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Microencapsulation of protein particles within lipids using a novel supercritical fluid process

I. Ribeiro Dos Santos [a], J. Richard [b,*], B. Pech [a], C. Thies [c], J.P. Benoit [a]

[a] *Inserm ERIT-M 0104, Ingénierie de la vectorisation particulaire, 10 rue André Boquel, 49100 Angers, France*
[b] *Mainelab S.A., Ethypharm Group, 8 rue A. Boquel, 49100 Angers, France*
[c] *Washington University, Biological Transport Laboratory, Campus Box 1198, St. Louis, MO 63130-4899, USA*

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Abstract

Solvent-free microparticles, loaded with a model protein (bovine serum albumin: BSA) have been produced using a novel coating process based on supercritical (SC) fluid technology. Coating material consists of either trimyristin (Dynasan® 114) or Gelucire® 50-02, two lipidic compounds having a high melting point. Microparticles were characterized as regards their morphology, protein content and in vitro release profile. A discontinuous coating made of crystalline micro-needles was obtained using Dynasan® 114. It led to particles with an initial burst release of about 70% in 30 min at 37 °C. However, a prolonged release of the protein has been achieved over a 24 h period from particles coated with Gelucire® 50-02, which produces a more homogeneous, film-forming coating. Furthermore, it was shown that BSA does not undergo any degradation after SC CO_2 treatment under the SC conditions used in the coating process.

Keywords: Supercritical carbon dioxide; Coating of microparticles; Protein stability; Prolonged release; Lipidic coating

1. Introduction

The use of peptides and proteins in pharmaceutical formulations is undergoing a rapid growth due to the high therapeutic potential of these substances, such as insulin for diabetic disorders (Pillai and Panchagnula, 2001) and erythropoietin for anemia (Krantz, 1991; Spivak, 2000). Conventional microparticle production techniques have shown promising results in the preparation of delivery systems for many bioactive peptides and proteins (Cleland et al., 2001). However, a serious drawback of these techniques is the extensive use of organic solvents to dissolve the coating materials. Most of these organic solvents are toxic (ATSDR, 1999) and moreover, are suspected to be responsible for protein denaturation (biological inactivation) (Sah, 1999). Other coating methods reported in the literature, that do not use any organic solvent, are based on hot-melt procedures, involving heat that can also have a detrimental effect on heat-sensitive materials, hence including the majority of proteins (Rothen-Wein-

* Corresponding author. Tel.: +33-241-730-202; fax: +33-241-732-273
E-mail address: richard.joel@ethypharm.com (J. Richard).

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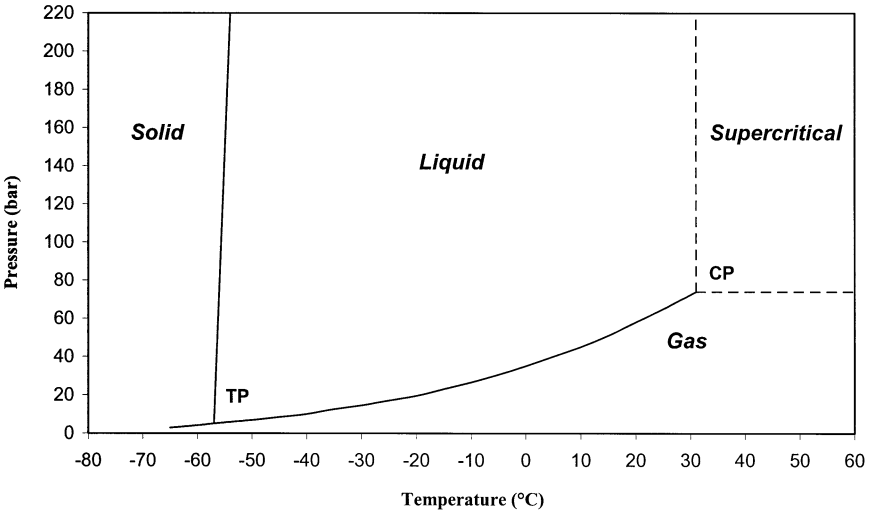


Fig. 1. Pressure/temperature phase diagram for pure carbon dioxide.

hold et al., 1999). Accordingly, there is a strong interest in solvent-free microencapsulation methods, carried out under conditions that have minimal detrimental effect on the active material being coated, especially in the case of fragile molecules like peptides and proteins.

Supercritical fluids, especially supercritical carbon dioxide (SC CO_2), are one of the most promising issues to develop new encapsulation techniques that completely eliminate organic solvents or minimize their use. It offers a lot of advantages over the classical microencapsulation processes. In the pressure–temperature diagram, supercritical fluids are characterized by a temperature and a pressure simultaneously higher than those of the critical point, that is the final point of the liquid–gas phase transition curve (Fig. 1). CO_2 is readily available, environmentally acceptable and non-flammable. It has a critical point which can be easily reached, that allows work at moderate temperatures, and leaves no toxic residues since it turns back in a gas phase at ambient conditions. Moreover, its solvent power can be significantly varied and adjusted by simply varying temperature and pressure conditions (Fig. 2): this original behavior of adjustable solvent makes CO_2 very attractive as a substitute for organic solvents in microencapsulation processes.

SC CO_2 based processes have been previously reported for the preparation of drug-loaded microspheres: these processes currently use either CO_2 as a solvent (Rapid Expansion from Supercritical Solutions: RESS process) (Debenedetti et al., 1993) or a non-solvent (Aerosol Solvent Extraction System: ASES process) (Bleich and Müller, 1996) of the coating material to produce matrix particles throughout which the active and

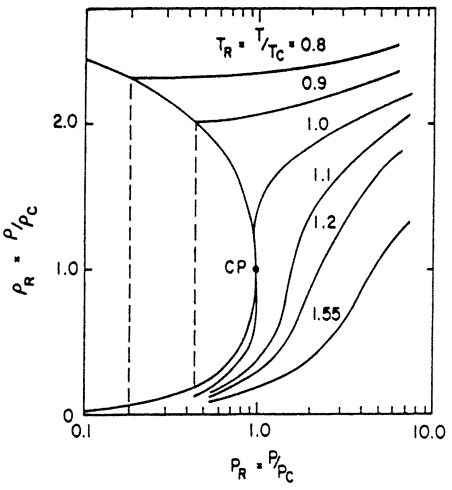


Fig. 2. Phase diagram of the fluid region of a pure substance in reduced density–pressure coordinates.

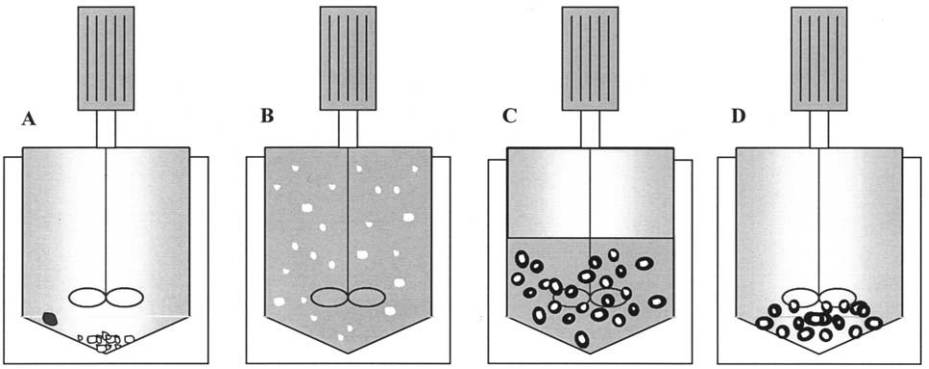


Fig. 3. Schematic representation of the coating process. Principle of the SC CO_2 based coating method. (A) Filling step: BSA crystals represented in white, coating material in black. (B) Solubilization of the coating material in the SC CO_2 (Step I). Insoluble BSA crystals are dispersed in the medium. (C) Decompression phase (Step II): insolubilization of the coating material. (D) Coated particles are harvested.

coating materials are intimately mixed. A novel coating process based on SC CO_2 technology without any organic solvent has been developed recently in our laboratory (Benoit et al., 1994). Our approach consists in solubilizing the coating material in SC CO_2 and gradually reducing the solvent power of CO_2 to make the coating material precipitate onto drug particles dispersed in the medium. Our process is totally different from the previously described SCF based processes since it does not lead to the in situ formation of microspheres but to the production of microcapsules composed of a preformed core of active material surrounded by a shell of coating material

The aim of this work was to implement this novel process to produce solvent-free microparticles for sustained release of proteins. Recent results concerning the preparation and characterization of microparticles containing a model protein (BSA) are presented and discussed, as regards their morphology, protein stability and content, as well as in vitro release kinetics.

2. Experimental methods

2.1. Materials

BSA crystals (fraction V) were obtained from Sigma Chemicals (St. Louis, MO, USA). They were either grinded until thin crystals were obtained (< 50 μm) or sieved so as to produce four fractions with different particle size distributions: < 125, 125–250, 250–500, > 500 μm. Gelucire® 50/02, which is a mixture of glycerides and fatty acid esters (Gattefossé SA, St-Priest, France) and trimyristin Dynasan® 114 (Condea, Hambourg, Germany) were used as coating materials. They have a melting point above human body temperature, namely 45 and 50 °C for, respectively, Dynasan® 114 and Gelucire® 50/02.

Carbon dioxide (CO_2) was obtained from L'Air Liquide (Paris, France) and had a purity exceeding 99.7%.

All materials were used as received without any further purification.

2.2. Microparticle production

Previous solubility experiments established that 1.5 g of Gelucire® 50-02 and 1.0 g of Dynasan® 114 dissolve completely in 1 l of SC CO_2 in 1 h at, respectively, 45 °C/200 bar and 35 °C/200 bar (Ribeiro Dos Santos, 2000).

Microparticle production is illustrated in Fig. 3. The process basically consists of three successive steps as follows (Fig. 4).

Coating material and BSA crystals were placed in a 1 l autoclave equipped with an impeller rotating continuously at 350 rpm. The autoclave was sealed, heated and pressurized with CO_2 over a ~ 45 min period until the desired SC conditions

were reached, i.e. temperature typically ranging between 35 and 45 °C and pressure about 200 bar. The system was allowed to equilibrate at these conditions for 1 h, so as to solubilize the coating material (step I). Then, cooling the autoclave induced a pressure decrease and a phase change from SC to liquid state (Fig. 5), therefore insolubilizing the coating material that precipitated upon the insoluble BSA crystals dispersed in the medium (step II). Afterwards, the autoclave was vented to ambient conditions and the coated particles were collected from the bottom of the autoclave (step III), stored at 4 °C and characterized.

2.3. Characterization

2.3.1. Protein analysis

The stability of the protein during the process was assessed using capillary electrophoresis (Spectraphoresis™ 1000, ThermoQuest, San Jose, CA, USA) before and after SC CO_2 treatment. An uncoated fused-silica capillary with a 75 μm internal diameter was used as a separation column. It exhibited a 58.5 cm effective length (to the UV detector). Migration of the protein was achieved by applying an electric field of 15 or 20 kV. Samples were prepared at a 0.2 or 0.5 mg/ml

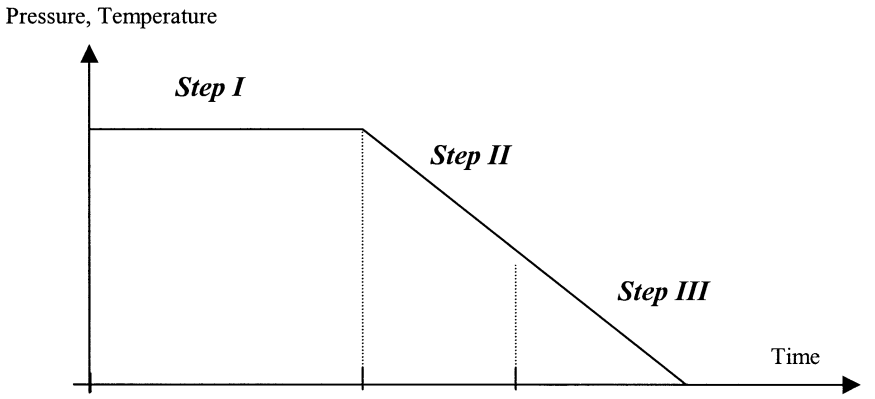


Fig. 4. Schematic steps for the coating process. Step I: Solubilization of the coating material (35–45 °C, 200 bar, 1 h). Step II: Desolvation and deposition of the coating material (decompression phase). Step III: Venting step (to atmospheric conditions).

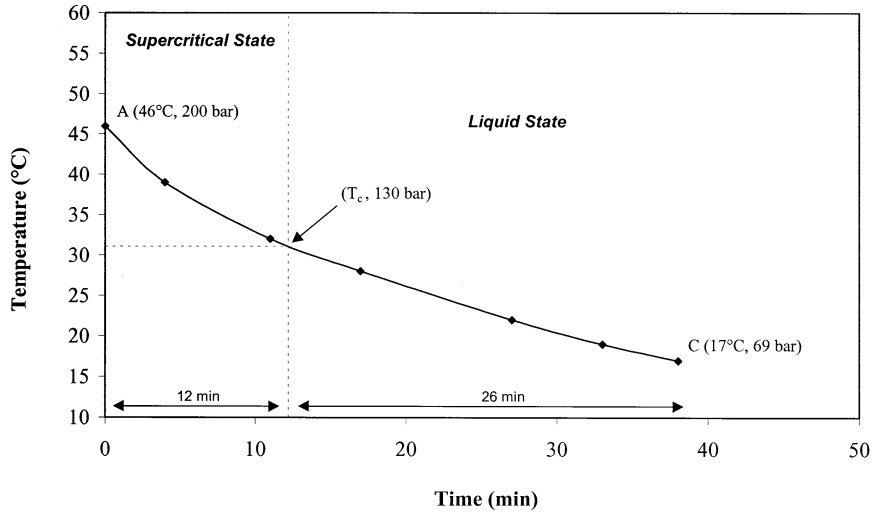


Fig. 5. Decrease in temperature during the depressurization step.

concentration and then introduced into the capillary by hydrodynamic injection for 5 s. UV detection was performed at 195 nm. Capillary was rinsed using HCl 0.1N, NaOH 0.1N and pure H_2O, and equilibrated with the running electrophoretic buffer (50 mM, pH 8.5 borate buffer). Injections were run in triplicate.

2.3.2. Morphology and surface appearance

The morphology of bovine serum albumin (BSA) crystals and microparticles was investigated using optical microscopy (stereomicroscope SMZ-U, Nikon Corporation, Tokyo, Japan) and scanning electron microscopy (SEM) (JEOL JSM 6301F, Tokyo, Japan, and Hitachi S-4500, Tokyo, Japan). Samples for SEM evaluation were vacuum coated with gold.

2.3.3. Particle size analysis

The mean size of coated particles was determined using optical microscopy with Saisam software (Microvision Instruments, Evry, France), which allows the following measurements: counting the particles, determining length, width, equivalent diameter of the particles and perimeter by particle contour analysis.

2.3.4. In vitro BSA release study

In vitro release kinetics of BSA microcapsules were recorded at 37 °C, in a 10 mM pH 7.4 phosphate buffered aqueous solution, under stirring at 100 rpm, using a USP dissolution apparatus (Sotax AT 7, Sotax, Basel, Switzerland) and spectrophotometry (Uvikon 922, Kontron, St-Quentin-en-Yvelines, France) either at 208 nm (microparticles coated with Gelucire® 50-02) or at 595 nm using a Bio-Rad standard protein microassay (Bio-Rad Laboratories, Hercules, CA, USA) (microparticles coated with Dynasan® 114). All release experiments were run as independent duplicate or triplicate analyses.

2.3.5. Protein content

The actual core loading of the samples was determined by melting the coating in PBS (60 °C for 10 min) at the end of the 37 °C release experiment, which led to a complete release of the protein. BSA concentration in PBS was determined by spectrophotometry. Each experiment and measurement was carried out in duplicate.

3. Results

3.1. Protein analysis

It is a key issue to check that conformation of the protein (BSA in the present case) is not altered during the SC CO_2 coating procedure. The stability of BSA was evaluated by performing capillary electrophoresis.

The electrophoregrams, presented in Fig. 6, show that there is no apparent effect of the encapsulation conditions on the structural integrity of the protein, since BSA samples treated in SC CO_2 at 200 bar at either 35 or 45 °C, showed the same electrophoretic pattern, in terms of migration times, peak area and shape, as the native BSA. So, no structural or conformational damage of the protein occurs during the SC treatment. These results suggest that the encapsulation conditions (35 °C/200 bar or 45 °C/200 bar for 1 h) do not affect the stability of BSA.

However, specific analyses, such as biological assays or immunoassays, need to be performed in the case of proteins having a therapeutic effect, such as cytokines (Mire-Sluis, 1999), to confirm that the biological activity is preserved after SC CO_2 treatment.

3.2. BSA particles coated with Dynasan® 114

Microparticle production experiments were performed using Dynasan® 114 at 35 °C and 200 bar for 1 h, with small (< 50 μm in diameter) BSA crystals of irregular shape. Depending on the experimental conditions (initial BSA and Dynasan® 114 weights), protein content was found to range between 13 and 62% (Table 1).

The most interesting release profile has been obtained for microparticles loaded with 15 wt% protein. Fig. 7 shows the in vitro release kinetics of microparticles from sample D5 compared to uncoated BSA crystals. Release kinetics of sample D5 exhibits an high initial burst of 35% over a 5 min period. Then, 70 and 85% of protein were

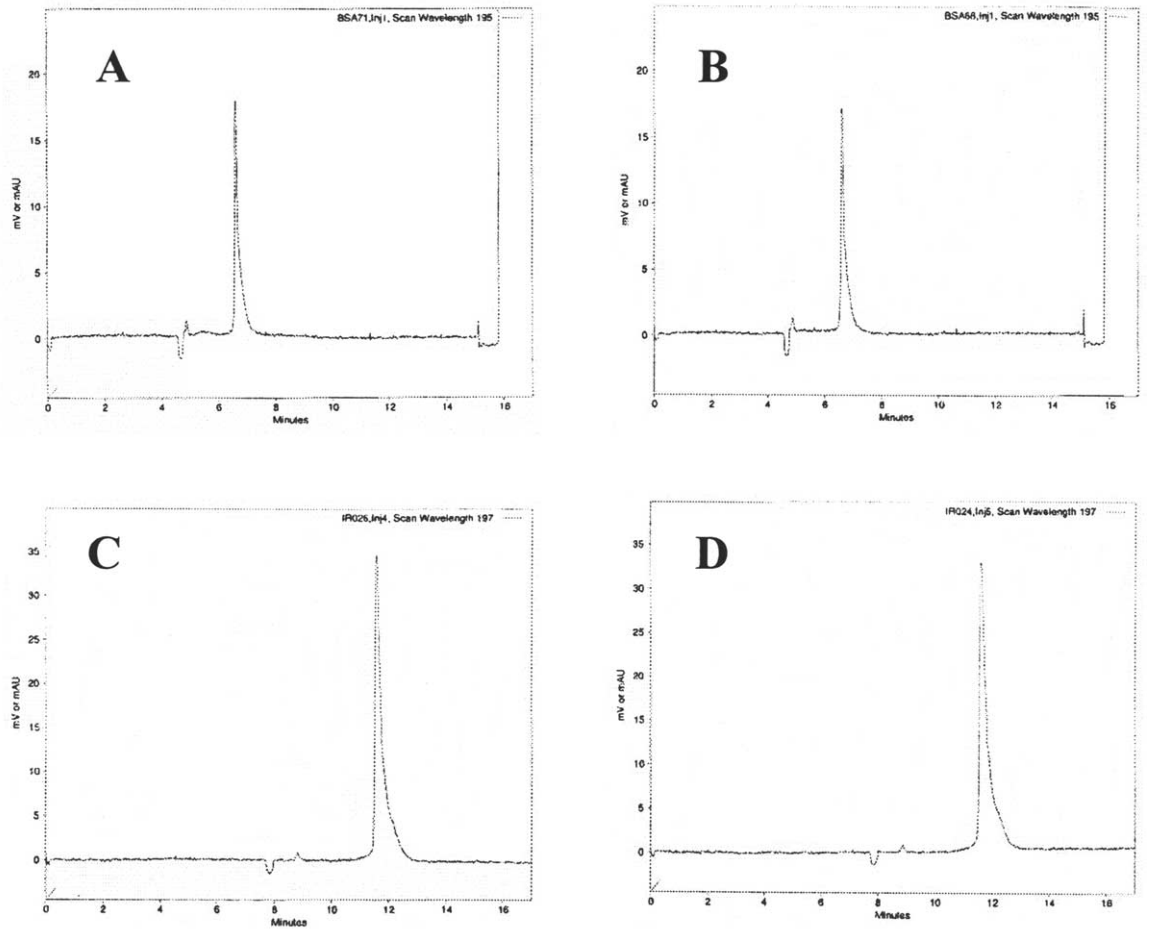


Fig. 6. Electrophoregrams of BSA: A, untreated; B, treated in SC CO_2 at 35 °C and 200 bar during 1 h; C, untreated; D, treated in SC CO_2 at 45 °C and 200 bar during 1 h. Electrophoregrams A and B: electric field of 20 kV, sample concentration of 0.2 mg/ml; electrophoregrams C and D: electric field of 15 kV, sample concentration of 0.2 mg/ml.

Table 1
Operating conditions used to prepare BSA samples coated with Dynasan® 114

Sample	Initial weight of BSA (g)	Initial weight of Dynasan® 114 (g)	Stage I time (min)	Stage II time (min)	Actual BSA content (wt%)
D1	0.50	0.27	70	40	62
D2	0.50	0.50	138	72	43
D3	0.33	0.67	122	41	27
D4	0.25	0.75	112	48	24
D5	0.15	0.85	125	108	13

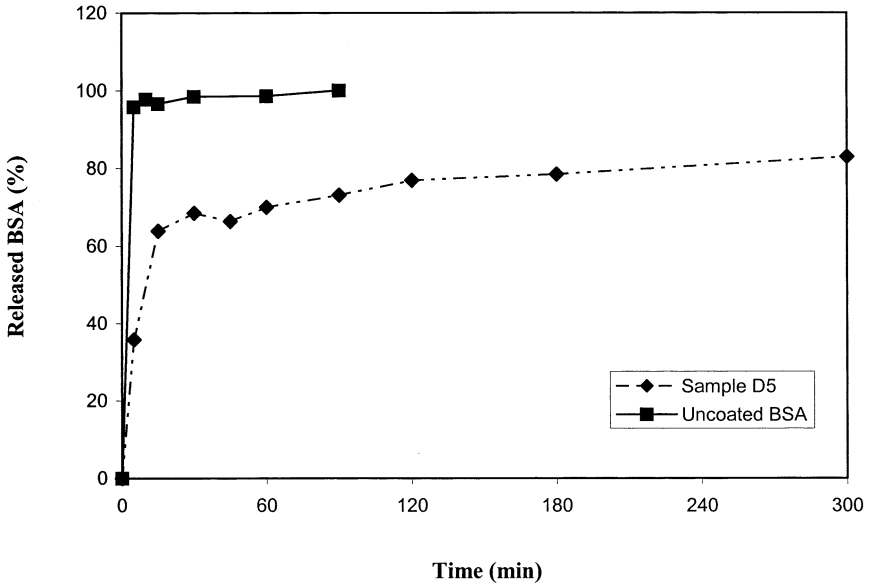


Fig. 7. Release kinetics of uncoated BSA crystals and BSA crystals coated with Dynasan® 114 (sample D5).

released over 30 min and 5 h, respectively. SEM observations (Fig. 8) showed a discontinuous coating made of disordered micro-needles (> 10 μm long) of Dynasan® 114. This morphology appears to be consistent with the fast in vitro release behavior of these particles. The coating, which consists of a hydrophobic porous network of crystalline needles deposited at the protein surface is likely to limit the wettability of BSA crystals by the aqueous phase, and hence to restrict the immediate dissolution of BSA, thus resulting in a slower release compared to uncoated crystals. However, precipitation of Dynasan® 114 is uneasy to control: it mainly gives rise to long crystalline needles (> 50 μm) that do not form an homogeneous and continuous coating.

3.3. BSA particles coated with Gelucire® 50-02

Experiments were carried out using sieved BSA crystals (four fractions) at 200 bar and 45 °C for 1 h, so as to solubilize Gelucire® 50/02. Stimulating results were obtained for BSA crystals whose size ranges between 125 and 500 μm, larger crystals being difficult to disperse efficiently in the autoclave at 350 rpm.

Depending on the initial BSA fraction, protein content ranges between 36 and 67% (Table 2), but finally the initial BSA particle size was not found to have a significant influence on the in vitro release kinetics (Fig. 9). Release kinetics of samples G1, G2, G3 and G4 show a prolonged release of the protein over a period of more than 24 h. In addition, these samples retain about 20% of their initial BSA payload after a 1-day immersion in PBS at 37 °C. In contrast, uncoated BSA dissolves completely in 5 min (Fig. 10). The burst effect

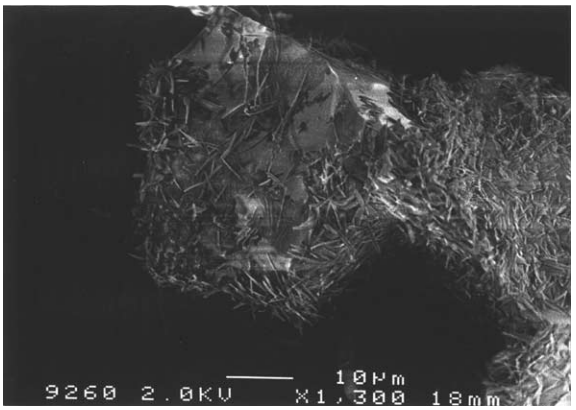


Fig. 8. SEM photomicrograph of a BSA crystal coated with Dynasan® 114.

Table 2
Operating conditions used to prepare BSA samples coated with Gelucire® 50-02

Sample	Initial weight of BSA (g)/sieved fraction[a]	Initial weight of Gelucire® 50-02 (g)	Stage I time (min)	Stage II time (min)	BSA content (%)
G1	0.4/III	1.5	33	36	66.5
G2	0.4/II	1.5	37	45	39.5
G3	0.8/III	1.5	38	38	67.0
G4	0.4/I	1.5	36	39	36.0

[a] I: <125 μm fraction; II: 125–250 μm fraction; III: 250–500 μm fraction.

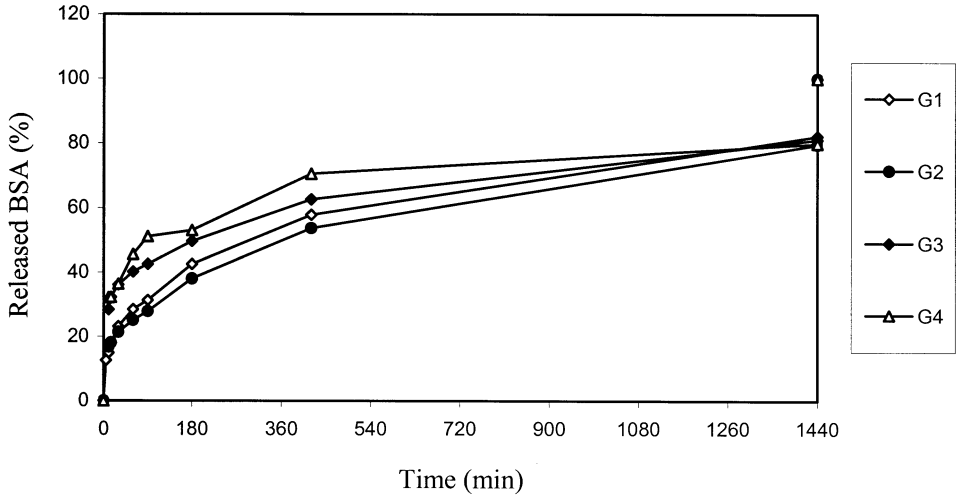


Fig. 9. Release kinetics of BSA crystals coated with Gelucire® 50-02. (samples G1, G2, G3 and G4).

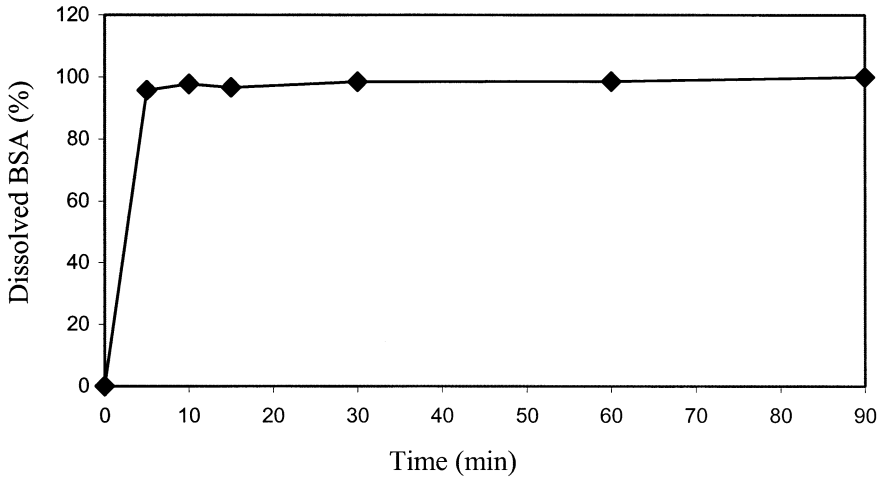
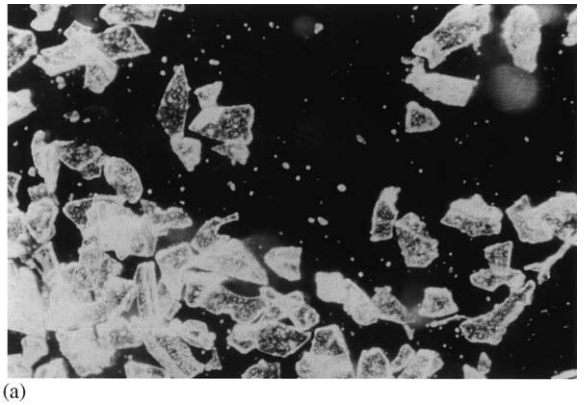


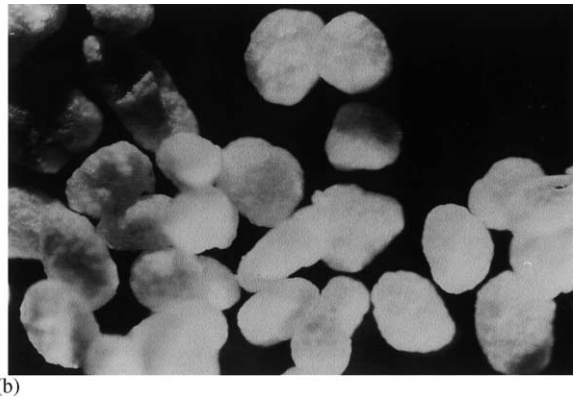
Fig. 10. Release kinetics of uncoated BSA crystals.

observed for Gelucire® 50-02 coated particles is limited compared to Dynasan® 114 coated particles. This suggests that a rather efficient coating was obtained during the coating process of BSA crystals with Gelucire® 50-02. It was confirmed using optical and scanning electron microscopy.

Optical photomicrographs (Fig. 11) show that uncoated BSA crystals are transparent, flat platelets with sharp edges. In contrast, BSA crystals coated with Gelucire® 50-02 are completely opaque and exhibit a round-shaped morphology, suggesting a complete coating of the BSA crystals. SEM confirms that protein crystals coated with Gelucire® 50-02 have a rather homogeneous coating. However, it was not found to be perfectly uniform: the outer surface of the coated particles exhibits some discontinuities and defects. Fig. 12A illustrates this morphology for a particle

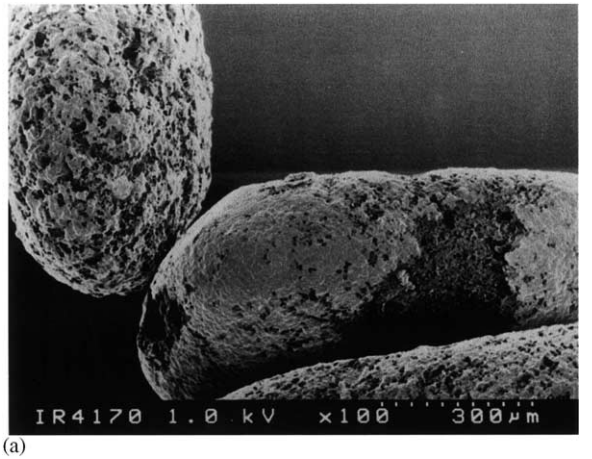


(a)

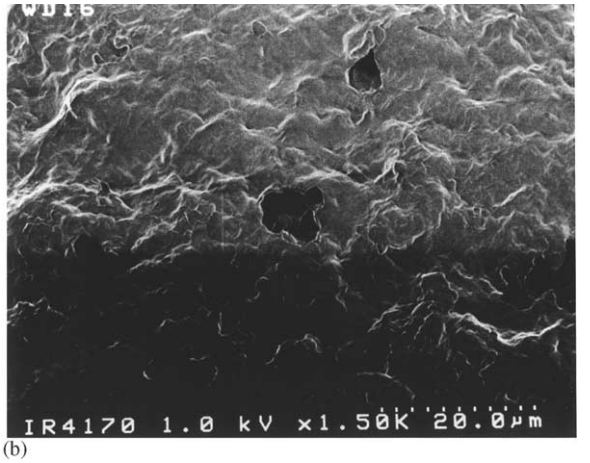


(b)

Fig. 11. A, Optical photomicrograph of uncoated 125–250 μm BSA crystals; B, Optical photomicrograph of coated 125–250 μm BSA crystals.



(a)



(b)

Fig. 12. SEM photomicrographs of BSA crystals coated with Gelucire® 50-02 (sample G1).

from sample G1. Although the coating seems to be porous, Fig. 12B suggests that, beneath the outer coating, a second layer of Gelucire® 50-02 exists. It is likely that the surface discontinuities and defects observed in Fig. 12A do not extend entirely through the coating.

Furthermore, optical microscopy indicates that minimal aggregation occurs during the coating process of sieved fractions of the smaller BSA crystals (fractions I and II). In order to determine the average number of BSA crystals per coated particle, the coating layer of about 30 particles of sample G2 were dissolved in methylene chloride. The average number of BSA crystals contained in each particle of sample G2 determined by this

simple method is found to be two. This is consistent with the results obtained from the contour particle size analysis: the mean particle size of the coated particles is 543 μm, whereas the uncoated BSA particles have a size ranging between 125 and 250 μm.

4. Conclusion

This work shows that our novel process can be used to efficiently coat preformed solid protein particles and get sustained-release solvent-free microcapsules, without any alteration of the structural integrity of the protein. Gelucire® 50/02 appears to be a good candidate as a coating material for implementation of the process. As it consists of a mixture of glycerides and fatty acid esters, Gelucire® 50-02 does not crystallize, whereas a pure triglyceride, such as Dynasan® 114 does. For this reason, its precipitation upon BSA crystals led to a rather homogeneous coating. Thus, a prolonged release associated with a limited burst effect was achieved at 37 °C over a 24 h period. The best advantage of this process compared to other SC CO_2 based processes is that the active material being coated stays along the whole process in its solid state, since no solubilization is required. Furthermore, exposure to heat or adsorption at water/solvent interface, which are the main sources of protein inactivation in classical microencapsulation processes, are avoided. For these reasons, beyond the present work performed with a model protein, this process should be well adapted to fragile biomolecules such as cytokines and growth factors.

Acknowledgements

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