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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.