A Supercritical Fluids Extraction Process for the Production of Drug Loaded Biodegradable Microparticles

BY

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ABSTRACT


The purpose of this thesis was to develop methods suitable for the incorporation of drug substances such as proteins into microparticles intended for controlled release. In particular a novel technique for the preparation of microparticles using supercritical fluids was investigated.

Supercritical fluids offer a considerable promise as extraction media for the formation of microparticles of drugs and pharmaceutical excipients. There are two main reasons for using this technique. Firstly, the selective solvating power of supercritical fluids makes it possible to separate a particular component from a multi-component mixture. Secondly, the favourable mass transfer properties and high solubility of solvent in supercritical fluid make the formation of the microparticles rapid and efficient.

The Solution Enhanced Dispersion by Supercritical fluids process (SEDS) was used for the production of microparticles from several different biodegradable polymers. Briefly, particles were formed by the extraction of solvent from a solution which was sprayed into a supercritical fluid.

The use of a combination of supercritical N\textsubscript{2} and CO\textsubscript{2} in the SEDS process, improved the dispersion of polymer solutions, as compared with CO\textsubscript{2} alone. This resulted in reduction of the particle size of discrete microparticles produced from amorphous biodegradable polymers. Proteins (lysozyme and urease) were successfully incorporated into the poly(d,l-lactide-co-glycolide): copolymer composition 50:50 (DL-PLG) microparticles. The particles showed high entrapment efficiencies and the incorporated proteins retained a high degree of biological activity. Compared with conventional technologies for the preparation of such drug delivery systems, e.g. solvent-evaporation emulsion techniques, this new technique is environmentally superior, and suitable for up-scaling. Moreover the higher degree of control as indicated by the high reproducibility, makes validation of the process feasible. In conclusion, the SEDS process is an attractive way of incorporating proteins and peptides into biodegradable microparticles for controlled release.

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To my parents
and
my youngest brother Kambiz
CONTENTS

PAPERS DISCUSSED 7

INTRODUCTION 8

METHODS OF MICROCAPSULATION 9
   Single emulsion-solvent evaporation method 9
   Double emulsion-solvent evaporation method 11
   Particle precipitation by non solvent addition (Coacervation) 12
   Particle precipitation by solvent partitioning 12
   Spray drying 13
   Supercritical fluid extraction methods 14

SUPERCRITICAL FLUIDS 14

PHASE BEHAVIOUR 17
   Phase diagram types for binary mixtures 18
      Type I 18
      Type II 18
      Type III 19
      Type IV 20
      Type V 20
      Solid- supercritical fluid systems 20
   Phase diagrams for ternary mixtures 21

PARTICLE PROCESSING WITH SUPERCRITICAL FLUIDS 23
   Rapid Expansion of Supercritical Solutions (RESS) 23
   The Gas Anti-Solvent (GAS) 23
   Solution Enhanced Dissolution by Supercritical Fluids (SEDS) 25

PAPERS 27
   Effect of preparative parameters on the characteristics of poly (d,l-lactide-co-glycolide) microspheres made by the double emulsion method (Paper I) 27
      Results and discussion 27
   Biological activity of Lysozyme after entrapment in poly (d,l-lactide-co-glycolide)- microspheres (Paper II) 30
      Results and discussion 30
   Preparation of biodegradable microparticles using solution-enhanced dispersion by supercritical fluids (SEDS) (Paper III) 31
      Results and discussion 32
   A new method for preparing biodegradable microparticles and entrapment of hydrocortisone in DL-PLG microparticles using supercritical fluids (Paper IV) 33
      Results and discussion 33
A new process for the incorporation of proteins into DL-PLG microparticles using supercritical fluids (Paper V)

Results and discussion

CONCLUSIONS

ACKNOWLEDGMENTS

REFERENCES
PAPERS DISCUSSED


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INTRODUCTION

Interest in the advantages of using controlled drug delivery systems is increasing in modern pharmaceutical development. In practice, the advantages of these systems include

• location of the drug at the site of action, e.g. in antitumour therapy (Bastian et al., 1998; Benoit et al., 2000 and Taguchi et al., 1992)

• prolongation of drug release, e.g. in hormone therapy (Zoladex ®)

• incorporation of sensitive drugs such as peptides and proteins into polymeric microparticles to protect them from chemical or enzymatic degradation (Cohen and Bernstein, 1996; Jalil and Nixon, 1990; Langer and Folkman, 1976; Vanberver et al., 1999 and Winters et al., 1996)

• increasing the immunogenic response of antigens entrapped in oral microsphere vaccine formulations; the particulate nature of these formulations can markedly increase the immunogenic response compared with that to solutions of free antigens (Service, 1994; Morris and Steinhoff, 1994).

In the development of drug delivery systems, biodegradable polymers such as poly (d,l- lactide-co-glycolide) (DL-PLG) microspheres are frequently considered as drug carriers for future pharmaceutical products. There are two main reasons for this. Firstly the material is biocompatible and degrades in vivo by forming the nontoxic monomers, lactic acid and glycolic acid. Secondly, the release rate of the entrapped drugs can be controlled by varying the molecular weight and the copolymer ratio. Furthermore, administration of the drug by injection is possible if it is dispersed into microspheres (Service, 1994; Aftabroushad and Doelker, 1994 and Morris and Steinhoff, 1994).

Biodegradable microparticles can be prepared by various methods but perhaps the most thoroughly investigated methods are the single and double emulsion evaporation methods. Because of various problems that have been encountered in emulsion evaporation methods, drug-loaded microparticles have recently been produced using supercritical fluid extraction technology. Supercritical fluids are used as extraction media to form microparticles of drugs and pharmaceutical excipients (MacHugh and Krukonis, 1994; Fredriksen et al., 1997; Subramaniam et al., 1997 and Eckert et al., 1996). This method offers the advantage of being a one-step process, and appears to be superior to other conventional incorporation methods such as emulsion evaporation methods.
METHODS OF MICROENCAPSULATION

A wide range of microencapsulation techniques have been developed. The selection of a particular technique depends on the nature of the polymer and the drug to be incorporated.

Single emulsion- solvent evaporation method

Microencapsulation of the polymer can be prepared using either oil-in-water (O/W) or oil-in-oil (O/O) emulsion systems. In the O/W emulsion technique, the polymer is dissolved in an organic solvent (the oil phase) such as methylene chloride or chloroform. The drug is either dissolved or suspended in this solution, which is then emulsified by adding a larger volume of water containing a suitable emulsifier. The organic solvent is then removed by evaporation or extraction, resulting in phase separation of the polymer and the drug to produce solid microparticles, suspended in an aqueous phase. A flow diagram for this technique is shown in figure 1. This technique has been used for the incorporation of various steroids by many research groups (Cavalier et al., 1986; Smith and Hunneyball, 1986 and Benita et al., 1984). However, the entrapment efficiency (EE) of hydrophilic drugs is poor using this technique since they partition out from the organic phase into the aqueous continuous phase.

![Flow diagram for the O/W single emulsion-solvent evaporation method.](image)

**Figure 1:** Schematic diagram showing the preparation of drug loaded microparticles by the O/W single emulsion-solvent evaporation method.
Successful entrapment of drug within the microspheres is therefore highly dependent on the solubility of the drug in the aqueous phase (Bodmeier and MacGinity, 1987a.). Water soluble drugs such as caffeine, theophylline and salicylic acid, for example, cannot be entrapped within polymer microspheres produced by an O/W emulsion technique. In contrast, drugs such as hydrocortisone, which are poorly soluble in water, have been successfully entrapped within microspheres using the O/W emulsion technique (Bodmeier and McGinity, 1987b. and Fong et al., 1986). Diffusion of ionised drugs such as quinidine sulphate from the organic phase to the aqueous phase can be minimised by adjusting the pH of the aqueous phase to suppress ionisation (Bodmeier and MacGinity, 1987a.). The un-ionised drug has a higher partition coefficient and remains in the organic phase, improving the degree of microsphere loading.

Alternatively, the drug can be prevented from partitioning out of the organic phase by saturating the aqueous phase with the drug before emulsification. The type and concentration of the surfactant agent (the emulsifier) in the aqueous phase affects the size and shape of the microspheres and can even affect the EE of the drug (Bodmeier and MacGinity, 1987a. and Wakiyama et al., 1981).

**Figure 2:** Schematic diagram showing the preparation of drug loaded microparticles by the O/O single emulsion-solvent evaporation method.

O/O emulsion systems can be used to improve the loading of water-soluble drugs into polymers. In these systems, the polymer is dissolved in acetonitrile (the dispersed phase) which is subsequently emulsified with liquid paraffin (continuous phase)
containing an oil-soluble emulsifier such as a sorbitan ester with a low HLB value. Removal of the volatile solvent (acetonitrile) by heat results in coprecipitation of the polymer and drug to form drug-loaded microparticles. A schematic diagram of this method is shown in figure 2.

The properties of microspheres can be affected by changing various processing parameters, such as emulsifier concentration, emulsifier type, polymer concentration, viscosity, stirring rate and solvent evaporation rate. If polymers of high molecular weight are used, precipitation will be rapid and the resultant microspheres will be porous. The more hydrophilic polymers result in smoother and less porous microspheres with the O/O emulsion method (Jalil and Nixon, 1990).

Double emulsion- solvent evaporation method

Sensitive water-soluble drugs such as proteins can be incorporated into microspheres using an alternative method: the double emulsion method. This method decreases contact between the organic phase and the active substance and improves the EE of water-soluble drugs. This preparation method could be also used for incorporation of exclusive substances since it may be performed in a small-scale (Jalil and Nixon, 1990; Morris et al., 1994; Paper II and Sturesson et al., 1999). Briefly, the water soluble drug is initially dissolved in a small aqueous aliquot, which is subsequently emulsified with the organic polymer solution to form a water-in-oil (W/O) emulsion. In a second emulsification, this emulsion is added to an aqueous solution containing an emulsifier, and a water-in-oil-in-water (W/O/W) double emulsion is the result. The organic solvent is then removed to leave an aqueous suspension of microspheres containing the drug. The microspheres are isolated by centrifugation, washed in water three to five times depending on the size of the batch, and freeze-dried (see figure 3).

Figure 3: Schematic diagram showing the preparation of microparticles by the W/O/W double emulsion-solvent evaporation method.
Particle precipitation by nonsolvent addition (Coacervation)

In this method microparticles are produced by dispersing either the solid crystal particles or an aqueous solution of the drug in an organic solution of polymer, followed by a phase separation by adding a second organic solvent in which the polymer is not soluble (defined here as a “nonsolvent”). The water soluble drug naferaline acetate for example, has been incorporated in DL-PLG microparticles using this method (Sanders et al., 1984 and 1985). An aqueous solution of the drug was emulsified in a polymer solution of DL-PLG and methylene chloride to produce a W/O emulsion. The addition of a nonsolvent resulted in precipitation of the polymer around the aqueous solution of drug to form microparticles. The addition of a large volume of the non solvent completes the extraction of the polymer solvent and hardens the microspheres. A similar method has been used for incorporation of oxytetracycline, but in this case the solid drug particles were suspended in the organic polymer solution (Vidmar et al., 1984).

The particles produced by this method have a wide size distribution, which is not desirable for their intended clinical use. Microparticles produced by this method also tend to aggregate to a high degree. The outcome of this method can be altered by changing preparation parameters such as the drug: polymer ratio, the polymer solvent, the stirring rate, the temperature at which the process takes place, or the volume or type of nonsolvent.

Particle precipitation by solvent partitioning

In this method, a solution or suspension of the drug in the polymer/organic solvent solution is slowly injected into a stream of mineral oil. Since the organic solvent is soluble in the oil, but the drug and the polymer are not, coprecipitation of the drug and polymer occurs as the mixture partitions into the oil. The outcome will depend on the solubility of the drug. If the drug is soluble in the polymer solution, the drug and polymer precipitate together. If the drug is suspended in the polymer solution, the polymer will precipitate around the solid drug particles.

Hydrocortisone has been incorporated into polylactide polymer microparticles using this method. The microparticles were however, relatively large. The particle size ranged from 144 \( \mu \text{m} \)-412\( \mu \text{m} \), depending on the flow rate and the diameter of the needle through which the drug/polymer mixture was injected (Leelarasamee et al., 1988).

With this method, preparation parameters that affect the size of the microparticles include the diameter of the needle, the drug:polymer ratio, the flow rate of the mineral oil, and the choice of polymer solvent.
Spray drying

In this technique the drug is dissolved or suspended in the organic polymer solution and the resultant mixture is spray dried to form microspheres (Bodmeier and Chen, 1988; Shell, 1978 and Wise et al., 1976). The advantage of this technique is that water soluble and insoluble compounds can be incorporated into the spheres, in contrast to the single O/W emulsion evaporation system which is unsuitable for water soluble compounds. Progesterone and theophylline have been incorporated into polylactide microparticles using this method (Bodmeier and Chen, 1988).

This system is, however, associated with some drawbacks. For example, needle-shaped crystals were formed when caffeine was incorporated using this method into a polylactide polymer, possibly the result of incompatibility between the drug and the polymer (Bodmeier and Chen, 1988). Similarly, fibres may be formed because of insufficient dispersion force to break up the polymer solution. The choice of organic solvent is also important; the polymer should be dissolved in solvents such as methylene chloride, ethyl acetate or an expensive fluorinated (hexafluoroisopropanol) solvent, because these solvents evaporate quickly in the heated air in the drying phase and because the polymers used are often insoluble in common organic solvents.

Furthermore, since the particles are exposed to a large volume of heated air during the extraction step, the stability of oxidation-sensitive or thermolabile drugs may be affected. Although nitrogen would avoid oxidation of the drugs if substituted for air in this phase, the heat conductivity of nitrogen is less than that of air, which would affect the outcome. Particles of 5 to 125 µm in diameter are produced using this method.
Supercritical fluid extraction methods

Micronisation and particle size reduction are fascinating areas in pharmaceutical technology that have been used to overcome problems involving the solubility or targeting of the drug.

Conventional size reduction methods required crystallisation of the substances before the process could proceed. During this phase, the crystals can grow in size uncontrollably. When mechanical force is used to reduce the size of the crystals, the particles often develop charged surfaces and become more cohesive. Other disadvantages associated with crystallisation include:

• the processes are time-consuming and costly
• the resultant particle size distribution is polydispersed with a wide size range
• toxic organic solvents are used in the crystallisation process and residual solvent in the recrystallised drugs may exceed the authorised levels.

The use of supercritical fluids as extraction media is a promising alternative for the formation of microparticles of drugs and pharmaceutical excipients (Fredriksen et al., 1997; Eckert et al., 1996; Hanna et al., 1995; Marr and Gamse, 1999; McHugh and Krukonis, 1994; Reverchon, 1999; Subramaniam et al., 1997 and York, 1999). Pioneering studies on the production of microparticles of biodegradable polymers using different supercritical fluid extraction methods have been reported in the literature (Bleich et al., 1993; Bodmeier et al., 1995; Pablo et al., 1993; Paper III; Thies and Müller, 1997 and Tom et al., 1993). There are two main reasons for using this technique. Firstly, the selective solvating power of supercritical fluids makes it possible to separate a particular component from a multicomponent mixture. Secondly, the favourable mass transfer properties and high solubility of solvents in the supercritical fluid make the drying of the microparticles rapid and efficient with low level of residual solvent as requested by the authorities (Folker et al., 1996).

In a recent study, it was shown that the supercritical carbon dioxide can be used for inactivation of a wide variety of bacterial organisms. This means that the final product (microparticles) produced by supercritical carbon dioxide are sterilised (Dillow et al., 1999).

In addition, solvents and the anti-solvent gas involved in the supercritical fluid extraction processes can be recycled, thus minimising waste, an attractive prospect from both environmental and economical points of view. The extractive supercritical fluids used are generally non-toxic, e.g. CO₂, N₂ etc.

The properties of supercritical fluids are explained in more detail below.

SUPERCRITICAL FLUIDS

For every substance, there is a critical temperature (Tc) and pressure (Pc) above which no applied pressure can force the substance into its liquid phase (the critical point, see figure 4). If the temperature and pressure of a substance are both higher than the Tc and Pc for that substance, the substance is defined as a supercritical fluid. At the critical point, the density of the gas and liquid phases is the same; there is no distinction between the phases.
Supercritical fluids are highly compressible with densities that are liquid-like and transport properties that are gas-like (see table I). The range of densities of the most common supercritical fluids is from 0.1-0.9 g/ml under normal working pressures (75-450 bar). The changes in density of a pure substance near its critical point can be seen in figure 5 (relative critical pressure).

**Figure 4:** Pressure-temperature diagram for a pure component.

Small changes in pressure or temperature near the critical point can greatly modify the density and, hence, the solubilising power of the supercritical fluid. Thus the physicochemical properties of supercritical fluids can be varied significantly without changing the molecular structure of the substance. This ability to change the properties of the supercritical fluid by changing the temperature and pressure in the vicinity of the critical point provides the equivalent of a series of different solvents, thus providing selective extraction properties.

**Table I:** Density, viscosity and diffusivity of gases, liquids and supercritical fluids.

<table>
<thead>
<tr>
<th>Physical state</th>
<th>Density (g/ml)</th>
<th>Viscosity (g/cm x s)</th>
<th>Diffusivity (cm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas</td>
<td>$10^{-3}$</td>
<td>$10^{-4}$</td>
<td>$10^{-1}$</td>
</tr>
<tr>
<td>Liquid</td>
<td>1</td>
<td>$10^{-2}$</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Supercritical fluid</td>
<td>0.2-0.9</td>
<td>$10^{-4}$</td>
<td>$10^{-5}$</td>
</tr>
</tbody>
</table>
The most useful extraction region is that in which the relative pressure \( (P_R = \frac{P}{P_C}) \) ranges from 1.01-1.5 and the relative critical temperature \( (T_R = \frac{T}{T_C}) \) ranges from 1.01-1.1 (Brenneck and Eckert, 1989).

Although the density of supercritical fluids is liquid-like under normal working pressures, these fluids exhibit gas-like transport properties (viscous with a very low surface tension). This allows easy penetration of the liquid into microporous materials.

\[ \rho_R = \frac{\rho}{\rho_C} \]
\[ T_R = \frac{T}{T_C} \]
\[ P_R = \frac{P}{P_C} \]

\( \rho_R \) = Relative critical density.
\( T_R \) = Relative critical temperature.
\( P_C \) = Critical pressure.
\( T_C \) = Critical temperature.
\( \rho_C \) = Critical density.

**Figure 5:** Variation of the reduced density of the a pure compound in the vicinity of its critical point.
Many supercritical fluids offer the advantage of separating compounds from a mixture by using either vapour pressure or polarity differences between the compounds. The solubility of a solute in supercritical fluids can also be altered tremendously by adding one or more extra solute and/or solvent. For example, the polarity of supercritical fluids can be significantly increased without affecting the supercritical condition by adding 1 to 10 volume percent of a polar substance (Clifford, 1999). An enhancement factor (a dimensionless measure of solvent power defined as the measured solubility of the substance in the solvent divided by the solubility in an ideal gas) of $10^4$ to $10^6$ is common (McHugh and Krukonis, 1994). Page and coworker have made a comprehensive review of modifiers, which are commonly used (Page et al., 1992).

This particular property of supercritical fluids has been utilized for purification and separation purposes in food processing and distillation industries as well as in analytical chemistry applications for many years (Bleich et al., 1993; Hawthorne, 1990; Braun et al., 1985 and Hubert and Vitzhum, 1980). Decaffeination of tea and coffee beans and purification and fractionation of polymers from residual solvent and monomers are examples of its use (McHugh and Krukonis, 1994).

Mechanisms reported in the literature that have been used to enhance the solubility of supercritical fluids include

- Hydrogen bonding
- Charge transfer complex formation
- Dipole-dipole interactions
- Dipole-induced dipole interactions
- Induced dipole-induced dipole interactions
- Solute-solute, solute-cosolvent, cosolvent-cosolvent and solvent-solvent interactions.

The critical temperature of the most common supercritical fluids is near ambient temperature, thus allowing their use with heat-sensitive compounds (see Table II). Carbon dioxide (CO$_2$) is one of the most extensively used supercritical fluids because of its relatively low critical temperature and pressure ($T_c=31.1$ °C, $P_c=73.8$ bar). The low critical temperature of CO$_2$ makes it attractive for processing heat-sensitive flavours, pharmaceuticals and labile lipids. In addition, CO$_2$ is nontoxic and nonflammable, prevents oxidative degradation and formation of artefacts, is inexpensive and has a relatively high dissolving power compared to other supercritical fluids.

**PHASE BEHAVIOUR**

Phase diagrams are used to indicate the possible solubility levels of a compound in a supercritical fluid over a wide range of temperatures and pressures.

There are five types of phase diagram to describe binary mixtures consisting of a single supercritical fluid solvent and a single solute (see below). If the phase behaviour is understood for this limited situation, it is possible to form a basis for
generalisation of the phase equilibrium principles that exist during the supercritical fluid solvent extraction of mixtures. In addition, knowledge of the types of phase diagrams applicable to the mixture under investigation can save time and effort when determining the solubility of a compound in a supercritical fluid over a wide range of pressures and temperatures.

For a full understanding of phase diagrams for mixtures, it is important to consider the geometric constraints on their topology imposed by the phase rule (Streeft, 1983), as follows:

Degrees of freedom = number of components + 2 – number of phases

This equation provides the number of independent variables that must be set in an equilibrium system. For example, for a two-phase binary mixture the regions of the equilibrium phases can be obtained either by experiment or calculation if the temperature and pressure are fixed. In a two-phase ternary mixture, one more variable (usually the overall mixture composition) must also be fixed.

Phase diagram types for binary mixtures

Type I

The simplest binary mixture behaviour is seen in type I phase diagrams. The two components are similar in molecular diameter, size, and intermolecular forces, and are miscible in all proportions. Only a single liquid phase exists throughout the phase diagram (Rowlinson and Swinton, 1982).

The type I phase diagram of a binary mixture with critical points $C_1$ and $C_2$ of the respective components, is illustrated in figure 6a. As can be seen, in type I diagrams, the critical mixture curve runs continuously from the critical point of the more volatile component to that of the less volatile component. A binary mixture of CO$_2$ and propane is an example of this type of behaviour.

Type II

Type II phase diagrams apply to binary mixtures in which the two components are not miscible in all proportions, despite a critical mixture curve similar to that in type I diagrams (i.e. running continuously between the critical points of the two pure components). Typically, an upper critical end point (UCEP) will exist. At this point, the liquid and gaseous phases of one of the components become critically identical in the presence of the liquid phase of the second component.

Figure 6b illustrates the UCEP at which the liquid-liquid ($L_1L_2$) and liquid-liquid-vapour ($L_1L_2V_2$) curves meet at a point lower than $C_1$ or $C_2$.

The CO$_2$ -n-octane system is an example of type II binary mixture behaviour.
**Figure 6:** Phase diagrams for binary mixtures. Point $C_1$ and $C_2$ are the critical points of components 1 and 2, respectively. The dashed curve in each figure is the critical mixture curve for the binary mixture. The open asterisks are critical end points.

**Type III**

In type III phase diagrams, a lower critical solution temperature (LCST) is observed (figure 6c). At the LCST point the two liquid phases of the LLV line become identical (e.g. density) in the presence of the component with the lower critical point ($C_1$). Thus, at temperatures lower than the LCST, the liquids are no longer miscible in all proportions. It should be keep in mind that the critical properties of any substance are a function of the molecular weight, molecular structure and intermolecular forces.
Type III behaviour occurs when the difference in molecular size between the components reaches a certain value. Since similar phase behaviour is observed in polymer-solvent systems, the occurrence of an LCST is very important (Zeman et al., 1972 and Zeman and Patterson, 1972).

Type IV

Type IV phase behaviour is indicated in the diagram (figure 6d) by discontinuity of the critical mixture curve between the critical points of the components. This type of behaviour is seen if there is a large difference in size, molecular structure or strength of the intermolecular forces between the pure components of the mixture. Although the shape of the critical mixture curve from the C₂ point depends on the nature of the components of the mixture, most end at high pressures. A typical critical mixture curve in a type IV binary mixture phase diagram would start from C₁ and meet the LLV curve at the UCEP (McHugh and Krukonis, 1994). The CO₂-hexadecane system provides an example of this type of behaviour.

Type V

The type V phase behaviour is very similar to type III as can be seen in figure 6e. The only difference between types III and V is the absence of the region of liquid immiscibility at temperatures below the LCST.

Solid-supercritical fluid systems

Figure 7 shows an example of the simplest pressure-temperature diagram (P-T diagram) for a solid-supercritical fluid system. For these types of binary mixtures, the melting temperature of the solid is usually higher than the critical temperature of the supercritical fluid.

![Graph showing a simplified P-T diagram for a solid-supercritical fluid system.]

Figure 7: Pressure-temperature diagram for a heavy solid-supercritical fluid system.
In general, the melting point of the pure solid increases with increasing pressure. In contrast, in the presence of a supercritical fluid, the melting point of the solid is decreased as the pressure increases. The solid-liquid-vapour (SLV) line in figure 7 indicates the depression of the solid melting point. The reason for this depression in the melting point is that, as the pressure increases, more and more of the gas dissolves in the liquid phase and therefore the temperature needed to melt the heavy component (solid) decreases. Curves MH and CD in figure 7 are the pure component vapour pressure curves of the heavy component (solid) and light component (supercritical fluid), respectively. The melting curve and the sublimation curve of the pure heavy component are indicated as MN and EM, respectively. Points D and H represent pure component critical points. The critical mixture curve runs continuously between the critical points of the two components in this type of phase diagram.

Phase diagrams for ternary mixtures

The single largest collection of ternary phase diagrams for mixtures with liquid CO₂ at a pressure of 800 bar and temperature of 25 °C were prepared by Francis in 1954. Despite the fact that these studies were not extended to the supercritical region for CO₂, these phase diagrams provide the basis for the qualitative estimation of the solubility of various compounds in supercritical CO₂. Nonetheless, there is a limited number of ternary phase diagrams reported in the literature for mixtures including a supercritical fluid as one of the components. The problem is the complexity of the system.

Figure 8: Phase diagram of Carbon dioxide- methanol- water at 50°C and 200 bar. Predicted from Peng-Robinson equation state.
Some studies of ternary mixtures have indicated that the solubility of the first component in the supercritical (second) component increases if the third component is soluble in the supercritical component (co-solvents) (Kurnik et al., 1981; Gopal et al., 1983). Figure 8 provides an example of such a system for a ternary mixture of supercritical CO₂- methanol- water at 50 °C and 200 bar.

Table II: Critical temperatures and pressures of commonly used supercritical fluids

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Critical Temperature (°C)</th>
<th>Critical Pressure (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene</td>
<td>9.3</td>
<td>50.4</td>
</tr>
<tr>
<td>Ethane</td>
<td>32.2</td>
<td>48.8</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>31.1</td>
<td>73.8</td>
</tr>
<tr>
<td>Propane</td>
<td>96.7</td>
<td>42.5</td>
</tr>
<tr>
<td>Ammonia</td>
<td>132.5</td>
<td>112.8</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>235.2</td>
<td>47.6</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>280.3</td>
<td>40.7</td>
</tr>
<tr>
<td>Benzene</td>
<td>289.0</td>
<td>220.5</td>
</tr>
</tbody>
</table>

This phase diagram shows that the supercritical CO₂ is miscible in all proportions with methanol, since the binodal curve no longer intersects the supercritical CO₂- methanol binary axis of the ternary diagram. At the same time, it is apparent that the supercritical CO₂ is not soluble in water, because the binodal curve intersects the supercritical CO₂- water binary axis at two locations. The tie line for the ternary system indicates that a liquid phase, mainly a mixture of methanol and water, is in equilibrium with a fluid phase, mainly a mixture of the supercritical CO₂ and methanol. The slope of the tie line indicates that the methanol, which is miscible with water at low pressures, is now selectively extracted from the methanol- water mixture with supercritical CO₂. The point CP on the graph indicates the only point at which a supercritical phase occurs at this fixed temperature, pressure and composition of methanol: water. At 50 °C and 200 bar, a single supercritical fluid is formed from mole fraction of almost 0.97 CO₂ and a mole fraction of 0.03 in a composition of methanol and water.

The literature provides examples of thermodynamic models for the prediction and estimation of the solubility of various compounds in supercritical fluids, but these are beyond the scope of this thesis.
PARTICLE PROCESSING WITH SUPERCRITICAL FLUIDS

There are various methods of processing materials using supercritical fluids. The choice of method depends largely on the solubility of the material of interest in the appropriate supercritical fluid. The following methods have been used (Pablo et al., 1993).

Rapid Expansion of Supercritical Solutions (RESS)

The Rapid Expansion of Supercritical Solutions (RESS), can be used when the substance of interest is highly soluble in the supercritical phase. The substance is dissolved in the supercritical phase and the solution is then expanded rapidly by depressurising the system, so that the substance precipitates as very small particles (Matson et al., 1987 and Tom and Debenedetti, 1991).

Gas Anti-Solvent (GAS)

The Gas Anti-Solvent (GAS) process can be used for crystallisation of substances which are only slightly soluble or not soluble at all in the supercritical fluid. Briefly, the process is performed by first dissolving the substance of interest in the organic solvent. When the supercritical fluid, which has a low solvent capacity with respect to the substance but is completely miscible with the organic solvent, is added to the solution the substance precipitates. The key to the process is the volumetric expansion of the organic solvent due to its contact with the supercritical fluid. The increasing amount of anti-solvent (supercritical fluid) in the solution and the evaporation of the organic solvent along with its extraction into the supercritical fluid eventually cause the precipitation of the solid substance (Gallagher et al., 1990; Gallagher-Wetmore et al., 1994 and Yeo et al., 1993).

Several methods based on the latter approach have been studied for application to material processing, especially particle formation. Among these are the Supercritical Anti-solvent (SAS) process (Winters et al., 1996 and Werling and Debenedetti, 1999), the Precipitation with Compressed Anti-solvents (PCA) process (bodmeier et al., 1995 and Thies and Müller, 1997), the Aerosol Solvent Extraction System (ASES) (Bleich et al., 1993, 1994) and the Solution Enhanced Dispersion by Supercritical fluids (SEDS) process (Hanna et al., 1998, 1995 and Paper III). The main differences among these processes involve the manner in which the substance solution is introduced into the supercritical fluid (see figure 9a, b, c).
Figure 9: Schematics of the (a) RESS, (b) GAS, and (c) PCA/SAS/SAES processes.
Solution Enhanced Dispersion by Supercritical fluids (SEDS)

A schematic diagram of the modified SEDS apparatus used for the formation of microparticles is shown in figure 10. In this thesis the process has been modified in order to improve the dispersion properties for noncrystalline polymers by using a combination of two supercritical fluids.

Briefly, in the original version of the process, a suitable anti-solvent gas is fed from source (A) to a cooler (B), in order to ensure the liquefaction of the gas and to prevent cavitation. The anti-solvent is then fed by a conduit from the cooler to a high pressure pump (C). From there it is pumped to the high pressure vessel (D) via a coaxial nozzle (G). A saturated solution of polymer, in a suitable organic solvent, is drawn from
source (H) by a conduit to the high pressure pump (C) and is fed to the high pressure vessel (D) via the coaxial nozzle (G). The supercritical anti-solvent leaves the high pressure vessel and flows to the back pressure regulator (I) which controls the pressure discharge in the system. The organic solvent is extracted into the supercritical fluid, resulting in the formation of solid microparticles in the vessel (D).

In the modified version, CO$_2$ is fed from its source (A) to a cooler (B) in order to liquefy the gas and to prevent cavitation. The CO$_2$ is then fed by a conduit from the cooler to a high pressure pump (C). From there it is pumped to the high-pressure vessel (D). The supercritical N$_2$ is fed from its source (E) by a conduit through a fine metering valve (F) and then into the high-pressure vessel (D). G is the dispersing nozzle. A saturated solution of polymer, in a suitable organic solvent, is drawn from its source (H) by a conduit to the high-pressure pump (C) and fed to the high-pressure vessel (D) through the nozzle (G). The supercritical CO$_2$ and N$_2$ mixture leaves the high-pressure vessel and flows to the back-pressure regulator (I) which controls the pressure discharge in the system. The organic solvent is extracted into the supercritical fluid, resulting in the formation of solid microparticles in the vessel (D). The particles are retained in the vessel by a suitable glass fibre paper filter. For further information regarding the details of the nozzle we refer to the SEDS-patent (Hanna and York, 1994).
## PAPERS

The purpose of this thesis was to develop methods suitable for the incorporation of drug substances such as proteins into microparticles intended for controlled release. In particular a novel technique for the preparation of microparticles using supercritical fluids was investigated.

### Effect of preparative parameters on the characteristics of poly(d,l-lactide-co-glycolide) microspheres made by the double emulsion method (paper I)

In this study we have incorporated small amounts of mannitol$^{14}$C into poly(d,l-lactide-co-glycolide) (DL-PLG) microspheres using a small-scale double-emulsion method. The inner aqueous phase volumes used for dissolving the model drug, mannitol$^{14}$C, were between 50 and 400 $\mu$l and the total volume of the double emulsion was about 7 ml. The use of a small-scale method is an advantage when incorporating substances such as highly purified proteins or antigens. In the study, it was shown how the variation of some experimental parameters in this method, influenced the characteristics of the resulting microspheres i.e. size distributions, entrapment efficiency, morphology and drug release kinetics. The DL-PLG concentration in the middle phase of the w/o/w emulsion was varied from 4.3% (w/w) to 43%. Different volumes, 50 to 400 $\mu$l, of the inner aqueous phase were employed. The time interval of the second emulsification was varied from 5 seconds to 80 seconds. The effects on microsphere characteristics of using phosphatidylcholine (PC) as stabiliser were also investigated.

No specific interactions between the model drug mannitol$^{14}$C and the polymer were expected and a low degree of loading was employed. Under these conditions changes in the release of drug between different preparations were expected to reflect differences between the polymer matrices obtained.

### Results and discussion

Table III shows how the particle size distributions were affected when using different concentrations of DL-PLG in the oil phase at varying internal water phase volumes. The particle size increased linearly with increasing polymer concentration. The results are in agreement with earlier findings using a similar method of preparation (Yan et al., 1994). The higher viscosity of the oil phase with the higher polymer concentration leads to a less effective emulsifying process resulting in larger droplets producing larger microspheres.

Changing the volume of the internal aqueous phases had no effect on the size distributions of the microspheres.

The reduction in the size of the microparticles on addition of PC was expected, given its emulsifying and stabilising effects. The lower concentration of PC (0.1% w/v) was sufficient for stabilising the emulsion (Florence and Whitehill, 1982). Increasing the concentration of PC from 0.1 to 0.5% in the oil phase did not result in any further size reduction. However, when the concentration of DL-PLG in the oil phase was 4.3%,
PC 0.1% reduced the size of microspheres by 40% and PC 0.5% reduced them by 60%. Obviously, the smaller emulsion droplets obtained using a lower DL-PLG concentration results in a larger interfacial area requiring a higher amount of PC to be stabilised. Thus the effect of PC on the size of microspheres depends on the concentration of DL-PLG.

Increasing the DL-PLG concentration in the oil phase leads to an increase of the EE of mannitol\textsuperscript{14}C (see table IV).

**Table III:** Size distributions of microspheres prepared with different PLG concentrations in the oil phase using different internal aqueous phase volume, \( V_{\text{i(aq)}} \).

<table>
<thead>
<tr>
<th>( V_{\text{i(aq)}} ) (µl)</th>
<th>Diameter( ^a ) (µm) at which indicated fraction (%) of microspheres was larger</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.3% PLG</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>7.1(0.1)</td>
</tr>
<tr>
<td>100</td>
<td>7.6(0.9)</td>
</tr>
<tr>
<td>200</td>
<td>8.0(2)</td>
</tr>
<tr>
<td>400</td>
<td>8.1(0.4)</td>
</tr>
</tbody>
</table>

\( ^a \) Standard deviation given in brackets

The main cause for loss of mannitol is thought to be transport of droplets of the internal aqueous phase to the external aqueous phase. As the double emulsion is formed, progressive loss of the organic solvent eventually leads to solidification of the microspheres causing cessation of this transport. With an increase in the concentration of DL-PLG in the oil phase, the time for reaching solidification will be shorter. Also the increased viscosity in the oil phase caused by the increased concentration of DL-PLG, will decrease the loss of mannitol\textsuperscript{14}C by this process and contribute to the enhanced EE. The time for reaching solidification of the microspheres is therefore critical for the EE of the double emulsion method. By changing the solubility of the organic solvent in the external aqueous phase or by changing its volume, the rate of organic solvent removal can be controlled (Alonso et al., 1993; Bodmeier and McGinity, 1988 and Albertsson et al., 1994, 1996).

During the release studies a strong burst effect was observed for the microspheres prepared with the lowest concentration of DL-PLG. This was probably the result of the diffusion of surface-localised mannitol\textsuperscript{14}C. For the other two preparations, mannitol\textsuperscript{14}C was continuously released (see figure 11).
Table IV: Entrapment efficiencies of mannitol $^{14}$C in microspheres prepared with different DL-PLG concentrations in the oil phase using different internal aqueous phase volumes, $V_{i(aq)}$.

<table>
<thead>
<tr>
<th>$V_{i(aq)}$ (µl)</th>
<th>Average entrapment efficiency$^a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.3% PLG</td>
</tr>
<tr>
<td>50</td>
<td>1.3(0.0)</td>
</tr>
<tr>
<td>100</td>
<td>1.6(0.5)</td>
</tr>
<tr>
<td>200</td>
<td>0.8(0.0)</td>
</tr>
<tr>
<td>400</td>
<td>0.5(0.0)</td>
</tr>
</tbody>
</table>

$^a$ Standard deviation given in brackets

The release rate increased with increasing internal aqueous volume used during preparation. The porosity also increased with increasing internal aqueous phase volume during preparation. Thus there appears to be a correlation between drug release and the porosity of the microspheres.

![Cumulative release of mannitol $^{14}$C from microspheres](image)

Figure 11: Cumulative release of mannitol $^{14}$C from microspheres prepared with different concentration PLG in the oil phase, but the same volume of the internal aqueous phase (100µl) as a function of time; 4.3% PLG (□), 27% PLG (▲) and 43% PLG (●).
The release of drug from microspheres prepared in the presence of PC was associated with a higher burst effect. This indicates that, when PC was present during the preparation of the microparticles, the distribution of mannitol within the matrix was modified and more incorporated substance was located on the surface of the microspheres.

**Biological activity of Lysozyme after entrapment in poly (d,l-lactide-co-glycolide)- microspheres (paper II)**

In this study, lysozyme as the model peptide was incorporated into DL-PLG microspheres using a small-scale double-emulsion method. The inner aqueous phase volume used for dissolving the model peptide, lysozyme, was 100 µl and the total volume of the double emulsion was about 2 ml.

The concentration of DL-PLG in the middle phase of the W/O/W emulsion was increased from 4.5% (w/w) to 37% (w/w). Different compositions of the organic phase were employed. The concentration of lysozyme within the inner water phase was increased in four steps from 1.2% (w/w) to 30%(w/w).

In this paper we attempted to evaluate the effect of changing the process parameters in order to achieve the maximum degree of retained biological activity (RBA) and a high degree of EE during the preparation process.

**Results and discussion**

The particle size increased with increasing polymer concentrations. The results were in agreement with earlier findings using a similar method of preparation (Paper I and Yan et al., 1994).

The RBA and EE of lysozyme were improved when the PLG concentration in the organic phase of the emulsion was increased (see table V). As the concentration of polymer in the organic phase was increased, the EE increased more than tenfold. A high lysozyme concentration in the inner water phase of the emulsion resulted in decreased EE and an increase in the proportion of fragmented particles. Furthermore, the external porosity of the microspheres increased with increasing concentrations of lysozyme. The RBA of lysozyme in the microspheres varied between 30 and 80% with changes to the process (see table V).

When the DL-PLG concentration was increased from 4.5 to 37% the RBA of the entrapped lysozyme was increased from 59 to 83% (see table V). The improvement was probably caused by the higher rate of solidification of the microspheres as a result of the higher DL-PLG concentration.
Table V: The entrapment efficiency (EE) and retained biological activity (RBA) of lysozyme entrapped in PLG-microspheres prepared by different methods

<table>
<thead>
<tr>
<th>Concentration</th>
<th>% PLG (w/w)</th>
<th>% Lysoz. (w/v)</th>
<th>% RBA</th>
<th>% E.E.</th>
<th>% E.E. x RBA</th>
<th>% M.F. x RBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5a</td>
<td>1.2</td>
<td>59 (0.5)</td>
<td>5 (1)</td>
<td>3.0 (0.7)</td>
<td>0.06 (0.02)</td>
<td></td>
</tr>
<tr>
<td>4.5b</td>
<td>1.2</td>
<td>72 (12)</td>
<td>14 (2)</td>
<td>10 (2.3)</td>
<td>0.21 (0.05)</td>
<td></td>
</tr>
<tr>
<td>4.5c</td>
<td>1.2</td>
<td>32 (8.5)</td>
<td>4 (0.1)</td>
<td>1.3 (0.4)</td>
<td>0.03 (0.01)</td>
<td></td>
</tr>
<tr>
<td>4.5a</td>
<td>30</td>
<td>55 (5.0)</td>
<td>0.6 (0.2)</td>
<td>0.34 (0.1)</td>
<td>0.17 (0.07)</td>
<td></td>
</tr>
<tr>
<td>37a</td>
<td>1.2</td>
<td>63 (4.0)</td>
<td>61 (8)</td>
<td>38 (2.4)</td>
<td>0.09 (0.01)</td>
<td></td>
</tr>
<tr>
<td>37b</td>
<td>1.2</td>
<td>62.9 (0.6)</td>
<td>27 (5)</td>
<td>17 (3)</td>
<td>0.04 (0.01)</td>
<td></td>
</tr>
<tr>
<td>37c</td>
<td>1.2</td>
<td>38.1 (1.1)</td>
<td>6 (1)</td>
<td>2.4 (1)</td>
<td>0.01 (0.00)</td>
<td></td>
</tr>
<tr>
<td>37a</td>
<td>30</td>
<td>83 (10)</td>
<td>12 (6)</td>
<td>11 (6.4)</td>
<td>0.65 (0.39)</td>
<td></td>
</tr>
</tbody>
</table>

* 0:1 acetone : CH2Cl2
b 1:1 acetone : CH2Cl2
c 2:1 acetone : CH2Cl2
da Standard deviation given in brackets.
e Mass Fraction (lysozym / lysozyme+PLG).

As reported earlier, during the incorporation process, proteins may lose their activity in the presence of organic solvents (Sah, 1999).

It was found that dichloromethane (MC) in the organic phase had the greatest influence on the biological activity of lysozyme. We also found that the longer the contact time between MC and lysozyme, the more enzyme activity was lost.

We further checked this hypothesis by increasing the rate of solidification of the microparticles by adding acetone, which is more soluble in water than MC, to the organic phase. It was observed, however, that when the volume of acetone added to the organic phase was over a certain proportion, the lysozyme no longer had a high RBA (see table V). This may have been caused by disturbed particle formation, since the proportion of fragmented microspheres was increased, resulting in decreased protection for the lysozyme from the organic solvent.

Preparation of biodegradable microparticles using solution-enhanced dispersion by supercritical fluids (SEDS) (paper III)

In this study, we have explored the use of a new process involving solution-enhanced dispersion by supercritical fluids (SEDS) (Hanna et al., 1995 and Shekunov et al., 1997) for the preparation of microparticles of several biodegradable polymers. The following polymers were used: Polycaprolactone (PCL), Poly (d,l-lactide-co-glycolide) : copolymer composition 50:50 (DL-PLG), Poly (L-lactide) (L-PLA) and Poly (DL-lactide) (DL-PLA). The polymer solutions were initially dispersed and atomized by the supercritical CO2 using the unique nozzle construction of the SEDS
process under this investigation. The organic solvents for the polymers, which are
soluble in the supercritical CO₂, were extracted, and solid microparticles formed. The
particles were characterised by differential scanning calorimetry (DSC), scanning
electron microscopy (SEM) and particle size measurements.

In addition, attempts were made to reduce or eliminate the use of toxic organic
solvents such as dichloromethane (MC) which are commonly used in both
conventional emulsion-based and alternative supercritical fluid extraction processes
for producing microparticles (Bleich et al., 1993; Bleich and Müller, 1996; Bodmeier
et al., 1995; Ruchatz et al., 1997 and Tom et al., 1993).

Results and discussion

The use of saturated polymer solutions enhanced particle formation for all polymers
after spraying into the supercritical CO₂. This was probably due to a more rapid
solidification rate than when using unsaturated polymer solutions. Microparticles
were obtained from all polymers. The mean particle size and shape varied with the
polymer used. The morphology of the particles was strongly affected by the choice of
polymer solvent. In contrast to earlier studies (Bleich et al., 1993 and Bodmeier et al.,
1995), we succeeded in producing discrete microparticles from DL-PLG with the
supercritical fluid technology using the SEDS process. These microparticles of DL-
PLG had a mean volumetric diameter of 130 µm. The microparticles prepared from
the semicrystalline L-PLA were almost spherical, and their size increased from 0.5 to
5 µm as the density of the supercritical CO₂ decreased (see table VI).

Table VI: Mean volumetric particle size distribution of L-PLA microparticles
prepared with different densities of CO₂. The results were based on data
from 3 batches (standard deviation in brackets).

<table>
<thead>
<tr>
<th>Pressure (bar)/</th>
<th>Diameter (µm) of microparticles</th>
<th>CO₂ density (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>10%</td>
<td>50%</td>
</tr>
<tr>
<td>130 / 35</td>
<td>0.13(0.0)</td>
<td>0.72(0.1)</td>
</tr>
<tr>
<td>130 / 40</td>
<td>0.3(0.0)</td>
<td>5.3(0.4)</td>
</tr>
<tr>
<td>185 / 35</td>
<td>0.14(0.0)</td>
<td>0.5(0.1)</td>
</tr>
<tr>
<td>185 / 40</td>
<td>0.14(0.0)</td>
<td>0.6(0.0)</td>
</tr>
</tbody>
</table>

PCL formed microparticles with diameters of 30-210 µm and showed a strong
tendency to form films at high pressure. The solvent compositions for the different
polymers were chosen with regard to the solubility of each polymer and
environmental aspects related to the toxicity of the solvents.

The DSC thermograms for the polymers before and after the SEDS process indicated
a higher degree of homogeneity and purity for the processed polymers (i.e. they had
narrower glass transition and melting temperature peaks) than for the unprocessed
polymers.
A new method for preparing biodegradable microparticles and entrapment of hydrocortisone in DL-PLG microparticles using supercritical fluids (paper IV)

The purpose of this study was twofold. Firstly, we wanted to improve control of the particle size and morphology by modifying the SEDS process. Our second purpose was to find out whether the SEDS process could be used to incorporate drugs into biodegradable microparticles (Bleich and Müller, 1996 and Bodmeier et al., 1995).

A combination of two supercritical fluids (CO₂ and N₂) was used to improve particle formation. Several biodegradable polymers (PCL, DL-PLG, L-PLA and DL-PLA) were used to prepare microparticles intended for use in the controlled release of drugs, particularly proteins and peptides (Bleich and Müller, 1996; Bleich et al., 1994; Bodmeier et al., 1995 and Denbenedetti et al., 1993). As before, solutions of the polymers in organic solvents were dispersed by using the atomising nozzle construction of the SEDS equipment (Hanna et al., 1995; Hanna and York, 1994 and Paper III). The organic solvents, which are soluble in the supercritical fluid phase, were then extracted, resulting in the formation of solid microparticles. Finally, a model drug, hydrocortisone was incorporated into the DL-PLG microparticles.

I characterised the microparticles by scanning electron microscopy (SEM) and particle size measurements and determined the degree of hydrocortisone entrapment. As in our previous studies we minimised the use of toxic organic solvents (Paper III).

Results and discussion

We improved the SEDS process for the production of biodegradable microparticles by using a supercritical mixture of CO₂ and N₂. For amorphous materials however, due to

![Figure 12: Scanning electron micrograph of DL-PLG (i. v. 0.78) prepared at 130 bar and 38°C.](image)
their lower rate of solidification, an increase of the CO₂ density is accompanied by a reduced rate of mass transfer. In this case, from our results on the increased dispersive effect by combining N₂ with CO₂ the aggregation effects were reduced which may occur before the particles are completely dried. This increased dispersion effect resulted in a reduced particle size for the amorphous polymers. Optimisation of the dispersion phase during particle formation is essential in order to control the particle size.

In contrast to earlier studies using supercritical fluid extraction methods (Bleich et al., 1993; Bodmeier et al., 1995; Denbenedetti et al., 1993 and Paper III), we succeeded in producing discrete microparticles from DL-PLG [inherent viscosity (i.v.) 0.78] with a volumetric diameter less than 10 μm.

The characteristics of the microparticles varied with the i.v. of the DL-PLG used. The microparticles formed from DL-PLG with i.v. 0.78 were smaller and had a narrower size distribution than those produced from DL-PLG with i.v. 0.63 (see table VII). These differences are due to the lower solubility of polymers with higher molecular weight, since this affects the rate of solidification. The microparticles obtained using DL-PLG with i.v. 0.78 were discrete, spherical in shape, had volumetric diameters between 6 and 10 μm (see figure12), and were more than 10 times smaller than those produced previously (Paper III).

Table VII: The mean volumetric particle size distribution of microparticles prepared from biodegradable polymers under varying process conditions by two different methods.

<table>
<thead>
<tr>
<th>Diameter (μm) of microparticles</th>
<th>Pressure (bar)</th>
<th>130</th>
<th>160</th>
<th>185</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL, 35°C</td>
<td>55.2</td>
<td>106</td>
<td>25.6</td>
<td>189</td>
</tr>
<tr>
<td>PCL, 40°C</td>
<td>58.0</td>
<td>27</td>
<td>83(13)</td>
<td>212</td>
</tr>
<tr>
<td>DL-PLA, 35°C</td>
<td>9.9</td>
<td>135</td>
<td>10.2(0.4)</td>
<td>216</td>
</tr>
<tr>
<td>DL-PLA, 40°C</td>
<td>8.6</td>
<td>178</td>
<td>8.8</td>
<td>105</td>
</tr>
<tr>
<td>L-PLA, 35°C</td>
<td>7.7</td>
<td>0.7(0.1)</td>
<td>7.2</td>
<td>0.5(0.1)</td>
</tr>
<tr>
<td>L-PLA, 40°C</td>
<td>7(2)</td>
<td>5.3(0.4)</td>
<td>7.9</td>
<td>0.6(0.0)</td>
</tr>
<tr>
<td>DL-PLG (0.78)b, 33°C</td>
<td>9.8</td>
<td>140</td>
<td>7.2</td>
<td>130</td>
</tr>
<tr>
<td>DL-PLG (0.78)b, 38°C</td>
<td>5.6</td>
<td>126</td>
<td>8(2)</td>
<td>136</td>
</tr>
<tr>
<td>DL-PLG (0.63)b, 33°C</td>
<td>15.3</td>
<td>-</td>
<td>56.6</td>
<td>151</td>
</tr>
<tr>
<td>DL-PLG (0.63)b, 38°C</td>
<td>39(3)</td>
<td>173</td>
<td>39.3</td>
<td>158</td>
</tr>
</tbody>
</table>

*Standard deviations given in brackets are based on measurement from three batches.

*b Inherent viscosity.

*c Data from particles produced by the modified SEDS equipment.

*d Data from particles produced by the original SEDS equipment (Paper III).
Microparticles from the DL-PLG with i.v. 0.63 were between 15 and 57 \( \mu \text{m} \) in diameter and were 2-3 times smaller than those produced by the original SEDS process (see table VII) (Paper III). The L-PLA microparticles produced by the modified method were a combination of almost spherical particles with a smooth surface and some more irregular particles, with a volumetric diameter of 7-10 \( \mu \text{m} \) (see table VII).

The DL-PLA microparticles produced using the modified SEDS process were more than 10 times smaller than those produced by the original SEDS process (see table VII). The particles produced by the modified process had a volumetric particle size between 9-10 \( \mu \text{m} \). The DL-PLA microparticles were almost spherical and, in contrast to our previous study (Paper III), the surfaces of these microparticles were very smooth and nonporous, under all the investigated experimental conditions.

The PCL particles obtained were a mixture of discrete and agglomerated particles, with a volumetric particle size between 25 and 85 \( \mu \text{m} \) (see table VII). The particles were 2-4 times smaller than those produced by the conventional SEDS process (Paper III).

Hydrocortisone was successfully entrapped in the DL-PLG (i.v. 0.78) microparticles. The EE of hydrocortisone was 22%. This result was quite promising, since no attempts were made to optimise the entrapment process (Bodmeier et al., 1995; Bleich and Müller, 1996). The remaining hydrocortisone was completely recovered from the washing medium. This remaining material had probably been loosely adsorbed onto the surfaces of the microparticles since no discrete crystals were seen in the SEM examination. There was no significant differences in EE for microparticles which were produced at two different pressures (130 and 160 bar).

A new process for the incorporation of proteins into DL-PLG microparticles using supercritical fluids (paper V)

The purpose of this study was to develop and apply the novel processing method for production of drug loaded microparticles using supercritical fluids. In particular, the retained biological activity and release kinetics of incorporated proteins were studied. We employed our previously developed and improved particle formation process based on using a combination of the two supercritical fluids \( \text{CO}_2 \) and \( \text{N}_2 \) (Paper IV). This method has demonstrated its suitability for producing discrete drug loaded microparticles with narrow size distribution and controllable morphology. Microparticles such as these are ideal for use in the controlled release of drugs, particularly proteins and peptides (Bleich and Müller, 1996; Denbenedetti et al., 1993 and Pablo et al., 1993). We prepared drug loaded microparticles of the biodegradable polymer, poly (d,l-lactide-co-glycolide) : copolymer composition 50:50 (DL-PLG). Three model substances, hydrocortisone, lysozyme and urease were incorporated into the microspheres.

The mixture of solutions of polymer and drug was dispersed and atomised using a co-axial nozzle construction as before (Paper III and IV). The organic solvents of the
polymer and drug solution were extracted with the supercritical fluid phase and solid drug loaded DL-PLG microparticles were the result.

The microparticles were characterised using SEM and particle size measurements. The degree of drug entrapment and the release rate of drug from the microparticles were also determined. The RBA of the enzymes (urea and lysozyme) was determined by bioassay. As in our previous studies, the use of toxic organic solvents was kept to a minimum (Paper III).

Results and discussion

Discrete spherical microparticles were obtained from DL-PLG with a mean volumetric diameter of less than 10 µm. The microparticles loaded with proteins (lysozyme and urease) were smaller than those loaded with hydrocortisone (see table VIII).

The EE for all three model drugs was directly affected by the drug/polymer ratio. Increasing the proportion of drug resulted in a decrease in EE, as was expected. The EE values for the model substances were between 25 and 55%. These findings show that the drug/polymer ratio has a significant effect on the EE and RBA of the drugs incorporated into DL-PLG microparticles.

The degree of loading for lysozyme is approximately 10 times higher with the modified SEDS process than with the double emulsion method (Paper II).

Table VIII: The mean volumetric particle size distribution of drug-loaded microparticles prepared from biodegradable polymer using various methods and process conditions.

<table>
<thead>
<tr>
<th>Polymer / Drug</th>
<th>Diameter a (µm) of microparticles</th>
<th>Pressure(bar)/Temp. (°C)</th>
<th>% EE</th>
<th>% RBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-PLG / HC c (8.5:1.5)b</td>
<td>2 (0) 10 (1) 30 (4)</td>
<td>135 / 38</td>
<td>33 (1.0)</td>
<td></td>
</tr>
<tr>
<td>DL-PLG / HC c (9.5:0.5)b</td>
<td>3 (0) 9 (1) 30 (8)</td>
<td>135 / 38</td>
<td>51 (1.0)</td>
<td></td>
</tr>
<tr>
<td>DL-PLG / HC c,d (9.5:0.5)b</td>
<td>2 (0) 5 (1) 12 (3)</td>
<td></td>
<td>3 (0)</td>
<td></td>
</tr>
<tr>
<td>DL-PLG / Lys.e (9.0:1.0)b</td>
<td>3 (0) 7 (1) 17 (2)</td>
<td>160 / 38</td>
<td>23 (1) 58(0)</td>
<td></td>
</tr>
<tr>
<td>DL-PLG / Lys.e (9.5:0.5)b</td>
<td>3 (0) 7 (1) 17 (7)</td>
<td>160 / 38</td>
<td>54 (10) 91(7)</td>
<td></td>
</tr>
<tr>
<td>DL-PLG / Ure.f (9.0:1.0)b</td>
<td>3 (0) 7 (1) 19 (4)</td>
<td>160 / 38</td>
<td>34 (3) 56(2)</td>
<td></td>
</tr>
<tr>
<td>DL-PLG / Ure.f (9.5:0.5)b</td>
<td>3 (0) 6 (1) 18 (4)</td>
<td>160 / 38</td>
<td>46 (3) 82(4)</td>
<td></td>
</tr>
</tbody>
</table>

a Standard deviations given in brackets are based on measurements from three batches, b Fraction polymer: Drug, c Hydrocortisone, d microspheres produced by emulsion method, e Lysozyme, f Urease.

The RBA of lysozyme and urease after incorporation, which varied inversely with the enzyme/polymer ratio (see table VIII), was between 60 and 90%. 

36
The RBA of urease was followed during the whole period of the release study and it was compared with the RBA obtained from a comparable urease solution, which also was incubated at 37 °C. We added a similar amount of empty DL-PLG microparticles, produced by the SCF-method, to the urease reference solution in order to mimic the denaturation of urease catalysed by the particle surfaces.

It was shown that the RBA for the urease reference solution declined much faster than the RBA of urease released from DL-PLG microparticles. After only 6 days the RBA of urease, from the urease reference solution, decreased to less than 15%, while the RBA of released urease from microparticles was 26% and 33%, respectively, for the high and low fractions of urease used during the incorporation process (see figure 13). This shows that the urease released after 25 days is still biologically active and has not been deactivated inside the microparticles.

Keeping the proportion of polymer higher than that of the enzyme appeared to protect the enzyme during the preparation process, as was reflected in higher RBA values.

During the in vitro release study for hydrocortisone in DL-PLG microparticles produced by the single emulsion and the SCF methods, a stronger burst effect was observed for the microparticles produced by the SCF method than by the single emulsion method. This might be explained by the tenfold higher EE of hydrocortisone obtained using the SCF method.

![Figure 13](image-url): RBA of urease from reference solution (□), microparticles prepared by low polymer/drug ratio 9.5:0.5 (◊) and high polymer/Drug ratio 9:1 (◇).
Figure 14 shows the release rate for urease from DL-PLG microparticles with polymer / urease ratios (9.0: 1.0 and 9.5: 0.5).

Microparticles containing the higher amount of urease were observed to have a stronger burst effect than those containing the smaller amount, as was expected. This is probably the result of diffusion of the higher amount of urease localised on the surface of the microparticles. The strong burst effect, was followed by the constant and continuous release of urease.

Figure 14: Cumulative release of urease from DL-PLG microparticles prepared with two different polymer/drug ratios. 9:1 (Δ) and 9.5:0.5 (□).
CONCLUSIONS

In this thesis, it has been shown (paper I) that the higher the internal volume of microparticles produced by the double emulsion method, the faster the enclosed drug is released. It was also shown (papers I and V) that increasing the proportion of polymer to drug improves the entrapment efficiency and decreases the release rate for both the double emulsion and supercritical fluid methods.

Paper II showed how important it is to optimise the process of manufacturing microspheres in order to give the entrapped peptide maximum protection against chemical degradation and denaturalisation during the preparation process. It is not sufficient to ascertain the value of the EE alone for biological substances (peptides, proteins, antigens, etc.) entrapped in a controlled release system. Both the entrapment efficiency and the retained biological activity should be determined for these systems. It was shown that between 0.3 and 38% of the bioactive lysozyme entrapped in DL-PLG microspheres was recovered, depending on simple variations in the process parameters. A reliable evaluation of these drug delivery systems is gained by multiplying the RBA with the EE to obtain the biological entrapment efficiency (BEE).

Paper III demonstrated that SEDS can be used for the production of microparticles of biodegradable polymers. The ability to precipitate these polymers was the first step towards reaching the goal of incorporating drugs into such microparticles for controlled drug delivery. Great care must be exercised in the selection of suitable solvents and operating conditions in order to optimise the formation of well defined microparticles. Changing the process parameters (temperature and pressure) did not result in any clear differences in the size distribution of microparticles prepared from PCL, DL-PLA and DL-PLG.

The size of the microparticles prepared from the semicrystalline L-PLA was dependent on the density of the supercritical CO$_2$ phase. Thus, although microparticle formation from amorphous polymers by supercritical extraction processing is more difficult than that from crystalline polymers such as L-PLA, we were able to produce discrete particles of a relatively uniform size from at least one of the DL-PLG polymers. The results of this study also show that the use of toxic solvents can be greatly reduced when the SEDS process is used for the preparation of microparticles from commonly used polymers.

To further optimise the properties of the microparticles a combination of supercritical N$_2$ and CO$_2$ was used in the SEDS process (Papers IV and V). This improved the dispersion of the polymer solutions in the supercritical fluid over the use of CO$_2$ alone and resulted in a reduction in the size of discrete microparticles produced from amorphous biodegradable polymers. The general robustness of this process for producing particles was evident from the small statistical variations in particle size obtained from three successive batches.

The relatively mild processing conditions involved make the process especially suitable for the incorporation of peptides and proteins.
The modified SEDS process (paper V) was used for the production of DL-PLG microparticles containing hydrocortisone, lysozyme or urease. This process resulted in high EE values and the proteins incorporated exhibited a high degree of retained biological activity. The drug/polymer ratio has a significant effect on the EE and RBA of the drugs incorporated into DL-PLG microparticles. The process is an attractive way of incorporating proteins and peptides into biodegradable microparticles for controlled release. Compared with conventional technologies for the preparation of such drug delivery systems, e.g. solvent-evaporation emulsion techniques, this new technique is environmentally superior, and suitable for up-scaling. Moreover the higher degree of control as indicated by the high reproducibility, makes validation of the process feasible.
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