# PREPARATION OF LIPOSOME PRECURSORS BY SUPERCRITICAL ANTI-SOLVENT TECHNIQUES

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**Abstract** The possibility of preparation of hydrogenated phosphatidylcholine (HPC) liposome precursors (LP) using supercritical anti-solvent technology (SAS) was evaluated. Vitamin D (VD) and coenzyme Q10 (CoQ10) were chosen as the model drugs, and HPC was used as the wall material. The influences of operation conditions (pressures, concentrations and etc.) on the HPC were studied. The structure of the LP was characterized by TEM. The size of the HPC and LP were also analyzed. The results show SAS can be as a simple and effective method to prepare LP. The product is in a dry form, which may solve the stability-associated problems.

**Keywords**: supercritical anti-solvent; liposome precursor; hydrogenated phosphatidylcholine.

## 1. INTRODUCTION

In recent years, liposomes have strongly influenced the development of drug and gene delivery to diagnostics, cosmetics, long-lasting immunocontraception [1, 2] and the food industry [3]. Many methods have been developed for the preparation of liposomes, including the Bangham method, the organic solvent injection method, and the reverse phase evaporation method. However, these techniques require large amounts of organic solvent, which are harmful both to the environment and to human health [4-7]. What is more, all of the current methods consist of many steps which limit their application in the mass production of liposomes.

Supercritical fluid (SCF) technology, which has many advantages such as mild operation temperature to avoid thermal degradation, obtaining production in a single step, and low residual solvent, has recently gained great attention as a new method of preparing liposomes. A method called supercritical reverse phase evaporation (SCRPE) method [8-13] has been developed for generating liposomes, in which $CO_2$ is used as a solvent. But the product of this method is suspension, which is inconvenient in transportation and storage than in the dry and reconstitutable form. What is more, the existence of a certain amount of ethanol is known to prevent liposome formation. Thus another method named aerosol solvent extraction system (ASES) which uses SC-$CO_2$ as an anti-solvent has been developed in the preparation of liposomes [14-16]. The direct production in a dry form can eliminate stability-associated problems which are encountered in the traditional aqueous dispersion of liposome preparation. Actually, the production in a dry form is not the real liposome we want, and it needs to be hydrated simply to form liposomal vesicles. It can be called liposome precursor (LP).

In this work, we tried to prepare the LP using supercritical anti-solvent technology (SAS). The hydrogenated phosphatidylchline (HPC), which has the similar properties as

phosphatidylcholine (PC) used by Sarinnate Kunastitchai [16], but is more stable than PC, was chosen as the wall material. VD and CoQ10 were chosen as model drugs. The influences of operation conditions on the HPC were studied. The processing conditions and the structure of LP were also examined.

## 2 MATERIALS AND METHODS

### 2.1 Materials

The hydrogenated phosphatidylcholine (99.9 % purity, average molecular weight of 790 g/mol) was obtained from Toshisun Enterprise Co., Ltd (Shanghai, China).VD(40MIU/g) was obtained from Shanghai Beirum Pharm-Chem Co., Ltd (Shanghai, China). CoQ10 (99.9 % purity, average molecular weight of 863.34g/mol) was obtained from Shanghai Kayon Biological Technology Co., Ltd (Shanghai, China). $CO_2$ with the purity of 99.95% was obtained from SJTU Gas Station (Shanghai, China). Ethanol, n-hexane and methylene chloride were purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd (Shanghai China), and they are all analytical reagents.

### 2.2 Precipitaition apparatus and procedure

The schematic diagram of the experimental apparatus used in this work is shown in Fig. 1. The experiment was performed by the following procedure. Liquid $CO_2$ was pumped with a high-pressure pump after being cooled by a refrigerator. The $CO_2$ was introduced into the precipitation vessel and heated to the desired temperature by the air bath. At the beginning of the experiment, pure $CO_2$ was introduced to get the experimental pressure. During this time, the valve A was opened while the valves of B and C were closed. After the experimental pressure was obtained, the valve C was opened to maintain a constant pressure in the vessel. The pressure in the vessel can be measured by the pressure gauge. When the pressure was constant, the valve B was then opened, and the solution pumped by the HPLC pump was sprayed through a capillary. Once the solution was finished, the valve of A and B were closed and the valve C was opened in order to depressurize the vessel at the operating temperature. The samples were collected on the filter at the bottom of the precipitator.

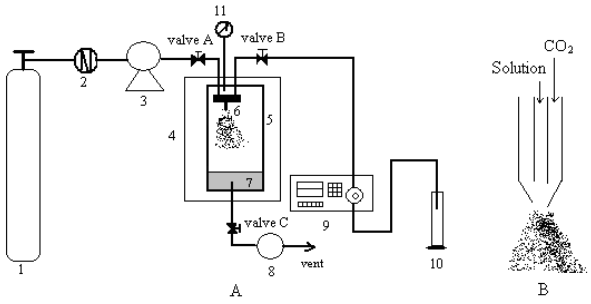



**Fig.1 A: The flow chart of SAS experiment: (1) $CO_2$ cylinder; (2) refrigerator; (3) pump; (4) air bath; (5)view vessel; (6) nozzle; (7) filter; (8)wet gas meter; (9)HPLC pump; (10) graduated flask; (11)pressure gauge. B: The sketch map of nozzle.**

### 2.3 Study on HPC and LP processing conditions

Before preparing LP, we must make sure that a certain amount of HPC, which was the basic component of LP, could be obtained by the SAS. So the HPC processing conditions were studied at first. Regarding the phase transition temperature of HPC (55℃), the

temperature was chosen at 45℃ near the value. The critical processing conditions to be studied were pressures in order to adjust the precipitation properties for the LP production. The pressure was varied from 10MPa to 19MPa.

The flow rate of solution was at 0.5ml/min, and $CO_2$ flow rate was at 6kg/h, since these conditions were demonstrated to have no direct effect on the properties of productions [16]. Varying concentrations of solution containing HPC in the solvent mixture of n-hexane and ethanol (2：1,v/v) were 40mg/ml and 10mg/ml. The effect of solute compositions containing HPC and VD on the drug content of LP was examined. Varying concentrations of such mixtures were 10%, 50%, 200% (VD：HPC, w/w).

### 2.4 Characterization

#### 2.4.1 Yield

The yield was determined by weighing the product recovered in the precipitation vessel and calculating the percentage of yield with respect to the initial amount which had been added into the SAS processing system.

#### 2.4.2 Particle size and size distribution

The particle size and size distribution of the products were measured by Laser diffraction spectrometry (Nanosizer series 2000, Malvern, Britain). The dried powder samples were suspended in deionised water and sonicated for 10s before measurement. Each sample was analyzed three times.

#### 2.4.3 Drug content in LP

The drug content was analyzed by Ultraviolet spectrophotometry detector (765PC, Shanghai Spectrum Instruments, Shanghai, China). The maximum absorbance of VD is at 265nm, and the maximum absorbance of CoQ10 is at 276nm. The amount of drug was determined based on the standard curve of an external standard. All samples were analyzed in triplicate.

#### 2.4.4 TEM

The structure of the encapsulated nanoparticles was examined by Analytical Transmission Electron Microscope (TEM, JEM-2010, Jeol, Japan). The dried powder samples were suspended in deionised water and sonicated for 30s. Then the suspension was dripped in copper mesh with carbon film and dried before TEM observation.

## 3 RESULTS

### 3.1 Study on HPC processing conditions

Because the product should be at an acceptable yield, the yield could be one of the parameters for researching the processing conditions. As shown in table 1(experiment 2~5), a pressure increased from 10 to 19MPa caused an increase in the yield. That was because $CO_2$ acted as an anti-solvent, the higher pressure made the HPC precipitate from the system more easily. In other words, more $CO_2$ decreased the dissolving power of solvent so that more HPC precipitated from the system as pressure increased.

Another parameter for reaching the processing conditions is particle size. The smaller the particle size of HPC is, the larger the specific surface area is. In other words, the smaller HPC

has the better absorbability, which can adsorb more drugs when preparing LP. So the smaller HPC would make more drugs be encapsulated possibly. As shown in table 1, from experiment 1 to 5, the particle sizes were all smaller after SAS processing, and as the pressure increased, the particle size increased. But the effect of pressure on particle size was not so obviously. Only when the pressure was at 10MPa, the particle size decreased greatly; when pressure was above 13MPa, the particle size decreased a little as the pressure increased.

We also investigated the effect of concentration on the yield and particle size roughly. As shown in experiment 5 and 6, when the concentration decreased, the yield and the particle size all decreased. This could be explained by the supersaturation degree. When the concentration increased, the superaturation degree increased during the SAS process so that it was more easily for HPC to precipitate. The more easily for HPC to precipitate, the more HPC could be obtained by SAS. But the more HPC precipitated, the more easily for HPC to conglomerate, which led to larger particle size.

**Table 1 Optimum experiment**

| Experiment | C(mg/ml) | P(MPa) | Yield (%) | Particle Size (nm) |
|---|---|---|---|---|
| 1 | | raw material | | 739.5 |
| 2 | 40 | 10 | 18.50 | 264.5 |
| 3 | 40 | 13 | 43.81 | 471.5 |
| 4 | 40 | 16 | 51.34 | 478.1 |
| 5 | 40 | 19 | 56.39 | 536.6 |
| 6 | 10 | 19 | 44.80 | 411.5 |

### 3. 2 Study on LP processing conditions

#### 3.2.1 The effect of HPC and VD compositions on the drug content

Considering the VD was more soluble at higher pressure [17], and the yield and particle size were important indexes, 13MPa was chosen as the processing condition for preparing LP. As shown in table 2 and figure 1, the effect of solution compositions on the LP obtained at 13MPa and 45℃ was examined. From the table 2, the recovery efficiency of VD increased as the ratio of HPC to VD increased. This may be due to more HPC was helpful for the precipitation of VD. The particle size of LP increased with the increase of concentration of HPC in the solute mixture, which was consistent with the trend of the HPC (table 1). That is to say, the particle size of LP was influenced mainly by the particle size of HPC. What is more, the content of VD increased with the increasing concentration of VD in solution as expected. Considering the recovery efficiency of VD, the ratio of HPC to VD should be chosen at 10%.

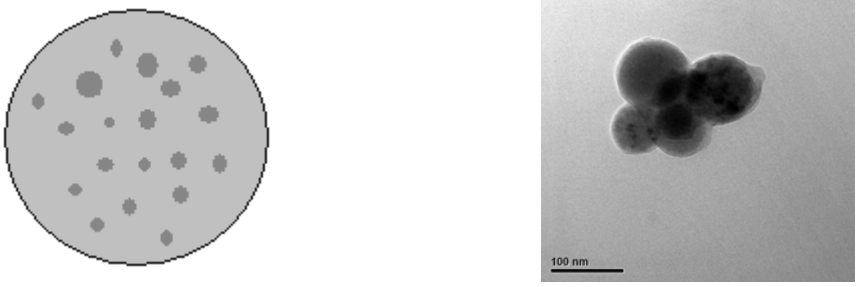
**Table 2 Effect of solution compositions**

| Experiment | VD/HPC (weight/weight, %) | Particle Size (nm) | Content of VD (%) | Recovery Efficiency of VD (%) |
|---|---|---|---|---|
| 1 | 10 | 632.4 | 10.76 | 53.98 |
| 2 | 50 | 492.1 | 11.31 | 9.12 |
| 3 | 200 | 432.4 | 24.90 | 5.60 |

#### 3.2.2 The structure of LP

As shown in figure 2, the structure of LP (VD/HPC=10%) was examined by TEM. It is

clear that the VD nanoparticles are coated with HPC. The VD in the LP distributed as matrix (Fig.2 (a)), which was not mentioned by Sarinnate Kunastitchai. The coated primary particle size is estimated to be about 100 nm from the scale bar, and the LP agglomerated obviously (Fig.2 (b)). The particle size was much smaller than that examined by Laser diffraction spectrometry. That may be because when LP was examined by Laser diffraction spectrometry, the microparticles were suspended in the water causing the agglomeration more seriously.




(a) ( b )

**Fig. 2 The structure of LP: (a) Matrix structure; (b) TEM micrographs of LP**

### 3.3 The preparation of CoQ10 LP

The CoQ10 LP was prepared at 13MPa with the ratio of CoQ10 to HPC at 10%. Then the content of CoQ10 was examined by UV at 276nm. The content of CoQ10 was 3.78%, which means the CoQ10 could be encapsulated by HPC possibly. However, there are still a lot of researches such as pressures, concentrations, characterizations and so on should be done in the future.

## 4 CONCLUTIONS

The LP could be prepared by SAS in the dry form, which will make the application of liposomes more convenient. The conditions of preparing LP of HPC encapsulating VD were at 13MPa, 45 . The recovery efficiency of VD increased with the increasing ratio of HPC to VD. The content of VD was found to increase with the increase of VD concentration in the solution as expected. However, the recovery of VD decreased with the increasing of VD. The VD in the LP distributed as matrix and the LP agglomerated. The LP of HPC encapsulating CoQ10 also could be prepared by SAS.

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