A Novel Preparation Method For PLGA Microspheres Using Non Toxic Solvent Emulsified In Supercritical CO_2

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ABSTRACT
Purpose. To generate biodegradable poly(lactic-co-glycolic acid) (PLGA) microparticles without using volatile organic solvent for a sustained protein release.
Methods. The formulation of microparticles is based on the formation of an emulsion of polymer solution in CO_2 medium. Polymer solution was prepared in injectable non-volatile solvent such as glycofurol or isosorbide dimethyl ether. Moreover, encapsulation experiments were carried out using lysozyme as model protein. An experimental design was built-up to go further in the understanding of the system and to better predict the encapsulation yield.
Results. Spherical microparticles were successfully generated. Encapsulation yield of lysozyme can reach up to about 85% within our chosen ranges of parameters.
Conclusion. This method with the use of non-toxic solvents is suitable for the preparation of PLGA microspheres. Further characterization step will be performed to confirm its utility in controlled release of therapeutic protein.

Key words: PLGA microparticles, protein encapsulation, CO_2, non-toxic solvents.

INTRODUCTION

Thanks to advances in biotechnology, many therapeutic peptides and proteins have been discovered. Most proteins have limitations such as they are incapable of diffusing through biological membranes and they are not stable in gastro intestinal tract. Hence, their oral bioavailability is low. Consequently, they are mostly administered via intravenous (i.v.) route for the treatment of chronic diseases that requires daily injections due to their short plasma half-life, which makes it clinically undesirable for patients. Thus, there is of great interest to encapsulate these therapeutic proteins for sustained-release purpose.
The poly-lactic-co-glycolic acid (PLGA) polymers with their biocompatibility and biodegradability, which is highly desirable for parental form, are subject of numerous studies of drug delivery. Various methods have been used for encapsulation of proteins in PLGA microspheres. Among them, the most popular is the double-emulsion method W/O/W (water/oil/water), which includes all the processes using this principle as starting point such as emulsification-evaporation[1], supercritical fluid[2], spray-drying [3]processes. Still, the critical challenge in this method is the inevitable adsorption of protein at the water/oil interface, which is deleterious for protein stability, even though many suggestions have been made to improve protein stability in this classical method [4, 5]. Consequently, another approach named S/O/W (including emulsification-evaporation[6], supercritical fluid [2], spray-drying [7]processes) where proteins in solid-state are dispersed in the oil phase was developed. In this method, proteins are not in molecular form, so the consequent stability in contact with oil phase is enhanced. The critical point is the preparation of proteins particles obtained traditionally by spray-drying or freezing which potentially might be harmful for proteins stability[8, 9]. Another well-known and widely used method involves phase separation phenomenon of polymer solution. This method was described to entrap peptide,

protein drugs and was subject of several patents application [10-15]. However, its main drawback lies in not only the volatile solvent used to dissolve PLGA polymers but also the toxicity of residual coacervating and hardening agents remaining in the final product[15].
The common point of these methods mentioned above is the use of volatile solvents, which are considered toxic for the body and the environment, to dissolve polymer. Therefore, their use is being regulated and the residual amount of these solvents must be strictly limited. Hence, it is of great importance to find ways of avoiding the use of these solvents in the encapsulation process. Many works have been undertaken to achieve this goal. A.Jain *et al*. have used triacetin to dissolve PLGA for the preparation of microglobules of PLGA using a novel coacervation process [16]. However, this process requires a heating step to 65°C, which could denature thermolabile drugs like proteins and peptides. Aubert-Pouesssel *et al*. utilized glycofurol, a non-volatile injectable solvent, as polymer solvent for the preparation of PLGA particles by an emulsification-extraction process[17]. Unfortunately, the encapsulation yield of active protein attained a maximum of 40%, which needs to be improved for a possible application. Recently, using glycofurol as polymer solvent to entrap lipophilic drugs, Allhenn and Lamprecht reported their work on the microspheres preparation and obtained a maximum of encapsulation yield of 60% with a method named quasi emulsion-extraction[18]. Besides of the concept using a non-toxic solvent, an interesting approach was developed based on the PGSS (Particle Gas Saturated System) technique which allows PLGA microparticles to be formed in CO_2 medium without using any organic solvent in a mild condition of temperature[19]. Moreover, it was reported that there was no significant loss of activity of proteins encapsulated by this technique. However, according to the authors, it is difficult to control the size, shape and drug release kinetics of this type of PLGA microparticles. Therefore, this technique requires further improvements to prove its utility in controlled protein release.
Our preparation method presented in this paper is based on the formation of an emulsion in CO_2 medium. More importantly, non-toxic solvents were used to dissolve PLGA polymer. In this current work, we focus on the formulation of PLGA microspheres using glycofurol as the polymer solvent and discuss the underlying mechanism of microparticles formation. Besides, the feasibility of using isosorbide dimethyl ether to dissolve PLGA for microparticles preparation will be also briefly presented. To our best knowledge, glycofurol (GF) and isosorbide dimethyl ether (DMI) are two injectable water-miscible non-volatile solvents used in different drug products for parenteral or other therapeutic uses [20-25]with a consistently low toxicity demonstrated through many scientific studies[25-28].

MATERIALS AND METHOD:

2.1 Materials:

Lysozyme (chicken egg-white), Micrococcus lysodeikticus, glycofurol (tetraglycol or α-[(tetrahydro-2-furanyl) methyl]-ω-hydroxy-poly(oxy-1,2-ethandiyl), isosorbide dimethyl ether were obtained from Sigma-Aldrich (Saint Quentin Fallavier,France). Uncapped 75/25 PLGA provided by Phusis (Saint-Ismier, France) had a mean molecular weight of 21,000 Da (polydispersity index I=1.8) as determined by size-exclusion chromatography (standard: polystyrene). Micro-BCA protein assay reagent kit was purchased from Pierce (Bezons, France).

2.2 Protein precipitation:

Protein precipitation method had been optimized by A.Giteau[29]. Condition of precipitation of experiment number 12 was chosen from the set of 13 experiments carried out by the author to perform the precipitation step. Precisely, 0.9 mg of lysozyme was dissolved in 46 µl of

NaCl solution 0.3M. This solution was then mixed into 954 µl of glycofurol to obtain a suspension of precipitated protein for further use (suspension 1).

2.3 Microparticle preparation:

2.3.1 Preparation of polymer solution:

A stock solution of 15% (w/v) PLGA in glycofurol was firstly prepared. In fact, after adding PLGA to glycofurol, the mixture was left under stirring for about 96 hours and then was left to stand for at least 3 days before any further use.

Solution of PLGA in isosorbide dimethyl ether can be simply prepared about 2 hours before any use.

2.3.2 Microparticles preparation in CO_2 medium:

A scheme of the experimental setup used for microparticle preparation is shown in Figure 1. 0.3 ml of a polymer solution or a suspension of lysozyme precipitates in polymer solution was first introduced in a 14 ml view-cell (E1) which was kept at the operating temperature by a thermostated water bath. The suspension of lysozyme precipitates in polymer solution was prepared by mixing the suspension 1 (c.f 2.2 part) and the stock solution at 1/2 (v/v) ratio. In this work, unless otherwise stated, the final concentration of polymer was 10%. CO_2 was then delivered to the cell by means of a membrane pump (E2). Once desired pressure was reached, the mixture of these two phases was stirred at 1500 rpm. A certain amount of ethanol (E4) was then injected to the cell by a HPLC pump (E5). Thereafter, HPLC circuit was washed with distilled water and 1% Lutrol F68 solution before 2 ml of 1% Lutrol F68 solution (E6) was added into the cell. The stirring was kept for 25 minutes before a depressurizing step. A suspension of microparticles was then collected.

2.4 Experimental design:

To better understand the system and to define eventually the optimum conditions of the process, an experimental design was used. Encapsulation yield of total protein and active protein were chosen as responses to be measured and three parameters (temperature, pressure and volume injected of ethanol) were chosen to study their influence on the responses. According to our miscibility test between polymer solution with CO_2 supercritical fluid previously carried out at different conditions of temperature and pressure, it was noted that pressure should not exceed 100 bar in the range of temperature of 32°C to 40°C. Temperature was chosen to not exceed 40°C to make sure the activity loss of protein was not caused by excessive temperature. Therefore, in order to explore the entire constrained region of process operability, a face-centered composite design was chosen rather than a classical central composite design[30].

In our first attempt to fit model to data, we studied the encapsulation yield of total protein over the three parameters within the ranges: A-temperature (33-40°C), B-pressure (80-100bar) and C-volume of ethanol (0.2-1 ml). The low levels of temperature and pressure were chosen to make sure CO_2 was under supercritical conditions.

A face-centered composite design was built up and it was divided in 2 blocks:

-Block 1: 12 runs composed of 8 two-level factorial combinations and 4 center points.

-Block 2: 8 runs composed of 6 axial points (α= 1) and 2 center points.

After conducting the experiments, data collected were then analyzed using Design-Expert software. Predictive model suggested by the software is based on an analysis process called “sequential model sum of squares”. The objective of this analysis is to add a higher-level source of terms only if it explains a significant amount of variation beyond what is already accounted for. While keeping the hierarchy of the selected model, model terms were selected or rejected based on the p value with 95% confidence level using ANOVA analysis.

2.5 Microparticle characterization:

2.5.1 Morphology and size:

The surface morphology of the microparticles was investigated by scanning electron microscopy (SEM) (JSM 6310F, JEOL, Paris, France).Freeze-dried microparticles were mounted onto metal stubs using double-sided adhesive tape and then vacuum-coated with a film of carbon using a MED 020 (Bal-Tec, Balzers, Lichtenstein).

Average particle size was determined using a Coulter Multisizer® (Coultronics, Margency, France). In case of particles prepared using phase separation method, the particle size was measured using a Nanosizer® (Malvern Instruments, Worcestershire,UK). Suspensions of particles in distilled water before freeze-dried step were used for these analyses.

2.5.2 Protein encapsulation yield:

2.5.2.1 Active protein:

The biologically active entrapped protein was determined using Micrococcus lysodeikticus. Briefly, lysozyme-loaded microparticles obtained after preparation were dissolved in 0.9 ml of DMSO. After 1 hour, 3 ml of 0.01M HCl was added. The solution was left to stand for 1 more hour and then diluted to an appropriate range of concentration before being incubated at least 4 hours with a suspension of Micrococcus lysodeikticus. Lysozyme activity determination was based on turbidity measurement at 450 nm. The amount of encapsulated active protein was calculated using a standard curve.

2.5.2.2 Total protein:

The total protein was quantified by using micro-BCA protein assay. Briefly, the solution (1) described in 2.5.2.1 was used to determine the total quantity of protein. The absorbance was measured at 580 nm. The amount of protein was calculated using a standard curve.

2.5.3 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. The measurements were carried out at 5°C.min^{-1} under nitrogen flow. The sample was firstly heated to 70°C. Thereafter the sample was cooled down to 0°C before was reheated to 70°C. Thermal data were taken from the second heating step using the supplied software.

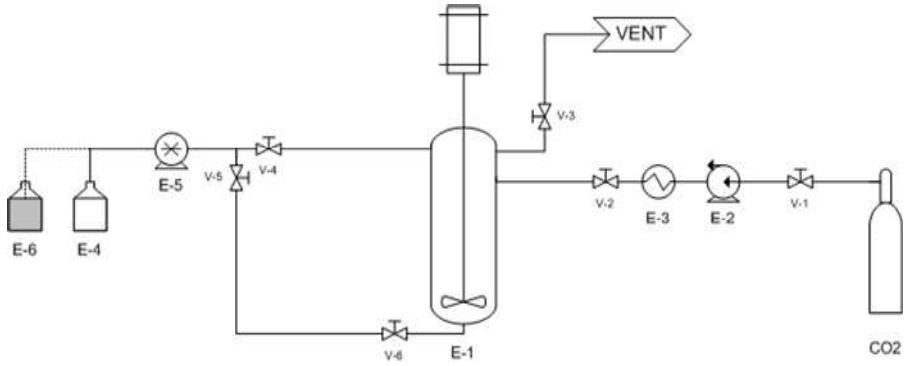

Figure 1: Schematic diagram of the experimental setup

RESULTS AND DISCUSSION

*Microparticles preparation :

The microparticles preparation process was inspired by the emulsification/extraction method which requires a formation of an emulsion followed by an extraction step. In our case, the polymer solution was emulsified in CO_2 fluid in the presence of ethanol. It was found that adding of a certain quantity of ethanol, which depends on the CO_2 conditions, supports the emulsion formation. Ethanol, miscible with CO_2 fluid and polymer solution in glycofurol , might decrease interfacial tension between these two phases or the viscosity of glycofurol phase, which could explain a better mixing between two phases. To confirm this hypothesis, density, viscosity and interfacial tension of glycofurol in CO_2 media need to be measured. Beyond the scope of this paper, it is the subject of our ongoing work.
Extraction step was carried out using a solution of Lutrol F68. It was found in literature that the presence of carbonyl group in PLGA structure and ether group in glycofurol structure favor the interaction between CO_2 and polymer solution[31], which can lead to the diffusion of certain amount of CO_2 into the polymer solution. Further experiments are needed to measure the solubility of CO_2 in the polymer solution under different conditions of temperature and pressure. Hence, in order to reduce CO_2 amount dissolved in the polymer solution, Lutrol F68 solution was prepared in glycin buffer pH 10. This solution was added into the emulsion formed in the first stage to extract glycofurol, which is necessary for the formation of solid particles. After depressurization, it was observed under optical microscopy that the obtained particles were spherical and isolated. However, freeze-drying of this suspension did not allow obtaining a free-flowing powder but highly aggregated powder of particles (observed under SEM). This fact might be explained by the presence of certain amount of residual glycofurol in the obtained particles at this stage. Therefore, a supplementary volume of Lutrol 1% solution needed to be added to achieve a complete glycofurol extraction.

*Experimental design:
Within the previously defined domain of key inputs, an experimental design was built up and the total protein yield chosen as output was quantified (Table 1). Response surface analysis was then carried out in order to fit model to data collected. The model suggested by Design-Expert package was a two factors interaction model (2FI) using sequential sum of squares approach. Analysis of variance (ANOVA) was performed to test the significance of model coefficients (Table 2). It was shown that temperature, pressure and ethanol volume had a significant effect on the encapsulation yield output. Although the final equation shows a lack of fit, which means it cannot be used for the prediction of response in the chosen ranges of factors, we better understand the system and the way the factors interact with each other to affect the output. Indeed, it is noteworthy to point out that the top three significant terms C, AB, B suggest that C should be at its low level; A,B should be at the same time either at their high or low levels while B should be at its high level for further optimization study. Besides, the remark drawn from ANOVA analysis about interaction between temperature and pressure corroborates our observation in miscibility test (data not shown). Effectively, when temperature is at its low level, pressure should not be at its high level in order to reduce the miscibility between CO_2 and polymer solution and then supports the dispersion of polymer solution in CO_2.

Table 1 : Experimental data showing total protein yield in function of experiment conditions

Std	Run	Block	A-temperature (°C)	B-pressure (bar)	C-C2H5OH volume (ml)	encapsulation yield (%)
4	1	Block 1	40,0	100	0,2	88

2	2	Block 1	40,0	80	0,2	20
10	3	Block 1	36,5	90	0,6	70
7	4	Block 1	33,0	100	1,0	0
9	5	Block 1	36,5	90	0,6	56
8	6	Block 1	40,0	100	1,0	40
12	7	Block 1	36,5	90	0,6	55
1	8	Block 1	33,0	80	0,2	84
11	9	Block 1	36,5	90	0,6	58
5	10	Block 1	33,0	80	1,0	26
3	11	Block 1	33,0	100	0,2	89
6	12	Block 1	40,0	80	1,0	0
17	13	Block 2	36,5	90	0,2	89
16	14	Block 2	36,5	100	0,6	86
19	15	Block 2	36,5	90	0,6	66
20	16	Block 2	36,5	90	0,6	71
13	17	Block 2	33,0	90	0,6	85
14	18	Block 2	40,0	90	0,6	15
15	19	Block 2	36,5	80	0,6	41
18	20	Block 2	36,5	90	1,0	39

Table 2 : ANOVA for the applied response surface model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	770,133	1	770,133			
Model	13524,375	6	2254,063	9,697	0.0005	Significant
A-temperature	1464,100	1	1464,100	6,298	0.0274	
B-pression	1742,400	1	1742,400	7,496	0.0180	
C-C2H5OH	7022,500	1	7022,500	30,211	0.0001	
AB	2080,125	1	2080,125	8,949	0.0112	
AC	780,125	1	780,125	3,356	0.0919	
BC	435,125	1	435,125	1,871	0.1963	
Residual	2789,291	12	232,441			
Lack of Fit	2632,041	8	329,005	8,368	0.0283	Significant
Pure Error	157,250	4	39,3125			
Cor Total	17083,800	19				
Final equation (coded factors)	Yield= 55,17 -12,1A + 13,2B - 26,5C + 16,12AB + 9,88AC -7,38BC					

Moving key inputs over their chosen range involved the modification of different characteristics like solubility of CO_2 in polymer solution, solubility of glycofurol in CO_2 phase, density of these two phases,...Hence, the measured output might have resulted from different underlying phenomena such as the miscibility between polymer solution and CO_2 phase, the dispersion ability of polymer solution in CO_2, a possible glycofurol extraction in CO_2 phase or a phase separation of polymer solution in the presence of ethanol,…which shows the complexity of the process. At this stage of response surface modeling, it would be reasonable to narrow down the experimental domain for prediction and optimization purposes. Therefore, another face-centered composite design was built up to inspect the active protein and total protein yield over the following ranges of the three factors: A-temperature (36.5-39.5°C), B-pressure (90-100 bar), C-volume of ethanol (0.15-0.75 ml).

Responses quantified were shown in Table 3. ANOVA table shows the chosen model is the three factors interaction (3FI) one. A particularly useful characteristic of central composite design is the fact that this type of design allows experimenters to take a sequential approach. In fact, a factor analysis was carried out to analyze data collected in block 1. This analysis permitted to discover the significance of ABC term normally neglected by the analysis process in central composite design which is not conceived to support the estimation of 3rd degree term. Indeed, p-value of ABC term shown in table 4 is highly significant. Hence, it seems fair for us to add this term into the model. Besides, it was observed that the final equation does not show a lack of fit, which allows the response to be predicted within the chosen ranges of the parameters. To illustrate this equation, active protein yield was mapped over the experimental domain (Figure 2A). The highest yield 87% was found at 39.5°C, 90bar and 0.15 ml of ethanol.

With intention of using equation (*) (cf. Table 4) to find the maximum of active protein yield, an extrapolation was carried out in the periphery of the previously chosen experimental domain. The tested condition suggested by the software was at 40°C, 90 bar, 0.1 ml of ethanol where temperature was moved upward 0.5°C and ethanol volume was moved downward 0.05 ml from their extremes points in experimental domain. Active protein yield was found to be about 75 %, which is lower than the maximum value previously obtained. Hence, it would be reasonable to stay inside the previously chosen experimental domain.

Table 3: Experimental conditions and results

Std	Run	Block	A-temperature (°C)	B-pressure (bar)	C-C2H5OH volume (ml)	activity yield (%)	total protein yield (%)
6	1	Block 1	39,5	90	0,75	41,1	60
2	2	Block 1	39,5	90	0,15	87,8	88,9
7	3	Block 1	36,5	100	0,75	63,3	73,3
8	4	Block 1	39,5	100	0,75	28,9	48,9
10	5	Block 1	38,0	95	0,45	58,9	78,9
4	6	Block 1	39,5	100	0,15	45,6	56,7
12	7	Block 1	38,0	95	0,45	58,9	78,9
3	8	Block 1	36,5	100	0,15	67,8	75,6
1	9	Block 1	36,5	90	0,15	34,4	42,2
9	10	Block 1	38,0	95	0,45	51,1	77,8
11	11	Block 1	38,0	95	0,45	58,9	71,7
5	12	Block 1	36,5	90	0,75	64,4	80,0
15	13	Block 2	38,0	90	0,45	46,7	65,9
18	14	Block 2	38,0	95	0,75	25,6	43,3
13	15	Block 2	36,5	95	0,45	48,9	68,9
14	16	Block 2	39,5	95	0,45	41,1	60,0
19	17	Block 2	38,0	95	0,45	57,8	78,9
16	18	Block 2	38,0	100	0,45	44,4	63,3
17	19	Block 2	38,0	95	0,15	57,8	56,7
20	20	Block 2	38,0	95	0,45	53,3	73,3

*Propagation of error:

This part consists of calculating the transmitted variation of responses via the noise of input factors. The idea is to seek out the region where responses do not get affected much by variations in factor settings[30]. Propagation of error of active protein yield is shown in Figure 2B. Unfortunately, the highest error was found to be at 39.5°C, 90 bar, 0.15 ml of ethanol where the best value of active protein yield was obtained. Therefore, at this condition

of formulation, we may obtain a highest yield but at the same time a lowest robustness due to variations in the input factors.

*Influence of ethanol on encapsulation yield and mechanism of microparticles formation:
Analyzing results obtained from two mentioned experimental designs reveals that encapsulation yields are always better when the volume injected of ethanol stays at its low level, which does not coincide with our observation about the minimum of ethanol volume needed for a good mixture of polymer solution in CO_2 phase. Center points in the first design and run number 1 in the second design were intentionally placed at conditions where optimal emulsification would happen, but it turned out that encapsulation yield obtained at these points were by far not the best. This fact suggests that there was another underlying mechanism implied in the microparticles formation and thus the subsequent encapsulation process of protein. To our knowledge, it might involve a phase separation process of polymer solution. At this stage, it would be reasonable to carry out a set of experiments to confirm our hypothesis. In fact, our results about encapsulation process using phase separation without the use of CO_2 show a high encapsulation efficiency, which confirms our hypothesis about the underlying mechanism of particles formation at low quantity of ethanol. Details in experimental design and results about phase separation process will be presented in another paper. Figure 3D. shows a SEM image of particles prepared by this method. Some characteristics of these particles were summarized in (Table 5) and were compared to those of microparticles prepared in CO_2 medium.

Table 4 : ANOVA showing the significance of the chosen model and lack-of-fit test

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	318,176	1	318,176			
Model	3118,661	7	445,523	9,756	0.0006	Significant
A-temperature	117,649	1	117,649	2,576	0.1368	
B-pressure	59,536	1	59,536	1,303	0.2778	
C-C2H5OH	491,401	1	491,401	10,760	0.0073	
AB	939,611	1	939,611	20,575	0.0008	
AC	987,901	1	987,901	21,633	0.0007	
BC	2,531	1	2,531	0,055	0.8182	
ABC	520,031	1	520,031	11,387	0.0062	
Residual	502,328	11	45,666			
Lack of Fit	446,573	7	63,796	4,576	0.0802	not significant
Pure Error	55,755	4	13,938			
Cor Total	3939,165	19				
Final equation (coded factors)	Yield= 51.02 - 3.43A - 2.44B - 7.01C - 10.84AB - 11.11AC - 0.56BC + 8.06ABC (*)					

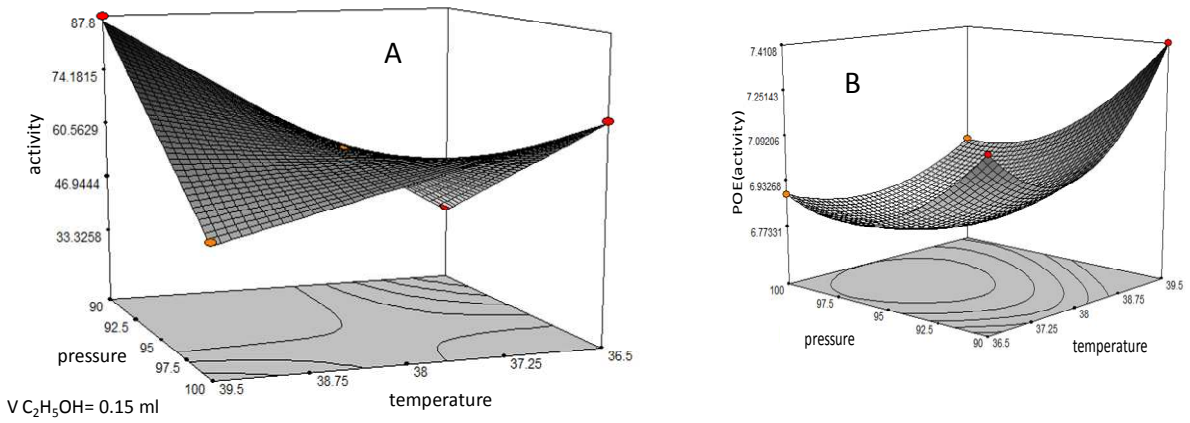

Figure 2: illustration of activity yield and the propagation of error of activity in the chosen ranges of parameters

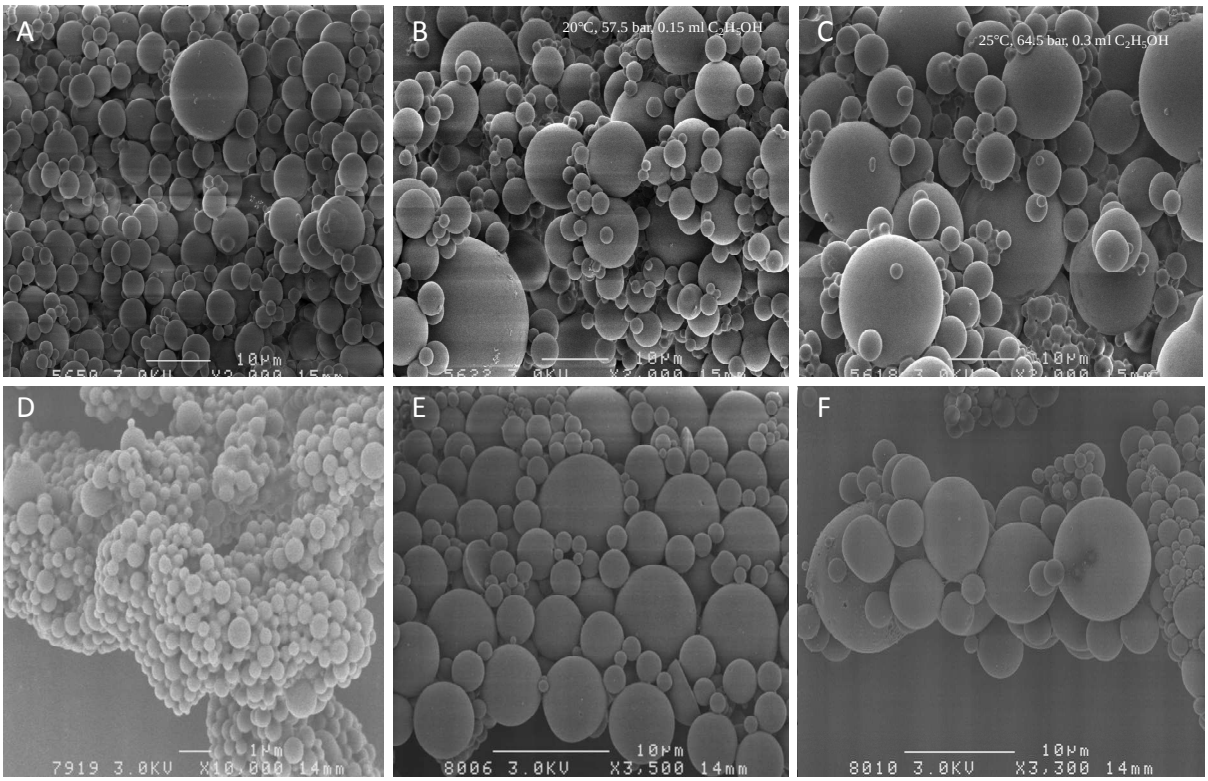

Figure 3: PLGA microparticles observed under electronic microscopy. (A) microparticles prepared under supercritical condition, (B,C) under two-phase region liquid-vapour, (D)using phase separation. In A,B,C,D, glycofurol was used to dissolve PLGA.(E) microparticles generated using DMI as polymer solvent, (F) using the mixture of DMI and glycofurol.

Table 5: Some characteristics of particles prepared using CO_2 or phase separation

Preparation method	Average diameter in number(µm)	Tg (°C)
Emulsification in CO_2*	2.14	30.5
Phase separation	0.44	27.5

*: run 1 in the second experimental design

When going further in active protein versus total protein ratio, it can be seen that increasing volume of ethanol decreases this ratio. It could be supposed that ethanol has a harmful effect on protein activity at its medium and high level. Therefore, it would be of great interest to reduce ethanol volume but still ensure a good emulsification of polymer solution in CO_2 phase. Recently, we have tried running our experiments outside of supercritical region (Figure 3.B and 3.C.). It seems interesting that at these conditions the ethanol volume needed for a good emulsification is low, which might be suitable for protein encapsulation, and besides enables one to modify microparticle size to suit different application purposes. Further experiments on encapsulation of protein need to be carried out to prove it.

*Preparation of microparticle using isosorbide dimethyl ether (DMI):

To better understand critical points in solvent properties and their interaction with CO_2 fluid, suitable for microparticle formation in CO_2 media, we have tried working on another injectable non-volatile solvent. After having screened solvents which had been described in literature for a parenteral use such as diethylene glycol dimethyl ether, N-methyl pyrrolydone, DMI,… based on their toxicity and their ability to dissolve PLGA, DMI was chosen to be tested for microparticles generation. In DMI's case, although ijR value is 11.2, which is outside of PLGA (75:25) solubility sphere (iR = 7.8) (Table.6), DMI can easily dissolve PLGA (75:25) polymer. This phenomenon was already observed in DMI's case with other polymers such as poly(vinyl butyral) (iR = 10.6, ijR =13.4), poly(ethyl methacrylate) (iR =10.6, ijR =17.2), cellulose acetate (iR =7.6, ijR =16),…[25]According to Van Krevelen, mutual solubility only occurs if degrees of hydrogen bonding δh of polymer and solvent are about equal and δd,δp of polymer and those of solvent do not differ too much[25, 32]. It seems that DMI satisfies these conditions.

In this paper, only our preliminary results of DMI will be presented. Figure 3E. shows an image of microparticles prepared in CO_2 medium at 82 bar; 39,5°C; 0,1 ml of ethanol. Moreover, a mixture of DMI, GF at 2/1 ratio (v/v) was also used to dissolve the polymer to anticipate our experiments on encapsulation of protein (Figure 3F). It seems fair for us to say these microparticles with their polydisperse size keep the "fingerprint" of an emulsification step, which proves our formulation concept and theoretically permits to tune particle size by modifying the stirring rate.

It is noteworthy that using phase separation process for particles formation was unsuccesful. Any attempt to use Lutrol solution as coacervating agent would lead to strong polymer desolvation. According to Thomasin *et al.*, three types of interactions may play a role in a coavervation process, i.e., polymer-solvent (ΔintE1), solvent-coacervating agent(ΔintE2), polymer-coacervating agent(ΔintE3)[15]. Unfortunately, at the time of this writing, all these interactions could not be calculated due to the lacking in literature of Drago parameters of GF and DMI. We suppose that ΔintE2 in the DMI's case is much larger than ΔintE1 leading to precipitation of solid polymer in contact with coacervating agent[15].

Table 6:Hansen parameters of different components used in microparticles preparation

	$\delta d(MPa^{1/2})$	$\delta p(MPa^{1/2})$	$\delta h(MPa^{1/2})$	Radius of solubility sphere iR	ijR
PLGA (75:25)	17.4	8.3	9.9	7.8 [33]	
Glycofurol[25]	19.1	5.9	13.4		5.43
Isosorbide dimethyl ether[25]	22.5	12.2	12		11.12
$ijR=[4(\delta d_i-\delta d_j)^2+(\delta p_i-\delta p_j)^2+(\delta h_i-\delta h_j)^2]^{1/2}$					

CONCLUSION

It has been shown that PLGA microparticles can be formulated using glycofurol and isosorbide dimethyl ether as the polymer solvents, which enables the replacement of toxic volatile solvents to be achieved. Moreover, encapsulation of model protein has been tested on microparticles prepared with glycofurol and the yield of encapsulation process can reach up to about 85%, which might allow this process to find its utility in sustained-release purpose of therapeutic protein. Therefore, we have demonstrated our formulation concept which is based on a formation of an emulsion in CO_2 fluid. Here, CO_2 plays a role of dispersing medium and the following extraction step needs an addition of an aqueous solution. Having said that, other processes using CO_2 as anti-solvent might also successfully generate PLGA particles since the miscibility between these solvents and CO_2 varies with temperature and pressure. Besides, this work has shown that PLGA microparticles could be prepared with a simple way using a phase separation process. Finally, further characterization step will be needed to help us to better understand the encapsulation process and to prove the utility of our formulation concept.

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