Biotechnol. Bioprocess Eng. **2001**, 6: 213-230

Microencapsulation Methods for Delivery of Protein Drugs

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Abstract Recent advances in recombinant DNA technology have resulted in development of many new protein drugs. Due to the unique properties of protein drugs, they have to be delivered by parenteral injection. Although delivery of protein drugs by other routes, such as pulmonary and nasal routes, has shown some promises, to date most protein drugs are administered by parenteral routs. For long-term delivery of protein drugs by parenteral administration, they have been formulated into biodegradable microspheres. A number of microencapsulation methods have been developed, and the currently used microencapsulation methods are reviewed here. The microencapsulation methods have been divided based on the method used. They are: solvent evaporation/extraction; phase separation (coacervation); spray drying; ionotropic gelation/polyelectrolyte complexation; interfacial polymerization; and supercritical fluid precipitation. Each method is described for its applications, advantages, and limitations.

Keywords: protein, peptide, drug delivery, microparticle, microencapsulation

INTRODUCTION

Significant advances in biotechnology have brought ever-increasing availability of recombinant peptide protein drugs in large quantities. The successful completion of the human genome project will undoubtedly lead to the explosion of new protein drugs with exquisite bioactivities. Since protein drugs carry out biological processes and reactions with high specificity and potency, protein drugs will continue to be drugs of choices for treating various diseases. Formulating protein drug delivery systems, however, poses several problems. Due to extremely low bioavailability of protein drugs by oral administration, which is the most convenient mode of drug delivery, protein drugs are usually administered by parenteral route. One way of minimizing discomfort and improving patient compliance is to produce sustained-release formulations that deliver protein drugs continuously over long periods of time. Another challenge in protein drug delivery is to maintain the tertiary protein structure, which is essential to bioactivity. Exposure of protein drugs to unfavorable conditions during formulation tends to reduce their bioactivities.

Most widely used approach for long-term delivery of protein drugs has been parenteral administration of protein drugs in microspheres made of biodegradable polymers. Preparation of protein drug-containing microspheres without reducing bioactivity has been the goal of microencapsulation methods. A number of microencapsulation methods have been developed through the years, but none of the methods has been ideal for loading protein drugs. Each method has its own advantages as well as limitations. For further improvement in microencapsulation technologies, it is important to understand the strengths and drawbacks of each method. Table 1 lists examples of microencapsulation processes found in the literature. Microencapsulation processes have been frequently classified either chemical or mechanical [1]. Chemical processes refer to methods that utilize the change of solvent property, chemical reaction between monomers, or complexation of polyelectrolytes. In mechanical processes, a gas phase is utilized at some stage to provide force to break up materials to small particles. There are many situations, however, where such a distinction is not clear.

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MICROENCAPSULATION METHODS

SOLVENT EVAPORATION/EXTRACTION

Solvent evaporation/extraction methods have been widely used to prepare microspheres loaded with various drugs, especially hydrophobic drugs. For the encapsulation of peptide and protein drugs, oil/water (o/w), oil/oil (o/o) and water/oil/water (w/o/w) emulsification methods have been used. Depending on the number of emulsions produced during the preparation of microspheres, solvent evaporation/extraction can be divided into two methods, single emulsion and double emulsion (Fig. 1).

Methods

Single Emulsion (o/o or o/w) Methods

In single emulsion methods, peptides/proteins are pre-

Table 1. Examples of microencapsulation methods

1.	***Chemical processes***
	Solvent evaporation and extraction
	Crygenic solvent extraction
	Phase separation (Coacervation)
	Non-solvent addition
	Temperature change
	Incompatible polymer or salt addition
	Polymer-polymer interaction (complex coacervation)
	Polyelectrolyte complexation
	Interfacial polymerization
2.	***Mechanical processes***
	Spray drying
	Spray chilling
	Spray desolvation
	Supercritical fluid precipitation

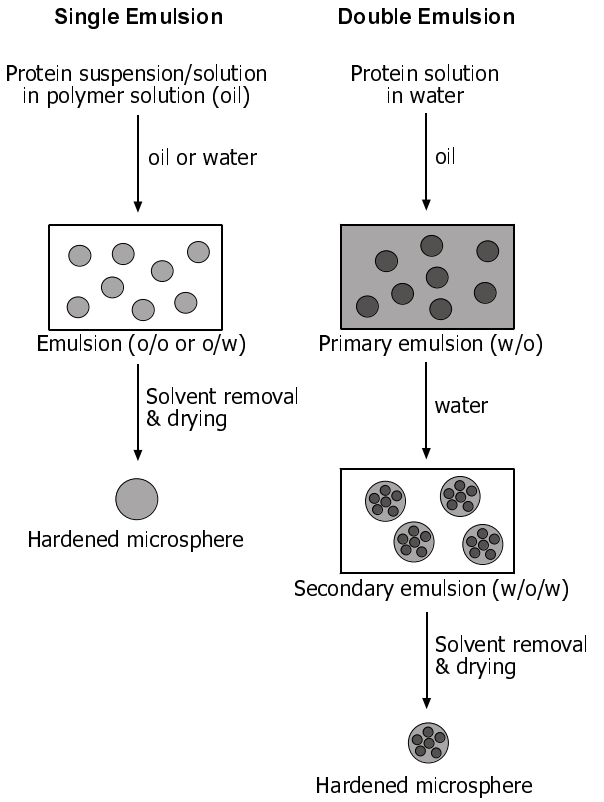

Fig. 1. Single emulsion (left) and double emulsion (right) solvent evaporation methods for microencapsulation of proteins.

sent in a dispersed phase, which is a polymer solution in organic solvent such as dichloromethane or ethyl acetate. Polylactic acid (PLA) and poly(lactide-*co*-glycolide) (PLGA) are the most widely used biodegradable synthetic polymers for sustained-release preparations. The release kinetics of active components can be controlled by changing molecular weight and/or copolymer ratio of those polymers. Drugs can be dispersed as solid particles or dissolved in polymer solution. The drug solution or suspension is added into a continuous phase, which can be mineral oil (o/o) or aqueous solution (o/w) containing emulsifiers. Emulsification is carried out by agitation, homogenization, or sonication. Emulsifiers in the continuous phase stabilize o/o or o/w emulsions being produced. Emulsifiers such as Span 85 [2], sorbitan sesquioleate [3], aluminium tristearate [4] have been used for o/o interface, and Carbopol® 951 [2], methyl cellulose [5], polyvinyl alcohol (PVA) [6] have been used for o/w interface.

Organic solvent in dispersed phase is removed by solvent evaporation or by solvent extraction. In solvent evaporation process, hardening of emulsion occurs when volatile organic solvent in dispersed phase leaches into continuous phase and evaporates from continuous phase at atmospheric pressure. Using vacuum or a moderate increase in temperature can accelerate the evaporation of organic solvent. In solvent extraction process, the emulsion is transferred to a large amount of water or other quenching medium, and the extraction of organic solvent occurs faster than in solvent evaporation process. Thus, microspheres produced by solvent extraction process are more porous than the ones produced by solvent evaporation. The porous structure usually results in faster release of peptide/protein drugs. For long-term sustained release, solvent evaporation is preferred. The prepared microspheres are collected by centrifugation or filtration, and freeze-dried.

Double Emulsion (w/o/w) Methods

In double emulsion methods, an aqueous drug solution is first emulsified in a polymer-dissolved organic solvent. The w/o emulsion is then added into an aqueous phase that contains emulsifier, thereby forming w/o/w emulsion. Then, the organic solvent is removed by extracting into external aqueous phase and evaporation.

Applications

Single Emulsion

Single emulsion solvent evaporation method has been used to prepare microspheres/nanospheres containing various peptide and protein drugs. Insulin solution in organic solvent was used in emulsification to prepare nanospheres [7]. Other researchers used solid particles of bovine serum albumin (BSA) [2] or ovalbumin (OVA) [4], instead of protein solution, in a hope that protein activity can be preserved in organic solvent. High encapsulation efficiency was obtained using protein particles. The prepared microparticles tend to show initial burst release profiles. Table 2 lists some examples of microparticle preparation using protein particles. A novel approach of protein particle preparation was used for the encapsulation of bovine superoxide dismutase (bSOD) into PLGA/PLA microspheres [5]. Protein spherical particles were prepared by lyophilization of protein-polyethylene glycol (PEG) aqueous mixture. This approach resulted in high encapsulation efficiency (88%) and high activity of the loaded enzyme (95%). In

Table 2. Encapsulation of protein particles by single emulsion/solvent evaporation methods

Protein particles	Polymer	Type	Encapsulation efficiency	Initial release in 1 day	Enzyme activity	Reference
Spray-dried BSA	PLGA	o/o o/w	>90% 20-50%	<10% <10%	- -	[2]
Micronized OVA	PLGA	o/o	89%	40%	-	[4]
Lyophilized bSOD/PEG	PLGA + PLA	o/w	88%	<5%	100%	[5]

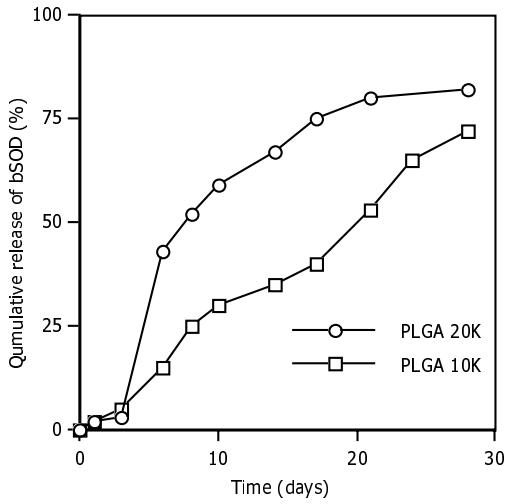

Fig. 2. Release of bovine superoxide dismutase (bSOD) from PLGA/PLA (19/76) microspheres. Microspheres prepared from PLGA (MW 10K) and PLA resulted in zero-order release of bSOD for 4 weeks. From reference [5].

addition, constant release of bSOD for 28 days was achieved without significant initial burst release (Fig. 2). Molecular weight of PLGA seemed to be an important factor in the release kinetics, since changing molecular weight of PLGA from 10K to 20K did not result in constant release of bSOD.

Double Emulsion

Table 3 lists some examples of microparticles prepared by the double emulsion methods. Leuprolide acetate, a luteinizing hormone-releasing hormone (LHRH) agonist, was encapsulated by the w/o/w emulsion method using PLGA(75/25) MW 14K. Constant release of leuprolide acetate without lag time or burst release was observed in *in vitro* and *in vivo* experiments using rats [8,9]. Currently, this product is on the market. Carbonic anhydrase [10] and BSA [11] were also encapsulated within PLGA microspheres. The release pattern of those proteins was biphasic. The first phase was initial burst release, and the second phase was slow release. The protein release from microsphere was incomplete due to protein aggregation and adsorption within the

Table 3. Encapsulation of proteins by double emulsion/solvent evaporation methods

Protein	Polymer	Encapsulation efficiency	Initial release in 1 day	Enzyme activity	Reference
Carbonic anhydrase	PLGA	45-48%	<10%	-	[10]
BSA	PLGA	56-85%	30-50%	-	[11]
rhGH	PLGA	40-66%	20%	-	[12]
rhEPO	PLGA	43-90%	15%	-	[14]
rhEPO	PLGA/PEO block	72-68%	17%	-	[17]
Urease	PLGA	17-55%	17-45%	60-89%	[13]
Lysozyme	PEG/PBT block	89%	<5%	-	[15]

microspheres. Recombinant human growth hormone (rhGH) also showed initial burst release of about 20% followed by a continuous release for 30 days [12].

Protein stability can be maintained by using stabilizers. High concentration (15%) of Pluronic F-127 was shown to increase the urease activity from 63% to 89%; however, it decreased the encapsulation efficiency from 55% to 17%, and increased burst release [13]. Recombinant human erythropoietin (rhEPO) was aggregated during the homogenizing step to form w/o emulsion [14]. EPO aggregates increased from 4% to 15% when sonication or vortex mixing was used in preparing the w/o emulsion. Protein stabilizers, such as BSA or hydroxypropyl-β-cyclodextrin, decreased the aggregates in the released EPO significantly. It is noted, however, that EPO was not released after the initial burst release, suggesting the formation of non-covalently bound high molecular weight aggregates. Multiblock copolymers of poly(ethylene glycol) (PEG) and poly(butylene terephthalate) (PBT) were used to encapsulate lysozyme [15]. When 4% PVA was used as a stabilizer in the external aqueous phase, most of the enzyme loaded was released at a constant rate for 3 weeks with no initial burst release. Encapsulation efficiency was 89%. In addition, by varying the composition of copolymer and the size of the microspheres, in vitro release profiles of the lysozyme could be further adjusted [16]. For example, increasing PEG molecular weight and decreasing PBT percentage resulted in faster release of lysozyme within several days. Fig. 3 shows the effect of PBT percentage on the release rate of lysozyme.

Advantages

Solvent evaporation/extraction method has been widely used for the delivery of small molecule drugs. Small peptide drugs with low aqueous solubility can be successfully encapsulated into polymer microspheres for sustained delivery. Since biodegradable polymers such as PLA and PLGA are generally used in this method, the release kinetics of drugs can be controlled by using PLGA with different degradation kinetics. Higher con-

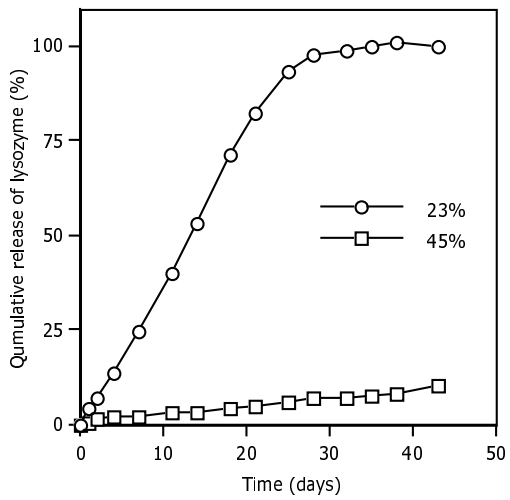

Fig. 3. Release of lysozyme from PEG-PBT microspheres. Molecular weight of PEG segment was 600. Polyvinyl alcohol (4%) was used as a stabilizer in the external aqueous phase. Increasing percentage of PBT in PEG-PBT block copolymer resulted in slower release of lysozyme. From reference [16].

tents of glycolide in PLGA polymers result in rapid degradation of the polymer, resulting in faster release of the loaded drugs. The release profile can also be controlled by the processing factors, such as shear forces, since high shear forces tend to produce smaller microparticles which release drugs at a faster rate due to higher surface area and shorter diffusion path length.

Limitations

While solvent evaporation/extraction methods have been used widely in preparing microparticles for peptide/protein delivery, the methods are still not ideal and have many aspects to be improved. First, the drug encapsulation efficiency into microspheres is not high. Although protein drugs can be produced in larger scale due to development of genetic engineering technique, the cost is still high. Thus, increasing the encapsulation efficiency is still quite important for preparing microspheres. Second, the solvent evaporation/extraction methods require use of toxic organic solvents, such as dichloromethane and ethyl acetate, as a solvent for dissolving biodegradable polymers. To meet the regulatory requirement regarding the residual solvent in the final products, the use of toxic organic solvent should be minimized or avoided. Third, in most situations, the loaded proteins are released with the initial burst release, and in many situations the loaded proteins are not released completely. The initial burst release may cause abnormal response in patients due to abnormally high drug concentration in blood. Incomplete release of the loaded proteins is often related to protein stability. Proteins have to maintain their 3-dimensional conformation to keep their bioactivity. Proteins, however, are prone to be denatured and to form aggregates by various factors, including exposure to large interface between organic and aqueous phases, shear force in the processing steps (*e.g.*, homogenization, and sonication), or protein-polymer interaction. Some reports showed that the loss of protein activity or denaturation/ aggregation of protein could be minimized by using stabilizers such as poloxamer, BSA or hydroxypropyl-β-cyclodextrin [13,14,18]. Fourth, although continuous release of peptide/protein drugs is desirable, there are situations where pulsatile release is preferred as in the case of insulin delivery. Furthermore, the timing and the exact amount of the released insulin need to be controlled carefully. This type of release can not be obtained by biodegradable microspheres prepared by solvent evaporation/extraction methods.

Modifications of Solvent Extraction

In an effort to protect proteins from denaturation during microencapsulation processes, alternative methods have been developed to minimize exposure of protein drugs to denaturing conditions, such as high temperature and water-oil interfaces [19].

Spray-desolvation

Spray-desolvation involves spraying a polymer solution onto desolvating liquid. For example, microdroplets were made by spraying PVA aqueous solution onto acetone bath, where the polymer solvent (water) was extracted into acetone and PVA precipitated to form solid microparticles [20]. This method was also applied to encapsulate peptides and BSA as model drugs in PLGA. The micronized drug was suspended in a PLGA-acetone solution and atomized ultrasonically into ethanol bath, where microspheres were precipitated [21].

Cryogenic Solvent Extraction

PLGA is dissolved in an organic solvent such as dichloromethane, together with protein powders. As shown in Fig. 4, the polymer/protein mixture is atomized over a bed of frozen ethanol overlaid with liquid nitrogen, at a temperature below the freezing point of the polymer/protein solution. The microdroplets freeze upon contacting the liquid nitrogen, then sink onto the frozen ethanol layer. As the ethanol layer thaws, the microparticles which are still frozen sink into the ethanol. Dichloromethane, the solvent in the microparticles then thaws and is slowly extracted into ethanol, resulting in hardened microparticles containing proteins and polymer matrix.

This process was used to produce a microsphere formulation for human growth hormone (hGH) [23-26]. Prior to the encapsulation process, hGH is formulated with zinc to produce insoluble Zn:hGH complex. The encapsulation of this complex contributed to stabilize hGH during the fabrication process and within the microspheres after hydration [23,24]. An *in vivo* study of this formulation demonstrated a lower C_{max} and an extended serum level for weeks, but a similar bioavailability, as compared with the protein in solution [26]. The

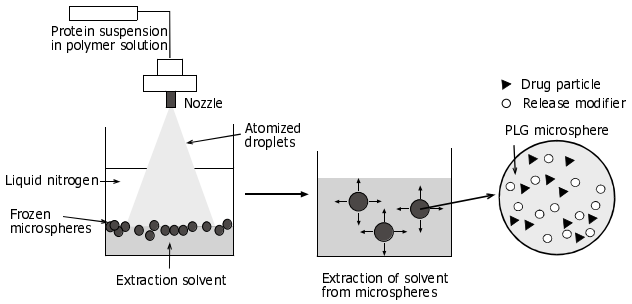

Fig. 4. Schematic diagram of the ProLease® encapsulation process. From reference [22].

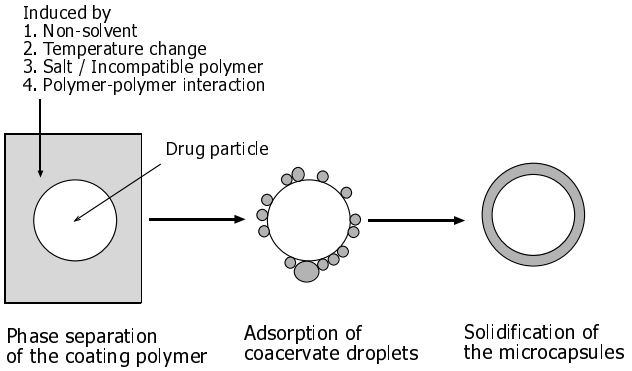

Fig. 5. Schematic diagram of the formation of a coacervate microcapsule. From reference [30].

hGH formulation using this method (referred to as ProLease®) was approved by the FDA in December 1999, as an injectable suspension for once- or twice-a-month adminitration, under a brand name of Nutropin depot®.

In this process, all manipulations are performed at low temperatures, therefore thermal denaturation of protein drugs can be prevented. There are no aqueous phases, thus the protein is not subjected to oil-water interfaces where some proteins may denature. In addition, the process utilizes solvents in which most proteins are insoluble (dichloromethane as a polymer solvent, ethanol as an extraction solvent), therefore a high encapsulation efficiency can be achieved [22,27]. However, this system is not easy to apply to various protein drugs. The use of liquid nitrogen also makes scale-up rather difficult. The system still utilizes toxic organic solvents, and thus it has similar problems associated with solvent evaporation/extraction methods.

PHASE SEPARATION (COACERVATION)

Methods

Microencapsulation by phase separation is basically a three-step process: (1) phase separation of the coating polymer to form coacervate droplets; (2) adsorption of the coacervate droplets onto the drug surface; and (3) solidification of the microcapsules [28], as shown in Fig. 5. Phase separation techniques can be classified according to the method to induce phase separation; non-solvent addition, temperature change, incompatible polymer or salt addition, and polymer-polymer interaction [29].

Non-solvent Addition

The polymer to be coated is dissolved in a good solvent, and drug may be dissolved or suspended or emulsified in the polymer solution. Then the first non-solvent (also called 'coacervating agent' or 'phase inducer') which is miscible with the good solvent but does not dissolve the polymer is slowly added to the polymer-drug solution system. The polymer is concentrated as the solvent for the polymer is slowly extracted into the first non-solvent. The polymer is then induced to phase separate and form coacervate droplets that contain the drug (Fig. 6(A)). The coacervate droplet size can be controlled by adjusting the stirring speed. At this point of the process, the coacervates are usually too soft to be collected, and thus they are usually transferred to a large body of a second non-solvent (also called 'hardening agent') to harden the microparticles [31].

When polyesters, such as PLA and PLGA, are used, most widely used solvents are dichloromethane, ethyl acetate, and acetonitrile. The first non-solvents for the polymers are low-molecular weight liquid polybutadiene, low-molecular weight liquid methacrylic polymers, silicone oil, vegetable oil, and light liquid paraffine oils. Aliphatic hydrocarbons, such as heptane, hexane, and petroleum ether, have been used as the second non-solvent.

Temperature Change

A system comprised of a polymer and a solvent exists as a single phase at all points above the phase boundary. Drug is dispersed in the polymer solution with stirring. As the temperature is decreased below the curve (Fig. 6(B)), phase separation of the dissolved polymer occurs in the form of immiscible liquid and the polymer coalesces around the dispersed drug particles, thus forming microcapsules. Further cooling accomplishes gelation and solidification of the coating. The microcapsules are collected from the solvent by filtration, decantation, or centrifugation techniques [32].

Incompatible Polymer Addition or Salt Addition

When two chemically different polymers dissolved in a common solvent are incompatible and do not mix in solution, phase separation takes place. This phenomenon is well described by the phase diagram of a ternary system consisting of a solvent, and two polymers, X and Y (Fig. 6(C)). A drug is dispersed in a solution of polymer Y (point a in Fig. 6(C)) and polymer X is added to the system, denoted by the arrowed line. When the phase boundary is passed with the further addition of polymer X, polymer rich phase begins to separate to form immiscible droplets containing the drug, and coa,-

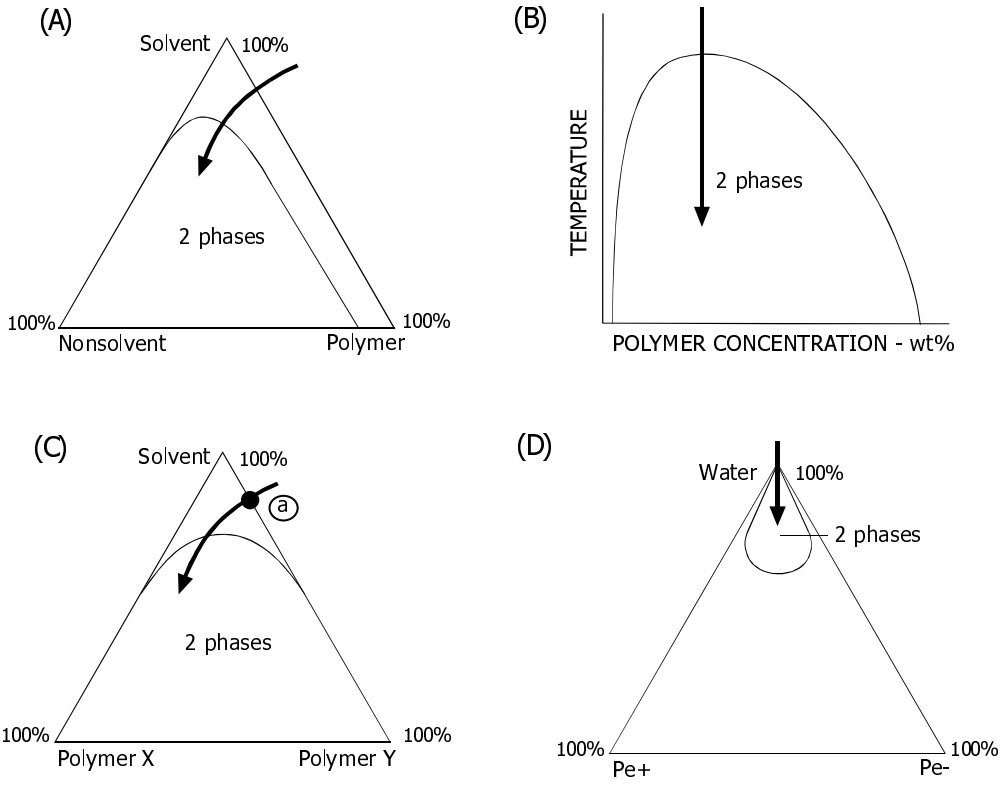

Fig. 6. General phase diagrams of phase separation techniques based on solvent (A), temperature (B), incompatible polymers (C), and interpolymer interactions (D). From reference [32].

lesce to make microcapsules [32]. In a similar manner inorganic salts can be added to aqueous solution of water-soluble polymers to cause phase separation.

Polymer-polymer Interaction (Complex Coacervation)

The interaction of oppositely charged polyelectrolytes can result in the formation of a complex with reduced solubility leading to phase separation (Fig. 6(D)). This method is often called complex coacervation [1]. The method differs from incompatible polymer addition in that both polymers become part of the final capsule wall. Gelatin is normally used as a cationic polymer at pH below its isoelectric point. A variety of natural and synthetic anionic polymers are available for interaction with gelatin to form complex coacervates. A well known example of natural polyanion for making complex coacervates with gelatin is gum arabic. Aqueous solutions of gum arabic and gelatin are adjusted to pH 4.5 and warmed to 40-45°C. The pI of gelatin is 8.9. The oppositely charged macromolecules interact to undergo phase separation, to which a water insoluble liquid core material is added and emulsified to yield the desired droplet size. The mixture is then slowly cooled for an hour. During the cooling cycle, phase separation is further enhanced, resulting in the microcapsules formed on the core material with thin film of coacervate [32]. To harden the microcapsules, the capsules are normally cooled further and treated with glutaraldehyde. This method is mainly for water insoluble materials, includeing inks for carbonless paper, perfumes for advertising inserts, and liquid crystals for display devices.

Applications

Since the non-solvent addition method does not include an aqueous continuous phase as in w/o/w double emulsion methods, the water-soluble drugs may be protected from partitioning into the water phase. This makes the non-solvent addition method one of the most widely used techniques for encapsulating water-soluble compounds [28,33-41]. Triptoreline (luteinizing hormone releasing hormone analogue)-containing microcapsules were prepared by the non-solvent addition method, and the effect of the physicochemical nature of the polymer on the stability of the coacervate droplets was examined [34,35]. Triptoreline was suspended in PLGA-dichloromethane solution, and silicone oil was then added to the suspension as the first non-solvent. The embryonic microspheres were further hardened in n-heptane, the second non-solvent. It was found that as the copolymer became more hydrophobic, it dissolved better in dichloromethane and thus more silicone oil was necessary to desolvate the copolymer. The average diameter of the microspheres increased with the volume of silicone oil, thereby lowering the initial burst effect. It was also observed from *in vivo* experiments. With op-

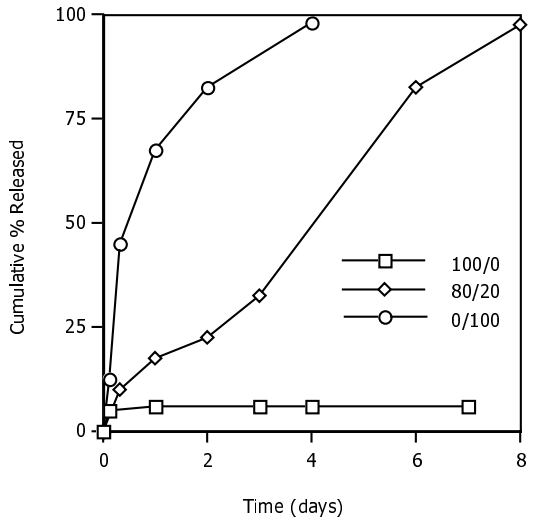

Fig. 7. Release of GM-CSF from microspheres prepared from 100% PLA (MW 6,100) (0/100), 80%/20% mixture of PLGA (MW 40,400)/PLA (80/20), and 100% PLGA (100/0). From reference [41].

timized microspheres, the *in vivo* peptide release reached the peak level on day 15 and the effect lasted for 1 month.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was encapsulated in different blends of PLGA and low molecular weight PLA by the non-solvent addition method, and the prepared microspheres were characterized both *in vitro* and *in vivo* [41]. The microspheres with high encapsulation efficiency were obtained within a size range between 20 μm and 80 μm. The *in vitro* release kinetics was dependent on the ratio of blended polymers. Steady release of GM-CSF could be achieved over a period of one week without significant burst effect using blend of low molecular weight PLA and high molecular weight PLGA (Fig. 7). GM-CSF released from the microspheres was found to be physically intact and biologically active both *in vitro* and *in vivo*.

To avoid the aggregation of microparticles often encountered in non-solvent addition, a modified method was developed [42]. Instead of adding non-solvent to a polymer-drug mixture, the polymer solution was added to an emulsion of aqueous ovalbumin in the non-solvent silicon oil. The obtained microspheres appeared smooth and remained individual, and displayed a small initial release of entrapped ovalbumin over the first 12 hours, with the subsequent release profile close to a zero order kinetics for a month. Another modification of the non-solvent addition method was introduced to protect protein entities from denaturation at the organic/aqueous interface or unfolding and/or aggregation within hydrophobic polymer matrix [40,43,44]. Drug-containing hydrophilic nanoparticles were prepared prior to encapsulation with hydrophobic polymer. Gelatin [40], agarose [43], and poly(vinyl alcohol) (PVA) [44] were used as nanoparticle matrices for BSA or insulin. Nanoparticles were suspended in PLGA-dichloromethane solution. Microparticles embedded with nanoparticles were made by phase separation method using silicone oil as the first non-solvent and heptane as the second non-solvent. The average diameter of the hydrophilic nanoparticle-hydrophobic microsphere composites was 150-180 μm. The protein release from the microspheres was prolonged to nearly two months, without degradation nor aggregation of the protein as shown by size exclusion chromatograms [44].

Phase separation by salt addition was adopted in preparation of ionotropic hydrogel in an attempt to control the size distribution of microspheres [45]. Prior to cross-linking with calcium ions, salts of monovalent ions (*e.g.*, sodium chloride) were added to aqueous solution of poly [di(carboxylatophenoxy) phosphazene] (PCPP) to form coacervate microdroplets with a size in the range 1-10 μm. Upon addition of calcium chloride, the microdroplets were stabilized via cross-linking between PCPP and calcium salts without changing the size and shape. The microsphere size increased linearly with the sodium chloride concentration, incubation time, and polymer concentration. This method avoided the use of organic solvents, heat, and complicated manufacturing equipment. Gelatin/Chondroitin 6-sulfate (CS6) microspheres were prepared to encapsulate proteins using complex coacervation for the joint therapy [46]. Gelatin (a polycation) and CS6 (a polyanion) solutions containing model proteins were mixed and vortexed at 37°C, pH 5.5 to form coacervates. The resulting coacervate microspheres were crosslinked with glutaraldehyde. It was claimed that proteins could be encapsulated with high encapsulation efficiency, retaining high bioactivity. The release of protein depended on the level of human matrix metalloprotease (MMP), since gelatin/CS6 coacervate was degraded by its gelatinase activity. The MMP concentration is one of the factors responsible for the joint pain, and thus the MMP-responsive release property is of distinctive advantage for the delivery of MMP inhibitors or the receptors for cytokines stimulating the synthesis of MMP to diseased joints. Gelatin/CS6 showed no significant cytotoxic effect *in vitro*.

Advantages

The non-solvent addition method is preferred in encapsulation of water-soluble drugs, such as proteins, peptides, and vaccines, since drug-polymer mixtures are not exposed to the continuous aqueous phase. This minimizes the loss of water-soluble drugs to the water phase and results in the high encapsulation efficiency. Another advantage of the phase separation method is that it enables efficient control of the particle size with a narrower size distribution by simply varying the component variables, such as concentration of added salts [45] or the viscosity and amount of the non-solvent and/or molecular weight of polymer used [47].

Limitations

Although many successful phase separation systems have been demonstrated, each method has several limitations. The microspheres tend to aggregate and the scale-up for mass production is difficult [48]. Agglomeration occurs when the coacervate droplets are sticky and adhere to each other before the solvent is completely removed or before the droplets are hardened [31]. To produce well-defined microspheres, wide 'stability window' is required. The stability window is defined as a step where addition of non-solvent does not induce agglomeration but forms stable coacervates [34]. The stability window is affected by the physicochemical nature of polymers used and/or the viscosity of the non-solvent added. The presence of residual solvents is a concern in the non-solvent addition method. The method includes at least three different solvents, *i.e.*, the polymer solvent and two polymer non-solvents. In general, the polymer solvents are toxic compounds, such as halogenated hydrocarbons (*e.g.*, dichloromethane), for which the exposure limits are regulated by the authority. For example, the concentration limit for dichloromethane is 600 ppm [49]. In addition to the polymer solvent, two non-solvents are generally known to remain in a substantial quantity. A study showed that depending on the type of solvent and polymer used, the residual solvents could be minimized by appropriate drying methods [38]. The solvents, however, were in general hard to remove. High levels of residues may alter the microsphere morphology due to their plasticizing effect. Moreover, it may cause a toxicity problem and influence crucial properties such as release profiles and drug stability. Undoubtedly, the search for toxicologically and technologically acceptable solvents and non-solvents should continue for the non-solvent addition method to be more useful.

In a polymer-polymer interaction (complex coacervation), the limited pH range and the use of cross-linking agent can be potential problems. Since the technique is based on electrostatic interactions between two polyelectrolytes, pH of the medium is an important factor. For example, in a gelatin-acacia system, the pH should be below the isoelectric point of gelatin (pH 8.9) to make it positively charged. The pH range suitable for the gelatin-acacia system is between 2.6 and 5.5 [30]. This condition, however, is not appropriate for encapsulation of acid-labile drug entities. The pH range could be extended by the addition of water-soluble nonionic polymers, such as polyethylene glycol [50,51]. Glutaraldehyde and formaldehyde are commonly used as cross-linking agents to harden the microcapsules in this technique. A condensation reaction occurs between the amino groups of the protein (*e.g.*, gelatin) and the aldehydes. The cross-linking agents may diffuse into the core and also react with drug entities, therefore this method may not be appropriate for the protein drugs. Potential toxicity problems arise as well [30].

SPRAY DRYING

Spray drying has been widely used in the pharmaceutical, food, and biochemical industries. The main applications in the pharmaceutical field include drying process, granulation, preparation of solid dispersions, alteration of polymorphism of a drug, preparation of dry powders for aerosols, and encapsulation of drugs for taste masking and protection from oxidation [52]. Since the late 1970s, the spray drying technique has been used for making microparticulate systems for controlled drug delivery.

Methods

Either hydrophilic or hydrophobic polymer is dissolved in a suitable solvent. Drug can be dissolved or suspended in the solvent. Alternatively, drug solution can be emulsified in the polymer solution. The mixture is then sprayed through a nozzle of a spray dryer, resulting in solid microspheres that precipitated into the bottom collector [53]. Sometimes, the process include the use of plasticizers to form microcapsules of regular spherical shape and smooth-surface by reducing the rigidity of polymer chain [54].

Alternative Technique

Spray-chilling (congealing): Spray congealing includes the dissolution or dispersion of the drug in a melted carrier without using solvent. This mixture fluid is then sprayed into a cold air stream to make small droplets and solidified by cooling at the temperature lower than the melting point of the carrier. This method was used to encapsulate bovine somatotropin (bST) in fat or wax [55]. bST was dispersed in molten fat or wax and sprayed through an air/liquid spray nozzle. The microspheres were formed as the molten droplets and were collected on sieves in the desired size range (45-180 μm). It was demonstrated that the microspheres were effective in maintaining increased level of bST in the blood of treated animals for 4 weeks, increasing weight gains and milk production.

Applications

Since spray-drying is relatively simple, fast and easy to scale-up, it has been tried as an attractive alternative to the conventional methods, such as coacervation and emulsion method to encapsulate protein drugs [48,56-65]. The spray drying technique was applied to produce PLGA microparticles containing thyrotropin releasing hormone (TRH) [48]. TRH in water and PLGA in acetonitrile were mixed to form a clear solution and then sprayed. Since the microparticles produced by conventional spray dryer tended to agglomerate and adhere to the surface of the chamber, they designed a double nozzle system such that additional nozzle might spray a mannitol solution as an anti-adherent simultaneously (Fig. 8). Microparticles with mean particle size of 20 μm

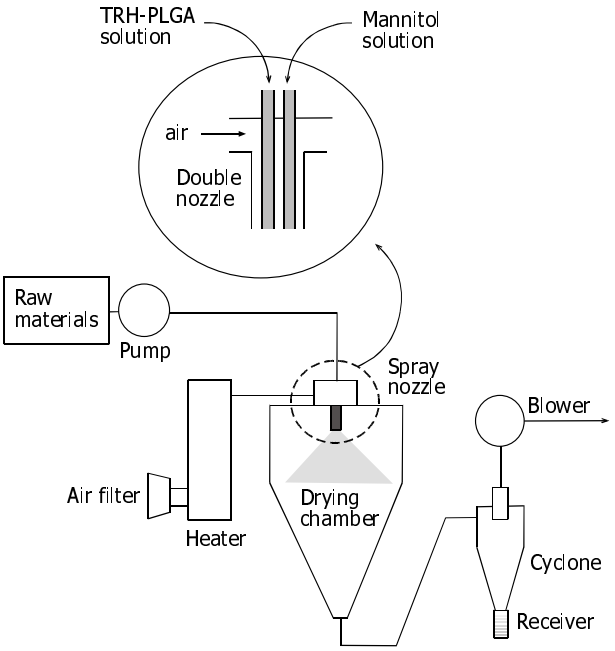

Fig. 8. Schematic diagram of a spray dryer used for preparation of PLGA microparticles containing TRH. From reference [48].

were obtained, from which zero order release of TRH continued for one month with a minimal initial burst. Table 4 lists some examples of microspheres prepared by spray drying.

Advantages

One of the major advantages of spray drying is its general applicability. Both hydrophilic and hydrophobic polymer can be used with proper selection of the solvent [52]. Spray drying is useful for encapsulating even heat-sensitive drugs, such as proteins or peptides, because it involves mild temperatures [52]. Although spray drying includes hot air stream, the temperature of the droplets may be maintained below the drying air temperature due to rapid evaporation of the solvent. The effective temperature applied to the drug itself is mild enough to be used for proteins. Moreover, spray drying is time effective. Spray drying can yield results equivalent to those of conventional methods in terms of size distribution, particle morphology, and release kinetics, yet with the advantage of high encapsulation efficiency and the short duration of the preparation procedure [53]. The spray drying equipment is easily available at the manufacturing site. Also, as a one-stage closed process, spray drying is ideal for production of sterile materials and good manufacturing practice (GMP) [30].

Limitations

During spray drying, considerable amounts of the material can be lost during the process due to sticking of the microparticles to the wall of the drying chamber. Spray drying process can often lead to change of polymorphism of the spray dried drugs [66,67]. For examples, progesterone crystal in its original alpha form (m.p. 402K) turned into beta form (m.p. 395K) when it was spray dried in combination with PLA. Fiber formation can be another major problem in spray drying PLA [67]. Fibers are formed due to insufficient forces present to break up the liquid filament into droplets. It depends on both the type of polymer and, to a lesser extent, the viscosity of the spray solution. For example, ethyl cellulose solutions made spherical droplets at relatively high concentrations, however PLA solutions of considerably low concentration and viscosity resulted in fibers. There are a number of process variables that should be optimized for drug encapsulation. They include feed material properties, such as viscosity, uniformity, and concentration of drug and polymer mixtures, feed rate, method of atomization, and the inlet and outlet temperatures [32]. The dependence on so many variables may become a problem in terms of reproducibility and a scale-up process. In addition, the amount of polymer and/or drugs for encapsulation may be limited, since fluid of high viscosity cannot be sprayed.

Table 4. Examples of microencapsulation of proteins by spray drying

Protein	Polymer	Solvent	Particle size	Release kinetics	Ref.
β-glucuronidase	Albumin/ Acacia with PVP as coacervate stabilizer	Distilled water	5 μm	Biphasic: initial burst (31%) within 6h followed by zero order release for 2 weeks	[60]
Bovine somatotropin	Poly anhydride	Dichloromethane	1-5 μm	90% release for 6 h due to the fast degradation of polymer and small particle size	[56]
Human erythropoietin	PLGA	Dichloromethane	10 μm	Initial burst during 24 h and no further release for 2 months, likely due to protein-polymer interaction and insoluble protein aggregates	[62]

IONOTROPIC GELATION / POLYELECTROLYTE COMPLEXATION

Ionotropic gelation (IG) is based on the ability of polyelectrolytes to crosslink in the presence of counter ions to form hydrogels. Since the use of alginate for cell encapsulation [68], ionotropic gelation has been widely used for both cell and drug encapsulation.

Method

Alginate is one of the most widely used polyanion for microencapsulation. Alginate is composed of 1,4-linked beta-D-mannuronic acid and alpha-D-guluronic acid

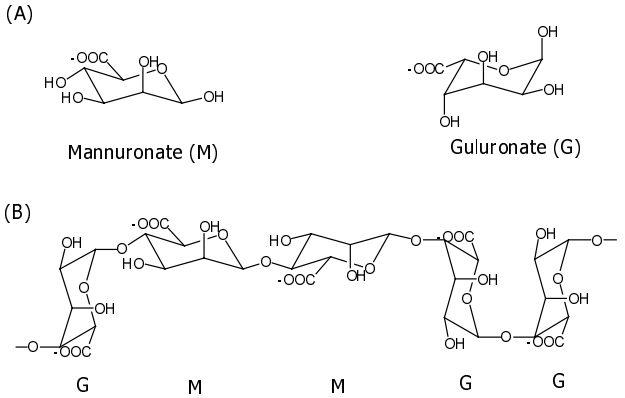

Fig. 9. Monomer units (A) found in alginate and the alginate polymer structure (B). From reference [69].

residues. Divalent and trivalent cations induce gelation by binding mainly to the guluronic blocks (Fig. 9). The micro- and macro-spheres are produced by dropping a cell- or drug-loaded alginate solution into aqueous calcium chloride solution. Calcium ions diffuse into the alginate drops, forming a three dimensional lattice of ionically crosslinked alginate. Polyelectrolyte complexation (PEC) with oppositely charged polyelectrolytes may be added to increase the mechanical strength of the hydrogel and/or to provide a permeability barrier. For instance, addition of polycation (usually poly-L-lysine) allows a membrane of polyelectrolyte complex to form on the surface of alginate beads [69]. A number of natural and synthetic polyelectrolytes have been investigated. Examples are listed in Table 5.

Since most of the droplets have been made with syringe needles, the particle sizes have been relatively large. Smaller droplets can be formed using a vibration system or air atomization method to extrude the alginate solution (Fig. 10) [30]. The latter involves a Turbotak air-atomizer. Pressurized air is fed to mix with the sodium alginate solution, forcing tiny liquid droplets out through the orifice of a nozzle. The calcium ions crosslink the droplets of sodium alginate on contact to form microgel droplets, which were further crosslinked by poly-L-lysine to form a membrane on the droplets. Microparticles obtained using this method were within the size range 5-15 μm [91].

Applications

IG/PEC has been widely used in microcapsule systems for cell encapsulation [82,87-89,92-94] and drug delivery systems [76,77,83,85,86,95,96]. Due to mild process conditions, IG/PEC has recently attracted a new attention for an application to protein delivery [71,78-81,84,90,97,98]. Calcium alginate hydrogels have been used for delivery of vascular endothelial growth factor (VEGF) [71]. The alginate beads demonstrated the ability to incorporate VEGF with efficiency between 30% and 67% and constant release (5%/day) of VEGF for up

Table 5. Examples of ionotropic gelation (IG) and polyelectrolyte complexation (PEC)

Polyelectrolytes	IG with	PEC with	References
Alginate (-)	Calcium (+)	-	[70-72]
Alginate (-)	Calcium (+)	Poly-L-Lysine (+)	[73]
Alginate (-)	Calcium (+)	Chitosan (+)	[74, 75]
Chitosan (+)	Tripolyphosphate (TPP)(-)	-	[76-81]
Carboxymethyl cellulose (-)	Aluminium (+)	Chitosan (+)	[82]
κ-carrageenan (-)	Potassium (+)	Chitosan (+)	[83]
ι-carrageenan (-)	Amines (+)	-	[84]
Pectin (-)	Calcium (+)	-	[85]
Gelan gum (-)	Calcium (+)	-	[86]
Alginate and cellulose sulfate (-)	Calcium (+)	Poly (methylene-co-guanidine) (+)	[87, 88]
Polyphosphazene (-)	Calcium (+)	Poly-L-Lysine (+)	[89, 90]

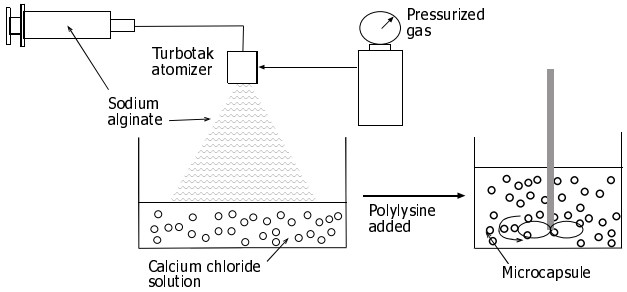

Fig. 10. Schematic diagram of the preparation of alginate-polylysine microcapsules.

to 14 days. The ionic interaction between VEGF and alginate contributed to maintaining the biological activity of VEGF.

Advantages

The main advantage of IG/PEC is the mild formulation conditions for maintaining cell viability and protein integrity. All of the polyelectrolytes are water-soluble, and thus proteins can be encapsulated without use of organic solvents or elevated temperatures, both of which may damage protein integrity. Also, this system is simple, fast and cost-effective.

Limitations

The biggest problem in applications of IG/PEC for controlled protein delivery, in spite of the great advantage of mild conditions, is that the matrix and membrane formed by IG/PEC is not capable of controlling the release rate for a long period of time. It does not matter in cell encapsulation, where the membrane should provide sufficient permeability for the cell prod-

uct, *i.e.*, a therapeutic protein. However, for IG/PEC to be used for controlled delivery of protein entities, a certain type of dense membrane (*e.g.*, biodegradable polymers), preferably capable of controlling the release rate, should be incorporated or alternative combinations of polyelectrolytes should be explored to control the permeability of the membrane, so that the release rate may be controlled over the desired span. Thus far, the release rate of the protein is likely to depend on the physicochemical properties of the model protein or the release medium in which the microparticles are placed [71]. In case of alginate-poly-L-lysine system, all parameters are tied to a single chemical complex, therefore it is not easy to optimize the capsule condition. For this reason, new combinations of polyelectrolytes were proposed to allow independent modification of capsule parameters including size, wall thickness, mechanical strength, permeability, and surface characteristics [99].

Another issue in IG/PEC is that some polyelectrolytes might have biocompatibility problem. There have been contradictory arguments concerning the biocompatibility of alginates [100-102]. A host foreign-body reaction (fibrosis) occurred on account of the implanted alginate-based particles and mannuronic acid block was found to be responsible for the fibrotic response, as a potent stimulator of IL-1 and TNF-α production. Use of alginates of high guluronic acid content was suggested to minimize the cytokine response. On the other hand, another study indicated that alginate particles with high guluronic acid contents provoked stronger response than the high mannuronic acid alginate particles [102]. In general, polyionic hydrogels obtained by IG/PEC have rather low mechanical strength. A few papers have suggested ways to overcome this problem by exploring alternative combinations of polyanions and polycations [87,99], introducing a different process [103], or applying additional coating [82,98].

INTERFACIAL POLYMERIZATION

Interfacial polymerization features in situ polymerization of reactive monomers on the surface of a droplet or particles.

Method

Two reactive monomers (typically dichloride and diamine) are respectively dissolved in immiscible solvents and mixed to form o/w emulsion (dichloride in oil phase and diamine in water phase). The monomers diffuse to the o/w interface where they react to form a polymeric membrane (Fig. 11). A typical example of this method is making nylon microcapsules [104]. Nonaqueous phase (chloroform/cyclohexane) containing surfactant (*e.g.*, sorbitan trioleate) and aqueous buffer containing drugs to be incorporated (enzymes or proteins), protective protein (*e.g.*, BSA or hemoglobin) and diamine are prepared respectively. Two phases are mixed and stirred to make an w/o emulsion in an ice bath until the desired droplet size is reached. Another nonaqueous phase containing acid chloride is added to the emulsion for interfacial polymerization. Polymerization is quenched by addition of excess nonaqueous phase. Microcapsules are allowed to sediment and collected and then washed in saline several times to remove organic solvents and byproducts.

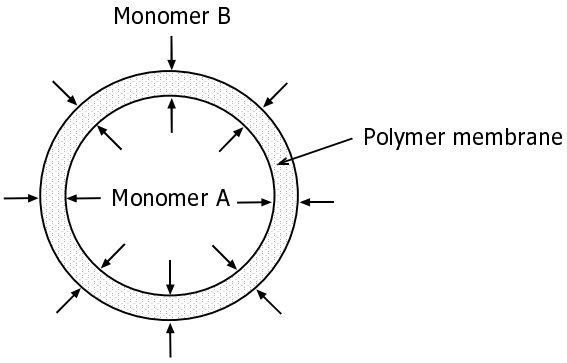

Fig. 11. Microencapsulation by interfacial polymerization method. From reference [104].

Various combinations of monomers can be used to obtain a range of polymer membranes [104]. Sebacoyl chloride and 1,6-hexane diamine can be used to form the polyamide nylon 6, 10. The microcapsules using this system, however, tend to be fragile and difficult to handle. Terephthaloyl chloride and 1,6-hexane diamine can yield polyester membrane. Alternatively terephthaloyl chloride can be used in combination with L-lysine to yield poly(terephthaloyl L-lysine). Typically acid chlorides and diamines are reactive monomers; however, isocyanates can be used instead of or as a partial substitute for acid chloride [105]. Alternatively, the polymer composed of only one type of monomer can be formed on the interface (Interfaicial addition polymerization) [104]. Polyalkylcyanoacrylate belongs to this type. Aqueous drug solution and oil phase containing cyanoacrylate monomer are mixed to form an w/o emulsion. The polymerization is initiated by water in the aqeous phase with cyanoacrylate dissolved in the oil phase.

Application

Recently insulin nanoparticles were prepared using interfacial addition polymerization techniques [106]. Nanoparticles were prepared by addition of ethyl 2-cyanoacrylate to a stirred w/o microemulsion consisting of aqueous insulin solution, oil and surfactant. To avoid exhaustive washing steps after polymerization reaction, biocompatible oils (caprylic/capric triglycerides and mono-/diglycerides) and surfactants (polysorbate 80 and sorbitan monooleate) were used to formulate the microemulsions. The obtained poly(ethyl 2-cyanoacrylate) is known to be biodegradable. Since insulin was confined to the aqueous phase in w/o emulsions, a high encapsulation efficiency (86%) could be

achieved. Insulin was released with initial burst over the first 30 minutes followed by a constant release up to 3 h, after which the release rate declined.

Limitations

Although this method was initially proposed for enzyme encapsulations, it has some serious problems in practice. First, large w/o interface is produced during the reaction, where proteins or enzymes are likely to be inactivated. Second, large amounts of proteins may participate in the polymerization reaction changing its biological activity. Third, it is often hard to control the polymerization reaction. The yield and quality of the membrane obtained by interfacial polymerization may be controlled by a number of factors, such as chemical natures of reactive monomers, and reaction conditions. The monomer concentrations, temperature, the mixing rate, and the reaction time are likely to be important parameters [105]. Fourth, exhaustive washing steps are required to remove monomers, byproducts, organic solvents, and surfactants. At the same time, several washing steps may lead to further loss of water soluble drugs. Fifth, pH change due to HCl byproduct formed by reaction of an acid chloride and an amine can damage the acid labile drugs.

SUPERCRITICAL FLUID PRECIPITATION

Supercritical fluid is defined as a fluid of which temperature and pressure are simultaneously higher than at the critical point, *i.e.* critical temperature Tc and critical pressure Pc, at which the density of gas is equal to that of the remaining liquid and the surface between the two phases disappears (Fig. 12). In practice, the term is often used to describe a fluid in the relative vicinity of the critical point, where a gas can be highly compressed with a small change of pressure into a fluid which is not a liquid but almost close to liquid in density. These two distinctive features of supercritical fluids (*i.e.*, a high compressibility and a liquid-like density) enabled supercritical fluids to attract considerable interest recently as vehicles for microparticle production.

Methods

There are two main routes to particle formation with supercritical fluids: the rapid expansion of supercritical solutions (RESS); and supercritical antisolvent crystallization (SAS) routes [108]. RESS exploits the liquid-like solvent power of the supercritical fluids while SAS uses supercritical fluid as an antisolvent. Carbon dioxide is most commonly used since the critical conditions are easily attainable, *i.e.*, Tc=31℃ and Pc=73.8 bar. CO_2 is environmentally benign, relatively non-toxic, non-inflammable, inexpensive, and has a reasonably high dissolving power. [109]

RESS(Rapid expansion of supercritical solutions): In RESS, the drug and the polymer are dissolved in a supercritical fluid at high pressure and precipitated by reducing the solvent's density through a rapid decompression (expansion) [110]. The actual solubility of the solutes in supercritical fluids can be higher than the value calculated assuming ideal gas behavior at the same temperature and pressure by factors of up to 10^6 [107]. Rapid expansion of supercritical solutions, therefore, can lead to very high supersaturation ratios. Since the supercritical fluid is a gas after expansion, the solid product is recovered in pure, solvent-free form.

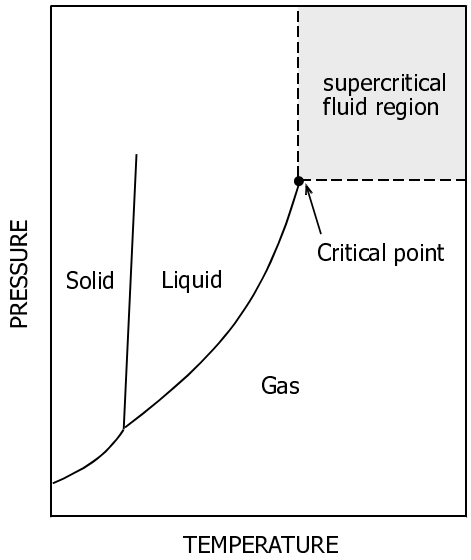

Fig. 12. Pressure-temperature projection of the phase diagram for a pure component. From reference [107].

SAS(supercritical antisolvent crystallization): The method is also called as ASES (aerosol solvent extraction system) or PCA (precipitation with a compressed fluid antisolvent). In SAS, a supercritical fluid is used as an anti-solvent that causes precipitation of solids. The solutes are dissolved in a suitable organic phase. At high pressures, anti-solvent enters into the solution and lowers the solvent power, thereby precipitating the solutes. The precipitation is followed by a large volume expansion and the solid product is collected. SAS process is illustrated in Fig. 13. As a modification of the SAS, the organic phase can be sprayed into a supercritical fluid, which causes the solutes to precipitate as fine particles. This method is also called gas-antisolvent (GAS) method. SAS is useful for processing of solids which are difficult to solubilize in supercritical fluids, such as peptides and proteins.

Applications

RESS offers distinctive advantages over the conventional methods, such as no requirements of surfactants, yielding a solvent-free product, and moderate process conditions [107]. However, the very low solvent power

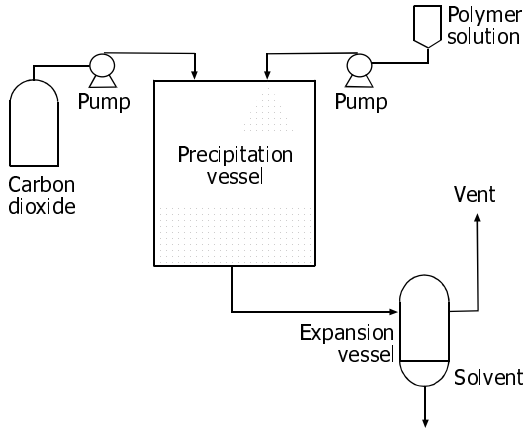

Fig. 13. Schematic diagram of the SAS method. From reference [111].

of common supercritical solvents toward the high molecular weight polymers and the solutes of therapeutic importance such as proteins has restricted RESS to a few low molecular weight polymers and a limited range of drugs, *e.g.*, lovastatin [107,112]. Lovastatin was dissolved in supercritical CO_2 together with DL-PLA and then subjected to RESS. The morphology of the coprecipitation product was found to be very sensitive to the relative amounts of drug and polymer, ranging from bicontinuous networks at high drug loading to drug needles encapsulated within polymer at low drug loadings.

SAS has been applied to protein drugs for size reduction [113] and bioerodible polymers for microencapsulation of pharmaceuticals in a polymer matrix [114]. Recently, this technique was used to produce PLGA microspheres containing lysozyme [115]. PLGA in dichloromethane solution with suspended lysozyme was sprayed into a CO_2 vapor phase through a capillary nozzle to form droplets which solidified after falling into a CO_2 liquid phase. In this study, several problems previously encountered in SAS process were overcome. Particle size (70 μm) large enough to encapsulate protein particles was achieved by delayed precipitation using CO_2 vapor phase above a CO_2 liquid phase. Agglomeration due to plasticization of the polymer by CO_2 was minimized at the optimum temperature of –20°C. Since many proteins are insoluble in organic solvents and are less likely to be denatured when suspended, this method can be extended to other proteins.

Advantages and Limitations

Supercritical fluids, particularly CO_2 offers various advantages when compared with widely used organic solvents. It is relatively non-toxic, environmentally acceptable, non flammable, and inexpensive. In general, particles with narrow size distribution can be achieved using supercritical fluid methods. Potential advantages also include mild process temperatures, the potential for scale-up and the possibility for aseptic preparation of

Table 6. Examples of micorencapsulation of maromolecular drugs by IG/PEC

Protein	IG/PEC system	Encapsulation efficiency	Particle size	Release kinetics	Ref.
Salmon calcitonin	Chitosan-TPP	54-59%	0.9 mm	Initial burst (<20%) within 1day followed by zero order release for a month	[81]
Insulin	Chitosan-TPP	> 87%	300-400 nm	Fast release within 1 h (~100%)	[78]
Bovine serum albumin	Chitosan/ PEO/PPO-TPP	Varies with formulations	200-1000 nm	Zero order release for 1 week	[80]
Dextran	Alginate-Calcium-Chitosan	49-89%	0.91 -1.07 mm	Zero order release for 6 h	[97]
Horseradish preoxidase	ι carrageenan - amines	1-72% (depending on amine employed)	50 μm	Zero order release (98%) for 100 mins	[84]

the microspheres [116]. In RESS, it is possible to achieve solvent free solids in a single processing step. Complicated recovering process is not required because the solvent (CO_2) expands to a gas and is separated from solid particle by gravity. The process does not include toxic organic solvents nor produce w/o interface where many proteins may be denatured. However, RESS is limited by the constraint that all solutes be soluble in the supercritical fluid. For this reason, RESS may not be used for protein encapsulation using high polymers, because of their low solubility in common supercritical fluids. Moreover, many process variables affect the morphology of RESS powders, and thus the morphology of the precipitate can be difficult to control and predict [108]. SAS, as compared with RESS, is more flexible in solvent choice. This technique allows the processing of more concentrated polymer solution and also the use of drug suspensions. One of the disadvantages of SAS is that as a result of plasticization of the polymer by CO_2, polymers with low glass transition temperatures often agglomerate even at low temperatures [115].

FUTURE OF MICROENCAPSULATION TECHNOLOGIES

Since the concept of controlled drug delivery was introduced in 1970s, great progresses have been made in the microencapsulation areas thanks to the achievement of polymer chemistry and evolution of intelligent microencapsulation techniques. Protein drugs have been one of the most attractive candidates of controlled drug delivery, ever since the explosive growth of biotechnology and genetic engineering. As reviewed previously, a number of efforts were devoted to develop a microcapsule system ensuring constant release rate over desired period and structural integrity of the protein. However, most of the current methods have several limitations such as unfavorable conditions for the drug entity, complicated procedure, low encapsulation efficiency,

burst effect and incomplete release behavior. As an ideal encapsulation technique, one should satisfy the following conditions. First, the manufacturing conditions should be mild enough to conserve the protein stability during the process. Oil-water interface, shear forces, or heat may be major causes of protein denaturation and aggregation. Second, high encapsulation efficiency is important. Even if the progress of genetic engineering made many therapeutic proteins available at a lower price than before, protein drugs are in general much more expensive than other low molecular drugs. Third, it is preferable to minimize the exposure to toxic organic solvents. Fourth, the manufacturing process should be reproducible and easy to scale up to the commercial scale. Also, aseptic manufacturing should be available. Fifth, in order to ensure product quality as an injectable formulation, uniform size distribution of the microparticles should be achievable.

Considering numerous novel protein drugs coming from the genome projects, new microencapsulation systems that minimizes use of toxic organic solvents and protein denaturating conditions will be invaluable tools in the future, and this is an exciting time to be involved in the microencapsulation technology.

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[Received May 22, 2001; accepted July 24, 2001]