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Review

Progress involving new techniques for liposome preparation



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ABSTRACT

The article presents a review of new techniques being used for the preparation of liposomes. A total of 28 publications were examined. In addition to the theories, characteristics and problems associated with traditional methods, the advantages and drawbacks of the latest techniques were reviewed. In the light of developments in many relevant areas, a variety of new techniques are being used for liposome preparation and each of these new technique has particular advantages over conventional preparation methods. However, there are still some problems associated with these new techniques that could hinder their applications and further improvements are needed. Generally speaking, due to the introduction of these latest techniques, liposome preparation is now an improved procedure. These applications promote not only advances in liposome research but also the methods for their production on an industrial scale.

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1. Introduction

Liposomes are enclosed vesicles formed by lipid materials, such as phospholipids, dispersed in an aqueous medium. One or more bilayers are formed, which have a similar structure to

the cell membrane, separating the inner water phase from the outer [1].

Because of their special structure, liposomes have some excellent advantages when used in drug delivery systems. First of all, the enclosed vesicles can separate the inner phase from the outside one and, thus, improve the stability of the

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encapsulated drug. Secondly, when loaded into liposomes, the poorly water-soluble drug exhibits enhanced bioavailability. In addition, a sustained or controlled drug release profile can be achieved after encapsulation. Also, liposomes possess a good cell affinity with excellent biodegradability. Furthermore, the target effect of liposomal preparations can change the *in vivo* distribution of the loaded drug, improving the therapeutic index of certain drugs [2–5]. Also, many ligands can be modified on the surface of the liposomes and these modifications can alter the characteristics and biological behavior of the final product.

Liposomes, with their special characteristics such as modifiable morphology, particle size, Zeta potential, encapsulation efficiency, and drug release profile can enhance the properties of certain drugs. For instance, liposomes with a particle size under 120 nm can produce better tumor accumulation [6]. Moreover, the encapsulation efficiency and stability of liposomes should be as high as possible to produce high drug concentrations as well as preventing drug leakage. Today, a great variety of liposome preparation methods are available and each of them has particular features and special advantages. If suitable methods are selected and corresponding new techniques are used, new liposomal formulations can be prepared easily and efficiently. With the recent advances, more and more novel technologies have been applied to the manufacture the wonderful vesicles in recent years. However, liposome researchers sometimes have a problem when faced with the huge variety of new technologies. In the light of this, we have produced a systematic review of the new technologies to help in the selection of suitable preparation methods to meet different needs.

2. Conventional methods of liposome preparation

Since the pioneering discovery of Bangham, a variety of methods have been reported in the literatures for liposome preparation including thin-film hydration or the Bangham method [7], reversed phase evaporation [8], solvent-injection techniques [9], and detergent dialysis [10]. These are the most commonly used ones. Some techniques have been employed to help reduce the size of vesicles, for instance, sonication [11], high pressure extrusion [12] and microfluidization [13].

Unfortunately, those conventional preparation methods have a number of problems which can be classified into the following four categories: (1) The particle size of liposomes is too large or has a broad distribution so there is a need for post-processing granulation. (2) The organic solvent remaining in the final product is also a serious issue since it not only affects the stability of some protein or polypeptide drugs, but also adversely affects clinical treatment. (3) Since many lipids are sensitive to temperature, the sterilization of liposomal preparations can be a problem. So there is a preference for preparation processes which can be carried out in an ultraclean environment. However, conventional methods do not always fulfill this requirement. (4) In some procedures careful monitoring is needed and this subjective technique might influence reproducibility.

To solve these problems, many novel preparation technologies have been applied for the preparation of liposomes.

3. Novel technologies for liposome preparation

Nowadays, supercritical fluid technology, dual asymmetric centrifugation, membrane contactor technology, cross-flow filtration technology and freeze drying technology have been employed for liposome preparation.

3.1. Liposome preparation methods based on supercritical fluid technology

Supercritical fluids are non-condensable fluids, which are very dense at certain temperatures and pressures beyond the critical values. As the line between the liquid and gas phase disappears, supercritical fluids have many particular characteristics compared with conventional fluids. Among these characteristics, solvents with special properties have attracted a great deal of interest from researchers. Remarkably, supercritical carbon dioxide (scCO₂) is an excellent organic solvent substitute. In spite of its low cost, it is non-toxic and is not inflammable. In addition, it has a relatively low critical temperature and pressure (31 °C and 73.8 bar) with the dissolution properties analogous to those of nonpolar solvents [14].

3.1.1. Supercritical anti-solvent (SAS) method

Regarding classical thin-film dispersion methods, lipids are always dispersed on the inner surface of the glass flask to form a lipophilic thin film. Similarly, the supercritical anti-solvent (SAS) method is being used to achieve a fine and homogenous dispersion of lipid materials. Briefly, in the SAS method, lipids dissolve readily in scCO₂ and then precipitate in the form of ultrafine particles. The experimental procedure for SAS is shown in Fig. 1A.

Phospholipid and cholesterol is dissolved in an organic solution and placed in a glass container which, together with a source of CO₂ gas, is connected to pumps linked to a precipitation vessel. Gaseous CO₂ is pumped into a high-pressure precipitation vessel by spraying through capillary tubes and then transformed into a supercritical phase because of the sudden change in temperature and pressure. Subsequently, the lipids are extracted into the supercritical phase as soon as the evaporation of the organic solvent is completely introduced, which lead to supersaturation of the solute in the scCO₂ phase and then the lipid materials precipitate. Afterwards, the organic solvent is removed by CO₂ continuously pumped into the vessel to produce fine lipid particles. Finally, liposomes are obtained by directly adding aqueous phase [15].

Data obtained show that SAS liposomes have no significant differences compared with conventional Bangham liposomes in terms of the particle size, encapsulation efficiency and stability [15]. Nonetheless, organic solvent can be removed completely using SAS, which is of great value.

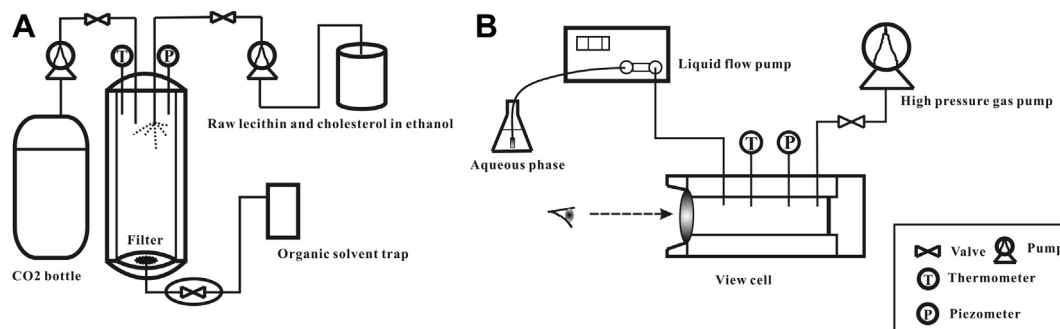


Fig. 1 – Experimental set-up for supercritical fluid technology-based liposome preparation methods: (A) the Supercritical anti-solvent (SAS) method and (B) the Supercritical reverse phase evaporation (SPER) method.

3.1.2. *Supercritical reverse phase evaporation (SPER) method*
In the conventional reverse phase evaporation method, organic solvents are usually used to dissolve lipid materials. Because of the dissolution properties of scCO_2 , the supercritical fluid might be an excellent substitute for organic solvents.

In 2001, Otake and his colleagues [16] first reported that liposomes can be prepared by a supercritical reverse phase evaporation method using scCO_2 as the solvent for lipids. The apparatus used is shown in the Fig. 1B.

Briefly, the apparatus consists of three parts: a viewing cell with a variable volume, an HPLC pump for feeding aqueous solution into the viewing cell and a high-pressure pump for CO_2 and pressure control by moving the piston in the viewing cell. The ethanol solution of lipid materials is measured with an electronic balance and fed into the cell. After the lipid materials are placed in the viewing cell the cell is sealed, and a magnetic tip is used for stirring inside the viewing cell and gaseous CO_2 is introduced into the cell. The temperature is then raised to a chosen value, which could reach both the phase transition temperature of the phospholipids and the supercritical temperature of carbon dioxide. The pressure is also kept above the supercritical value. After several seconds to reach equilibration, an aqueous solution of the model drug is slowly introduced into the cell through the HPLC pump, until a sufficient amount of solution is reached. Finally, the pressure is reduced to release CO_2 and a homogeneous liposomal dispersion is formed.

Large unilamellar liposomes with ellipsoidal apsidal of 0.1–1.2 μm have been reported. There is also an increasing tendency for the shape of the liposomes to become spherical with decreasing liposome size [16]. It has been shown that the SRPE method allows efficient preparation of large unilamellar liposomes with a membrane structure that is quite similar to that of biological cells such as erythrocytes. The SRPE method has a particular advantage in that only a small amount of an organic solvent, like ethanol, can be used.

In 2006, Otake et al. developed the SRPE method in which they put an aqueous phase together with the solid lipid materials into a sealed viewing cell. The temperature and pressure were adjusted to suitable values and then the CO_2 was introduced. After equilibration, CO_2 escapes and liposomes are formed [17]. The liposomes prepared by the improved supercritical reverse phase evaporation (ISRPE) method have an enhanced entrapment compared with those obtained by

SRPE. Also, the process is shorter because of a reduction in the apparatus required. In addition, the ISRPE method does not require any organic solvents and thus completely avoids any problems caused by organic residues which is very significant. However, whether the former or the latter procedure is used, liposomes prepared by SRPE do not have a narrow particle size distribution. So post-procedures are needed to optimize this excellent method.

3.2. Dual asymmetric centrifugation (DAC)

Dual asymmetric centrifugation (DAC) is a special kind of centrifugation. Vials in DAC turn around the main rotation axis at a defined distance and speed, which is similar to that used in conventional centrifugation. However, the main difference between DAC and normal centrifugation is that the vials in the DAC process also turn around their own center (vertical axis) while they still turn around the center of the centrifuge as in the normal centrifugation process. In other words, the vial make two movements in the DAC process. Interestingly, this results in two overlaying movements of the sample material in the centrifugation vial. The main rotation pushes the sample material in an outward direction in response to centrifugal forces, while the rotation of the centrifugation vial around its own center pushes the sample material in the opposite direction due to adhesion between the sample material and the rotating vial. If there is sufficient adhesion of the sample material to the vial material and the sample material is viscous enough, the latter movement, the inward transport of the sample material, is effective. Both of these factors may significantly affect the transference of energy into the sample material [18]. Therefore, DAC is particularly appropriate for the homogenization of viscous materials.

Vesicular phospholipid gel (VPG) is a highly concentrated lipid dispersion system, which can be diluted by a suitable aqueous phase and form liposomes [19]. Since lipids are usually viscous materials, and VPG is sufficiently viscous. Hence, DAC can be used for the preparation of VPG and liposomes (Fig. 2).

Hirsch et al. [18] have carried out the homogenization in a dual asymmetric centrifuge in multiples of 5-min runs (since 5 min is the maximum runtime of the DAC that can be selected, the instrument is immediately restarted after every 5-min run until the total mixing time is reached) directly after

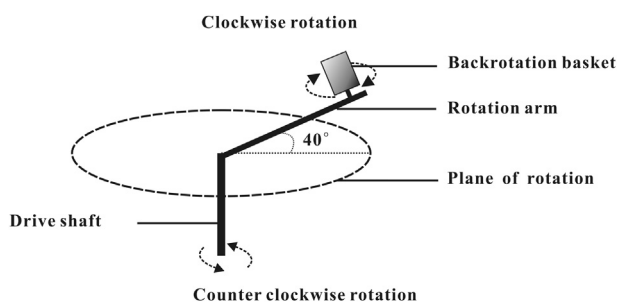


Fig. 2 – Schematic representation of the principle of dual asymmetric centrifuging.

weighing the constituents, namely lipids and 0.9% sodium chloride solution. After the DAC process is finished, the VPGs are redispersed in a double volume of 0.9% NaCl solution to produce a liposomal dispersion. In this way, conventional and stealth liposomal preparations can be made and their mean particle sizes were 70–100 nm and 100–120 nm, respectively. A model drug, calcein, and siRNA had encapsulation efficiencies of 60–80% and 43–71%, respectively [20].

Compared with conventional methods of liposome preparation, the DAC method has several significant advantages: (1) The DAC equipment is small in size, easy to operate and offers good reproducibility. (2) Using the DAC method, liposomes with a small particle size can be produced directly without requiring other processes for granulation or homogenization. (3) Water-soluble drugs have a high entrapment efficiency. (4) No organic solvents are used in DAC for the dispersing lipid, which is of particular significance for liposomal injections. However, there are some drawbacks associated with DAC. For instance, the formulations usually need to contain a high amount of phospholipids to obtain a sufficient viscosity. In order to take full advantages of DAC and its further applications, there is a need to overcome the aforementioned flaws by formulation optimization and method improvement or integration.

3.3. Liposome preparation methods based on membrane contactor technology

Membrane contactors can be used to mix two materials efficiently, and many kinds of dispersion systems can be

prepared using a membrane contactor. It has been reported that membrane contactors have already been applied to the preparation of emulsions, precipitates, polymeric and lipidic nanoparticles and, most recently, for the preparation of liposomes. Since homogenization of lipid and water phases is a key procedure in liposome preparation, this apparatus can be applied for preparing liposome. Based on the theoretical advantage of the ethanol injection method for liposomes, Laouini and co-workers [21] combined a membrane contactor with a hollow fiber module. This combination could increase the membrane area and thus offers improved efficiency.

As can be seen in Fig. 3, the aqueous phase is poured into the module by the action of a pump, while the organic phase is placed in the pressurized vessel. The nitrogen in the bottle can push the oil phase into the system. Then, the connecting valve to the nitrogen bottle is opened and the gas pressure is set at a fixed level. The aqueous phase is subsequently pumped through the membrane contactor module. When water reaches the inlet of the hollow fiber module, the valve connecting the pressurized vessel to the filtrate side of the membrane device is opened so that the organic phase can permeate through the pores of the hollow fibers. When the aqueous and lipid phases meet, liposomes are formed spontaneously. The experiment is stopped when air bubbles start to appear in the tube joining the pressurized vessel to the membrane module, indicating that the pressurized vessel is empty. Then, the liposomal suspension is stabilized under magnetic stirring. Finally, the ethanol is removed by rotary evaporation to complete the final preparation.

Basing on the forementioned system, in 2010, Jaafar-Maalej et al. [22] were the first to use an SPG (Shirasu porous glass) membrane and obtained a final product with a mean particle size of approximately 100 nm and 98% encapsulation efficiency for the model drug beclomethasone dipropionate. Later, in 2011, Laouini et al. [21] produced a similar product—100 nm and 93% (model drug: spironolactone). By adjusting the flow rate of the pump and the pressure of the nitrogen gas bottle, the process conditions can be easily controlled, which makes the preparation procedure easy to reproduce and increase to an industrial scale. Shortly after this, in the same year, Thi Thuy Pham et al. [23] reported their work on industrial scale liposome preparation using a membrane contactor. Their product could be obtained in a very reproducible manner.

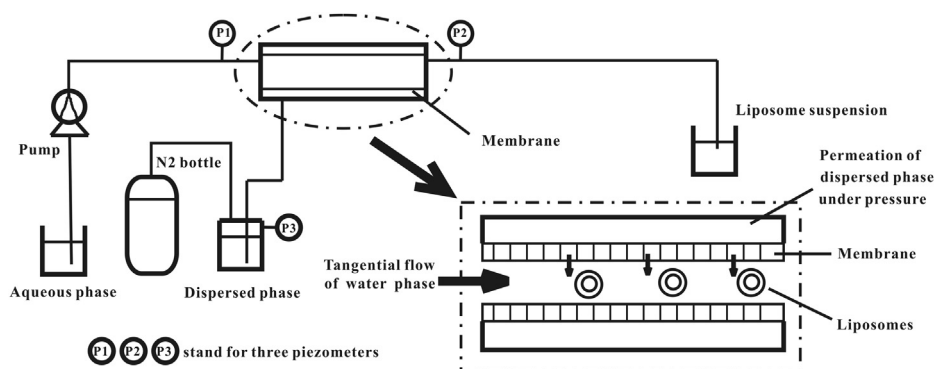


Fig. 3 – Schematic representation of liposome preparation based on a membrane contactor.

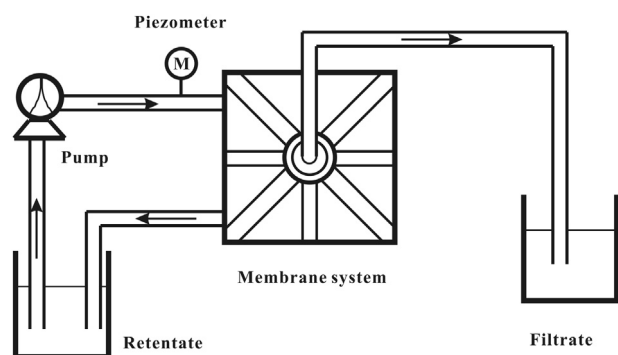


Fig. 4 – Schematic representation of the experimental setup of a cross-flow filtration unit.

Generally, liposomes prepared using membrane contactor-based techniques have the following characteristics: (a) homogeneous and small particle size, (b) multilamellar vesicles, (c) high encapsulation efficiency for lipophilic drugs and (d) simplicity for scaling up. However, if certain active drug-loading methods can be introduced successfully, drugs with a high water solubility can also undergo enhanced entrapment. This would make the technology even more attractive.

3.4. Cross-flow filtration detergent depletion method

Detergent-mediated liposome production is based on the solubilization of lipids with the aid of a suitable detergent resulting in the formation of mixed micelles. Then, the detergent is removed, which leads to the breaking up of micelles and repacking into lipid bilayers. The edge tension

increases and finally the curved bilayers shrink and form unilamellar vesicles [24]. However, the membranes still contain a great amount of detergent. The membrane-bound detergents may produce severe effects in the bilayer. Hence, fast removal of membrane-bound detergent is a crucial step in detergent removal techniques.

Therefore, a technique, which combines the advantages of known detergent removal methods with an accelerated and efficient removal of detergent, can markedly reduce the preparation time and heterogeneous liposome lamellarity. In addition, the procedure would be economical and preferable even if carried out continuously and under sterile conditions.

In 1998, Regine Peschka et al. [25] developed a combined conventional detergent depletion method with a cross-flow technique, a combination which can meet the demand for the fast removal of detergents. A schematic representation of the experimental setup of the cross-flow filtration unit is shown in Fig. 4.

The cross-flow filtration unit consists of a starting reservoir, a pump, a filtration device (membrane system) and tubing with an integrated rotary slide valve and a manometer to monitor the retentate pressure. By increasing the retentate pressure, the pressure on the membrane increases, leading to the fast removal of detergent. The starting reservoir contains the mixed micelle solution which is subjected to tangential filtration. The filtration unit is equipped with a single membrane or membrane cassettes with a cutoff of a selected molecular weight.

By using the cross-flow filtration process, liposomes of defined size, homogeneity and high stability can be obtained. Large quantities of liposomes can be produced in a significantly shorter time compared with other methods used for

Table 1 – Features of new techniques in liposome preparation.

New technique	Particle Size (nm)	Encapsulation efficiency	Features (advantages and drawbacks)
Supercritical fluid technology	100–10,000 [15,16]	10–20% [15] 20% (fluorescein isocyanate dextran) [16]	<ul style="list-style-type: none"> a. Organic solvent can be excluded completely, or even need not to be used. b. Postal procedures should be utilized to achieve a narrow particle size distribution. c. Efficiency of encapsulation has no significant improvement compared with Bangham method. d. The recovery of raw material is usually raw.
Dual asymmetric centrifugation	70–120 [20]	80% (calcein) 71% (siRNA) [20]	<ul style="list-style-type: none"> a. The equipment of DAC is small in size and easy in operation with a good reproducibility. b. Liposomes with small particle size can be achieved. c. Water soluble drugs could have an efficient entrapment. d. High phospholipids content should be in the formulation to obtain a sufficient viscosity.
Membrane contactor technology	~100 [21,22]	93% (spironolactone) [21] 98% (beclomethasone) [22]	<ul style="list-style-type: none"> a. Homogeneous and small multilamellar liposomes with high encapsulation efficiency for lipophilic drugs can be obtained. b. It is easy to scale-up. c. The encapsulation for hydrophilic drugs still need further investigation.
Cross-flow filtration detergent depletion method	~50 [25]	Data not shown	<ul style="list-style-type: none"> a. Liposomes of defined size, homogeneity and high stability can be prepared in a short time. b. Sterile products can be obtained by the presented methods. c. The waste filtrate can be recycled to minimize costs of production.
Freeze drying double emulsions method	<200 [26]	87% (calcein) 93% (flurbiprofen) 19% (5-fluorouracil) [26]	<ul style="list-style-type: none"> a. Sterile preparation with good storage stability can be achieved. b. Cryoprotectants are always carbohydrates, which limits the applications for patients with diabetes.

detergent removal. In addition, sterile products can be obtained by these methods when starting with sterile filtered mixed micelles and autoclaved devices. In addition, the waste filtrate can be recycled to minimize the production costs.

3.5. Freeze drying double emulsion method

Based on the traditional double emulsions method, in the freeze drying double emulsion method, different kinds of cryoprotectants are added to the inner or outer water phase of the liposome formulation. After the formation of a W/O/W type multiple emulsion, a sterilization process is carried out. Subsequently, liposomes in the form of lyophilized powder can be obtained via a freeze drying procedure. Before use, the powder should be hydrated to the original volume of the water phase to be transformed into a liposome suspension.

Using this procedure, the liposomes usually have a small size below 200 nm. If appropriate cryoprotectants are chosen, we can obtain a liposome preparation with a small mean particle size (approximately 100 nm), and a highly efficient encapsulation (87% for calcein, 93% for flurbiprofen and 19% for 5-fluorouracil), good reproducibility and high stability. In addition, a sterile product can be also achieved with a good storage stability by the application of freeze drying as in other drug delivery systems [26–28].

However, this method also has its drawbacks. The used organic solvents might pose critical problems such as safety issues associated with the disposal of residual solvents and damage to the freeze dryer. In addition, cryoprotectants are always carbohydrates which limits their use for patients with diabetes.

4. Future

Combined with the basic preparation principles of liposomes, a great many unique new technologies have been used to prepare liposomes. They can be used to solve certain problems posed by new preparation procedures. In summary, every newly-applied technique has its own pros and cons and their features are listed in Table 1. Although deficiencies or limitations still exist in various technologies, the desired effects can be obtained by drawing on the strengths of individual methods. We expect that researchers will be helped to make a choice with the aid of this review to fulfill their particular requirements.

The unique applications of new technologies will also help in the pursuit of industrial-scale pharmacy applications—simplified operation, high repeatability, suitability for industrial-scale up. The introduction of new technologies will produce more liposome formulations from the laboratory, which will be suitable for industrial-scale production and even more effective clinical applications.

REFERENCES

- [1] Sessa G, Weissmann G. Phospholipid spherules (liposomes) as a model for biological membranes. *J Lipid Res* 1968;9:310–318.
- [2] Mohammed AR, Weston N, Coombes AGA, et al. Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. *Int J Pharm* 2004;285:23–34.
- [3] Oussoren C, Eling WMC, Crommelin DJA, et al. The influence of the route of administration and liposome composition on the potential of liposomes to protect tissue against local toxicity of two antitumor drugs. *Biochim Biophys Acta (BBA) – Biomembranes* 1998;1369:159–172.
- [4] Kimelberg HK, Tracy TF, Biddlecome SM, et al. The effect of entrapment in liposomes on the in vivo distribution of [3H] methotrexate in a primate. *Cancer Res* 1976;36:2949–2957.
- [5] Senior J. Fate and behavior of liposomes in vivo: a review of controlling factors. *Crit Rev Ther Drug* 1987;3:123.
- [6] Barenholz Y. Relevancy of drug loading to liposomal formulation therapeutic efficacy. *J Liposome Res* 2003;13:1–8.
- [7] Bangham A. Properties and uses of lipid vesicles: an overview. *Ann N. Y Acad Sci* 1978;308:2–7.
- [8] Szoka F, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc Natl Acad Sci* 1978;75:4194–4198.
- [9] Deamer DW. Preparation and properties of ether-injection liposomes. *Ann N. Y Acad Sci* 1978;308:250–258.
- [10] Zumbuehl O, Weder HG. Liposomes of controllable size in the range of 40 to 180 nm by defined dialysis of lipid/detergent mixed micelles. *Biochim Biophys Acta (BBA)-Biomembranes* 1981;640:252–262.
- [11] Saunders L, Perrin J, Gammack D. Ultrasonic irradiation of some phospholipid sols. *J Pharm Pharmacol* 1962;14:567–572.
- [12] Hope M, Bally M, Webb G, et al. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta (BBA)-Biomembranes* 1985;812:55–65.
- [13] Pradhan P, Guan J, Lu D, et al. A facile microfluidic method for production of liposomes. *Anticancer Res* 2008;28:943–947.
- [14] Lesoin L, Boutin O, Crampon C, et al. CO₂/water/surfactant ternary systems and liposome formation using supercritical CO₂: a review. *Colloids Surf Physicochem Eng Asp* 2011;377:1–14.
- [15] Lesoin L, Crampon C, Boutin O, et al. Preparation of liposomes using the supercritical anti-solvent (SAS) process and comparison with a conventional method. *J Supercrit Fluids* 2011;57:162–174.
- [16] Otake K, Imura T, Sakai H, et al. Development of a new preparation method of liposomes using supercritical carbon dioxide. *Langmuir* 2001;17:3898–3901.
- [17] Otake K, Shimomura T, Goto T, et al. Preparation of liposomes using an improved supercritical reverse phase evaporation method. *Langmuir* 2006;22:2543–2550.
- [18] Massing U, Cicko S, Ziroli V. Dual asymmetric centrifugation (DAC)—A new technique for liposome preparation. *J Control Release* 2008;125:16–24.
- [19] Brandl M, Drechsler M, Bachmann D, et al. Preparation and characterization of semi-solid phospholipid dispersions and dilutions thereof. *Int J Pharm* 1998;170:187–199.
- [20] Hirsch M, Ziroli V, Helm M, et al. Preparation of small amounts of sterile siRNA-liposomes with high entrapment efficiency by dual asymmetric centrifugation (DAC). *J Control Release* 2009;135:80–88.
- [21] Laouini A, Jaafar-Maalej C, Sfar S, et al. Liposome preparation using a hollow fiber membrane contactor—Application to spironolactone encapsulation. *Int J Pharm* 2011;415:53–61.

-
- [22] Jaafar-Maalej C, Charcosset C, Fessi H. A new method for liposome preparation using a membrane contactor. *J Liposome Res* 2011;21:213–220.
- [23] Pham TT, Jaafar-Maalej C, Charcosset C, et al. Liposome and niosome preparation using a membrane contactor for scale-up. *Colloids Surf B Biointerfaces* 2012;94:15–21.
- [24] Schubert R, Beyer K, Wolburg H, et al. Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate. *Biochemistry* 1986;25:5263–5269.
- [25] Peschka R, Purmann T, Schubert R. Cross-flow filtration—an improved detergent removal technique for the preparation of liposomes. *Int J Pharm* 1998;162:177–183.
- [26] Wang T, Deng Y, Geng Y, et al. Preparation of submicron unilamellar liposomes by freeze-drying double emulsions. *Biochim Biophys Acta (BBA) – Biomembranes* 2006;1758:222–231.
- [27] Van Winden E, Crommelin D. Long term stability of freeze-dried, lyoprotected doxorubicin liposomes. *Eur J Pharm Biopharm* 1997;43:295–307.
- [28] Chen C, Han D, Cai C, et al. An overview of liposome lyophilization and its future potential. *J Control Release* 2010;142:299–311.