Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 344 (2007) 16–25
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Improvement of a bovine serum albumin microencapsulation process by screening design

L. Al haushey [a,b,c], M.A. Bolzinger [a,b,c], C. Bordes [a,d], J.Y. Gauvrit [a,d], S. Briançon [a,b,c,*]

[a] Université de Lyon, Lyon F-69003, France
[b] Université Lyon 1, ISPB, Lyon F-69003, France
[c] CNRS, UMR 5007, Laboratoire d'Automatique et de Génie des Procédés (LAGEP), Villeurbanne F-69622, France
[d] CNRS, UMR 5180, Sciences Analytiques, Villeurbanne F-69622, France

Received 1 February 2007; received in revised form 26 May 2007; accepted 29 May 2007
Available online 13 June 2007

Abstract

The first objective of this study was to prepare microspheres containing a model protein by double emulsion-solvent evaporation/extraction method. This method was modified to consider the fragile nature of proteins. These modifications related to the reduction of polymer loss, of agitation duration and of contact time between protein and solvent. The polymer used was poly(ε-caprolactone) and the model protein was bovine serum albumin. The control of the microsphere properties constituted a second objective of this project. A screening design methodology was used to evaluate the effects of the process and formulation variables on microsphere properties. Twelve operating factors were retained, and the particle properties considered were the mean size, the encapsulation efficiency, and the surface state. The statistical analysis of the results allowed determining the most influent factors. Considering the whole results, it appeared that the polymer concentration, the osmotic pressure equilibrium and the volume of the inner, outer and organic phases were the most important parameters. Following this screening study, it was possible to produce particles of small size with high entrapment efficiency (near to 80%) and smooth surface. A good batch to batch reproductibility was obtained.

Keywords: Microspheres; Bovine serum albumin; Double emulsion; Solvent evaporation; Screening design

1. Introduction

Therapeutic proteins have become available with the development of the biotechnology industry and the advances in DNA recombinant technology (Couvreur et al., 1997; Putney, 1998; Wang et al., 2004). As they possess multiple biological functions like ligands, enzymes, receptors or antibodies (Sinha and Trehan, 2003), proteins may help to treat many diseases. Unfortunately the use of peptides/proteins as therapeutic agents is severely restricted by many problems associated with their high molecular weight, their sensitivity to environmental conditions which make them instable during preparation, storage (Liu et al., 1991) and release (Couvreur et al., 1997). Furthermore, proteins have poor oral and transdermal bioavailabilities, they are consequently often administered by parenteral routes (Putney, 1998). Most proteins having short half lives, they need to be frequently injected. This could limit the widespread use of protein therapeutics. The development of sustained release therapies that reduce the dosing schedule of the drugs should greatly increase patient compliance (Putney, 1998). In this regard, microencapsulation technology could be used to deliver the required dose of the drugs for prolonged time periods by a single shot (Kim and Park, 2001) while reducing the toxicity (Cleland, 1997). Biodegradable polymeric microparticles have been investigated successfully for such sustained delivery applications (Wang et al., 2004). Microencapsulation of proteins and peptides improves the therapeutic efficiency of these bioactive materials, protects them against the degradation and enhances the sustained drug release. However, the difficulties mentioned above about the proteins properties reduce the choice of an encapsulation method and impose to carefully control the external conditions to maintain the protein integrity during all the formulation steps.

* Corresponding author at: Université Lyon 1, ISPB, Lyon F-69003, France; LAGEP UMR CNRS 5007, France. Tel.: +33 4 72 43 18 64; fax: +33 4 72 43 18 64.
E-mail address: briancon@lagep.univ-lyon1.fr (S. Briançon).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.05.067

The methods generally used for proteins microencapsulation are spray drying, multiple emulsion and phase separation (Sinha and Trehan, 2003). The conventional double emulsion-solvent evaporation/extraction technique is the most common method to encapsulate proteins in microspheres. The polymers usually used are the poly(lactic acid) (PLA), its copolymer: poly(lactic-*co*-glycolic) acid (PLGA) and the poly(ε-caprolactone) (PCL). These poorly water-soluble polymers, which are completely biodegradable and biocompatible can be easily formulated into various types of delivery vehicles and administered by various routes. Proteins encapsulated by the double emulsion evaporation technique include the influenza A vaccine (Hilbert et al., 1999), the bovine serum albumin (Yang et al., 2001), the lysozyme (Nam et al., 2000) and the recombinant human epidermal growth factor (Han et al., 2001). Some microencapsulated protein drugs can now be found on the market (Lupron Depot®, Decapeptyl®, Enantone Depot®, Parlodel LA®) (Kostanski and Deluca, 2000). However, the major drawbacks of this method are the high shear stress necessary to emulsify the protein solution in the organic phase containing the polymer and the time of contact between the organic solvent necessary to dissolve the polymer and the protein, which could result in partial protein denaturing.

The goal of this research was to formulate microparticles loaded with BSA (a model protein) using the water-in oil-in water (W/O/W) emulsion technique. The double emulsion technique was modified to produce microspheres with variable properties (size distribution, encapsulation efficacy, porosity. . .) and to decrease the stresses imposed to the protein during process. The screening design methodology was used to investigate the parameters affecting the particles properties. The experiments for a screening model were structured by Plackett and Burman Algorithm (1946).

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) (M_w 67,000) as a model protein was purchased from Sigma Chemical Co. Poly(ε-caprolactone) (PCL) (M_w = 14,000) was obtained from ALDRICH. Methylene chloride (MC) from Laurylab was used as the polymer solvent. Isopropanol was from CarloErba reagenti. Polyvinyl alcohol 4–88 (PVA) from Fluka was used as a stabilizer in the external phase. Sucrose from Merck was used to equilibrate the osmotic pressure. The phosphate buffered saline tablets (PBS 0.01 M, pH 7.4) were obtained from ZYMED (USA). All other chemicals and solvents used were of analytical grade.

2.2. Microparticles preparation

The method of particle preparation is schematically represented on Fig. 1. BSA-loaded, poly(ε-caprolactone) (PCL) based microspheres were prepared by a modified W/O/W solvent evaporation/extraction method. Briefly, a solution (volume V_1) of the protein at different concentrations (C_1), in which PVA was added or not as a stabilizer (concentration C_2), was emulsified in a solution of PCL (quantity Q_3) in MC (volume V_2) using a rotor stator stirrer (T25 Ultra Turrax®, Ika). The stirring rate was variable (N_1 rpm) during (T_1 s). This primary emulsion W_1/O was then added to a variable volume of an aqueous solution (V_3) of PVA (C_4) under high speed mechanical stirring (T25 Ultra Turrax®, Ika) (N_2 rpm) during (T_2 s). The resultant double emulsion was transferred into a large volume of water, in which isopropanol was added, under gentle mechanical stirring for a given duration. In this aqueous phase, the osmotic pressure was equilibrated or not (P_1). Isopropanol allowed the extraction of MC from the droplets to form the microparticles; both solvents were then evaporated. The last stage of the process consisted in separating and purifying the microparticles by centrifugation and/or filtration, washing with water to eliminate residual PVA and finally drying at room conditions.

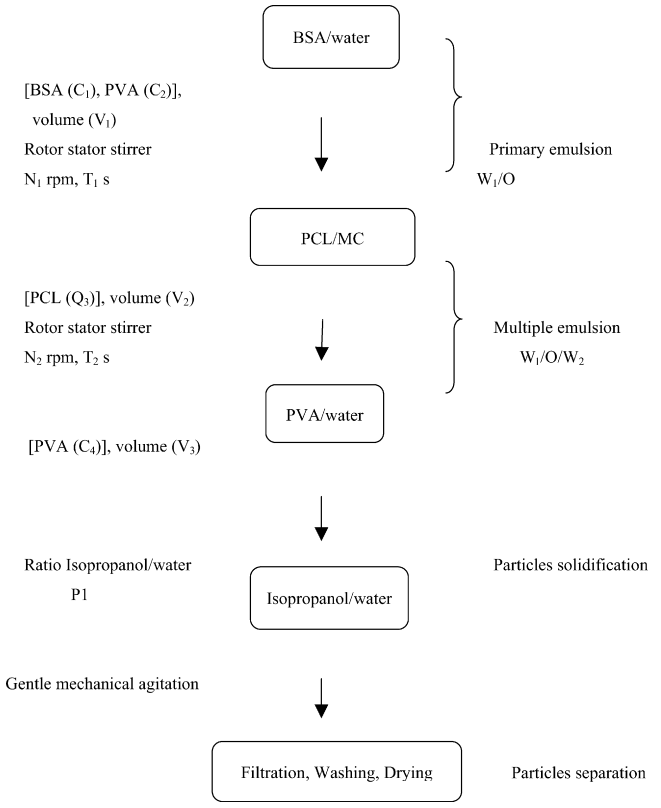

Fig. 1. Method of particle preparation by double emulsion-solvent evaporation/extraction technique.

2.3. Construction of the screening design

A preliminary study showed that the formulation parameters (volumes and concentrations of the phases, stabiliser concentration) as well as the process parameters (agitation rate and duration of the two emulsification steps) had an influence on the particle properties (size, morphology and encapsulation rate). Considering the great number of parameters, a screening design was constructed to estimate the effect of process and formulation variables on the particle characteristics (size, morphology and encapsulation efficiency). The results previously obtained were used to define the two levels of the factors involved in the

Table 1
Experimental factors and levels

Factor	Factor signification	Abbreviation	Level (−1)	Level (+1)
X_1	Stabilizer concentration/outer phase (%)	C_4	1	2
X_2	Polymer quantity (g)	Q_3	1.5	2.5
X_3	Inner phase volume (mL)	V_1	0.3	1
X_4	Protein concentration (mg/mL)	C_1	100	200
X_5	Outer phase volume (mL)	V_3	50	150
X_6	Speed rate: emulsion 1 (rpm)	N_1	8000	24,000
X_7	Speed rate: emulsion 2 (rpm)	N_2	9500	24,000
X_8	Duration agitation: emulsion 1 (s)	T_1	15	60
X_9	Duration agitation: emulsion 2 (s)	T_2	15	60
X_{10}	Stabilizer concentration/inner phase (%)	C_2	0	0.1
X_{11}	Organic phase volume (mL)	V_2	4	6
X_{12}	Osmotic pressure equilibrium	P_1	No	Yes

screening design. The experimental domain for each factor is summarized in Table 1.

Sixteen experiments, structured according to the Plackett and Burman Algorithm were carried out. The calculated model was of the form:

$$Y(\text{response}) = b_0 + b_1X_1 + b_2X_2 + \cdots + b_nX_n.$$

b_0: average of the responses for the 16 experiments. $b_1, b_2, \ldots, b_n$: coefficients of the factors $X_1, X_2, \ldots, X_n$ (representing the effect of each factor ordered within −1, +1) (Box GEP et al., 1978).

The following design of 16 experiments was obtained (Table 2).

The coefficients (b_i) were calculated by multiple linear regression and the results were analysed by NEMRODW® software Version 2000 (Nemrodw, LPRAI, Marseille, France).

The statistical analysis provided several informations:

- the regression quality was evaluated by some statistical indicators: the determination coefficient R^2 and the adjusted determination coefficient R^2a taking into account the freedom degree (FD) of the system.
- the response residual S.D.
- the coefficients values b_i and their S.D. The coefficient significativity was evaluated by using a Student test: the t Student value was thus given with its associated probability (or significativity). In this study, the coefficients whose significativity was lower than 10%, were assumed to be statistically different from 0, i.e. to have a significant influence on the response.

Table 2
Experimental design

Experiment	Factor											
	X_1 [C_4 (%)]	X_2 [Q_3 (g)]	X_3 [V_1 (mL)]	X_4 [C_1 (mg/mL)]	X_5 [V_3 (mL)]	X_6 [N_1 (rpm)]	X_7 [N_2 (rpm)]	X_8 [T_1 (s)]	X_9 [T_2 (s)]	X_{10} [C_2 (%)]	X_{11} [V_2 (mL)]	X_{12} [P_1]
1	2	2.5	1	200	50	24,000	9,500	60	60	0	4	Yes
2	1	2.5	1	200	150	8,000	24,000	15	60	0.1	4	No
3	1	1.5	1	200	150	24,000	9,500	60	15	0.1	6	No
4	1	1.5	0.3	200	150	24,000	24,000	15	60	0	6	Yes
5	2	1.5	0.3	100	150	24,000	24,000	60	15	0.1	4	Yes
6	1	2.5	0.3	100	50	24,000	24,000	60	60	0	6	No
7	1	1.5	1	100	50	8,000	24,000	60	60	0.1	4	Yes
8	2	1.5	0.3	200	50	8,000	9,500	60	60	0.1	6	No
9	2	2.5	0.3	100	150	8,000	9,500	15	60	0.1	6	Yes
10	1	2.5	1	100	50	24,000	9,500	15	15	0.1	6	Yes
11	2	1.5	1	200	50	8,000	24,000	15	15	0	6	Yes
12	1	2.5	0.3	200	150	8,000	9,500	60	15	0	4	Yes
13	2	1.5	1	100	150	24,000	9,500	15	60	0	4	No
14	2	2.5	0.3	200	50	24,000	24,000	15	15	0.1	4	No
15	2	2.5	1	100	150	8,000	24,000	60	15	0	6	No
16	1	1.5	0.3	100	50	8,000	9,500	15	15	0	4	No

Table 3
Experiment results

Experiment	Size (μm) Y_1[a]	Entrapment efficiency % Y_2[b]	Number of pores Y_3	Width of pores Y_4	Surface state Y_5
1	28.4 ± 0.8*	43	2	1	1
2	20.1 ± 1	15	3	2	2
3	28 ± 1.2	7	0	0	1
4	4.9 ± 0.06	48	0	0	1
5	9.5 ± 0.2	60	0	0	0
6	4 ± 0.4	39	1	1	1
7	3.3 ± 0.1	42	1	1	1
8	12.9 ± 0.3	26	0	0	0
9	19.1 ± 1.2	46	0	0	1
10	16.7 ± 0.6	51	1	1	0
11	2.5 ± 0.06	36	0	0	0
12	43.8 ± 3.3	53	0	0	0
13	30.8 ± 1	10	2	3	2
14	11.6 ± 0.7	46	0	0	1
15	11.4 ± 0.7	15	1	3	1
16	18.3 ± 0.2	34	1	1	2

[a] Mean value of three measurements.
[b] The S.D. was estimated at 4% after having processed two times the particles of experiments 2 and 16 and four times the particles of experiments 17 and 18.

2.4. Microparticle characterization

The microparticles were characterized by the mean particle size, the entrapment efficiency, the porosity and the surface state. These two latter properties are qualitative responses, derived from the microscopic observations. These characteristics constituted the considered responses (Y_i).

2.4.1. Particle size distribution

Particle size distribution was analysed by laser scattering (Coulter®LS230, Beckman Coulter, France). Average particle size was expressed as volume-average diameter in micrometer.

2.4.2. Encapsulation efficiency

The microspheres were assayed for BSA content using a HPLC detection method described by Yang et al., 2000. Briefly, a determined amount of the preparation was solved in 1 g of methylene chloride MC for 6 h. Then, the protein was extracted in 2 mL of PBS (pH 7.4) for 24 h under gentle agitation. The supernatant was centrifuged for 10 min at 2700 × *g* using an Eppendorf Centrifuge (model 5417C). The protein solution was filtered and then placed in special vials for HPLC assay. Aliquots of 20 μL from each vial were injected onto the HPLC system equipped with a Zorbax GF-250 column (4.6 mm × 25 cm – porosity 4 μm). The HPLC system consisted of an autosampler and an UV detector (Spectra System, Thermo Separation Products TPS, California). The BSA quantification was performed by integrating the peaks detected at 210 nm. The samples were chromatographed using a mobile phase consisting of PBS (pH 7.4) at a flow rate of 1 mL/min. A calibration curve (peak area versus drug concentration) was constructed by dissolution of BSA powder defined quantities in MC for 6 h and then extraction in PBS for 24 h (the same treatment as applied to the microspheres) Calibration curves were linear over the range of 25–400 μg/mL. The efficiency of encapsulation was expressed as a ratio of the experimental protein entrapment to the theoretical protein entrapment, i.e. the quantity of protein introduced initially in the inner aqueous phase.

2.4.3. Particle morphology

The particle surface morphology was estimated from scanning electron microscopy analysis (SEM, Hitachi S800, Japan). Three parameters related to the surface rugosity and the porosity were defined. A number from 0 to 2 was used to quantify the surface state (Table 3), 0 was attributed to a smooth surface and 2 to a very rough one. The porosity was characterized by the numbers and the width of the pores. The pores frequency and their width were scored from 0 to 3 according respectively to the following gradation:

- Number of pores present at the surface: 0 = no porosity, 3 = great number of pores.
- Width of the pores: 0 = small pores, 3 = big ones.

2.4.4. Osmotic pressure

The osmotic pressure of the inner and outer phases was measured with an automatic cryoscopic osmometer from Fisher Scientific Bioblock (France).

3. Results and discussion

The fabrication of microspheres by the W/O/W emulsion technique was modified to respect the fragile properties of proteins and to modulate the microparticle properties. To respect mild environmental conditions during the process illustrated on Fig. 1, the two main critical steps of this process which are respectively the stresses imposed during the two emulsification steps (agitation time and shear rate) and the contact time between protein and solvent were minimized.

The agitation time of the two emulsion steps (X_8 and X_9) was decreased until 15 s, (level −1, see on Table 1) reducing the shear stresses and the protein degradation risk. In the literature, the first emulsion is frequently obtained by sonication of the organic and inner phases, the duration is comprised between 20 s and few minutes (Panyam et al., 2003; Yang et al., 2001). The use of a rotor stator system instead of sonication limited the protein degradation risk by decreasing the heat generated in the system for the same operating duration. The rotor stator has been used in others studies to form the first and the second emulsions, using a higher stirring rate during a higher or an equivalent duration. As an example, Pistel et al. (2001) used 20,000 rpm during 30 s for the first emulsion and Benoit et al. (1999) used 8000 rpm during 2–15 min for the two emulsions. The stirring rate and the duration in our study were reduced until 8000 rpm (level −1) and 15 s, respectively to minimize the shear stress applied on the protein.

The contact time between the organic solvent and the protein was also decreased by adding isopropanol in the outer phase. The addition of isopropanol had the major advantage to enhance the MC diffusion, leading consequently to a rapid precipitation of the polymer in the preparation and therefore to a shorter contact time between the protein and the solvent. Finally, the polymer amount in the final microspheres composition was increased and the polymer precipitation apart from the particles was avoided by reducing the volume (X_5) of the outer aqueous phase.

As the protein integrity was not checked in the microspheres after encapsulation, it cannot be stated that these operating conditions entirely hinder the protein degradation. However, it can be assumed that this process is in favour of a decrease of the protein degradation risk as all the parameters were adjusted to limit the physical stresses imposed to the protein. Furthermore, previous studies demonstrated that the BSA primary structure was not affected by the double emulsion technique in similar conditions (Benoit et al., 1999; Jiang et al., 2002).

The second challenge of our research was to investigate the role of each experimental factor defined in Table 1 on the particle properties.

The results of the16 experiments carried out are summarized in Table 3. All the calculated values of the b_i coefficients are reported for each response on Figs. 1–3. The factors influencing strongly the response are those having the highest absolute value. The dotted line separates the statistical significant effects (the coefficients values are beyond the line) from the others. The sign of the coefficient shows how the factor influences the response: if the coefficient is negative, the response is decreased when the factor moves from level (−1) to level (+1); the contrary is obtained if the coefficient is positive. The sign and the value of the coefficients allow directing the choice of the best level for each factor.

3.1. Influence of the investigated parameters on the particle mean size (response Y_1)

The particle mean size ranged from 2.5 to 44 μm depending on the production variables employed (Table 3 and Fig. 2(a–c)).

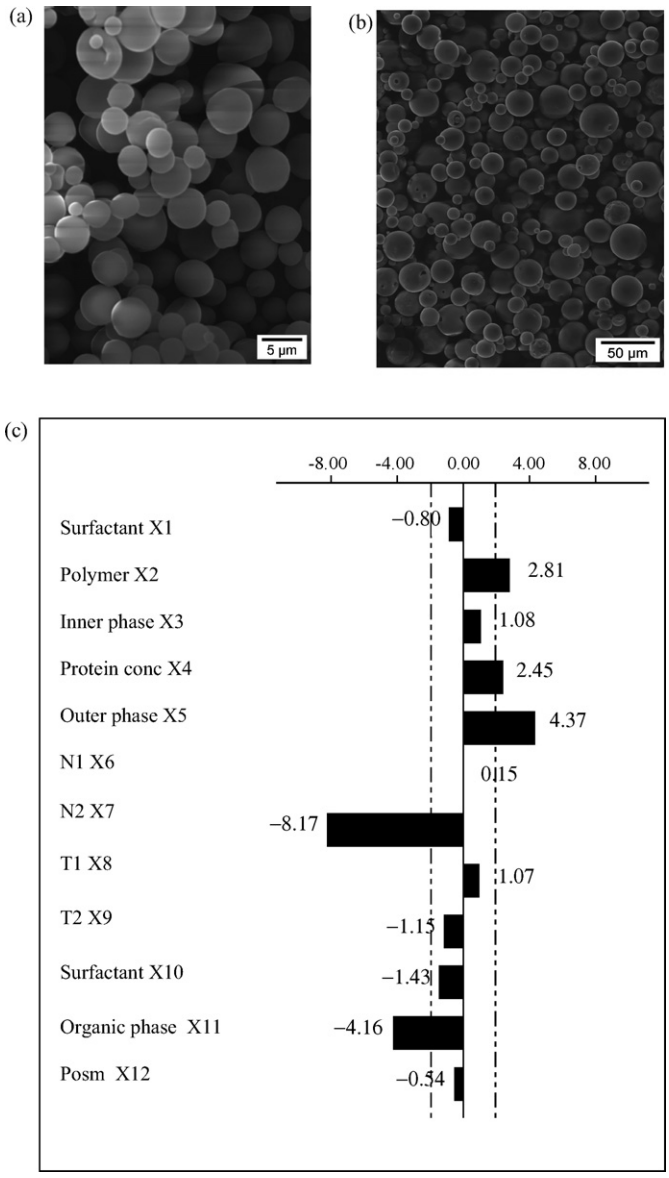

Fig. 2. Images of two different particles sizes obtained. (a) Experiment 11 magnification 3000×; (b) experiment 16 magnification 350×; (c) effect of the different factors on the response Y_1 (size).

The statistical analysis revealed the significant effects of the following parameters (Fig. 2(c) and Table 4):

- The stirring rate (X_7) during the second emulsification and the volume of the outer phase (X_5).
- The volume of the organic phase (X_{11}) during the fabrication of the W_1/O primary emulsion, the polymer quantity (X_2) and the protein concentration (X_4).

The other parameters studied did not have any significant effect on the particle size in the range of chosen operating conditions.

An increase in polymer quantity (X_2) led to an increase in particles size as also observed in other studies (Yan et al., 1994; Yang et al., 2000). This effect could be attributed to the increase of the organic phase viscosity (Blanco-Prieto et al., 1994; Yang

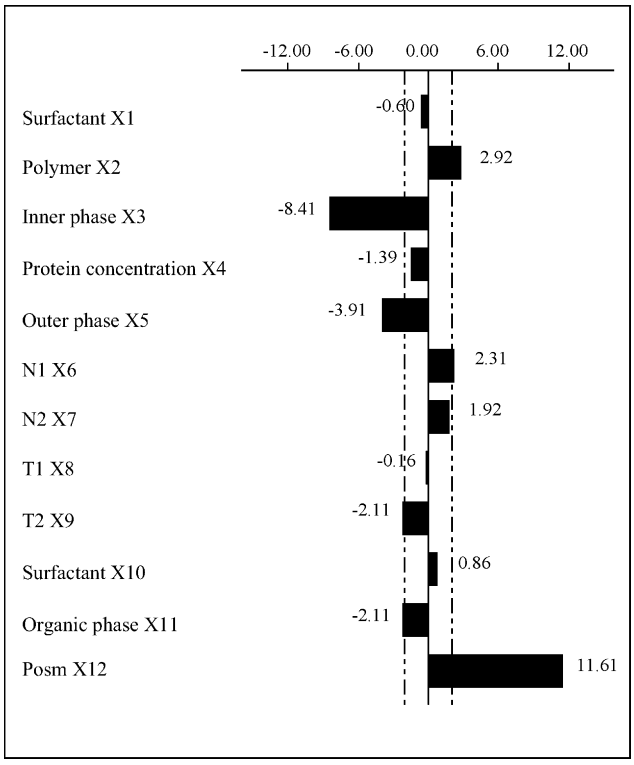

Fig. 3. Effect of the different factors on the response Y_2 (encapsulation efficiency).

et al., 2001) which results in turn in a reduction of stirring efficiency (Couvreur et al., 1997). The same effect was observed when using a low organic phase volume (X_{11}) leading to difficulties for the polymer solution to be broken up in smaller droplets (Yang et al., 2001). In addition, due to an increase in the polymer concentration, the frequency of collisions during the second emulsification and the droplet solidification might increase, resulting in the aggregation of semi-solids particles and particle size increase (Jeffery et al., 1991). The increase of particle size obtained by increasing the protein concentration (X_4) might be explained by the creation of an osmotic pressure gradient between the inner and outer phases (due to protein) that generated a flow of water from the outer phase. The osmolarities of the inner phase were 19 and 37 mosm/kg H_2O for the low and high protein concentrations, respectively. The increase of the outer phase volume (X_5) resulted in an increase of the particles size because of the reduction in the agitation efficiency associated with large volumes (Jeffery et al., 1993; Benoit et al., 1999). The stirring rate (X_7) had to be increased at the higher level (24,000 rpm) so that the particle size decreased down to 16 μm. This high stirring rate provided the necessary energy to divide the droplets into smaller ones (Yang et al., 2001). The Fig. 2(a) and (b) show microparticles obtained from the experiments 11 and 16. The mean particles sizes are 2.5 and 18.3 μm, respectively. Except for the protein concentration X_4, these experiments corresponded to factor levels combinations leading to a particle size increase (experiment 16, Fig. 2(b)) or decrease (experiment 11, Fig. 2(c)).

Surprisingly, neither the surfactant concentration in the outer phase nor the duration of agitation, respectively to their experimental domain, played a significant role to drive the particle size. These results could be surprising as it was shown in other studies that the particle size decreased when the surfactant concentration in the outer phase increased (Yang et al., 2001) and when the durations of the two emulsions increased (Benoit et al., 1999).

Table 4
Statistical analysis of Y1 results (mean particle size)

Name	Coefficient value	S.D.	$t_{exp.}$ Student	Significativity (%)
b_0	16.581	0.955	17.35	0.0417***
b_1	−0.806	0.955	−0.84	46.1
b_2	2.806	0.955	2.94	6.1
b_3	1.069	0.955	1.12	34.5
b_4	2.444	0.955	2.56	8.3
b_5	4.369	0.955	4.57	1.96*
b_6	0.156	0.955	0.16	88
b_7	−8.169	0.955	−8.55	0.336**
b_8	1.081	0.955	1.13	34
b_9	−1.144	0.955	−1.2	31.7
b_{10}	−1.431	0.955	−1.5	23.1
b_{11}	−4.144	0.955	−4.34	2.26*
b_{12}	−0.556	0.955	−0.58	60.1
R^2	0.978	Response residual S.D.	3.822	
R^2a	0.892	FD	3	

R^2: determination coefficient; R^2a: adjusted determination coefficient; FD: freedom degree; the asterisk in the significativity column show the coefficients of the most influent factors.

3.2. Influence of the investigated parameters on the particle encapsulation efficiency (response Y_2)

Entrapment efficiencies between 7% and 60% were obtained for the various factor level combinations (Table 3). The equilibrium of the osmotic pressure (X_{12}), the volumes of the inner phase (X_3) and outer (X_5) phases, the polymer quantity (X_2), the stirring rate (X_6), the agitation time (X_9), the volume of the organic phase (X_{11}) and the stirring rate (X_7) were the most influent parameters (Table 5 and Fig. 3) relatively to the others factors and their experimental domain.

To increase the entrapment efficiency, the volumes of the inner and outer phases (X_3 and X_5) had to be low; and the osmotic pressure (X_{12}) between the inner and the outer phases had to be equilibrated to hinder the protein leakage during the solidification of particles. At high polymer quantity (X_2), the solidification of microparticles was faster; this resulted in a viscous polymer layer at the droplet surface. This layer inhibited the protein diffusion towards the external phase (Yang et al., 2000) and thus an increase of the encapsulation efficiency was observed. Moreover, the increase of the system viscosity associated with a high polymer quantity tended to limit the migration of the protein from the inner phase towards the outer one (Raffati et al., 1997). When the volume of the outer phase (X_5) increased, the encapsulation efficiency decreased. This could be related to the increase of the pore size at the particle surface (see Section 3.3 and Fig. 4). The lower encapsulation efficiencies were obtained with experiments 2, 13, 15, 16 where porous microparticles were obtained,

Table 5
Statistical analysis of Y_2 results (encapsulation efficiency)

Name	Coefficient	S.D.	$t_{exp.}$ Student	Significativity (%)
b_0	35.688	0.695	51.35	<0.01***
b_1	−0.438	0.695	−0.63	57.4
b_2	2.813	0.695	4.05	2.72*
b_3	−8.313	0.695	−11.96	0.126**
b_4	−1.438	0.695	−2.07	13
b_5	−3.938	0.695	−5.67	1.09*
b_6	2.313	0.695	3.33	4.48*
b_7	1.938	0.695	2.79	6.9
b_8	−0.063	0.695	−0.09	93.4
b_9	−2.063	0.695	−2.97	5.9
b_{10}	0.938	0.695	1.35	27
b_{11}	−2.188	0.695	−3.15	5.1
b_{12}	11.688	0.695	16.82	0.0458***
R^2	0.994	Response residual S.D.	2.78	
R^2a	0.971	FD	3	

R^2: determination coefficient; R^2a: adjusted determination coefficient; FD: freedom degree; The asterisk in the significativity column show coefficients of the most influent factors.

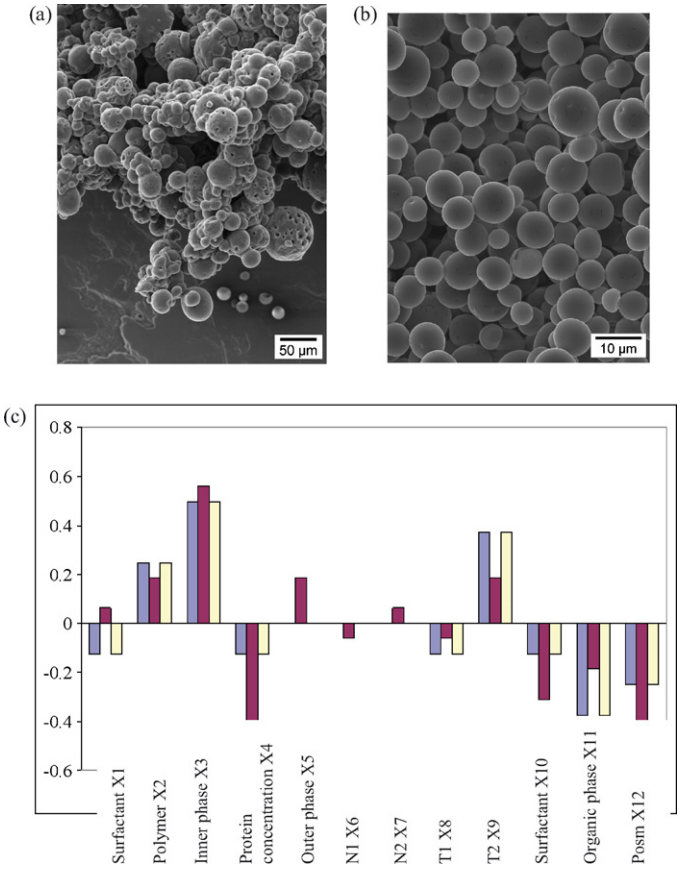

Fig. 4. Images of two different morphologies obtained. (a) SEM picture of very rough particles, with a great number of large pores (experiment 2, magnification 250×); (b) SEM picture of particles, with a smooth surface and no pores (experiment 2, magnification 1700×); (c) Effect of the different factors on the responses Y_3 (number of the pores), Y_4 (width of the pores) and Y_5 (rugosity).

Table 6
Conditions of experiments 17 and 18

Experiment	Factor											
	X_1 [C_4 (%)]	X_2 [Q_3 (g)]	X_3 [V_1 (mL)]	X_4 [C_1 (mg/mL)]	X_5 [V_3 (mL)]	X_6 [N_1 (rpm)]	X_7 [N_2 (rpm)]	X_8 [T_1 (s)]	X_9 [T_2 (s)]	X_{10} [C_2 (%)]	X_{11} [V_2 (mL)]	X_{12} [P_1]
17	1	2.5	0.3	100	50	13,500	24,000	15	15	0.1	5	Yes
18	1	2.5	0.3	100	50	13,500	24,000	15	15	0.1	5	Yes

Table 7
Properties of the particles fabricated in the optimal conditions

Experiment	Size (μm) Y_1[a]	Entrapment efficiency % Y_2[b]	Number of pores Y_3	Width of pores Y_4	Surface state Y_5
17	4.3 ± 0.6	77	0	0	1
18	4.3 ± 0.3	83	0	0	1

[a] Mean value of three measurements.
[b] The S.D. was estimated at 4% after having processed two times the particles of experiments 2 and 16 and four times the particles of experiments 17 and 18.

as already observed in other studies (Herrmann and Bodmeier, 1995). In these experiments, the osmotic pressure in the inner phase was greater than that of the outer phase (i.e. P1 = NO). The water flew from the outer phase and the particles were large and porous (Han et al., 2001). Lower encapsulation efficiencies were thus obtained. The volume of the inner phase (X_3) played an important role on the entrapment efficiency since high inner phase volume resulted in an enlargement of the surface pore sizes thus reducing the entrapment efficiency by drug loss through a porous structure (Han et al., 2001). Hollow microparticles have been obtained by Crotts and Park (1995) while increasing the inner phase volume from 0.5 to 2.5 mL. This peculiar structure was not observed in our study, probably because smaller X_3 volume variations were investigated (0.3–1 mL). As an example the experiment no. 5 which corresponded to the lower X_3 volume and a Posm at equilibrium yielded a high encapsulation rate.

3.3. Influence of the investigated parameters on the particle morphology (responses Y_3, Y_4 and Y_5)

The morphology of particles depended on the operating conditions as illustrated in Fig. 4.

Since the responses Y_3, Y_4 and Y_5 are rather qualitative than quantitative, the statistical analysis was not available. The b_i coefficients were calculated only to indicate the parameter tendency, they are represented in Fig. 4(c).

The number of pores (Y_3) and the particle rugosity (Y_5) were correlated with the volumes of the inner (X_3) and organic phases (X_{11}), the duration of agitation during the second emulsification (X_9) and relatively less correlated with the quantity of polymer (X_2), and the equilibrium of the osmotic pressure (X_{12}). At high polymer quantity, the solidification of microparticles was more rapid (Yang et al., 2000). The fast removal of the organic solvent caused by the increase of the polymer amount or the decrease of the organic phase volume could cause a local explosion inside the polymer droplets leading to the formation of porous structure and coarse surface (Arshady, 1991). When the organic phase volume increased, the surface of the particles became less porous. In fact, as explained by Yang et al. (2000), a high content of methylene chloride may result in a slow solvent removal, which could hinder the formation of porous skin and the methylene chloride may re-dissolve the skin of semi-solid particles leading to a less porous surface. The factors that increase the solvent removal kinetics (increase of polymer quantity and outer phase volume) might increase the porosity. We also notice that increasing the volume of the inner phase resulted in porous external and internal structure in accordance with Crotts and Park (1995). Fig. 4(b) shows smooth particles and the absence of pores could be noticed. It corresponds to an experiment in which the osmotic pressure between the inner and outer phases was at equilibrium and the X_3 and X_{11} volumes were at level (−1) and (+1), respectively. On the contrary, the particles on Fig. 4(a) (corresponding to the experiment no. 2) presented a great number of pores at their surface. In this experiment the volume X_3 was high (+1), the volume X_{11} was low (−1), and the osmotic pressure was not at equilibrium.

The parameters that modulate the width of the pores were the volume of the inner phase (X_3), the protein concentration (X_4), the surfactant presence in the inner phase (X_{10}) and finally the equilibrium of the osmotic pressure (X_{12}).

It appeared that the osmotic pressure played an important role on the surface quality. In fact, the equilibrium of this pressure could inhibit the flow of water from the external phase towards the inner one (due to the gradient of osmotic pressure created by the protein) leading to a denser material as described previously by Han et al. (2001). In addition, the number of pores and their width might decrease.

3.4. Overview

In order to prepare microspheres of a given size (variable between few micrometers and 50 μm), with maximal encapsulation efficiency, a smooth and non-porous surface, the coded factor levels had to be:

Table 8
Representation of the five most influent factors in the experimental domain on the particles properties

Factor	Effect on size Y_1	Effect on entrapment efficiency Y_2	Effect on the number of pores Y_3	Effect on the width of pores Y_4	Effect on the morphology state Y_5
↑ Quantity of polymer X_2	↑	↑	↑	↑	–
↑ Volume of inner phase X_3	↑	↓	↑	↑	Rough
↑ Volume of external phase X_5	↑	↓	–	↑	Rough
↑ Volume of organic phase X_{11}	↓	↓	↓	↓	Smooth
↑ Equilibrium of osmotic pressure X_{12}	↓	↓	↓	↓	Smooth

↑, increase of the property value; ↓, decrease of the property value; –, no effect.

- −1 for the stabiliser concentration in the outer phase (X_1), the volumes of the inner (X_3) and outer (X_5) phases, the protein concentration (X_4), the two durations of agitation (X_8, X_9).
- +1 for the others except the stirring rate during the first emulsification (X_6) and the volume of the organic solvent (X_{11}) that had to take middle values in order to respect the fragile properties of proteins.

The stirring rate during the second emulsion (X_7) had to be adjusted according to the desired particle size. A high value of this stirring rate allowed to get particles of a few micrometers diameter but the protein degradation might be higher.

With the parameter levels described above and considered as optimal conditions, two additional experiments were carried out (Table 6). The results are summarized in Table 7.

The obtained particles had a small size, a high encapsulation efficiency, a smooth and non-porous surface, these results were reproducible.

From these results, it could be concluded that five factors strongly influenced the particle characteristics, while the other factors could be neglected respectively to their experimental domain.

These factors and their influences on the particle properties when they took the level +1 are reported in Table 8.

4. Conclusion

In this study, the method of double emulsion was modified in order to respect the properties of proteins. The time of agitation was decreased to 1 min maximum, the contact time between the protein solution and the organic solvent was also reduced by the use of isopropanol to accelerate the diffusion of methylene chloride in the aqueous phase. This modified method also allowed to reduce the polymer loss by using a small amount of outer phase. Moreover, this reduction of the outer phase volume was a way to improve the particle properties (smooth surface state and high encapsulation efficiency). The effects of the different operating factors on the particle characteristics were investigated by an experimental screening design. To obtain high encapsulation efficiencies, the volumes of the inner and outer phases had to be decreased and the osmotic pressure had to be equilibrated. To modulate the particle size, the stirring rate during the second emulsification, the polymer concentration and the volume of the outer and organic phases could be considered as the control parameters. It should be noticed that the stirring rate during the second emulsion is the most influent parameter to control the particle size. It should be high to obtain small particle, but this could have a negative effect on the protein stability and a compromise could be needed. Finally, to modify the external structure, the volume of the inner phase, the duration of agitation during the two steps of emulsification, the volume of the organic phase and the equilibrium of the osmotic pressure must be adjusted. The osmotic pressure need to be at equilibrium to efficiently limit the diffusion of water from and to the inner phase and consequently to give a less porous structure and high encapsulation rate.

This first part of the study led to define good production conditions according to the particle properties. These conditions are also those supposed to be in favour of the protein protection (low agitation time, low volumes of the inner and outer phases, middle stirring rates). Two experiments were carried out using these conditions; the obtained particles had a mean size of 4 μm, a smooth surface and high encapsulation efficiency (almost 80%). A good batch to batch reproducibility was obtained.

Following this study, five parameters which have the greatest influence on the particle properties were determined. The next step actually in progress will consist to model the particle properties depending on these parameters. Additional particle properties could also be added in the factorial design and considered as pertinent responses, as in vitro release profile, or microsphere stability.

Acknowledgement

The authors acknowledge the Technologic Centre of Microstructures-University Claude Bernard Lyon 1 for SEM analysis.

References

Arshady, R., 1991. Preparation of biodegradable microspheres and microcapsules: 2. polylactides and related polyesters. J. Control. Release 17, 1–21.

Benoit, M.A., Baras, B., Gillard, J., 1999. Preparation and characterization of protein-loaded poly(ε-caprolactone) microparticles for oral vaccine delivery. Int. J. Pharm. 184, 73–84.

Blanco-Prieto, M.J., Delie, F., Fattal, E., Tartar, A., Puisieux, F., Gulik, A., Couvreur, P., 1994. Characterization of V3 BRU peptide-loaded small PLGA microspheres prepared by a $(W_1/O)/W_2$ emulsion solvent evaporation method. Int. J. Pharm. 111, 137–145.

Box, G.E.P., Hunter, W.G., Hunter, J.S., 1978. Statistics for Experimenters. John Wiley, NY.

Cleland, J.L., 1997. Protein delivery from biodegradable microspheres. In: Sanders, L.M., Hendren, R.W. (Eds.), Protein Delivery: Physical Systems. Plenum, New York, pp. 1–43.

Couvreur, P., Blanco-Prieto, M.J., Puisieux, F., Roques, B., Fattal, E., 1997. Multiple emulsion technology for the design of microspheres containing peptides and oligopeptides. Adv. Drug. Deliv. Rev. 28, 85–96.

Crotts, G., Park, T.G., 1995. Preparation of porous and nonporous biodegradable polymeric hollow microspheres. J. Control. Release 35, 91–105.

Han, K., Lee, K.D., Gao, Z.S., Park, J.S., 2001. Preparation and evaluation of poly(L-lactic acid) microspheres containing rhEGF for chronic gastric ulcer healing. J. Control. Release 75, 259–269.

Herrmann, J., Bodmeier, R., 1995. Effect of particle microstructure on the somatostatin release from poly(lactide) microspheres prepared by a W/O/W solvent evaporation method. J. Control. Release 36, 63–71.

Hilbert, A.K., Fritzshche, U., Kissel, T., 1999. Biodegradable microspheres containing influenza-A vaccine: immune response in mice. Vaccine 17, 1065–1073.

Jeffery, H., Davis, S.S., O'Hagan, D.T., 1991. The preparation and characterization of poly(lactide-*co*-glycolide) microparticles. I. Oil-in-water emulsion solvent evaporation. Int. J. Pharm. 77, 169–175.

Jeffery, H., Davis, S.S., O'Hagan, D.T., 1993. The preparation and characterization of poly(lactide-*co*-glycolide) microparticles. II. The entrapment of a model protein using a (water-in-oil-in-water) emulsion solvent evaporation technique. Pharm. Res. 10, 362–368.

Jiang, G., Thanoo, B.C., Deluca, P.P., 2002. Effect of osmotic pressure in the solvent extraction phase on BSA release profile from PLGA microspheres. Pharm. Dev. Technol. 7, 391–399.

Kim, H.K., Park, T.G., 2001. Microencapsulation of dissociable human growth hormonr aggregates within poly(D,L-lactic-*co*-glycolic acid) microparticles for sustained release. Int. J. Pharm. 229, 107–116.

Kostanski, J.W., Deluca, P.P., 2000. A novel in vitro release technique for peptide containing biodegrdabale microspheres. AAPS Pharm. Sci. Tech., 1, article 4.

Liu, W.R., Langer, R., Klibanov, A.M., 1991. Moisture-induced aggregation of lyophilized proteins in the solid state. Biotech. Bioeng. 37, 177–184.

Nam, Y.S., Song, S.H., Choi, J.Y., Park, T.G., 2000. Lysozyme microencapsultion within biodegradable PLGA microspheres: urea effect on protein release and stability. Biotechnol. Bioeng. 70, 270–277.

Panyam, J., Dali, M.M., Sahoo, S.K., Ma, W., Chakravarthi, S.S., Amidon, G.L., Levy, R.J., Labhasetwar, V., 2003. Polymer degradation and in vitro release of a model protein from poly(D,L-lactide-*co*-glycolide) nano-and microparticles. J. Control. Release 92, 173–187.

Pistel, K.F., Breitenbach, A., Zange-Volland, R., Kissel, T., 2001. Brush-like branched biodegradable polyesters, part III: protein release from microspheres of poly(vinyl alcohol)-graft-poly(D,L-lactic-*co*-glycolic acid). J. Control. Release 73, 7–20.

Plackett, R.L., Burman, J.P., 1946. Biometrika 33, 305–325.

Putney, S.D., 1998. Encapsulation of proteins for improved delivery. Curr. Opin. Chem. Biol. 2, 548–552.

Raffati, H., Coombes, A.G.A., Adler, J., Holland, J., Davis, S.S., 1997. Protein-loaded poly(DL-lactide-*co*-glycolide) microparticles for oral administration: formulation, structural and release characteristics. J. Control. Release 43, 89–102.

Sinha, V.R., Trehan, A., 2003. Biodegradable microspheres for protein delivery. J. Control. Release 90, 261–280.

Wang, J., Chua, K.M., Wang, C.H., 2004. Stabilization and encapsulation of human immunoglobulin G into biodegradable microspheres. J. Colloid Interface Sci. 271, 92–101.

Yan, Ch., Resau, J.H., Hewetson, J., West, M., Rill, W.L., Kende, M., 1994. Characterization and morphological analysis of protein-loaded poly(lactide-*co*-gycolide) microparticles prepared by water-in-oil-in-water emulsion technique. J. Control. Release 32, 231–241.

Yang, Y.Y., Chung, T.S., Bai, X.L., Chan, W.K., 2000. Effect of preparation condition on morphology and release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion method. Chem. Eng. Sci. 55, 2223–2236.

Yang, Y.Y., Chung, T.S., Ng, N.P., 2001. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 22, 231–241.