Preparation of PLGA Microparticles by an Emulsion-Extraction Process Using Glycofurol as Polymer Solvent

Anne Aubert-Pouëssel[1], Marie-Claire Venier-Julienne[1,3], Patrick Saulnier[1], Michelle Sergent[2], and Jean-Pierre Benoît[1]

Received April 13, 2004; accepted September 5, 2004

Purpose. To develop biodegradable poly(lactic-*co*-glycolic acid) (PLGA) microparticles prepared by an original emulsion-extraction process, with glycofurol, a nontoxic excipient, as polymer solvent.
Methods. The preparation of microparticles consisted in dissolving polymer in glycofurol. This solution was emulsified in a vegetable oil, and then amphiphilic agent was added into the emulsion to extract glycofurol and lead to microparticle formation. Physicochemical studies were carried out, and an experimental design was prepared in order to elucidate the impact of the formulation composition on the microparticle characteristics. Finally, encapsulation tests were made with a model protein.
Results. In a ternary diagram, a small feasibility area allowing particle formation was located. The resulting microparticles were spherical with a homogeneous, polymeric matrix structure. They exhibited a variable size from 3 to 15 μm, which was controlled by the different formulation parameters. Differential scanning calorimetry (DSC) analysis made it possible to detect their composition. Preliminary results showed that these particles were able to encapsulate a protein model, lysozyme.
Conclusions. This simple and convenient technique enabled us to obtain spherical, biodegradable microparticles from acceptable excipients. Moreover, the process conditions made possible the encapsulation of drugs, including proteins.

KEY WORDS: diffusion process; glycofurol; microparticles; poly(lactic-*co*-glycolic acid); protein.

INTRODUCTION

Biodegradable poly(lactic-*co*-glycolic acid) (PLGA) polymers show interesting properties for biotechnology through their biocompatibility and their authorization by the Food and Drug Administration (FDA) for drug delivery. Various polymeric drug delivery systems like microparticles or nanoparticles have been developed using these polymers for the delivery of a variety of drugs (1). However, the technology processes often use organic solvents to dissolve the water-insoluble PLGA. Usually, halogenated solvents, such as methylene chloride and chloroform, are used in the microencapsulation process. These solvents are rated as class 2 ("to be limited") according to the International Conference of Harmonization (ICH). The permitted daily exposure (PDE) for methylene chloride is 6.0 mg, and for chloroform the PDE is 0.6 mg (2). Furthermore, additional steps of desorption have to be considered to reduce the residual content of organic solvent in the resulting products. Even if a favorable benefit-risk ratio is taken into consideration for microspheres aimed at treating serious pathologies, no potential toxicity is tolerated for microspheres aimed at vaccination or minor disorders, where the risk is greater than the potential benefit. Moreover, the use of such solvents makes it difficult to respect safety conditions during production; thus, the development of halogenated solvent-free microencapsulation methods is desirable.

Efforts to avoid the use of halogenated solvents in PLGA microencapsulation processes have been undertaken. For example ethyl acetate (3,4), and ethyl formate (5), both belonging to class 3 entitled "low toxic potential" and limited to 50 mg/day, were used as alternative solvents. Equally, some encapsulation techniques, using supercritical fluids, especially supercritical carbon dioxide, were proposed in order to completely eliminate organic solvents or at least to minimize their use (6). Jain (1) described a technique based on phase separation using only oil phases (triacetin, Mygliol 812, etc.). However, in order to dissolve the polymer in the triacetin phase, a heating step (65°C) was required; this thermal treatment could both alter the polymer properties and also limit the encapsulation of delicate drugs such as proteins.

In the current work, excipients were chosen from those used in human medicine. Thus, in order to dissolve the polymer, a parenteral solvent, glycofurol, was used (7–9); it is a component of a marketed product (Mobic: meloxicam). Glycofurol has also been used in research as a vaccine adjuvant (10), and as a solvent in injectable polymer implants (11–13). In order to form an emulsion with the polymer solution, olive oil was chosen. This common vegetable oil has been used as an excipient in long-acting injectable preparations. Finally, different non-ionic amphiphilic excipients were used both in the extraction and washing steps: Labrafil M 1944 CS and poloxamer 188, respectively. Labrafil has been used as a solvent in an injectable gel delivering contraceptive steroids (14); poloxamer 188 has been commonly used in various marketed products, especially in injectable preparations (Norditropin). The strong interest in halogenated solvent-free methods is driven by the potential for a reduction of toxicological risk and an improved preservation of drug integrity, especially for proteins. Indeed, with the steady increase of recombinant protein-based pharmaceuticals due to the development of genetic engineering, there clearly existed a need for developing effective polymeric delivery systems. Therefore, to test the ability of the proposed method to encapsulate proteins, preliminary studies with a model protein, lysozyme, were carried out.

MATERIALS AND METHODS

Materials

Uncapped PLGA was obtained from Phusis (PLGA 75/25) (Saint-Ismier, France). The mean molecular weight ($\overline{M}w$) was 21,000 (polydispersity index $I = 1.7$) as determined by size-exclusion chromatography (polystyrene standard). Other polymers were used in a preliminary screening, such as PLGA

[1] INSERM U646, Ingénierie de la Vectorisation Particulaire, IBT, 10 rue André Boquel, 49 100 Angers, France.
[2] Laboratoire de Méthodologie de la Recherche Expérimentale, Université Paul Cézanne Aix Marseille III, avenue Escadrille Normandie-Niemen, 13 397 Marseille cedex 20, France.
[3] To whom correspondence should be addressed. (e-mail: venier@univ-angers.fr)

0724-8741/04/1200-2384/0

50/50 Resomer RG506, $\overline{M}w$ = 75,000 (BI Chimie, France) and poly(lactic acid), $\overline{M}w$ = 96,000 (Phusis Saint-Ismier, France). Glycofurol (tetraglycol or α-[(tetrahydro-2-furanyl) methyl]-ω-hydroxy-poly(oxy-1,2-ethanediyl) was purchased from Sigma (Saint Quentin Fallavier, France). Labrafil M 1944 CS (oleoyl macrogol-6 glycerides), Lutrol F68 (poloxamer 188: polyoxyethylene-polyoxypropylene), and Crodamol EO (ethyl oleate) were kindly supplied by Gattefossé (Saint Priest, France), BASF (Levallois-Perret, France), and Croda (Trappes, France), respectively. Olive oil was purchased from Coopérative Pharmaceutique (Melun, France). Lysozyme is a monomeric globular protein with 129 amino acids and an isoelectric point pI = 10.7. Lysozyme from chicken egg white (95%), its substrate, *Micrococcus lysodeikticus,* and buffer components were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). A Bio-Rad DC protein assay kit was obtained from Bio-Rad Laboratories (Marnes-la-Coquette, France).

Feasibility Domain

A ternary diagram was developed to consider the three major components used in the microparticle process (polymer-glycofurol solution, vegetable oil, and Labrafil M 1944 CS). The feasibility zone was defined as an area that allowed formation of micrometric-sized, nonaggregated, and spherical microparticles.

Microparticle Preparation

An initial phase containing glycofurol and PLGA polymer was prepared at room temperature. The polymer concentration used in glycofurol was 1%, 2%, or 3% w/w. The polymer solution was emulsified in an olive oil phase under mechanical stirring for 5 min at 500 rpm (Heidolph RGH 500, Merck Eurolab, Strasbourg, France). This step was carried out at 24°C in a thermostatic bath (Polystat 22, Bioblock Scientific, Illkirch, France). An amphiphilic agent, Labrafil M 1944 CS, was added to the emulsion under mechanical stirring (500 rpm) through a syringe for 10 min to obtain particle formation. Deionized water (100 ml) was added to the resulting mixture and magnetically stirred for a further 10 min. Finally, the formed microparticles were filtered through an 0.45-μm filter (HVLP type, Millipore SA, Saint Quentin Yvelines, France), washed with 100 ml 1% Lutrol F68 solution, rinsed five times with 50 ml of deionized water, and freeze-dried (RP2V Serail SGD, Argenteuil, France) to obtain a free-flowing powder (15). The dried particles were stored at +4°C until required. The excipient proportions were chosen with respect to the feasibility zone previously described; the total quantity of excipients (polymer solution, olive oil, Labrafil M 1944 CS) was fixed at 20 g.

Experimental Design

In order to determine the influence of the components on microparticle size (mode, diameter) and particle distribution (peak width), a mixture experimental design was planned. Three variables, corresponding to the amount of polymer solution, olive oil, and Labrafil M 1944 CS, respectively X_1, X_2, and X_3, were considered. These nonindependent variables were subjected to the constraint that their sum was 100%. Due to the constraints of the component proportions to be used, the region of interest was limited to a parallelogram, located into an equilateral triangle in one plan. Whatever the formulation, the proportion of polymer in the glycofurol was fixed at 1% w/w. To determine the best formulation according to the targets, we needed to obtain information of the entire domain of interest. For this purpose, we used an empirical model, this being a reduced cubic model, whose proposed equation is as follows (where η represents the response and β_i the coefficients of the model):

$$\eta = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3$$

To calculate the estimation of the coefficients, an experimental design with 13 experiments was carried out. Some experiments were replicated in order to estimate the reproducibility of the experimental results. To minimize the effect of systemic errors, experiments were carried out in a random fashion. Two responses were considered: the mode of the particle diameter (Y_1) and the peak width, which represented particle distribution (Y_2). All samples presented a monodisperse size distribution. If the mode classically characterized particle size, the peak width was preferred to the coefficient of variation, as it was more discriminating (it was measured as the peak width at half height).

From the experimental results, an estimation of the coefficients of the model was calculated using the least squares method. Construction of the experimental design, estimation of the coefficients of the models, and statistical study were achieved using the Nemrod software (16).

Particle Characterization

Morphology and Size

Each microparticle batch was observed by optical microscopy (BH2, Olympus, Tokyo, Japan). The surface and the internal morphology of the microparticles were investigated by using scanning electron microscopy (SEM; JSM 6310F, JEOL, Paris, France). Freeze-dried microparticles were mounted onto metal stubs using double-sided adhesive tape, vacuum-coated with a film of carbon (10-nm thick) using a MED 020 (Bal-Tec, Balzers, Lichtenstein). To characterize the internal morphology, the particles were embedded in Epon resin, and 20-nm-thick sections were sliced off using a cryocut at −26°C (Frigocut 2800, Reichert-Jung, Paris, France). Average particle size and their distribution were determined using a Coulter Multisizer (Coultronics, Margency, France). Microparticles were suspended in isotonic saline solution without surfactant and sonicated a few minutes prior to analysis.

DSC Analysis

Differential scanning calorimetry (DSC) was performed with a Mettler Toledo Star System (Mettler-Toledo, Viroflay, France). Approximately 10 mg of sample was placed in a sealed aluminum crucible; experiments were replicated (n = 3). The measurements were carried out in the range of −150 to 300°C at 10°C·min^{-1} under a nitrogen flow; two heating cycles were carried out.

Protein Encapsulation Efficiency

Encapsulation tests were achieved with lysozyme as a model protein. Lysozyme-loaded microparticles were prepared according to the previously described protocol. Protein (insoluble in glycofurol) was dispersed in the polymer solution (2% PLGA in glycofurol) to obtain encapsulation ratios between 0.5% and 2.0% w/w (with respect to the amount of PLGA). Entrapment efficiency was determined by using two techniques allowing the estimation of both the total and the active entrapped protein. Total protein was determined by using a method reported by Yan *et al.,* consisting of a basic digestion of microparticles followed by a Bio-Rad protein assay (17). Active entrapped protein was determined by measuring lysozyme activity in the presence of its substrate *Micrococcus lysodeikticus* following microparticle dissolution in a mixture of dimethyl sulfoxide and acidic solution. The biological activity of lysozyme was not impaired by the extraction method used as reported by Ghaderi *et al.* (18).

RESULTS AND DISCUSSION

Microparticle Preparation

The microparticle preparation process was based on a hydrophilic/lipophilic emulsion followed by an extraction step. The polymer solution (composed of PLGA and glycofurol) was emulsified in an oily phase; then to induce the particle formation, the oily phase properties were modified to allow the extraction of the polymer solvent. The first stage of the process was polymer dissolution in glycofurol at room temperature. PLGA (50/50 or 75/25) and PLA with various molecular weights from 14,000 to 96,000 Da were dissolved in glycofurol up to 10% w/w, beyond which rate gels were formed. Low PLGA 75/25 concentrations were used (1%, 2%, or 3% w/w) for improved polymer dissolution.

Then, in a second stage, an emulsion was formed by dispersing the polymer-glycofurol solution into a nonsoluble phase, selected after the screening of excipients used in human medicine. Two vegetable oils, olive and sesame, were chosen because they presented the advantage of forming with the polymer-glycofurol solution a relatively stable emulsion without the use of surfactant. This was partly due to the very low interfacial tension (<1 mN $\cdot$ m^{-1}) between polymer-glycofurol solution and vegetable oil.

Third, in order to extract glycofurol, an amphiphilic agent that was miscible with both vegetable oil and glycofurol, was selected. Crodamol EO (ethyl oleate) and Labrafil M 1944 CS (oleoyl macrogol-6 glycerides) fulfilled these conditions. By allowing the polarity of olive oil to change, and hence making glycofurol soluble in this phase, we attempted to promote the diffusion of glycofurol toward the oil, and hence to polymer desolvatation. Both selected excipients achieved polymer desolvatation, however only Labrafil allowed the formation of spherical particles. To confirm the assumption of the glycofurol diffusion phenomenon, an experiment was performed using a hydrophilic dye (methylene blue) mixed with glycofurol. After the formation of glycofurol drops in a drop of olive oil, the addition of Labrafil promoted the glycofurol diffusion toward the oil/Labrafil solution. This model allowed us to visualize the process under static conditions. We could therefore suppose that this phenomenon was increased in the dynamic conditions of the particle formation process. It should also be noted that the process up to this stage does not require water. Working in anhydrous conditions is advantageous for some drugs, including proteins, by favoring their stability (19). However, the resulting spherical particles suspensed in a mixture composed of oil, Labrafil, and glycofurol needed to be improved (morphology and homogeneity). Consequently, an additional step favoring glycofurol extraction was performed. It consisted of adding water or propylene glycol to the mixture under stirring (10 min). This last step of glycofurol extraction allowed both suitable particles to be obtained and a reduction in filtration time. Importantly, in the presence of water, but in the absence of Labrafil, no particles were formed, proving the key role of Labrafil in particle formation. We also studied the influence of the volume of water added on particle size. Results showed that size remained stable (about 5 μm) with a narrow distribution whatever the volume of water, except for volumes below 75 ml (Fig. 1). These low volumes appear to be insufficient to complete particle formation, leading to their agglomeration and increasing their final size.

Finally, at the end of the whole process, the microparticles were recovered via filtration and washed. Washing with water alone was not able to remove efficiently the oil residue (observation of powder aggregates using microscopy and Coulter Multisizer). Therefore, it was necessary to use a surfactant solution composed of Lutrol to obtain a free-flowing powder.

Further improvements in the process were made to improve reproducibility. It included the study of different parameters affecting particle formation, which were refined later. The stabilization of the temperature appeared to be important: at temperatures below 24°C, particle formation was incomplete. The temperature had important effects on component viscosity, solubility, and ease of exchange, factors at the heart of the process. Moreover, Labrafil partially crystallizes at temperatures below 20°C. Therefore, for reasons of drug thermal stability, the minimum temperature was fixed at 24°C. Also, it was found that particle formation was not possible when the stirring rate was below 250 rpm. Finally, by using different stirring rates and polymer concentrations, it

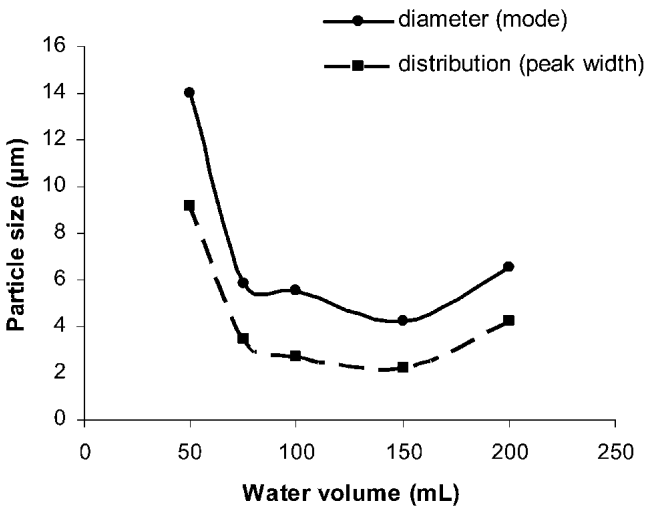

Fig. 1. Particle size evolution according to the volume of added water during microparticle preparation.

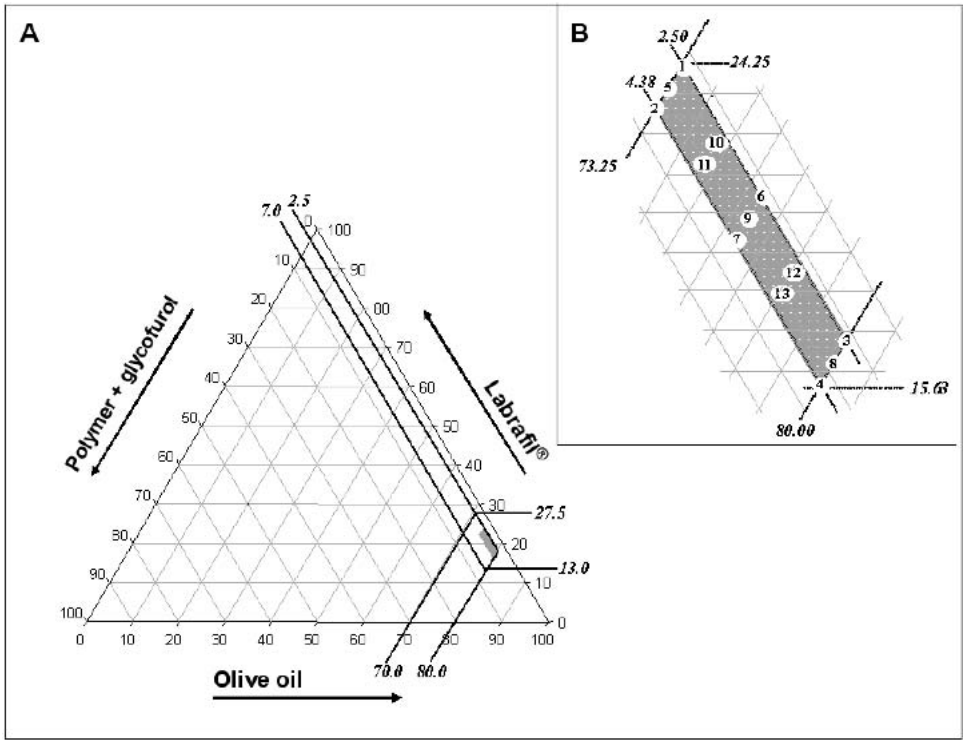

Fig. 2. (A) Illustration of the feasibility zone composed of a discontinuous domain and a continuous domain represented by the gray parallelogram. (B) Illustration of the experimental design (13 design experiments including 4 test points) inside the interest zone: the continuous domain.

was found that the particle size could be modified. For instance, between 250 and 1000 rpm, particle sizes varied from 10.0 to 4.8 μm at a constant concentration of polymer (2%). Between 1% and 3% w/w polymer concentration, particle size varied from 5.6 to 13.2 μm (mode) at a constant stirring rate (500 rpm).

To confirm the microparticle formation mechanism, we needed to show that particles had their origin in the polymer-glycofurol drops. Thus, we compared the size of polymer-glycofurol solution drops with that of the microparticles. Unfortunately, the size distribution of the drops could not be measured due to technical limitations, so average diameters were measured instead by observation under an optical microscope. Results showed a slightly inferior size of microparticles compared to the drop size, with around 6 μm and 10 μm, respectively. This size reduction confirmed the diffusion

Table I. Experiment Design and Corresponding Results for the Surface Response Analysis

	Weight fraction of excipient (%)			Measured response (μm)		Response estimated by the model (μm)	
Experiment no.	Glyco + Poly X_1	Olive oil X_2	Labrafil X_3	Mode Y_1	Peak width Y_2	Mode Y_1	Peak width Y_2
1	0.0250	0.7325	0.2425	9.78	10.40	10.35	10.43
2	0.0438	0.7325	0.2238	5.08	6.44	5.82	6.37
3	0.0250	0.8000	0.1750	9.53	10.15	9.39	10.47
4	0.0438	0.8000	0.1563	5.32	3.22	5.34	3.44
5	0.0344	0.7325	0.2331	9.04	8.17	7.74	8.21
6	0.0250	0.7662	0.2087	8.29	5.95	7.89	5.62
7	0.0438	0.7662	0.1900	4.58	4.21	5.08	4.98
	—	—	—	5.57	5.69	5.08	4.98
	—	—	—	5.82	5.20	5.08	4.98
8	0.0344	0.8000	0.1656	7.30	8.66	7.43	8.13
9	0.0344	0.7662	0.1994	7.30	6.44	6.28	5.63
	—	—	—	4.33	3.96	6.28	5.63
	—	—	—	5.82	6.19	6.28	5.63
	—	—	—	6.56	5.45	6.28	5.63
10	0.0297	0.7494	0.2209	8.29	7.43	7.57	6.52
11	0.0391	0.7494	0.2116	6.56	4.46	5.88	5.88
12	0.0297	0.7831	0.1872	8.54	7.92	7.30	6.68
13	0.0391	0.7831	0.1778	6.56	5.94	5.80	5.45

Table II. Validation of the Model Predictions (Three Test Points)

Weight fraction of excipient (%)			Y_1: mode (μm)			Y_2: peak width (μm)		
Glyco + Poly	Oil	Labrafil	$Y_{estimated}$	Confidence interval	$Y_{measured}$	$Y_{estimated}$	Confidence interval	$Y_{measured}$
0.0400	0.7900	0.1700	6.03	[3.59–8.47]	6.56	5.42	[3.12–7.72]	5.45
0.0391	0.7381	0.2229	6.52	[4.00–9.04]	7.06	6.54	[4.16–8.92]	6.19
0.0390	0.7750	0.1860	5.93	[3.56–8.29]	6.07	5.39	[3.16–7.62]	4.95

Confidence interval = $Y_{A/estimated} \pm t_{\alpha/2,N-p} \sqrt{[s^2 (1 + d_A)]}$, where d_A = function of variance at point A, S^2_{mode} = 1.001 (11 df), and $S^2_{peak\ width}$ = 0.896 (11 d*f*). t: Student's variable; α: significance level (0.05); N: number of data points; p: number of coefficients in the model (N-p: number of degrees of freedom)

of the glycofurol from the drops leading to polymer desolvation.

Determination of the Feasibility Zone

A ternary diagram was established in order to determine the experimental conditions that yielded microparticles. The formation of microparticles strongly depended on the proportions of the three components (polymer solution, olive oil, Labrafil M 1944 CS). The ternary diagram was rationally explored to define the feasibility zone. It was defined by a parallelogram with the following coordinates: 2.5% to 7.0% of polymer-glycofurol solution, 70% to 80% of vegetable oil (olive or sesame oil), and 13.0% to 27.5% of Labrafil (Fig. 2A). The feasibility zone contained two domains: a discontinuous and a continuous domain. In the discontinuous domain, both spherical or nonspherical objects such as fragments or fibers were obtained. On the other hand, in the continuous zone, only spherical microparticles were formed. To study the influence of components on microparticle size, we focused on this continuous

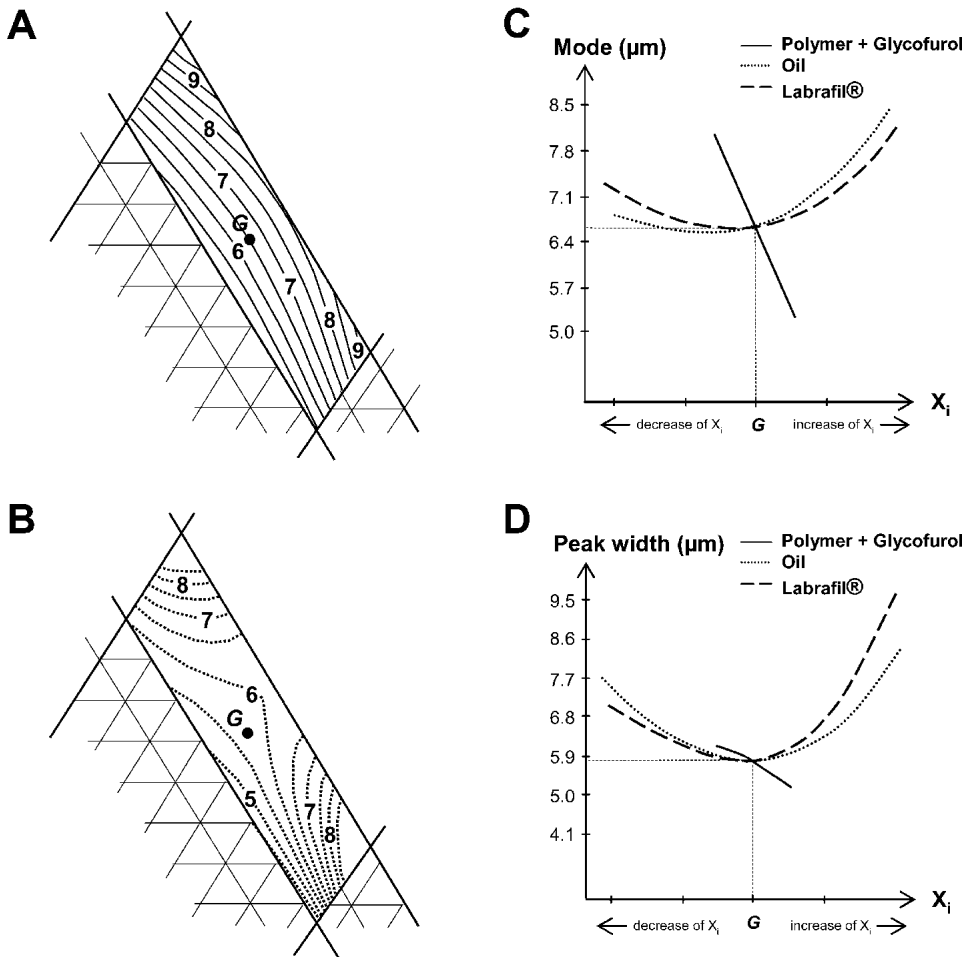

Fig. 3. Graphical representation of the proposed model for the two responses: (A, C) the microparticle diameter (mode) (Y_1) and (B, D) the microparticle distribution (peak width) (Y_2). (A) and (B) represent the contour plots allowing the prediction of the size and the distribution of the microparticles. (C) and (D) represent the respective influence of each component on mode diameter and peak width. X_i is the proportion of the considered component

zone, which was defined by the co-ordinates 2.50% to 4.38% of polymerglycofurol solution, 73.25% to 80.00% of olive oil, and 15.63% to 24.25% of Labrafil (Fig. 2B).

Experimental Design

Within the previously defined domain, a response surface analysis was carried out to predict the effect of excipients on particle size. Table I shows the results in terms of diameter (mode) and particle distribution (peak width) corresponding to the 13 experiments carried out. To predict these two responses within the experimental domain, a reduced cubic polynomial model was designed. A statistical analysis based on the lack of fit (differences between the measured responses and those calculated by the model (Table I) allowed us to accept the proposed models for the two responses. To test the model predictions, three additional experiments were carried out afterwards. Experimental test point values were included in the confidence interval for a given statistical significance ($\alpha = 0.05$) (Table II). The models could be used to predict the responses in each point of the domain of interest, and contour plot of the two responses are graphically represented in Fig. 3. Mode diameters were from 5 to 10 μm (Fig. 3A), which correspond to a mean diameter of 7 to 14 μm. Peak widths were between 4 and 8.5 μm (Fig. 3B). When superposing both graphical representations, it was possible to define a zone where particles presented the best homogeneity (peak width <5.5 μm) corresponding to a particle mode ≤6 μm.

The effect of each component is shown in Figs. 3C and 3D to enable comparisons to be made between their respective influences on mode diameter and peak width. The graph represents the variation of the responses when we shift along a line drawn between the center of the domain of interest and the pure component (top of the ternary diagram). From the center of the domain (point G), when the proportion of the considered component (X_i) varies into the limits defined by the domain, the proportions of the other components vary in a constant ratio (20). Considering both responses, the polymer-glycofurol solution appeared to be the most critical component. When the proportion of the dispersed phase was increased, particle diameter strongly decreased. A range of variation of 2% led to a variation of 3 μm of particle diameter (Fig. 3C). This result implied that perfect control of the amount of polymer-glycofurol solution was required for the robustness of the process. The vegetable oil and the surfactant were influential to a lesser extent. For a range of variation of 6.7% and 6.8%, the size variation was 2 and 1 μm for oil and Labrafil, respectively. The major effect of glycofurol on particle size and on homogeneity could be explained by the importance of the emulsion step. During the emulsion process, two contrary phenomena occur and influence the size of the drops: the shearing of the drops and their coalescence. By increasing the polymer-glycofurol solution volume (dispersed phase), the probability of breaking up drops is less important than coalescence, leading to larger droplets (21,22). Unfortunately, our results were at the opposite of this observation. It should also be noted that we did not evaluate drop size but the particle size. Particle formation requires an additional step of solvent diffusion. This process is governed by Fick's law; in this way, a higher ratio of dispersed phase to continuous phase leads to longer solvent diffusion time and also to a longer drop solidification time. This phenomenon was described by Tuncel (23). Therefore, by increasing the polymer-glycofurol solution volume (dispersed phase), the continuous phase allows a low and sustained diffusion of the solvent leading to smaller particles.

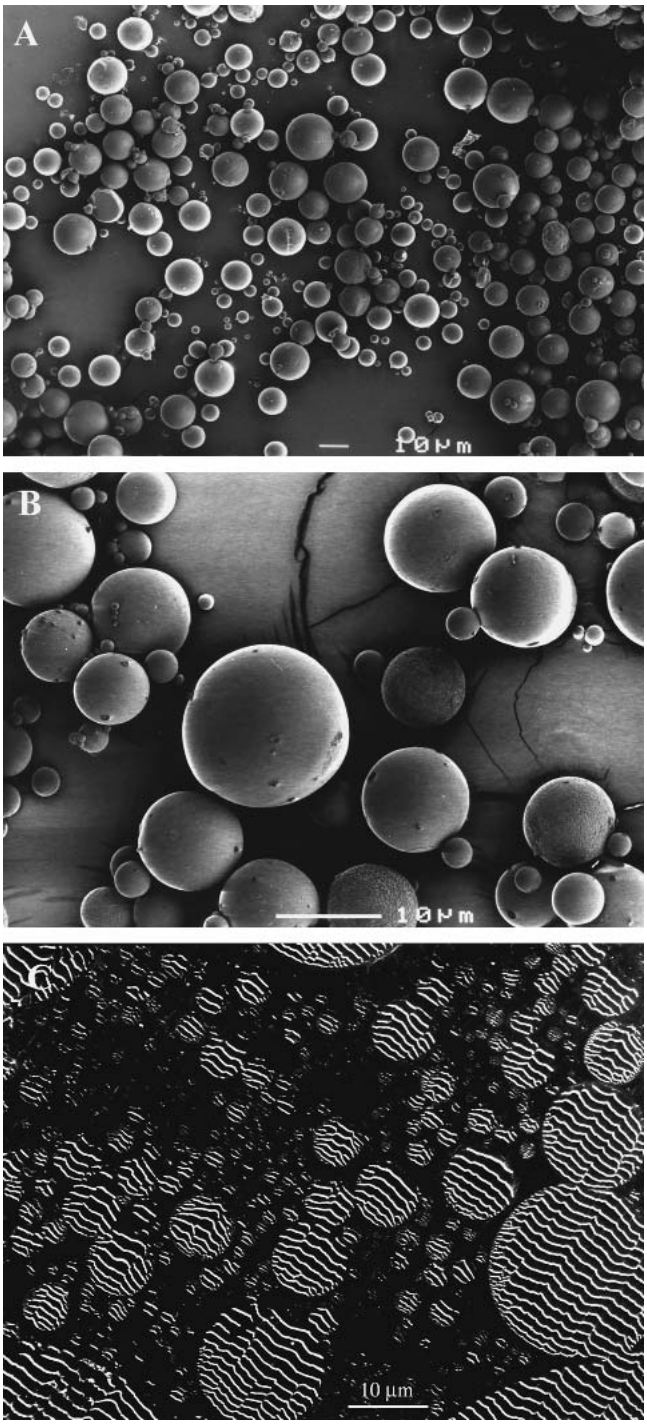

Fig. 4. Scanning electron micrographs (SEM) showing: (A, B) the surface morphology of microparticles and (C) the internal morphology of microparticles after section (the white lines correspond to the blade tracks of the cryocut).

Table III. Thermal Events Observed During DSC Analysis for Components Going into Microparticle Formulation and Physical Mixtures (Temperature in °C)

Components	Tg (midpoint)	Endothermal peak	Exothermal peak
Single components			
PLGA (P)	46		
Glycofurol (G)			–95/128
Labrafil (L)		–80/–65/–47/–24/–1	–74/–60/–40/220
Olive oil		–4	–38
Dispersed phase			
P-95/G-5	46		184
Continuous phase			
Oil-90/L-10		–6	–41
Oil-50/G-50		–5	–95/–42/133
Oil-33/L-33/G-33		–8	–95/–47/130
Continuous phase + PLGA			
Oil-4.5/L-0.5/G-5/P-90	45	–8	191
Microparticles			
	32	–7	182

Tg: glass transition.

Microparticle Characterization

Microscopy

Optical microscopy showed well-formed, spherical particles. Their size distribution was in accordance with formulations based on emulsions. Under SEM analysis, microparticles exhibited a smooth skin without pores. Particles were formed by a dense and homogeneous structure, supporting the hypothesis of particle formation (i.e., the desolvatation of the drops of polymer solution) (Figs. 4A–4C).

DSC Analysis

Unloaded microparticles as well as pure and mixed components were studied (Table III). All of the particle batches (n = 5) showed similar thermal events: a glass transition between 32°C and 34°C, an endothermal peak between –5°C and –7°C, and an exothermal peak at around 180°C. In order to identify these thermal events, single components were studied. PLGA (75/25) exhibited a glass transition at around 46°C. Glycofurol exhibited two exothermal peaks at around –96°C and 130°C. Olive oil and Labrafil exhibited a multitude of peaks related to the nature of their mixture. For olive oil, they included a crystallization and a fusion peak at –38°C and –4°C, respectively. Finally, Labrafil showed a series of crystallization-fusion peaks between –80°C and 0°C. By comparing the thermograms, we could observe that no thermal event belonging to a single component corresponded with those observed with microparticles. A study was then carried out to show the evolution of the thermal events according to the proportions of the components in the mixtures.

First, we studied the dispersed phase, a binary mixture of polymer-glycofurol by increasing PLGA proportions. The progressive increase of PLGA led to a shift of the glycofurol events, allowing a rough determination of glycofurol in PLGA (i.e., its second exothermal peak increased from 128°C to 184°C for a PLGA:glycofurol ratio varying from 0:100 to 95:5, respectively). PLGA did not exhibit any glass transition up to a 50:50 PLGA:glycofurol proportion. But, at 60:40 (PLGA:glycofurol), PLGA suddenly recovered its initial PLGA glass transition at 46°C. This value then remained unchanged from 60:40 to 95:5 PLGA:glycofurol. Therefore, considering microparticles, we can assume that the presence of an exothermal peak (182°C) indicates glycofurol residues in microparticles at levels up to 5% w/w with respect to the amount of PLGA. However it does not explain the decrease of PLGA Tg (from 46°C to 32°C).

Second, we studied the continuous phase of olive oil, Labrafil (Table III). Olive oil and Labrafil (L) were mixed in different proportions. Density measurements of the mixture demonstrated that olive oil-Labrafil mixtures behaved as real solutions (results not shown). DSC results showed multiple thermal events that only partially reflected each of both components. The addition of glycofurol to the olive oil-Labrafil mixture was also studied. Glycofurol did not change the thermal events, regardless of the added amount, suggesting an absence of interaction with the other components.

Finally, different physical mixtures were made with the polymer and the other components. The results obtained with a mixture of 90% polymer and a minimum amount of other components (due to technical limitations: 5% of glycofurol, and 5% of [oil-90/Labrafil-10]) showed thermal events close to those observed with microparticles: a fusion peak at –8°C, a glass transition at 46°C, and an exothermal peak at 180°C. This led us to assume that olive oil-Labrafil residues could remain in particles, at weak levels (disappearance of the crystallization peak). Hence, further investigation is required to quantify them. Unfortunately, the reduced PLGA glass transition (32°C instead of 46°C) could not be reproduced. It could be due to an intimate mixture with residues or to the process itself.

Lysozyme-Loaded Microparticles

Lysozyme-loaded particles showed the same characteristics as for unloaded particles: spherical particles with a smooth surface and similar sizes. Their encapsulation yields reached 100% as determined by Bio Rad assay (total protein) and a maximum of 40% (between 25% and 40%) by enzymatic assay (biologically active protein). These preliminary

results show that the new method can efficiently entrap a drug, but unfortunately with a loss of protein integrity. Lysozyme stability was tested in glycofurol, Labrafil, and olive oil at 24°C under shaking. Results showed that 100% of biologically active protein was recovered. Consequently, we can suppose that the environment inside the particles, and especially the presence of residues, can work against protein stability. Stabilizers such as albumin or sucrose, which were commonly used for protein preservation, appeared to be necessary to prevent protein denaturation. In this perspective, the optimization of protein encapsulation is still in progress.

CONCLUSIONS

This method allowed loaded PLGA microparticles to be prepared without the use of unacceptable organic solvents. The procedure is based on an emulsion-diffusion process and uses excipients such as glycofurol, vegetable oil, and Labrafil M 1944 CS to achieve different properties of miscibility. In a small feasibility zone, well-formed particles were obtained. They appeared spherical, with a matrix structure, and with micrometric sizes around 10 μm. Moreover, the particle size within the feasibility zone can be predicted according to the proportions of excipients used. Finally, this technique used simple equipment making it possible to consider industrial production. Preliminary tests allowed a model protein, lysozyme, to be encapsulated at a maximum yield of 40% of biologically active protein without the addition of stabilizers. This point needs to be optimized before considering the encapsulation of drugs such as proteins, peptides, and DNA.

ACKNOWLEDGMENTS

The authors thank Dr.Robert Filmon (SCIAM: Service Commun d'Imageries et d'Analyse Microscopiques) for the SEM photographs and Alexandra Giteau and Jacqueline Colleau for their technical assistance. We are also grateful to the Conseil Général de Maine et Loire for its financial support and to INSERM for its financial and scientific support.

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