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Review

Liposomal preparation by supercritical fluids technology

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Liposomes have been proved to be useful drug carriers in preclinical and clinical trials. Many methods of liposomal preparation have been developed, but there are still few methods that can completely remove the solvent when liposomes are used as drug carriers. Furthermore, most of the methods are not suitable for mass production. From 1970s, supercritical fluids technology (SCF) has been utilized in liposomal preparation because of its friendliness, nontoxicity to the environment and its possibility to achieve solvent-free liposomes and industrial-scale of liposome production under the conditions of current good manufacturing practice (cGMP). Therefore, SCF may become a dominant technology for the preparation of liposomes. Here, we reviewed and discussed the advancement of preparing liposomes by SCF.

Key words: Liposomal preparation, supercritical fluids, supercritical carbon dioxide, solvent free, industrial scale.

INTRODUCTION

Liposomes are vesicular colloid particles in which aqueous volumes are entirely enclosed by membranes usually consisting of phospholipids and cholesterol (Lasic, 1997). Since the liposomes were first used as drug carriers in 1970s (Gregoriadis andRyman 1972), liposomal products have been steadily entering the pharmaceutical market (Gregoriadis, 1995). Many methods for preparing liposomes have been developed, but these methods require large amounts of organic solvents like chloroform, ether, freon, methylenechloride and methanol that are harmful to the environment and the human body, and very few methods have been developed that yield liposomes that have a high trapping efficiency for water-soluble substances without using any organic solvent. Additionally, all these methods are not suitable for mass production of liposomes because they consist of many steps (Imura et al., 2002).

With the advent of Green Chemistry in the early 1990s, the surge of supercritical fluids (SCFs) increased vastly (Ginty, 2005). The supercritical state of a fluid is intermediate between that of gas and liquids. Supercritical fluids (SCFs) exhibit some solvent power which is adjustable in function of pressure and temperature. The SCF has been used widely in pharmaceutical industrial operations including crystallization, particle size reduction, drug delivery preparation, coating and product sterilization (Sunkaraand Kompella, 1999; Kompella and Koushik, 2001). In the pharmaceutical field, supercritical carbon dioxide ($scCO_2$) is by far the most commonly used gas (Delattre, 2007), which can become supercritical at conditions that are equal or exceed its critical temperature of 31.1°C and its critical pressure of 7.38 Megapascals (Mpa) (Castor, 2005).

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Abbreviations: SCF, supercritical fluids technology; **cGMP,** current good manufacturing practice; **SCFs,** supercritical fluids; **$scCO_2$,**supercritical carbon dioxide; Tc, critical temperature; **Pc,** critical pressure; **RESS,** rapid expansion of supercritical solutions**; RESOLV,** rapid expansion of a supercritical solution into a liquid solvent; **GAS,** gas anti-solvent process**; PCA,** precipitation from compressed antisolvent; **ASES,** aerosol solvent extraction system; **SAS,**supercriticleantisolvent process; **SEDS,** solution-enhanced dispersion by supercriticle fluids; **PGSS,** particle from gas-saturated solutions; **DELOS,** depressurization of an expanded liquid organic solution; **CAN-BD, CO_2**-assisted nebulization and bubble drying; **SAA,** supercritical assisted atomization; **SCRPE,** supercritical reverse phase evaporation; **ISCRPE,** improved supercritical reverse phase evaporation.

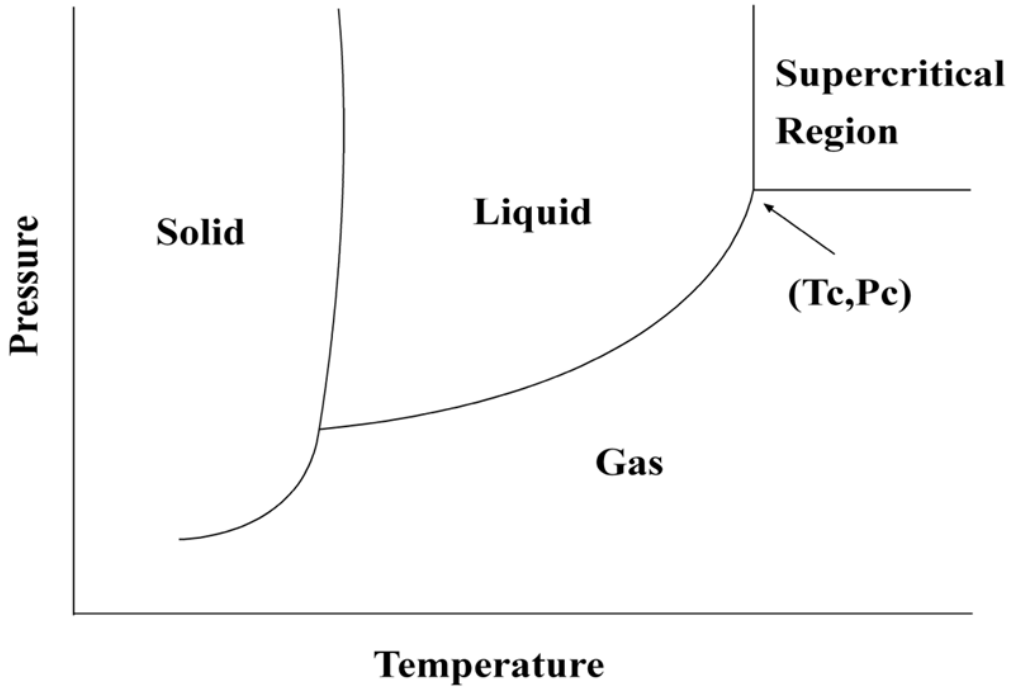

Figure 1. Small changes in temperature and pressure near the critical region will affect the density and solubility of SCFs.

Since the SCF was applied in the filed of crystallization/particle production, so many groups have began to investigate the possibility of using $scCO_2$ to prepare liposomes. In practice, $scCO_2$ was mainly used either as an alternative solvent or as an anti-solvent in the micronization process (Frederiksen et al, 1997; Castor and Chu, 1998; Magnan et al., 2000; Badens et al., 2001; Otake et al., 2001; Imura et al., 2002; Bridson et al., 2006). In this review, we outlined the advancement of liposome preparation using supercritical fluids technology.

THE OVERVIEW OF SUPERCRITICAL FLUIDS TECHNOLOGY

The definition and character of SCFs

Fluid is defined as being supercritical when the temperature and pressure are simultaneously higher than their critical values, Tc (critical temperature) and Pc (critical pressure). The phase diagram and the supercritical region of a pure substance are depicted in Figure 1. Small changes in temperature and pressure near the critical region will affect the density and solubility of SCFs. At the critical point, SCFs have the properties of density as a liquid and low viscosity with better flow property as a gas.

Commonly used supercritical solvents include ethylene, carbon dioxide, nitrous oxide, propylene, propane, ammonia, n-pentane, ethanol and water (Kompella et al., 2001). Table 1 lists the critical pressure and critical temperature of the aforementioned solvents. Most hydrocarbons' critical pressure is about 4MPa, and the critical temperature of homologues increases with increasing molar mass.

$scCO_2$ is currently being widely used for pharmaceutical application, because of its low critical pressure and low critical temperature. It has a high solvating power property in the near critical point. Similar to non-polar solvent, it can be adjusted by changing the pressure or temperature (Manosroi et al, 2007). $scCO_2$can be used to substitute organic solvents to form bilayer vesicles with the advantages of being environmental friendly, non-toxic, non-inflammable and inexpensive (Bunker et al., 1997; Heo et al., 2000; Cooper, 2000).

Main kinds of SCF and their variants

The first report about a supercritical fluid as a medium for particles production was published by Hannay and Hogarth (1879). To date, the kinds of SCF are not less than a dozen according to the process characteristics, but they are mainly divided into three types based on the role of SCFs in the process: 1, As a solvent; 2, as an anti-solvent; 3, as a solute. Table 2 lists many kinds of SCF.

Rapid expansion of supercritical solutions (RESS), gas anti-solvent process (GAS) and particle from gas-saturated solutions (PGSS) are the typical representatives

Table 1. Properties of some commonly used supercritical fluids.

Solvent	Critical pressure (MPa)	Critical temperature (K)
Carbon dioxide	7.37	304.2
Ethane	4.88	305.4
Ethylene	5.04	282.4
Propane	4.25	369.8
Propylene	4.62	365.0
Water	22.05	647.3
Ethanol	6.14	513.8
Nitrous oxide	7.15	309.0
Ammonia	11.1	405.0
n-Pentane	3.37	469.6

Table 2. Kinds of SCF.

Role of SCFs	Process (reference)	Abbreviation
As a solvent	Rapid expansion of supercritical solutions (Krukonis et al.,1984)	RESS
	Rapid expansion of a supercritical solution into a liquid solvent (Meziani et al., 2002)	RESOLV
As an anti-solvent	Gas antisolvent process (Gallagher et al., 1992)	GAS
	Precipitation from compressed antisolvent (Bodmeier et al.,1995)	PCA
	Aerosol solvent extraction system (Bleich et al., 1993)	ASES
	Supercritical antisolvent process (Elvassore et al., 2001)	SAS
	Solution-enhanced dispersion by supercritical fluids (Palakodatyand York, 1999)	SEDS
As a solute	Particle from gas-saturated solutions (Weider et al., 1997)	PGSS
	Depressurization of an expanded liquid organic solution (Ventosa et al., 2001)	DELOS
	Supercritical assisted atomization (Reverchon andPorta2003)	SAA
	CO2-assisted nebulization and bubble drying (Sievers et al., 2000)	CAN-BD

of the three kinds, respectively. Other variants are those that make some improvements based on these three typical representatives.

Rapid expansion of supercritical solutions (RESS)

This process consists of the saturation of the supercritical fluid with a solute; then, the depressurization of the solution through a heated nozzle into a low pressure chamber produces a rapid nucleation of the solute in the form of small particles that are collected from the gaseous stream (Byrappa et al., 2008). Figure 2 shows the equipment commonly used in RESS.

The RESS process can produce particles with different sizes and morphology. The solubility of solute in SCFs, pre- and post-expansion temperature and pressure, the diameter and length of nozzle are some of the factors that determine the size and morphology of the particles. So, how to get the optimal particle size and morphology is very important.

The main problem of RESS is that lots of solutes in thesolubility of SCFs are very low, although, a small amount of organic solvent can increase its solubility, it still cannot enter into the production application because of the low yield. Moreover, as the concentration of solution in the reactor is declining, the stable process will be hard to control, and the nozzle will often be clogged.

Gas anti-solvent process (GAS)

GAS process was developed later due to some materials that cannot be processed by RESS owing to their poor solubility in SCFs. GAS recrystallization uses a dense gas as an anti-solvent for precipitating a solute that has been dissolved in an organic solvent. For a solute to be processed by GAS, it must be dissolved in an organic solvent, and must be insoluble in SCFs. The solvent must be at least partially miscible with the SCFs. Figure 3 shows the equipment commonly used in GAS.

There are several other processing variables involved

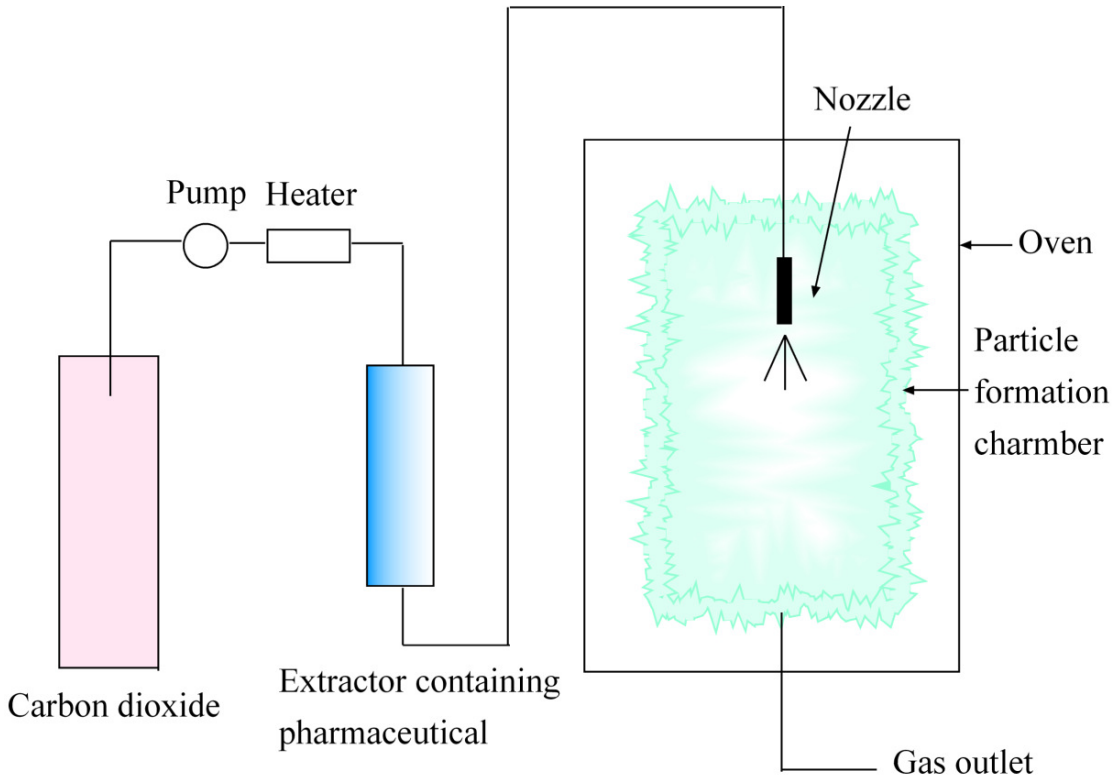

Figure 2. The equipment commonly used in RESS.

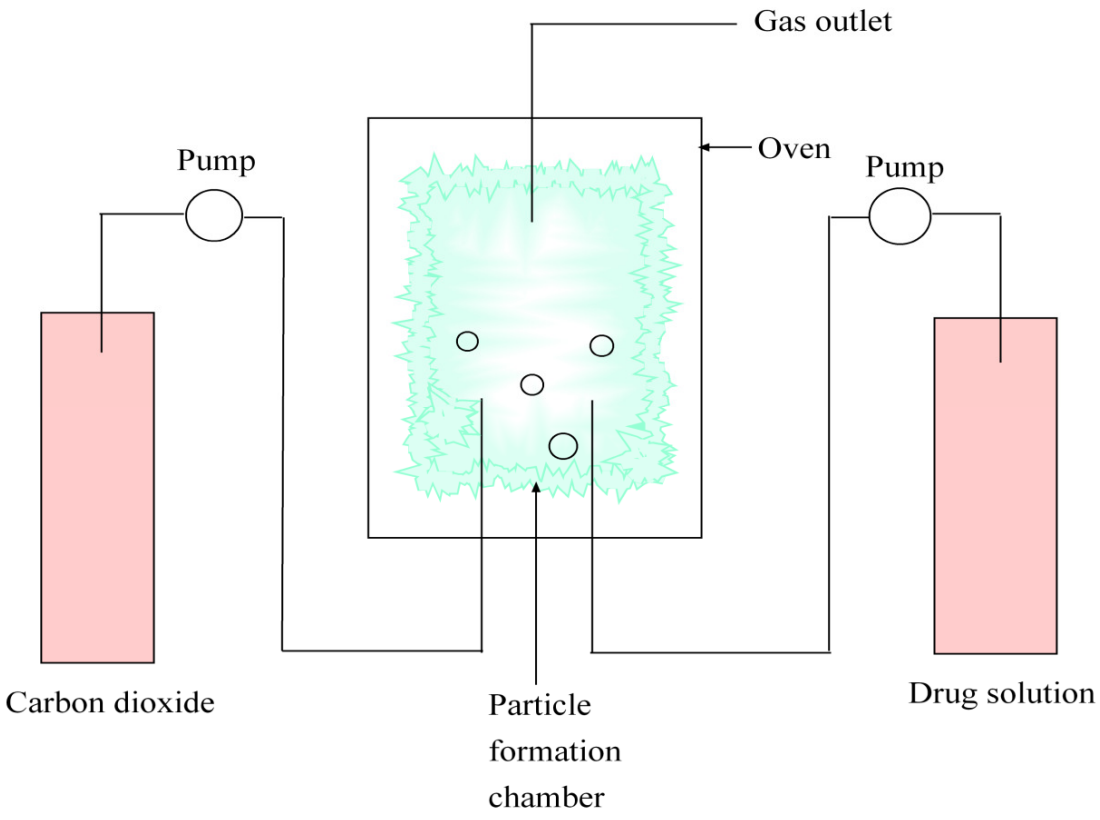

Figure 3. The equipment commonly used in GAS.

in this process for the efficiency such as temperature, pressure, stirring rate, concentration of the solute in feed solution, flow rate and nature of liquid solvents, and choice of the SCFs on the physical properties of the final product should be studied in order to obtain optimal particles. By this method, the nucleus is first formed in the liquid phase, then has to be dried using pure SCFs, so, it is only in batch or semi-continuous mode. It should be noted that the distribution of the particle size and crystalline quality are affected by the SCFs' re-importation accompanied by heat.

Particle from gas-saturated solutions (PGSS)

It involves the dissolution of SCFs into a melted material (usually a polymer) followed by rapid depressurization of the gas-saturated solution through a nozzle that causes the formation of particles (Graser and Wickenhaeuser, 1982). The interaction between SCFs and the polymer reduces the glass transition or melting point. This process is mainly for polymers used as coating agent, to produce foams, or to incorporate drugs. However, major drawbacks of this process are the difficulty to process heat-sensitive materials and the possible presence of residual solvent in the final product (Pasquali and Bettini, 2008). Figure 4 shows the equipment commonly used in PGSS.

THE CONVENTIONAL PREPARATIONSOF LIPOSOMES

Although, liposomes as drug carriers have been studied for a long time, there still exists a problem in putting liposomes to practical use because a suitable way for liposomal preparation has not been found.

Since liposomes' discovery (Bangham et al., 1965), numerous strategies have been available for preparing liposomes, which are suitable primarily for small scale production. These strategies include the Bangham method (1965), the organic solvent injection method (Batzriand Korn, 1973), the reverse phase evaporation method (SzokeandPapahadjopoulos, 1978) and so on. Unfortunately, no single method is ideal in all respects (Bridson et al., 2006).

Among these conventional preparations of liposomes that are noted earlier, the main disadvantage is the bulk use of organic solvent, which may be harmful to the human body and environment. Another problem is the limited industrial-scale production by conventional preparation methods.

LIPOSOMAL PREPARTION BY SCF

The advancement of preparing liposomes using SCF

Pioneering work in the preparation of liposomes using SCF was done by Frederiksen et al. (1997) based on an improvement of RESS process. The general aim of this project was to explore the use of SCFs as an alternative solvent to conventional organic solvents for preparation of liposomal formulations with a view to reducing the amount of organic solvent. However, $scCO_2$ has weak-solvent power (like phospholipids that are known to have a very poor solubility in $scCO_2$), therefore, it needs a co-solvent. Frederiksen et al., (1997) designed a set of sophisticated apparatus consisting of two main parts. In the high pressure part, phospholipids, cholesterol and other raw materials were solvated first by $scCO_2$ concurrently with a certain proportion of ethanol (because of its low toxicity and its accepted presence in injections described in the USP XXII) before introducing the aqueous systems. Then, in a low pressure part, the homogeneous super-critical solution was expanded and simultaneously mixed with the aqueous phase to yield liposomes encapsulating the water soluble drugs. Through this process, 200 nm size and 20% encapsulation efficiency of liposomes were obtained, which was a great success in the field of liposomal preparation. Noteworthy, this technique had scale-up possibilities and got the same encapsulation efficiency (~20%) but required 15-fold less organic solvent, as compared to the ethanol injection method of Batzri and Korn (1973).

Based on this foundation, the so-called supercritical reverse phase evaporation method (SCRPE) was invented by Imura et al. (2002), which form the similarity of the method with the conventional reverse phase evaporation method. This method used $scCO_2$ concurrently with ethanol as co-solvent to dissolve phospholipids before introducing aqueous systems and emulsion formation (Imura et al., 2002, 2003; Aburaiet al., 2011). The liposomes were obtained when the pressure was reduced to release CO_2. As compared to the methods noted earlier (Frederiksen et al., 1997), the SCRPE method was a physicochemical method, since colloidal structures such as water/CO_2 or CO_2/water emulsions directly influenced the physicochemical properties of liposomes. Nevertheless, since this system subjected to that of depressurization step was a heterogeneous aqueous dispersion, this process failed to separate the particles from the co-solvent which may prevent the liposome formation.

But lately, an improved method (ISCRPE) was developed to obtain higher trapping efficiency using fewer process steps, which can be easily scaled up for the mass production of liposomes (Otake et al., 2006). And the biggest progress was that the whole process did not use any organic solvent, including ethanol. Furthermore, liposomes prepared by the ISCRPE method have a unilamellar structure comprising a loosely packed phospholipid membrane that is highly stable. In the same year, the same method was used to successfully prepare chitosan-coated cationic liposomes for DNA transfection (Otake et al., 2006).

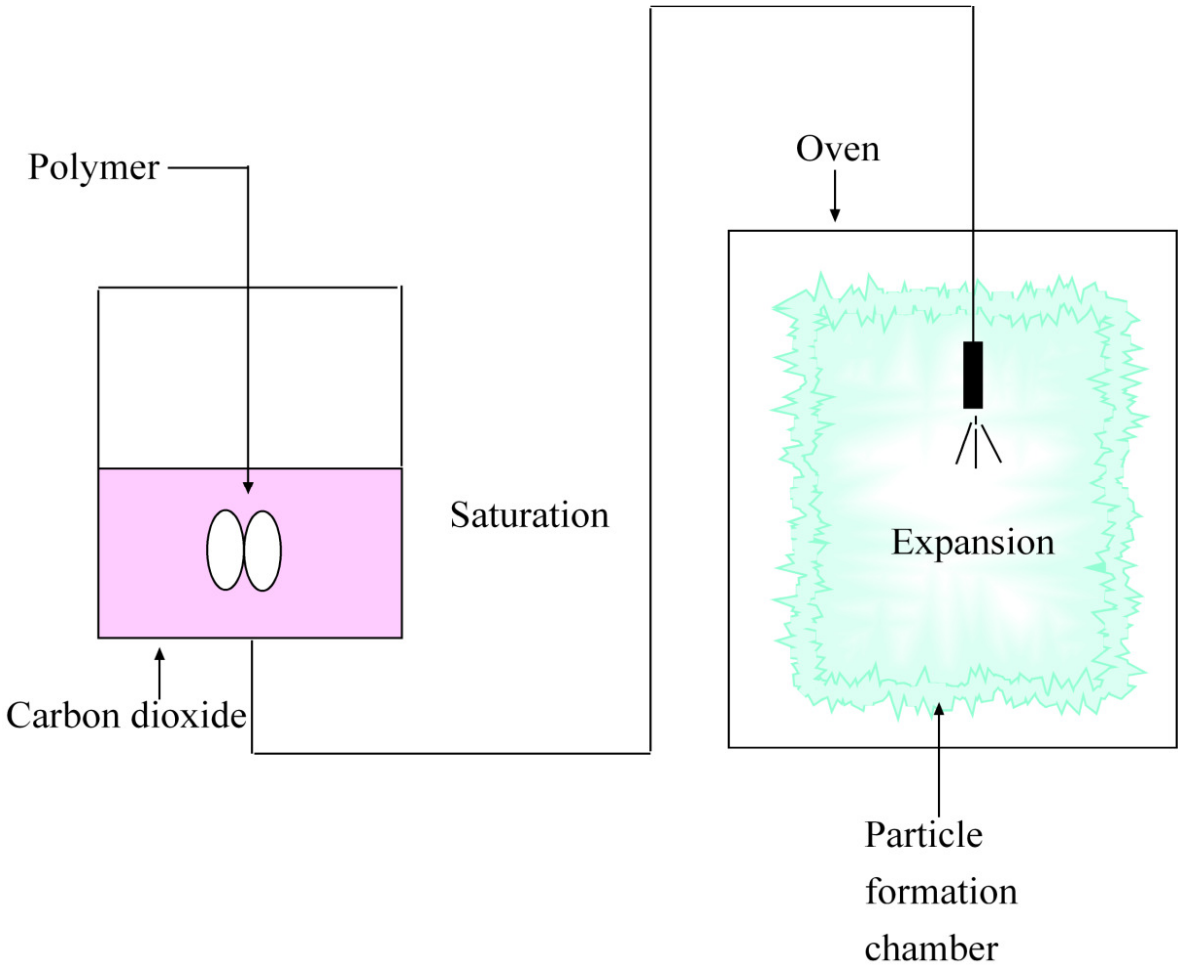

Figure 4. The equipment commonly used in PGSS.

Another strategy for the use of $scCO_2$ is SAS, which has received more attention in the preparation of liposomes. It overcomes the drawback of RESS process, that is, the poor solubility of the solid in the SCFs (Berends et al., 1994). This method requires the raw materials dissolving in auxiliary liquid solvent first, then spraying by using a high-pressure pump through a nozzle into $scCO_2$ filled in the high vessel or expanding by introducing $scCO_2$ to form droplets. The dissolution of $scCO_2$ into droplets reduces the solubility of the solid in the droplets and then precipitates to obtain particles. In the area of liposomal preparation, the exploratory work was made by Magnan et al. (2000) and Badens et al. (2001), micronizing various grades of soya phosphatedylcholine (raw materials of liposomes) by this method. Badens et al. (2001) even compared the RESS and the SAS process at the same time. The results showed that the RESS process failed to separate the particles formed from the co-solvent, and the SAS process seemed to be free of solvent by infrared analysis. Considering this reason, the SAS process may be a better choice to obtain solvent-free liposomes.

Similar work was done by Bridson et al. (2006), involving the production of phospholipid powders using $scCO_2$ as an anti-solvent to find the possibility of preparing liposomes directly from the powders hydrating with water. From a process point of view, it was found that this technique provided continuous operation and met cGMP requirements. In addition, it is likely that lipids that would have minimal or no solubility in modified carbon dioxide could be processed using this method.

After this, the real vesicle preparation was reported by Kadimi et al. (2007). They prepared amphotericin B-intercalated liposomes by the GAS process and the conventional sonication process. Liposomes prepared by these two methods had a spherical shape. Additionally, it was found that a narrow range of size distribution and higher intercalation efficiency were acquired by theGASprocess, but the solvents used in this experiment were chloroform and methanol, which were toxic and mayremain in the final products (dates were not shown). We hope that, the process should be free of solvent or atleast, the solvent had to be nontoxic, like ethanol.

The aerosol solvent extraction system (ASES) process

was also applied to prepare real liposomes which containedphosphatidylcholine, cholesterol and Poloxamer 407 in a dry and reconstitutable form (Kunastitchai et al., 2006). The ASES made some improvements on the basis of GAS. It involved spraying the solution through an atomization nozzle as fine droplets into compressed carbon dioxide (Bleich et al., 1993). This was very closely associated with SAS, but the solution and SCFs was spraying into the vessel in turns. The procedure aforementioned was roughly similar to that of Bridson et al. (2006), including the microparticle production and hydration of dry ASES powders. It was worth mentioning that the optimal of ASES process must be primarily at the temperature below phase transition temperature of phospholipids used, due to the yield of microparticles. Then, in the following steps, the pH of hydration medium influenced the drug entrapment efficiency as well as particle size of liposomes; the highest entrapment efficiency reached 100.2% when the pH was 7.2. On the basis of this procedure, organic liquids which were sprayed through a nozzle into a bulk of SCFs could rapidly be extracted, enabling fast and continuous precipitation of solutes out of the organic solution. After a relatively short period of drying with SCFs, the residual solvent could dramatically be removed from the precipitates produced (Ruchatz et al., 1997; Thiering et al., 2001). And the last advantage was that the ASES process seemed to be suitable for preparing pharmaceutical liposomes as dry and reconstitutable vesicles for large scale production, which could settle the stability problems which are encounted in the traditional method.

Overviewing the advancement of SCF for liposomal preparation mentioned earlier, the liposomes could be successfully prepared by RESS, GAS and their variants, like SAS and ASES. Every SCF process has its own advantages and disadvantages. The RESS process suffers a limitation related to the high levels of pressure that is generally involved because of the low solvent power of SCFs; typically, values up to 40 MPa may be required to ensure an acceptable solubility of the solute in the supercritical phase. Furthermore, both RESS and SCRPE failed to separate the particles formed from the co-solvent. When compared with the RESS, the SAS has three major advantages: (1) More concentrated solution may be operated; (2) Operating pressures are much lower, typical values are of 100 bar; (3) The absence of residual solvent in the final product. On the basis of SAS, the ASES makes the process better. It seemed to be suitable for preparing pharmaceutical liposomes as dryand reconstitutable vesicles for large scale production, which could settle the stability problems which areencounted in the traditional method.

The advantage of preparing liposomes using SCF

When compared with conventional technology, SCF has created great expectations due to its numerous great features that could be exploited:

1. Environmental friendliness is the most popular feature owing to the use of supercritical carbon dioxide ($scCO_2$), which is green, nontoxic and unexpensive (Kendall et al., 1999).
2. The whole operation controlled easily, is linked to the reduced steps of process. Therefore, the size distribution of liposomes and reproducibility could be controlled smoothly.
3. It is possible to achieve the industrial-scale of liposome production by SCF under the conditions of current good manufacturing practice (cGMP).
4. The whole operation is preceded at about 40°C. So, this mild process could avoid the phase transition of phospholipids, the degradation of partial lipid or drugs in liposomes, which has perspective in the pharmaceutical research of biological and natural medicine.
5. The liposomes once formed need not undergo further processing (such as freeze-drying,spray-drying and precipitation) to achieve liposomal dry powder formulations, which could be attained directly (Misra et al., 2009).

CONCLUSION AND PERSPECTIVES

In this review, we described the advancement of liposomal preparation using SCF. The current methods of liposomal preparation are not suitable for achieving free-solvent liposomes and industrial-scale production under cGMP conditions. However, the SCF could solve most of these problems and obtain liposomal dry powder formulations directly. Although, the SCF has been utilized in liposomal preparation for many years, it still has not become a mature technology in this field. There are few studies and slow progress on liposomal preparation using SCF, which may be as a result of the high costs of equipments. Actually, in the existing research, there still exist many questions in the process design and industrial problems, such as low level of continuous production, high investment costs, as well as the application of high-pressure decomposition or degradation materials. And even the conditions of making the optimal liposomes need careful selection. Noteworthy, the kinds of SCF mentioned earlier in the liposomal preparation are already very old. Many new kinds of SCF, like SEDS and SAA, have not been applied in the field of liposomal preparation. Therefore, much work is needed to make the SCF better in the future.

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