spherical shape. The solubility of acetone in water is high, if there is only acetone in the organic phase, acetone in the organic particles will dissolve into the water phase so quickly that it cannot form intact microsphere and therefore, irregular shaped particles will form. When organic phase is composed of water-soluble dissolvent acetone and water-insoluble dichloromethane, nano-sized emulsion particles can form gradually and precipitate because the dispersion of the water-soluble dissolvent results in the reduction of the surface tension and thus, the reduction of the particle sizes. The increase of the concentration of the water-soluble dissolvent will result in significant decrease of the size of the nanoparticles. In this study, we have used mixed dichloromethane and acetone dissolvent to fabricate nano-sized microspheres to obtain intact nano-spheres without porous on the surface while enhancing their drug loading ability.

3.2. Analysis of data

CCD constitutes an alternative approach since they offer the possibility of investigating a large number of variables at five levels after performing only a limited number of experiments. So, CCD is used here to determine the effect of the variables named in Table 1 on the characteristics of the resulting nanoparticles. The three factors selected are thought to influence mean particle size and the percentage of drug entrapment. The extreme values assigned to the independent variables were first checked in order to establish that nanoparticles could be prepared under these conditions.

The model was tested with the results obtained in the CCD. A multiphase curve fitting model was employed and the best fit for the experimental results from the CCD showed a curvilinear relationship. Fig. 1 display the predicted response surfaces of the size of PELGE1-DHAQ-NP as a function of the formulation variables. In Fig. 2(A), (B), (C) the effects of altering PELGE concentration and DHAQ concentration, PELGE concentration and the ratio of inner-phase/outer-phase, DHAQ concentration and the ratio of inner-phase/outer-phase respectively, on the size of nanoparticles have been illustrated. The combination of levels of each independent variable that gives the largest size was then determined. A combination so obtained gives an indication of the optimal conditions for the production of PELGE-DHAQ-NP with respect to its size and ER. It was found that optimal formulation of PELGE-DHAQ-NP requires use of PELGE 9 mg/ml, DHAQ 27.5 mg/L, and the ratio of inner-phase/outer-phase 17/2(v/v).

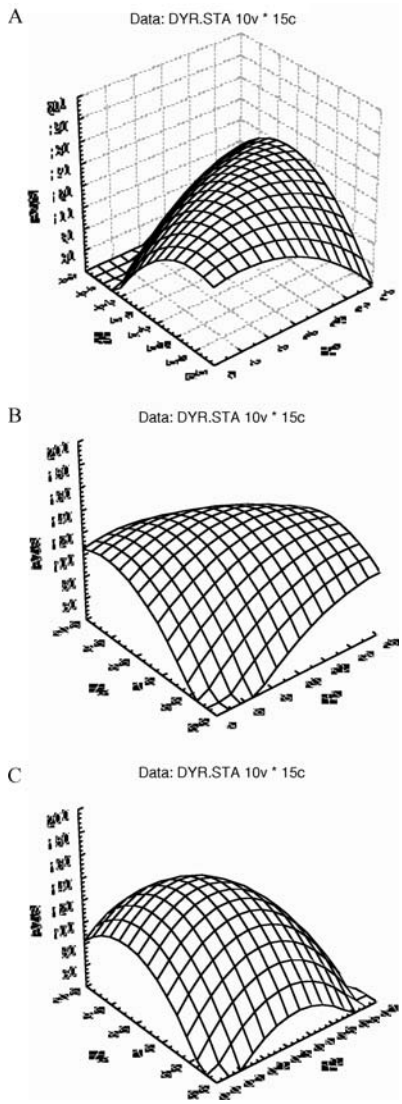

Fig. 1 Predicted response surfaces of the size of PELGE1-DHAQ-NP as a function of the formulation variables (A) X_1 and X_2, $X_3 = 8$, (B) X_1 and X_3, $X_2 = 0.325$, (C) X_2 and X_3, $X_1 = 10$.

Table 2 shows the average experimental results from the joint effects of the amounts of PELGE, the concentration of DHAQ, and the ratio of inner-phase/outer-phase on the mean size and entrapment efficiency ratio. In this study, the particles size ranged from 99.47 to 680.7 nm, but most of them ranged from 99.47 to 188.2 nm, thus very fine control of particles size can be achieved by combining the three independent variables. The drug entrapment percentage ranged from 40.00 to 90.23%. Drug entrapment percentage levels varied by about 50%, this result may have a relation with the concentration of DHAQ and PELGE that used in different levels.

In order to evaluate the predictive power of this model, PELGE-DHAQ-NP was prepared under the optimal conditions. The results comparing the experimentally derived and

Table 3 Comparison of the observed and predicted values of the response variables of the PELGE-DHAQ-NP prepared under the optimum conditions

Response variables	Predicted response	Observed response	Bias (%)
SIZE(nm)	171.61	127	25.99
ER (%)	92.42	90.25	2.35
OD(Overall Desirability)	0.71	0.745	−4.93

*Bias was calculated as (predicted-observed value)/predicted value ×100%.

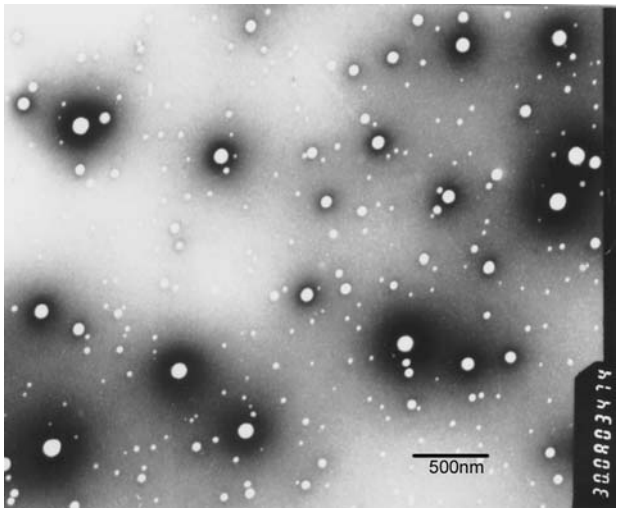

Fig. 2 TEM micrograph of PELGE nanoparticles containing DHAQ.

model predicted values of dependent variables are presented in Table 3. The predicted values for the size and entrapment efficiency of PELGE-DHAQ-NP demonstrated close agreement with the experimental date (Table 3). However, the size of nanoparticles demonstrated a less accurate prediction. These may possibly be due to the complexity of the response surfaces of this variable.

A transmission electron microphotograph of freeze-dried nanoparticles prepared was shown in Fig. 2. The figure shows that the nanoparticles were spherical, discrete particles without aggregation, and smooth in surface morphology, with a diameter of less than 200 nm.

4. Conclusion

In conclusion, this study demonstrates the use of central composite design in the optimized production of PELGE-load nanoparticles. This statistical technique not only allows the possibility of altering more than one independent variable at a time, but also reduces the experimental work load by a considerable extent. A high yield of PELGE-load nanoparticles is inversely related to their small size. Hence, determination of an optimal condition is imperative to obtain PELGE-load nanoparticles with desirable characteristics. Results indicate that all the included variables affect the production of PELGE-DHAQ-NP. It suggested that the method of CCD is simple and accurate. These particles may be used in intravenous administration and drug targeting.

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References

1. K. AVGOUSTAKIS, A. BELETSI and D. S. ITHAKISSIOS, *J. Contr. Rel.* **81** (2002) 123.
2. S. BRANCHU, R. T. FORBES and P. YORK, et al., *Pharm. Res.* **16** (1999) 702.
3. M. FORADADA, M. GALLARDO and J. EATELRICH, *J. Pharm. Biomed. Anal.* **12** (1994) 1495.
4. A. D. MCLEOD, F. C. LAM, P. K. GUPTA and C. T. HUNG, *J. Pharm. Sci.* **77** (1988) 704.
5. J. MOLPECERES, M. GUZMAN and L. BERGES, *J. Pharm. Sci.* **85** (1996) 206.
6. H. L. SHENG and J. S. TU, *J. Chi. Pharm. Univ.* **27** (1996) 211.
7. S. BOLTON, *J. Pharm. Sci.* **72** (1983) 362.
8. B. LU and W. WU, *Acta. Pharm. Sin. (In Chinese)* **34** (1999) 387.
9. T. DE BOER, R. BIJMA and K. ENSING, *Pharm. Biomed. Anal.* **19** (1999) 529.
10. Y. R. DUAN, Z. R. ZHANG and C. Y. WANG, *Key Eng. Mater.* **256** (2004) 887.
11. K. A. ABU-LZZA, L. GARCIA-CONTRERAS and D. R. LU, *J. Pharm. Sci.* **85** (1996) 144.
12. Z. R. ZHANG and J. X. WANG, *Acta. Pharm. Sin. (In Chinese)* **36** (2001) 456.
13. Y. R. DUAN, W. S. LIU and Z. R. ZHANG, *Key Eng. Mater.* **288** (2005) 163.
14. Y. I. JEONG, C. S. CHO and J. W. NAH, et al., *J. Appl. Polym. Sci.* **80** (2001) 2228.
15. R. GREF, Y. MINAMITAKE and R. LANGER, et al., *Science* **263** (1994) 1600.
16. H. FESSI, F. PUISIEUX and S. BENITA, et al., *Int. J. Pharm.* **55** (1989) R1.