Lysozyme encapsulation within PLGA and CaCO3 microparticles using supercritical CO2 medium

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\section*{1. Introduction}

Thanks to advances in biotechnology, many therapeutic peptides and proteins have been discovered. Owing to the many benefits that it can offer to patient comfort and therapeutic efficiency, protein encapsulation has been drawing the attention of researchers for decades [1,2], and it is still the subject of many publications in recent years [3,4]. The literature shows that numerous types of carriers, polymeric and inorganic, with different methods have been developed and used for protein encapsulation [5,6].

Concerning polymeric carrier systems, many encapsulation methods have been developed and among them can be cited the double-emulsion water/oil/water (W/O/W) method [7], the solid/oil/water (S/O/W) method [8], simple coacervation [9] and other derivative methods [10,11]. The negative aspect in the double emulsion method is the inevitable adsorption of proteins at the water/oil interface, which is deleterious for proteins, even though many suggestions have been made to improve protein stability [12,13]. Consequently, the solid/oil/water (S/O/W) method in which proteins in the solid-state are dispersed in the oil phase was developed. In this case, proteins are not in molecular form, so the stability of proteins when put in contact with the oil phase is enhanced. The negative aspect is the preparation of protein particles obtained traditionally by spray-drying or freezing which potentially might be harmful for protein stability [14,15]. Simple coacervation is very well known, has been widely used to entrap peptides and protein drugs, and has been the subject of several patent applications [1,16]. The common point of the methods mentioned above is the use of volatile solvents, which are considered toxic for the body and the environment. Nowadays, the use of volatile solvents is being regulated, and the residual amount of these solvents is strictly limited. Hence, it is of great importance to find ways of avoiding the use of these solvents in the encapsulation process. Much research has been undertaken to achieve this goal. One of these pieces of research was an interesting approach based on the particle gas saturated solution (PGSS) technique which allows PLGA microparticles to be formed in CO2 medium without using any organic solvents [17]. 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 improvement to prove its utility in controlled protein release.

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In this paper, we will present our preparation method for the formation of PLGA particles in CO₂ medium.

Concerning inorganic carriers, protein encapsulation has mostly been performed on porous materials. To date, different types of biocompatible porous material have been developed, for instance, mesoporous silicas [18], halloysite nanotubes [19] and calcium carbonate (CaCO₃) particles [20,21]. In most cases, the encapsulation of proteins or enzymes in the systems was achieved via an adsorption phenomenon [18,19] and can involve a layer-by-layer assembly of polyelectrolytes to form composite materials [22]. Calcium carbonate is widely present on earth and is industrially prepared from calcium hydroxide slurry or slaked lime [23,24], but it is also a by-product of sodium hydroxide production [25]. CaCO₃ possesses high loading capacity, thus it is an effective material for drug and protein encapsulation. In the literature, one can find reports on the loading of CaCO₃ with various molecules, including low-molecular weight drugs [26–28], DNA [29,30], enzymes and proteins. Proteins could be entrapped into CaCO₃ using different loading modes, the most effective one being the active loading or co-precipitation, whereby molecules of interest are entrapped during the process of CaCO₃ crystal growth resulting from the mixing of two aqueous solutions, CaCl₂ and Na₂CO₃ [31–33]. By contrast, in passive loading or impregnation, the molecules of interest are adsorbed from the solution onto preformed CaCO₃ [26,33,34]. Many other loading modes have been described such as chemical adsorption [35], phase transition [36] and interfacial reaction [29].

In the past we developed a process based on supercritical CO₂ for the synthesis of CaCO₃ particles. In our previous work we mainly focused on the synthesis and characterization of unloaded CaCO₃ particles [37]. It was shown that CaCO₃ microspheres of about 5 μm were constituted of an assembly of vaterite nanograins (approximately 40 nm width) which exhibited a specific surface of 16 m² g⁻¹ with a mesoporosity of around 11% [38].

In this work, protein encapsulation in PLGA particles and in CaCO₃ particles will be presented. In both cases, the preparation method was based on the formation of an emulsion in CO₂. Concerning the preparation of PLGA particles, a non-toxic solvent, glycofurol, was used for the formulation. To the best of our knowledge, glycofurol is a safe injectable water-miscible solvent with a consistently low toxicity, as demonstrated by many studies [39–42]. The optimization of protein encapsulation using experimental design and discussions on the mechanism of particle formation will be the focus of this part. Concerning CaCO₃ particles, the preparation was carried out in an aqueous medium, thus avoiding recourse to any use of organic solvents. Results presented in this paper follow our previous work which mainly focused on characterizing CaCO₃ particles using XRD, SEM, TEM, AFM, and nitrogen adsorption/desorption techniques [37]. Experiments on protein encapsulation and in vitro release will be shown. Comparisons between the protein-loaded system and the unloaded system were also investigated. The optimization of protein encapsulation in CaCO₃ particles involved in a study of surface functionalization will be presented in another publication.

This study is involved in a global project which aims at conceiving implantable synthetic extracellular matrices seeded with multifunctional particles for bone and cartilage tissue engineering. However, the application of the carriers can be extended to any field requiring protein encapsulation.

2. Materials and methods

2.1. Material

Lysozyme (chicken egg-white), Micrococcus lysodeikticus, glycofurol (tetruglycol or α-[(tetrahydro-2-furanyl)methyl]-ω-hydroxy-poly(oxy-1,2-ethanediyl)], isosorbide dimethyl ether, glycine, calcium hydroxide, and hyaluronic acid were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Uncapped 75/25 PLGA provided by Phusis (Saint-Ismer, France) had a mean molecular weight of 21,000 g mol⁻¹ (polydispersity index I = 1.8) as determined by size-exclusion chromatography (standard: polystyrene). The information about polydispersity index was provided by the supplier. Micro-BCA protein assay reagent kit was purchased from Pierce (Bezons, France). Non-ionic surfactant Lutrol F68 (poloxamer 188) was purchased from BASF (Laserson, France).

2.2. Methods

2.2.1. Preparation of PLGA particles

2.2.1.1. Protein precipitation. The protein precipitation method had been optimized by Giteau et al. using experimental design. In fact, a Doehlert matrix was used to study the influence of ionic strength, aqueous volume, and protein quantity on the activity yield of protein precipitates [43]. The condition of precipitation of experiment number 12, which is one of the optima found by the author, was chosen to perform the precipitation step. Precisely, 0.9 mg of lysozyme was dissolved in 46 μL of 0.3 M NaCl solution. This solution was then mixed into 954 μL of glycofurol, which played a role of an anti-solvent, to obtain a suspension of precipitated protein for further use (suspension 1).

2.2.1.2. 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 48 h and then left to stand for at least 7 days. Before any further use, the solution was filtered through a 0.2 μm pore size filter (Minisart RC 25, Sartorius Stedim).

2.2.1.3. Preparation procedure of PLGA particles in CO₂ medium. A scheme of the experimental setup used for microparticle preparation is shown in Fig. 1. 0.3 mL of a polymer solution or a suspension of lysozyme precipitates in polymer solution was first introduced into 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 (cf. Section 2.2.1.1) and the stock solution at 1/2 (v/v) ratio. In this work, unless otherwise stated, the final concentration of polymer was 10% (w/v). CO₂ was then delivered to the cell by means of a membrane pump (E2). Once the desired pressure was reached, the mixture of these two phases was stirred at 1500 rpm. The impeller is in the form of a cylindrical spindle. A certain amount of ethanol (E4) was then injected into the cell by an HPLC pump (E5). Thereafter, the 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 to extract glycofurol. The stirring was maintained for 25 min before a depressurizing step.

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(20–30 bar/min). A suspension of microparticles was collected and then added dropwise into 15 mL of glycine buffer solution pH 10 (0.00125 M). This final suspension was left to stand at ambient temperature (≈25 °C) overnight. Thereafter, the suspension was centrifuged at 2000 rpm (1000 × g) for 15 min, which allows the particles to be collected. The particles were washed once with distilled water before being freeze-dried.

2.2.1.4. Experimental design. To better understand the system and to define the optimum conditions of the process, an experimental design was used. The encapsulation yields of total protein and of active particle were chosen as responses to be measured, and three parameters (temperature, pressure and injected volume of ethanol) were chosen to study their influence on the responses. The range of pressures and temperatures included in this experimental design were chosen based on a preliminary experiment in which the ability of the polymer solution to be dispersed in CO2 media had been tested. The experimental procedure can be briefly summarized as follows: at a constant temperature, 0.3 mL of the polymer solution was added into the 14 mL view cell; pressure was then increased progressively under mechanical stirring until the polymer solution began to rest at the bottom of the cell and could not be dispersed in the cell. The pressure value was then noted and it was called \( P_{\text{max}} \). \( P_{\text{max}} \) values at different temperatures are summarized in Fig. 2. The temperature was chosen to not exceed 40 °C to make sure the activity loss of protein was not caused by excessive heat. 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 [44].

In our first attempt to fit a 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 (80–100 bar) and C—volume of ethanol (0.2–1 mL). A face-centered composite design was built up and it was divided into 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 (\( \alpha = 1 \)) and 2 center points.

After conducting the experiments, data collected were then analyzed using Design-Expert software (Stat-Ease, Minneapolis, U.S.). Predictive models were built based on an analysis process called the “sequential model sum of squares”. While keeping the hierarchy of the selected model, model terms might be selected or rejected based on the \( p \) value with 95% confidence level using ANOVA analysis.

2.2.2. Preparation of CaCO3 particles

2.2.2.1. Carbonation reaction media. CaCO3 microparticles were obtained by a supercritical CO2 process as described in our previous work [37]. This process, which is based on the formation of an emulsion of water in SC-CO2 (W/C), was developed in our laboratory and patent applications were filed [45]. Aqueous microdroplets act as microreactors where several reactions succeed. In this case, Sc-CO2 is a continuous (or external) phase and acts as reactant for the synthesis of CaCO3 particles. Sc-CO2 is dissolved in the aqueous solution \((\text{CO}_2(\text{aq}))\) leading to the formation of carbonic acid H2CO3, ionic species such as hydrogen carbonate \((\text{HCO}_3^-)\) and carbonate \((\text{CO}_3^{2-})\) according to Eqs. (1)–(3). Then, the CO\(_3^{2-}\) ions react with Ca\(^{2+}\) ions to form calcium carbonate (Eq. (4)) which may crystallize into different polymorphs.

\[
\begin{align*}
\text{CO}_2(\text{aq}) + \text{H}_2\text{O} & \rightleftharpoons \text{H}_2\text{CO}_3 \quad (1) \\
\text{H}_2\text{CO}_3 & \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \quad (2) \\
\text{HCO}_3^- & \rightleftharpoons \text{CO}_3^{2-} + \text{H}^+ \quad (3) \\
\text{CO}_3^{2-} + \text{Ca}^{2+} & \rightleftharpoons \text{CaCO}_3 \quad (4)
\end{align*}
\]

2.2.2.2. Preparation of aqueous solution. Calcium hydroxide (1%, w/v) was added in glycine buffer (NaCl 0.62 M, glycine 0.62 M) and filtered through a 0.45 μm filter. Then the pH was readjusted to 10 by adding dropwise 6 M HCl solution. The pH was measured using a Microph2001 pH-meter (Crison Instruments, Paris, France). Hyaluronic acid was then added (0.1%, w/v). Hylauronic acid plays a role of an anionic biopolymer directing the polymorph of CaCO3 particles. For the synthesis of loaded microspheres, lysozyme (1 mg/mL) was dissolved in the aqueous solution right before the encapsulation experiment was carried out.

2.2.2.3. Experimental set-up and procedure. A schematic diagram of the device used for CaCO3 synthesis is shown in Fig. 3. The stainless autoclave (1) with a capacity of 500 mL is heated at 40.0 ± 0.1 °C and pressurized with CO2 at 200 ± 1 bar. Liquid CO2 is pumped with a high pressure membrane pump (2) and preheated by a heat exchanger (3) before feeding the autoclave equipped with a mechanical stirring device. The axis of the magnetic stirrer is equipped with an anchor stirrer, and the stirring speed is 1200 rpm. Once equilibrium is reached (temperature and pressure constant), 25 mL of previously prepared aqueous solution are injected by means of an HPLC pump (4). The injection flow is...
fixed at 10 mL/min. Once addition is achieved, the final pressure is 240 ± 5 bar and stirring is maintained at 1200 rpm for 5 min. Thereafter, stirring is stopped and the autoclave depressurized at a rate of 40–50 bar/min.

Suspension of CaCO₃ microparticles is collected and centrifuged at 2400 × g for 10 min. Finally, microparticles are washed with 50 mL of ultrapure water, centrifuged and freeze-dried to obtain a dry powder of CaCO₃.

2.2.3. Microparticles characterization

2.2.3.1. Morphology, size and zeta potential. 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).

The average particle size was determined using a Coulter Multisizer® (Coultronics, Margency, France). Suspensions of particles in distilled water before being freeze-dried were used for these analyses. XRD analysis was carried out using an X-pert diffractometer (CuKα₂ doublet, λ = 1.54056 Å from 2θ = 10 to 70° in continuous mode with a step size of 0.07°). A Nanosizer ZS (Malvern Instruments, Orsay, France) was used to assess the zeta potential of the microparticles. Microparticles were redispersed in distilled water and vortexed prior to every measurement. The zeta cell was washed with ultrapure water between every sample. Results are presented as the average of 3 measurements.

2.2.3.2. Encapsulation yield of protein. Encapsulation yield is defined as the percentage of encapsulated protein compared to the initial amount of protein.

2.2.3.2.1. Active protein. The biologically active entrapped protein was determined using M. lysodeikticus [34, 33]. 30 mg of M. lysodeikticus were placed in 200 mL TRIS NaCl buffer (pH 7.4) and incubated at 37 °C for 1 h. In the case of CaCO₃, 5 mg of protein-loaded microspheres were dissolved by contact with 1.5 mL 0.1 M HCl for 10 min and diluted in TRIS NaCl buffer to reach a lysozyme concentration of 0.06–0.3 μg/mL. 100 μL of samples or standards (0.06–0.3 μg/mL of lysozyme) in triplicate were mixed with 2.9 mL of M. lysodeikticus suspension in glass tubes (Kimble, Thermo Fisher Scientific) and incubated for at least 4 h at 37 °C. Turbidity was finally assessed by measuring the absorbance at 450 nm (Kontron Uvikon 922, Northstar Scientific, Leeds, UK). For protein-loaded PLGA particles, the same quantification method was used except that lysozyme-loaded PLGA microparticles obtained after formulation were dissolved in 0.9 mL of DMSO and then after 1 h, 3 mL of 0.01 M HCl was added. The solution was left to stand for 1 more hour and then diluted to an appropriate range of concentration. The amount of encapsulated active protein was calculated using a standard curve after subtraction of the control value of a blank sample. The blank sample was prepared in the same conditions as the protein-loaded sample in the absence of protein precipitants. It should be pointed out that the blank sample did not show any lysis effect on M. lysodeikticus.

2.2.3.2.2. Total protein. The total protein was quantified by using a micro-BCA (bicinchoninic acid) protein assay according to the manufacturer’s guidelines. Briefly, a volume of sample (100 μL) or lysozyme standard was mixed with BCA reagent at the same volume (100 μL) in a 96 microwell plate (Nunc, ThermoFisher Scientific) and incubated at 37 °C for 2 h away from any light. Absorbance was finally measured at 580 nm (Multiskan Labsystems, PA). The amount of protein was calculated using a standard curve. The standard curve was created in the range of 5–100 μg/mL. The experiment was replicated for each batch of particles.

2.2.3.3. Protein release kinetics. CaCO₃ microparticles (10 mg) were dispersed in 10 mL phosphate buffer (PBS, prepared according to the European pharmacopeia, Ed 6), at either pH 4.5, 6.0 or 7.4 and incubated in a water bath at 37 °C under agitation (75 rpm). Samples were taken at 30, 60, 90, 120, 150, 180 min and 24 h. At each sampling time, the buffer was totally removed; the microparticles were washed once with ultrapure water (Millipore) before replacing the buffer for sink conditions. The samples were diluted to obtain a final concentration of 0.06–0.3 μg/mL of lysozyme. The quantification of active lysozyme was performed using a M. lysodeikticus assay as described previously in Section 2.2.3.2.1. Release profiles were performed in triplicate for each condition.

3. Results and discussion

3.1. PLGA particles

3.1.1. Particle formation

The preparation procedure in this work was designed to follow the emulsification/extraction method, which requires the formation of an emulsion followed by an extraction step. In this procedure, the polymer solution was emulsified in CO₂ fluid. It was observed that the presence of a certain quantity of ethanol enhanced mixing between the liquid phase and the CO₂ fluid, and hence, enhanced emulsion formation. In fact, under fixed conditions of pressure and temperature, optimal amounts of ethanol were determined by progressively injecting ethanol into the cell followed by observations via the view-cell (Fig. 4). In our definition, the optimal amount of ethanol under a predefined condition of pressure and temperature is the minimal amount of ethanol which allows the obtained emulsion with a white appearance to be dispersed along the whole length of the view cell.

In the extraction step, an aqueous solution of Lutrol was used to extract the polymer solvent. As mentioned earlier, glycofurol is a water-miscible solvent. Therefore, a hydrophilic non-solvent of PLGA such as glycerol, or polyethylene glycol can be used for extraction [46]. In this work, we did not study the influence of different external phases on particle formation. An aqueous phase was chosen because of its safety and its ability to dissolve surfactant agents. Fig. 5a is an image of particles prepared under an optimal amount of ethanol (temperature = 39.5 °C, pressure = 90 bar, C₂H₅OH = 0.65 mL). It can be seen that the particles look spherical.
and are well separated from one another. The mean average size of particles is about 2.1 μm (Fig. 5b).

In this study, PLGA particles were used for protein encapsulation and lysozyme was chosen as a protein model. The encapsulation yield of lysozyme in PLGA particles prepared under supercritical CO2 was studied and optimized using experimental design. Three factors chosen to study their influence on encapsulation yield were: temperature, pressure, and ethanol volume.

3.1.2. 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 the model to data. The chosen model was a two factors interaction model (2F) using the 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 (A), pressure (B), and ethanol volume (C) had a significant effect on the encapsulation yield. 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, based on the sign of the top three significant terms C, AB, B in the equation, it should be pointed out that C should be at its low level; A and 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 studies. Moreover, the results of the ANOVA analysis about the interaction between temperature and pressure corroborate our observation presented in Fig. 2. Effectively, when temperature is at its low level, pressure should not be at its high level since it will be greater than the \( P_{\text{max}} \) value, which means the ability of the polymer solution to be dispersed in CO2 fluid would be very limited.

At this stage of response surface modeling, it would be reasonable to narrow down the experimental domain for prediction and optimization purposes. As pointed out earlier, B should be at its high level and A, B should be at the same levels. 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), and C—volume of ethanol (0.15–0.75 mL). Experimental points and their outputs are shown in Table 3. The ANOVA table shows the chosen model is the three factor interaction (3F) one (Table 4). A particularly useful characteristic of central composite design is the fact

Table 1
Experimental data showing total protein yield as a function of experimental conditions.

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<th>Std</th>
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<th>Block</th>
<th>( A )—temperature (°C)</th>
<th>( B )—pressure (bar)</th>
<th>( C )—C(_2)H(_5)OH volume (mL)</th>
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Table 2
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<td>0.0180 Significant</td>
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<tr>
<td>C—C₃H₇OH</td>
<td>7022.500</td>
<td>1</td>
<td>7022.500</td>
<td>30.211</td>
<td>0.0001 Significant</td>
</tr>
<tr>
<td>AB</td>
<td>2080.125</td>
<td>1</td>
<td>2080.125</td>
<td>8.949</td>
<td>0.0112 Significant</td>
</tr>
<tr>
<td>AC</td>
<td>780.125</td>
<td>1</td>
<td>780.125</td>
<td>3.356</td>
<td>0.0919 Significant</td>
</tr>
<tr>
<td>BC</td>
<td>435.125</td>
<td>1</td>
<td>435.125</td>
<td>1.871</td>
<td>0.1963 Significant</td>
</tr>
<tr>
<td>Residual</td>
<td>2789.291</td>
<td>12</td>
<td>232.441</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>2632.041</td>
<td>8</td>
<td>329.005</td>
<td>8.368</td>
<td>0.0283 Significant</td>
</tr>
<tr>
<td>Pure error</td>
<td>157.250</td>
<td>4</td>
<td>39.3125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor. total</td>
<td>17083.800</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Final equation (coded factors) Yield = 55.17 – 12.1A + 13.2B – 26.5C + 16.12AB + 9.88AC – 7.38BC

Table 3
Experimental conditions and results in the second experimental design.

<table>
<thead>
<tr>
<th>Std</th>
<th>Run</th>
<th>Block</th>
<th>A—temperature (°C)</th>
<th>B—pressure (bar)</th>
<th>C—C₃H₇OH volume (mL)</th>
<th>Activity yield (%)</th>
<th>Total protein yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>1</td>
<td>39.5</td>
<td>90</td>
<td>0.75</td>
<td>41.1</td>
<td>60.0</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>1</td>
<td>39.5</td>
<td>90</td>
<td>0.15</td>
<td>87.8</td>
<td>88.9</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>1</td>
<td>36.5</td>
<td>100</td>
<td>0.75</td>
<td>63.3</td>
<td>73.3</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>1</td>
<td>39.5</td>
<td>100</td>
<td>0.75</td>
<td>28.9</td>
<td>48.9</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1</td>
<td>38.0</td>
<td>95</td>
<td>0.45</td>
<td>58.9</td>
<td>78.9</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>1</td>
<td>38.0</td>
<td>95</td>
<td>0.45</td>
<td>58.9</td>
<td>78.9</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>1</td>
<td>36.5</td>
<td>100</td>
<td>0.15</td>
<td>67.8</td>
<td>75.6</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>2</td>
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</tr>
<tr>
<td>9</td>
<td>14</td>
<td>2</td>
<td>38.0</td>
<td>95</td>
<td>0.75</td>
<td>46.7</td>
<td>65.9</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>2</td>
<td>38.0</td>
<td>95</td>
<td>0.75</td>
<td>25.6</td>
<td>43.3</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>2</td>
<td>36.5</td>
<td>95</td>
<td>0.45</td>
<td>48.9</td>
<td>68.9</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
<td>2</td>
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<td>95</td>
<td>0.45</td>
<td>41.1</td>
<td>60.0</td>
</tr>
<tr>
<td>13</td>
<td>18</td>
<td>2</td>
<td>38.0</td>
<td>100</td>
<td>0.45</td>
<td>57.8</td>
<td>78.9</td>
</tr>
<tr>
<td>14</td>
<td>19</td>
<td>2</td>
<td>38.0</td>
<td>95</td>
<td>0.45</td>
<td>44.4</td>
<td>63.3</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>2</td>
<td>38.0</td>
<td>95</td>
<td>0.15</td>
<td>57.8</td>
<td>56.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.45</td>
<td>53.3</td>
<td>73.3</td>
</tr>
</tbody>
</table>

that this type of design allows experimenters to take a sequential approach. In fact, a factor analysis was first carried out to analyze data collected in block 1. This analysis permitted us to discover the significance of the ABC term, normally neglected by the analysis process in central composite design which is not conceived to support the estimation of a 3rd degree term. Indeed, the p-value of the 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 (Fig. 6a). The highest yield of 87% was found at 39.5 °C, 90 bar and 0.15 mL of ethanol.

With the intention of using (Eq. (6)) (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 calculated based on a desirability function was at 40 °C, 90 bar, 0.1 mL of ethanol where the temperature was moved upward 0.5 °C and the ethanol volume was moved downward 0.05 mL from their extremes in

Table 4
ANOVA showing the significance of the chosen model and lack-of-fit test in the second experimental design.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F value</th>
<th>p-Value Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>3118.176</td>
<td>1</td>
<td>3118.176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>3118.661</td>
<td>7</td>
<td>445.523</td>
<td>9.756</td>
<td>0.0006 Significant</td>
</tr>
<tr>
<td>A—temperature</td>
<td>117.649</td>
<td>1</td>
<td>117.649</td>
<td>2.576</td>
<td>0.1368 Significant</td>
</tr>
<tr>
<td>B—pressure</td>
<td>59.536</td>
<td>1</td>
<td>59.536</td>
<td>1.303</td>
<td>0.2778 Significant</td>
</tr>
<tr>
<td>C—C₃H₇OH</td>
<td>491.401</td>
<td>1</td>
<td>491.401</td>
<td>10.760</td>
<td>0.0073 Significant</td>
</tr>
<tr>
<td>AB</td>
<td>930.611</td>
<td>1</td>
<td>930.611</td>
<td>20.575</td>
<td>0.0008 Significant</td>
</tr>
<tr>
<td>AC</td>
<td>987.901</td>
<td>1</td>
<td>987.901</td>
<td>21.633</td>
<td>0.0007 Significant</td>
</tr>
<tr>
<td>BC</td>
<td>2.531</td>
<td>1</td>
<td>2.531</td>
<td>0.055</td>
<td>0.8182 Significant</td>
</tr>
<tr>
<td>ABC</td>
<td>520.031</td>
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<td>11.387</td>
<td>0.0062 Significant</td>
</tr>
<tr>
<td>Residual</td>
<td>502.328</td>
<td>11</td>
<td>45.666</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>446.573</td>
<td>7</td>
<td>63.976</td>
<td>4.576</td>
<td>0.0802 Significant</td>
</tr>
<tr>
<td>Pure error</td>
<td>55.755</td>
<td>4</td>
<td>13.938</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor. total</td>
<td>3939.165</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Final equation (coded factors) Yield = 51.02 – 3.43A – 2.44B – 7.01C – 10.84AB – 11.11AC – 0.56BC + 8.06ABC

Please cite this article in press as: M.-K. Tran et al., Lysozyme encapsulation within PLGA and CaCO₃ microparticles using supercritical CO₂ medium, J. Supercrit. Fluids (2013), http://dx.doi.org/10.1016/j.supflu.2013.02.024
3.1.3. 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 [44]. The propagation of error of active protein yield is shown in Fig. 6b. Unfortunately, the highest error was found to be at 39.5 °C, 90 bar, and 0.15 mL of ethanol where the best value of active protein yield was obtained. Therefore, at this condition of formulation, we may obtain the highest yield but at the same time the lowest robustness due to variations in the input factors.

3.1.4. Influence of ethanol on encapsulation yield and discussion on the mechanism of particle formation

Analyzing results obtained from the two mentioned experimental designs reveals that encapsulation yields are always better when the injected volume of ethanol stays at its low level, which does not coincide with our observation of the optimal volume of ethanol needed for a good mixture of polymer solution in the CO2 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 not the best by far. The encapsulation yield of total protein was about 60%. This fact suggests that there was another underlying mechanism involved in the particle formation and the subsequent process of protein encapsulation. To our knowledge, it might involve a phase separation phenomenon of the polymer solution when put in contact with an aqueous solution. In fact, our results about the encapsulation process using phase separation without the use of CO2 show a high level of encapsulation efficiency [47], which indicates that phase separation might be the underlying mechanism of particle formation generated under low quantity of ethanol.

When going further in active protein versus total protein ratio, it can be seen that increasing the 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. It should be pointed out that the process described hereby was used for protein encapsulation, but it can serve as well for the encapsulation of other drugs, such as lipophilic anti-cancer agents.

3.2. CaCO3 particles

3.2.1. CaCO3 microparticles formation in supercritical CO2 medium

Generally, calcium carbonate is obtained by simple mixing of calcium and carbonate salts, only few research groups used supercritical CO2 for the synthesis of calcium carbonate, and none described drugs or protein encapsulation [48–51]. During the formulation process, we speculate that the water droplets emulsified within supercritical CO2 act as microreactors to prepare CaCO3 microparticles able to encapsulate proteins such as lysozyme, while the CO2 has a double role: it acts as an external phase for the emulsion and as a CO2 provider inside the water droplets.

The mechanism of formation of calcium carbonate is complex to determine. Just after the injection of the basic aqueous solution in the pressurized autoclave, it is assumed that the fast dissolution of CO2 molecules into the aqueous solution leads to the formation of several ionic species, mainly CO32− and HCO3− due to the used basic pH. Then, CO32− ions react with Ca2+ ions, resulting in CaCO3 particles which crystallize into the vaterite phase (Fig. 7) as previously shown by our research group [37], but the mechanism of the formation is still unclear.

Moreover, the addition of hyaluronic acid and lysozyme can have an impact on the CaCO3 particles. By comparison with the work of Zhang et al. [52] showing CaCO3 precipitation in the presence of a polyaniionic matrix (polysacartic acid), these species may interact with CaCO3 particles via electrostatic interactions and as a consequence may influence the morphology, the size or the surface of CaCO3 particles.

3.2.2. Morphology, size and zeta potential of CaCO3 microparticles

Lysozyme-loaded CaCO3 particles were characterized and compared to the unloaded ones. It is important to note that adding lysozyme has no influence on CaCO3 polymorphism (Fig. 7). Indeed, polymorphic composition (molar) is unchanged with around 98% of vaterite and 2% of calcite. Moreover, as shown on the SEM
images (Fig. 8), the spherical shape and the mean diameter of around 5 μm are not modified by the presence of lysozyme. However a careful analysis reveals that lysozyme-loaded microparticles show a more stratified surface in the SEM images (Fig. 8c and d) compared to the unloaded microparticles (Fig. 8a and b). It is supposed that the presence of lysozyme induces surface structural changes of CaCO₃ particles as observed by Jimenez-Lopez et al. [53] and Yang et al. [54]. To confirm this assumption, zeta potentials were measured. The unloaded particles bear a zeta potential of $-20.8 \pm 2.5$ mV against $-6.7 \pm 2.2$ mV for lysozyme loaded microparticles. Lysozyme-loaded particles are more positively charged compared to the unloaded ones which can be attributed to the presence on the surface of positively charged lysozyme (isoelectric point $pH_{i} \approx 11$). The size of CaCO₃ microparticles ranged from 1 to 10 μm and exhibited a unimodal distribution with a mean diameter of 4.9 ± 0.1 μm for unloaded microparticles, whereas lysozyme loading slightly increased microparticle size (4.5 ± 0.5 μm). There was almost no microparticle with a diameter higher than 10 μm.

3.2.3. Lysozyme encapsulation into CaCO₃ microparticles

The preparation and characterization of unloaded CaCO₃ microparticles have been studied and reported in our previous work [37]. In this study, we focused on the encapsulation of proteins into CaCO₃ microparticles to demonstrate their utility in the microencapsulation field. Lysozyme was chosen as model protein to provide a proof of concept. We successfully obtained CaCO₃ microparticles with a high protein loading (quantified amount of lysozyme/amount of CaCO₃ microparticles) and encapsulation yield (total amount of encapsulated lysozyme/initial amount of lysozyme in calcium solution). The highest lysozyme loading we could obtain was 7.1 ± 0.5% corresponding to an encapsulation yield of about 64.4 ± 4.1%, at a lysozyme starting concentration of 1 mg/mL, as quantified by BCA assay. These results were confirmed by M. lysodeikticus assay, active lysozyme loading was 6.4 ± 0.1% suggesting that above 90% of the encapsulated lysozyme remains active. These preliminary results are much higher than those obtained by co-precipitation [32], interfacial reaction [29] or phase transition method [36]. If we compare different loading modes it can be noticed that lysozyme was scarcely encapsulated into
CaCO$_3$ microparticles. In the case of co-precipitation (encapsulation yield < 0.01%) this could be explained by the repulsion of lysozyme during CaCO$_3$ crystal growth, due the fact that lysozyme and CaCO$_3$ (pH = 8) were both positively charged [32]. Fujiwara et al. described the interfacial encapsulation method to encapsulate various proteins of increasing molecular weight such as BSA, ovalbumin and papain within CaCO$_3$ microcapsules [29,36]. It is noteworthy to mention that the molecular weight of the protein to be encapsulated was proportional with the encapsulation yield. Thus, no inclusion of lysozyme, the smallest protein, into vacant CaCO$_3$ microcapsules was observed (encapsulation yield < 0.01%). However, the same research group later used phase transition from vaterite to calcite method to encapsulate lysozyme; the obtained loading was about 0.2% with an encapsulation yield of about 17% [36]. An impregnation (or physical adsorption) process of proteins onto preformed CaCO$_3$ microspheres has been studied by Volodkin et al. [20]. These authors demonstrated that protein adsorption is not only depending on protein molecular weight, but also on the charge of the protein and the microspheres. Due to the diffusion limitation, large molecules such as Dextran (2000 kDa) could not diffuse deeply inside CaCO$_3$ microspheres. Protein adsorption is governed by the electrostatic interactions; the pH of the medium plays an important role since proteins can bear positive or negative charges according to their isoelectric point. The comparison of physical adsorption and co-precipitation methods was performed by Petrovet et al. [31], it was demonstrated that co-precipitation-protein capture into CaCO$_3$ microspheres was five times more effective than the physical adsorption. Besides, $\alpha$-chymotrypsine recovered 85% of its native activity after being captured during CaCO$_3$ growth. Interestingly, a study described the encapsulation of small molecules as well as proteins within CaCO$_3$ nanoparticles, using both co-precipitation and absorption methods, but no loading nor encapsulation efficiency data were available in the case of the studied proteins [33].

In our strategy, we successfully obtained a high encapsulation efficiency of lysozyme into CaCO$_3$ microparticles. In addition, the SC-CO$_2$ process offers optimal conditions [55] in terms of protein stability and integrity and permitted to retention of 90% of the biological activity of lysozyme as proved by the M. lysodeikticus assays, which is in prefect agreement with a review from Wimmer et al. describing the effect of supercritical CO$_2$ on enzymes, which may even enhance the stability under denaturing conditions [56].

3.2.4 Discussion on the mechanism of lysozyme encapsulation into CaCO$_3$ microparticles

In our previous work, CaCO$_3$ particles were fully characterized [37]. It was demonstrated that CaCO$_3$ particles were formed from an aggregation of nanograins with an average diameter of 40 nm [37], which gave CaCO$_3$ particles a porous structure, and thus an available space for drug loading. As mentioned earlier, the zeta potential of CaCO$_3$ measured in distilled water is negative, which suggests that zeta potentials of CaCO$_3$ particles and CaCO$_3$ nanograins would also be negative in the basic synthesis medium which is buffered by glycine–NaOH. On the other hand, lysozyme is positively charged in the synthesis medium due to the fact that the pH of lysozyme is about 11. Therefore, it is believed that during the formation of CaCO$_3$ particles in the basic medium, there is electrostatic interactions between lysozyme and CaCO$_3$ nanograins, which

![Fig. 9. Lysozyme kinetic release profiles and the corresponding SEM observations of microparticles after release.](http://dx.doi.org/10.1016/j.supflu.2013.02.024)
allows lysozyme to attach themselves to the surface of CaCO3 nanoparticles, leading to the subsequent encapsulation of lysozyme within CaCO3 particles. By now, in situ pH measurements and in situ X-ray diffraction experiments in CO2 media have been carried out by our team to help us get more insight into the understanding of the mechanisms of particle formation and the dynamics of protein encapsulation within CaCO3 particles. This is beyond the scope of this study, and the results will be presented in another paper.

3.2.5. Kinetic release of Lysozyme

This study was carried out at three different pH values: 7.4, 6.0, 4.5. A burst release was observed during the first 30 min of release, followed by a more progressive release that started to stabilize after 3 h (Fig. 9). The amount of released protein increased while lowering the pH, due to the faster degradation of CaCO3 microspheres under acidic pH, and a maximum release of 89% was obtained after 24 h at pH 4.5. This degradation was observed at 24 h of kinetic release using scanning electron microscopy. It can be noted that after 24 h at pH 4.5, particles were totally dissolved and could not be observed anymore. It should be pointed out that one of the applications of CaCO3 particles we intend to use is to incorporate them into an implantable hydrogel system for bone and cartilage tissue engineering. Therapeutic protein will be encapsulated in CaCO3 particles and undoubtedly will be released much more slowly in such conditions.

4. Conclusion

In the present study, two protein encapsulation methods were designed based on the emulsification of a polymer solution or an aqueous solution in CO2 media. Two types of particles, polymeric PLGA and inorganic CaCO3, were successfully generated. The encapsulation yields in both cases can reach 60%. The mechanism of particle formation in the case of PLGA particles appears to be complex. It might involve an emulsification/extraction process or a phase separation phenomenon depending on the experimental conditions. This work can serve as an alternative for classic methods which use volatile toxic solvents. The mechanism of CaCO3 formation in the CO2 media and the mechanism of lysozyme encapsulation within CaCO3 particles were also discussed. Details about the mechanism of CaCO3 formation will be presented in the next publication by introducing results of in situ experiments in CO2 media. Lysozyme release profiles were also carried out in the case of CaCO3 particles. The release profile of lysozyme from CaCO3 particles reveals it to be pH-dependent. Further efforts will be provided for process optimization, particle characterization, encapsulation of therapeutic proteins, in vitro and in vivo release, and particle surface modification as necessary steps for the development of the final product in the form of hybrid biomaterials.

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