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Comparative study on preparative methods of DC-Chol/DOPE liposomes and formulation optimization by determining encapsulation efficiency

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ABSTRACT

Three most commonly used preparative methods, dry-film, reverse phase evaporation and ethanol injection were employed to prepare cationic liposomes composed of DC-Chol and DOPE, respectively. The resulting samples were contrasted through morphology observation, particle size and zeta potential analysis. Sephadex filtration method with high selectivity was developed to determine the encapsulation efficiency of plasmid DNA-loaded cationic vectors, on this basis, cationic liposomes formulation was further optimized by applying Box Behnken design with encapsulation efficiency as evaluation index. The results showed that liposomes prepared by dry-film method were of best quality and stability, moreover, the optimum formulation of cationic liposomes and optimal value of each influencing factors were quantitatively obtained, measured value was highly consistent with predicted results. These findings preliminarily clarified the effect of preparative methods on performance of cationic liposome, as well as formulation factors on encapsulation efficiency, and will provide important methodological reference for further study of liposomes carriers for gene delivery.

1. Introduction

In the last decade, cationic liposomes have been extensively studied and widely applied in gene therapy to transfer exogenous genes into cells. Despite a few drawbacks, such as low transfection efficiency in vivo, the liposome carrier is considered as a promising delivery tool, owing to its relative advantages over viral vectors (Chang et al., 2010; Li and Huang, 2006). In most cases, the most commonly used preparative methods of cationic liposomes include dry-film method, reverse phase evaporation method, ethanol injection method (Huang et al., 2006; Zhang et al., 2010; Maitani et al., 2007), and so on. Although the way to prepare liposomes was reported to have effect on activity of this vector (Tranchant et al., 2004), comparative studies concerning these different preparation methods are quite scarce so far.

For liposome preparation, encapsulation efficiency is most widely employed to evaluate and control its quality. Unlike common liposomes, as gene delivery carrier, cationic liposomes absorb nucleic acid molecules via electrostatic interactions to form bigger cationic complexes, lipoplexes (gene-loaded cationic liposomes), rather than entrap and package "gene drugs" into the lipid compartment or layer (Tranchant et al., 2004; Zuidam et al., 1999). Generally speaking, the amounts of nucleic acid fragments absorbed by liposomes will affect transfection result to a great extent. Meanwhile, being similar to transfection efficiency, encapsulation efficiency of cationic liposomes is dependent on several factors, for examples, the molar ratio of cationic lipid to neutral lipid in formulation, the total lipid contents or concentration in preparation, the cationic lipid/DNA charge ratio of the lipoplex, but there is virtually little information in the literature about researching encapsulation efficiency of cationic liposomes so far as we know.

Response surface methodology (RSM) is a collection of mathematical and statistical technique which quantifies the functional relationship between a number of measured response variables and several explanatory factors, hereby to acquire an optimal response by using a sequence of tests. The main advantage of RSM is to reduce the experimental runs required than would be needed in a full factorial design and it is already widely applied to optimize formulation design in pharmaceutics studies (Hatambeygi et al., 2011). Box Behnken design (BBD) is a popular form of RSM and is more effective than other response surface designs, which is acknowledged as one of the best statistical and analytical models (Zhou et al., 2009; Ghasemi et al., 2011).

In the present study, the cationic liposomes prepared by three different methods was investigated with particle size and zeta potential as assessment index, which composed of DC-Chol and DOPE, the most efficient cationic and neutral lipids in formulating liposome vectors (Farhood et al., 1995; Ciani et al., 2007). Furthermore, the effect of three important factors on encapsulation

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efficiency of cationic liposomes was studied in detail, on the basis of these result, we optimize the formulation to obtain liposome carriers with high encapsulation efficiency.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylethanolamine (DOPE), 3β-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). PureLink™ Genomic DNA Mini Kit was purchased from Invitrogen (USA). Sephadex G-50 was obtained from Shanghai JiXin Biotechnology Co. Ltd. Protamine sulfate was obtained from Shanghai Biochemical Pharmaceutical Co., Ltd. All other chemical reagents were of analytical grade. The pEGFP-C1 plasmid DNA (pDNA, 4.7 kb) was generously provided by Zhengzhou University School of Pharmaceutical Sciences (Zhengzhou, China). pDNA purity was determined by agarose gel electrophoresis and measuring the optical density (OD). The pDNA used in this study has $OD_{260}/OD_{280} > 1.8$ which means the pure and super-coiled form of DNA.

2.2. Preparation of liposomes

Cationic liposomes were prepared by three methods, described as follows.

2.2.1. Dry-film method (DF)

Briefly, cationic and neutral lipids (total lipids were 20 μmol) were dissolved in 5 ml chloroform and the solution was evaporated for about 50 min at 45 °C. After the dried film was formed, N_2 gas was used to remove the residuary solvent. The dried film was hydrated with deionized water. After sufficient hydration, the film was suspended by vortexing. The liposomes were then sonicated.

2.2.2. Reverse phase evaporation method (RPE)

A lipid mixture of DC-Chol/DOPE (20 μmol in total) was dissolved in chloroform, deionized water was added to the lipid solution to form an emulsification automatically, subsequently the organic solvent was dried under vacuum through attaching to a rotary evaporator.

2.2.3. Ethanol injection method (EI)

In brief, lipids (20 μmol in total) were dissolved in 4 ml ethanol, and the ethanol was removed in rotary evaporator leaving behind about 2 ml solution. Next, a constant volume of deionized water was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual solvent.

Finally, the liposome suspension was further disrupted by using ultrasonic probe. Resulting liposomes were sterilized by extruding through a 0.22 μm sterile filter with the final concentration of DC-Chol was 2 mg/ml. Cationic liposomes were prepared the day before the experiment, stored overnight at 4 °C.

2.3. Morphology observation

The morphologies of cationic liposomes were observed using transmission electron microscopy apparatus (JEM-200CX), samples were negatively stained with 1% phosphotungstic acid.

2.4. Particle size and zeta potential measurement

Blank liposomes prepared by different methods were stored at 4 °C for 2 months. Samples were taken on the 0 day, 1, and 2 months for particle size and zeta potential analysis to assess the stability of vesicles. For determination process, each sample was dispersed in deionized water to a final volume of 10 ml, its particle size and zeta potential were analyzed using laser particle analyzer (Malvern Zetasizer 3000HS, Malvern, UK). Volume-weighted Gaussian size distribution was fit to the autocorrelation functions and particle size values were obtained.

Table 1
Independent variables and their levels in coded and physical units.

Level	*A*	*B*	*C*
1	1:2	1.6	1:1
2	1:1	3.2	2:1
3	3:2	6.4	4:1

2.5. Determination of encapsulation efficiency

Sephadex filtration method was developed to separate free pDNA and liposomes, encapsulation efficiency was determined by using an ultraviolet spectrophotometer. Liposomes were diluted with deionized water to a final DC-Chol concentration of 1 μg/μl. Then the diluted liposomes and appropriate amount of pDNA were mixed, and incubated at room temperature for 30 min to form the final lipoplex. Taking 1.2 ml lipoplex into sephadex column, elution was conducted at optimal rate to separate free pDNA and lipids more efficiently with deionized water as eluent. DNA exuded before lipid constituents and was immediately collected, ultraviolet spectrophotometry was used to measure absorbance of DNA. The total and free amount of DNA were signed C_0 and C_1, respectively, encapsulation efficiency (EE) was calculated according to the formula: $EE = (1 - C_1/C_0) \times 100\%$.

2.6. Study and optimum design on formula of cationic liposomes

Box Behnken design was employed to optimize lipid formulation. Three parameters that have significant effect on encapsulation efficiency were selected to investigate, they were the mole ratio of cationic lipid to neutral lipid (DC-Chol/DOPE, *A*), the total lipid contents (mg/ml, *B*), and charge ratio of cationic lipid to pDNA (*C*). Three levels were choosed for each factor, which were cautiously defined on the basis of result of single factor experiment and feasibility of preparing liposome samples at the maximum and minimal levels. Designs of factors and levels were described in Table 1.

2.7. Data processing

Multiple linear regression and binomial fitting were performed for each factor (independent variable), with encapsulation efficiency as the only index (dependent variable). Multiple linear regression formula was: $R1 = b + b_1A + b_2B + b_3C$; binomial fitting formula was: $R1 = b_0 + b_1A + b_2B + b_3C + b_4AB + b_5AC + b_6BC + b_7A^2 + b_8B^2 + b_9C^2$, in which *R*1 was determination value of index, b_0 was intercept, b_1–b_9 were regression coefficients, *A*–*C* are factors investigated. The relationship between each factor and index was fitted by using data processing software Design-Expert trial version7.1.6 (Stat-Ease Inc., Minneapolis), following regression coefficients and constants were calculated, in addition, accuracy of regression formula obtained was evaluated by fitness and correlation coefficient. Response surfaces that exhibit the relationship between each factor and index were drew according to fitting equation. Finally, we selected optimization formula from response surface drawing to produce cationic liposomes and executed predictive analysis.

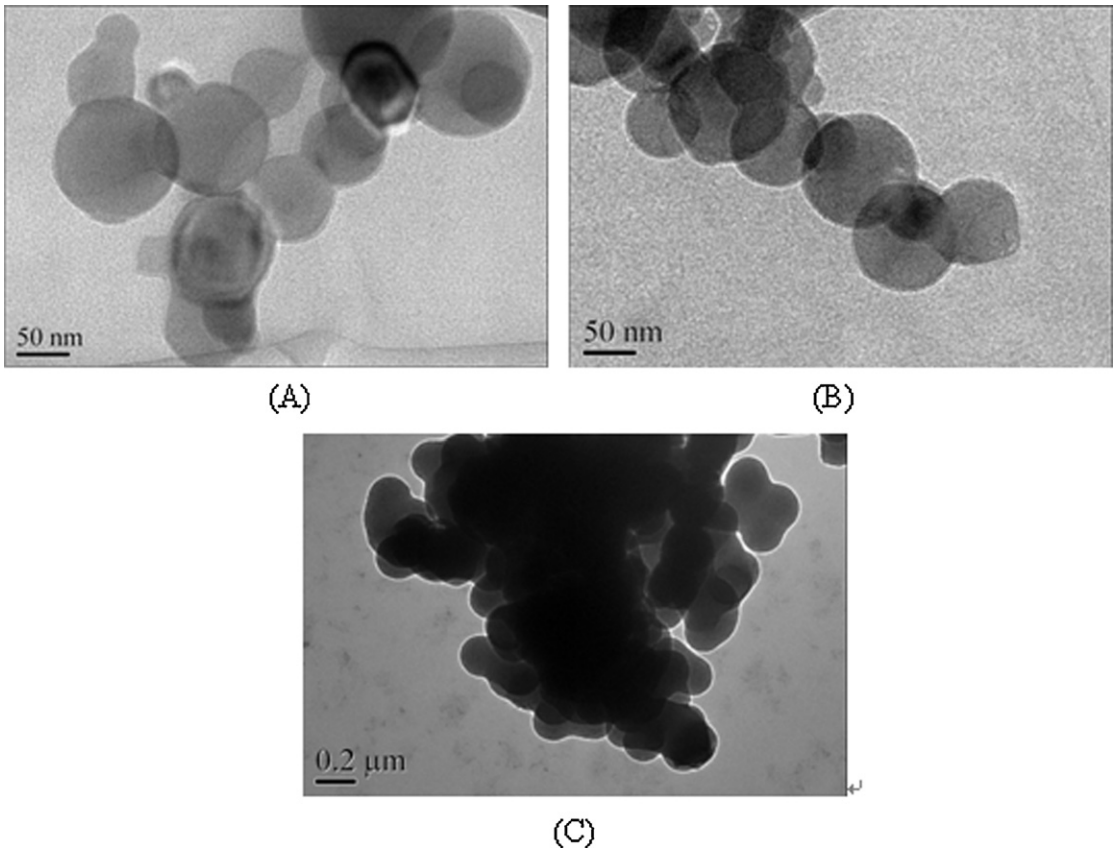

Fig. 1. TEM photographs of cationic liposomes prepared by (A) DF method, (B) RPE method and (C) EI method.

3. Results

3.1. Morphology of cationic liposomes

The morphologies of cationic liposomes were observed by transmission electron microscopy and the observation results were shown in Fig. 1. All the liposomes were spherical particles with integrated bilayers and fingerprint-like surface on the liposomes was distinct. There was no difference in the morphology of the samples prepared by different methods.

3.2. Characterization of liposomes prepared by three methods

As shown in Tables 2 and 3, for particle size analysis results, there is no significant difference between blank liposomes prepared by three methods, but samples produced by DF method showed better stability within 2 months, without the tendency to aggregate and become larger; for zeta potential analysis results, samples prepared by DF method possessed higher potential, as well as were more stable compared with their counterparts prepared by the other two methods. Based on an overall consideration, DF method was selected as the best alternative to prepare cationic liposomes.

Table 2
Results of particle size (nm) analysis for liposomes prepared by three methods ($n = 3$). Data represent average ± standard deviation.

Days	RPE	DF	EI
0	206.4 ± 14.4	172.6 ± 10.1	185.1 ± 5.7
30	252.6 ± 32.6	181.9 ± 7.5	217.4 ± 19.6
60	282.2 ± 27.6	177.9 ± 9.2	226.4 ± 28.3

Table 3
Results of zeta potential (mV) analysis for liposomes prepared by three methods ($n = 3$). Data represent average ± standard deviation.

Days	RPE	DF	EI
0	12.6 ± 3.4	24.8 ± 2.2	15.1 ± 1.7
30	9.4 ± 2.2	22.7 ± 4.5	17.1 ± 6.4
60	7.2 ± 5.0	17.3 ± 7.7	11.2 ± 3.5

3.3. Result of formulation design

According to BBD designs, a total of seventeen tests containing five replicates at the center point were carried out in random order (Table 4). Analysis of variance for the experimental results and quadratic model were shown in Table 5, in which the small p-value for the model (<0.0001) implied the model was significant, p-value less than 0.05 suggested model term was significant. The p-value for the 'lack of fit' test was 0.3055, indicating the quadratic model was adequate.

By statistically processed and fitting, multiple second-order equations were obtained as follows:

Final equation in terms of coded factors:

$$R1 = +83.16 - 3.06A + 1.04B + 10.70C - 2.28AB + 0.15AC - 0.95BC - 13.47A^2 - 7.82B^2 - 5.99C^2$$

Final equation in terms of actual factors:

$$R1 = -32.23083 + 108.69833A + 13.84549B + 21.30556C - 1.89583AB + 0.200000AC - 0.26389BC - 53.87000A^2 - 1.35720B^2 - 2.66333C^2$$

Table 4
Experimental design for three factors and experimental values of objective variables.

Std	Run	Factor 1 *A:A*	Factor 2 *B:B*	Factor 3 *C:C*	Response 1 *R*1 (%)
16	1	1	4	2.5	84.2
1	2	0.5	1.6	2.5	63.5
6	3	1.5	4	1	49.7
12	4	1	6.4	4	82.2
10	5	1	6.4	1	62.60
4	6	1.5	6.4	2.5	55.7
5	7	0.5	4	1	56.4
17	8	1	4	2.5	81.3
7	9	0.5	4	4	77.4
14	10	1	4	2.5	85.2
13	11	1	4	2.5	79.7
11	12	1	1.6	4	78
15	13	1	4	2.5	85.4
9	14	1	1.6	1	54.6
3	15	0.5	6.4	2.5	66.1
8	16	1.5	4	4	71.3
2	17	1.5	1.6	2.5	62.2

The analysis of fitting result was shown in Table 6.

The above regression equations quantitatively described the effects of three independent variables (*A*–*C*) on index and their correlation. The adjusted R^2 for the predictive model was 0.9438, as well as the statistical test results of equation parameters (Table 6), revealed that the experimental results adequately fitted the equation selected. It can be predicted to obtain response value of a random formula within the range of designed factor and level by regression equations.

3.4. Effect of formulation ingredients on encapsulation efficiency

To better comprehend the predicted models of the results, three-dimensional graphs were mapped by plotting the response versus two of the factors while the third was fixed at its central level, the response surface diagrams of encapsulation efficiency of cationic liposomes were shown in Fig. 2.

As shown in Fig. 2A, with an increase of mole ratio of cationic lipid to neutral lipid, encapsulation efficiency ascended in first and descended at last, the similar trend was observed as the total lipid contents increased, but the latter factor did not exhibit significant effect. Fig. 2B demonstrated that with increasing of molar ratio of DC-Chol to DOPE, encapsulation efficiency showed an earlier raised and later decreased state just like in Fig. 2A, while it continually increased as charge ratio of cationic lipid to DNA rose, and these two factors all exhibited significant effect. Finally, Fig. 2C indicated that encapsulation efficiency experienced an alternate increase and decrease again under the increasing total lipid contents, differently, this index showed the tendency of going up steadily as the third parameter increased, the former had significant effect but not the latter. At last, three was no significant influence was observed for mutual interaction between any two factors on encapsulation efficiency.

Table 6
The result of fitting second-order equations.

Std. Dev.	2.89	*R*-squared	0.9754
Mean	70.32	Adj *R*-squared	0.9438
C.V. %	4.10	Pred *R*-squared	0.7633
PRESS	561.30	Adeq precision	15.082

Table 7
Optimized values obtained by constraints applied on *R*1.

Variable	Nominal values	Response	Expected values	Observed values
A	0.89	*R*1	87.94	89.94 ± 3.64%
B	4.08			
C	3.80			

3.5. Formulation optimization of cationic liposomes

To obtain the liposomes carriers with high encapsulation efficiency, on the basis of regression equations and response surface models, the optimum formulation of preparing liposomes were defined: DC-Chol/DOPE = 0.89, total lipid contents = 4.08 mg/ml, and DC-Chol/pDNA = 3.80. Under the conditions optimized above, predictive value and actually measured value of index were shown in Table 7.

It can be concluded that actually measured value was quite close to predictive value from result of verification test (Table 7).

4. Discussion

Particle size and zeta potential are generally considered as most important parameters in liposomes quality evaluation, especially for cationic liposomes used for gene delivery (Ma et al., 2007; Ramezani et al., 2009). Stable particle size indicated liposomes did not show to aggregate and cluster swimmingly during the use or deposit, thus they could carry pDNA to enter the cells more effectively via clathrin-mediated endocytosis (Zuhorn et al., 2002), leading to more efficient transfection and the following transcription in cellular nucleus. On the other hand, cationic carriers with high zeta potential were more positively charged, which were capable of absorbing and binding to pDNA better, furthermore, more positive charges were likely left on lipid vesicles after electrostatic interaction with nucleic acids, allowing the forming lipoplexes to be internalizated more easily by fusion with cell membrane (Ewert

Table 5
Statistic analysis of variance for the experimental results.

Source	Sum of squares	df	Mean square	*F* value	*p*-Value Prob > *F*	
Model	2312.63	9	256.96	30.87	<0.0001	Significant
A–A	75.03	1	75.03	9.01	0.0199	
B–B	8.61	1	8.61	1.03	0.3429	
C–C	915.92	1	915.92	110.04	<0.0001	
AB	20.70	1	20.70	2.49	0.1588	
AC	0.090	1	0.090	0.011	0.9201	
BC	3.61	1	3.61	0.43	0.5312	
A^2	763.68	1	763.68	91.75	<0.0001	
B^2	257.32	1	257.32	30.91	0.0009	
C^2	151.20	1	151.20	18.17	0.0037	
Residual	58.26	7	8.32			
Lack of fit	32.57	3	10.86	1.69	0.3055	Not significant
Pure error	25.69	4	6.42			
Cor total	2370.89	16				

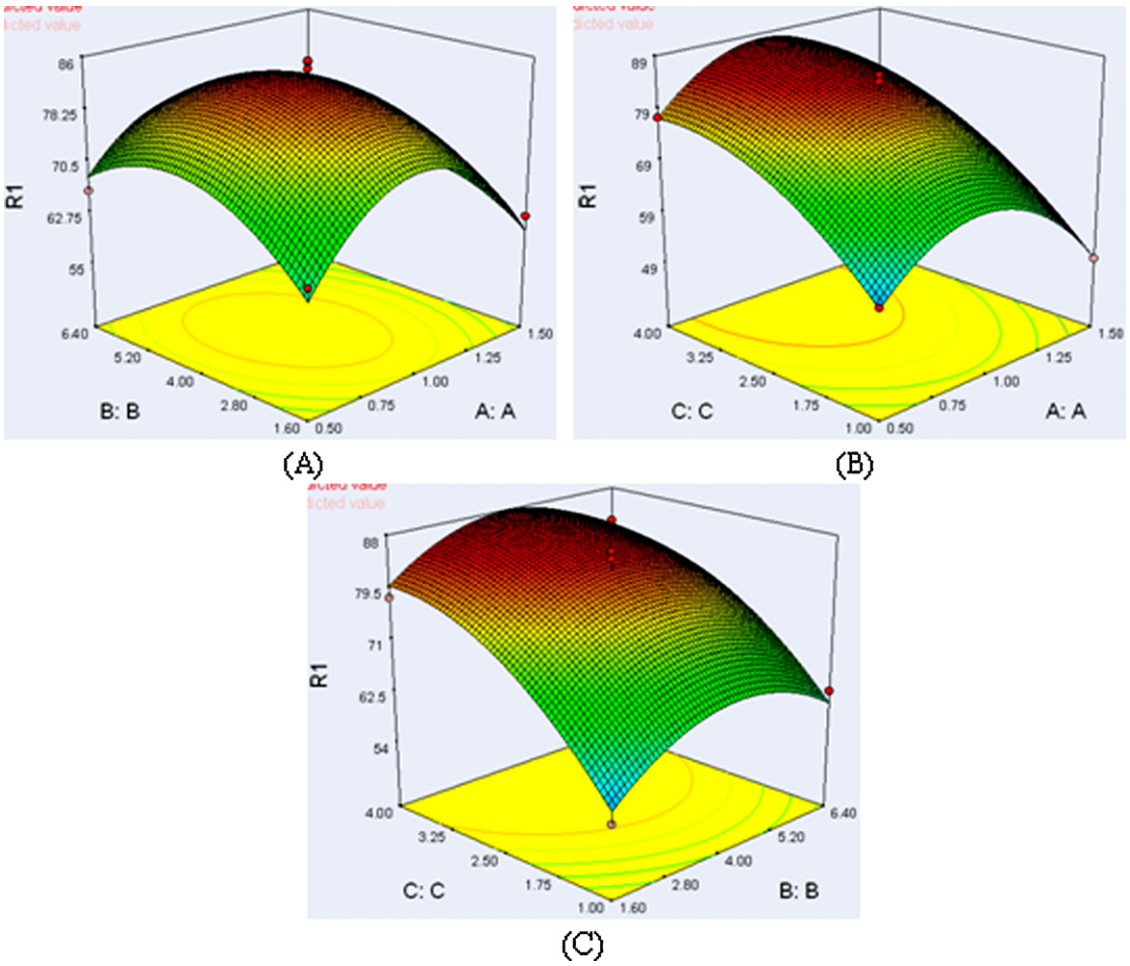

Fig. 2. Response surface plot showing the effect of (A) mole ratio of DC-Chol/DOPE and total lipid contents on the response of transfection efficiency while keeping the charge ratio of DC-Chol/pDNA. (B) Mole ratio of DC-Chol/DOPE and the charge ratio of DC-Chol/pDNA on the response of transfection efficiency while keeping the total lipid contents. (C) Total lipid contents and the charge ratio of DC-Chol/pDNA on the response of transfection efficiency while keeping the mole ratio of DC-Chol/DOPE.

et al., 2006). For the preparative methods selection, although as many as a dozen methods have been used to prepare cationic liposomes in recent research, most of them, such as extrusion method, freeze thawing method, etc., were less frequently employed in report or only used as supplement and substitution for the three methods investigated in the present study. It was suggested that cationic vectors prepared by DF method had more advantages over ones produced by the other two from the results of particle size and zeta potential analysis.

Encapsulation efficiency is one of the most important evaluation parameters for liposomes carriers. Due to the specificity of ways with that liposomes and "gene drugs" integrate (by electrostatic interactions automatically occurred on the surface of both sides but not commonly package directly), some researchers considered that "incorporation efficiency" or "carrying efficiency" was the better and more appropriate term to substitute "encapsulation efficiency" (Shi et al., 2005). Given the features of nucleic acid molecules, such as the high molecular weight, charged surface, being prone to be destructed by organic solvents, most of the encapsulation efficiency measurement methods including high speed centrifugation, equilibrium dialysis, protamine aggregation, cannot be used to separate nucleic acids and lipid carriers effectively or difficult to achieve this goal. Through large experimental validation and comparison with other methods, sephadex filtration method was selected to separate pDNA and gene-loaded cationic liposomes, which could determine encapsulation efficiency accurately.

Indeed, the three factors selected in this study were interrelated and interacted with each other. First, high total lipid contents used in preparation would result in more lipid carrier formation. Next, higher DC-Chol ratio in lipid formulation made liposomes possessed strong electrostatic attraction to interact with pDNA and target cells, whereas increasing DOPE proportion could aid to an inverted hexagonal phase transition from lamellar structure and therefore destabilize the liposome membrane, being favorable for fusion with cell membrane (Wasungu and Hoekstra, 2006), however, excessive DOPE composition would likely shield positive charges provided by cationic lipid, as a consequence, the binding activity of adhering pDNA and cells was weaken. Moreover, adding amounts of pDNA within a certain range while producing lipoplexes could effectively improve encapsulation efficiency, but the positively charged liposome surface would be inevitably neutralized by overmuch electronegative nucleic acid. Consequently, in order to comprehensively evaluate and elucidate the effect of the three factors, Box Behnken design was applied to formulation design and screening, at last, the optimum formulation of cationic liposomes encapsulating pDNA most efficiently was quantitatively obtained.

It was reported in recent literature that the best molar ratio of DC-Chol/DOPE liposomes was 1:2 or 3:2 (Farhood et al., 1994; Colosimo et al., 1999; Mukherjee et al., 2005), as well as the modified ethanol injection method was suggested to be better option, while transfection efficacy was used as evaluation index in these studies (Maitani et al., 2007). There are some differences between these results and our study, which indicated that encapsulation

efficiency correlated with transfection activity much intricately rather than linearly linked, therefore, it should be noted that the screening result could not be simply applied to gene transfer research. Despite its preliminary character, this study clearly showed the interrelations of formulation factors and encapsulation efficiency, and the application of response surface optimization would be contributive to in-depth research for cationic lipid vectors.

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