Supercritical CO_2 as highly efficient innovative process for protein encapsulation

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INTRODUCTION AND OBJECTIVES

The challenge in protein drug delivery is the formation of microcarrier with well-defined characteristics: size, morphology, composition and density, these characteristics are important to achieve high bio-availability with a particular administration route. Traditional methods used to formulate microcarriers frequently cause protein aggregation and inactivation. In this point of view, supercritical fluid process presents an innovative route, which can evade most of the drawbacks of the traditional ones, especially Supercritical CO_2 (SC-CO_2).

SC-CO_2-based processes may be considered as environmentally friendly as CO_2 is non-toxic, low-cost and may be recycled. Finally, its easy reachable critical point (T = 31.1 °C and P = 7.39 bar) makes SC-CO_2 an especially adequate and mild process to manipulate sensitive compounds such as therapeutic proteins.

The aim of this work is to bring out a proof of the concept of bioencapsulation of therapeutic proteins using water in SC-CO_2 emulsification within calcium carbonate $CaCO_3$ particles as microcarriers. We tried to compare this process to protein-$CaCO_3$ co-precipitation, in terms of protein loading, protein integrity and stability.

MATERIALS AND METHODS

Calcium carbonate ($CaCO_3$) microspheres containing lysozyme as model protein (1.0 g/L), were synthesized using an aqueous solution (25 mL) composed of calcium hydroxide ($Ca(OH)_2$ 21mM), hyaluronic acid (0,1% m/v), glycine buffer at final pH of 10, which was emulsified in SC-CO_2 at 40°C and 20 MP (Boury 2009).

Co-precipitation process was carried out by mixing the calcium solution ($Ca(OH)_2$ 21mM, 12.5 mL) containing lysozyme (1.0 g/L) and carbonate containing solution (Na_2CO_3 21 mM, 12.5 mL).

X-Ray Diffraction analysis was obtained by X-pert diffractometer . The surface morphology and size of the microparticles were investigated by scanning electron microscopy (SEM) (JSM 6310F, JEOL). Lysozyme loading and encapsulation efficiency were obtained by measuring lysozyme activity using *Micrococcus lysodeikticus* bioassay. This assay is based on *Micrococcus lysodeikticus* membrane lysis under the action of active lysozyme, which results in a decreased suspension turbidity.

RESULTS AND DISCUSSION

The preparation and characterization of unloaded $CaCO_3$ microparticles have been studied and reported in our previous work (Beuvier 2011).

The presence of hyaluronic acid (Dickinson and McGrath 2004) and glycine (Shivkumara 2006) may control the crystal growth of thermodynamically instable $CaCO_3$ vaterite. Also $CaCO_3$ crystallization is highly depending on the experimental parameters such as temperature, stirring, pressure and pH. All $CaCO_3$ particles obtained by either supercritical process or co-precipitation; consist of almost pure vaterite as revealed by XRD analysis (Figure I). These results suggest that the presence of lysozyme and the used process have no effect on the polymorph of the obtained $CaCO_3$.

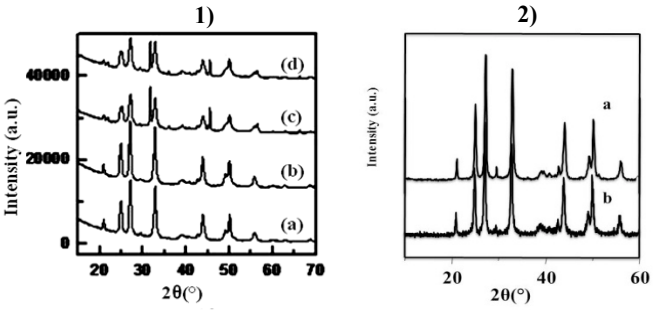

Figure I. XRD patterns of 1 a, b) unloaded and c, d) lysozyme loaded $CaCO_3$ vaterite microspheres obtained by co-precipitation mode.
2 a) unloaded and b) lysozyme loaded $CaCO_3$ vaterite microspheres obtained by SC-CO_2.

Morphologies of the $CaCO_3$ particles prepared with two different methods are shown in SEM images (**Erreur ! Source du renvoi introuvable.**). Unloaded $CaCO_3$ microspheres formulated by supercritical process (Fig. II-1-a and b) had spherical shapes with a size ranging from 1 to a maximum of 10 µm (average size of 4.9 ± 1.0 µm).
When lysozyme is encapsulated similar $CaCO_3$ particles with spherical shapes 4.9 ± 0.3 µm in diameter, were obtained (Fig. II-1-b).

For those prepared by co-precipitation process, the average size was 1.86 ± 0.4 µm (Fig. II-2-a and b).

Thus the presence of lysozyme has no effect on particles size, by contrast there is an important impact of the used process.

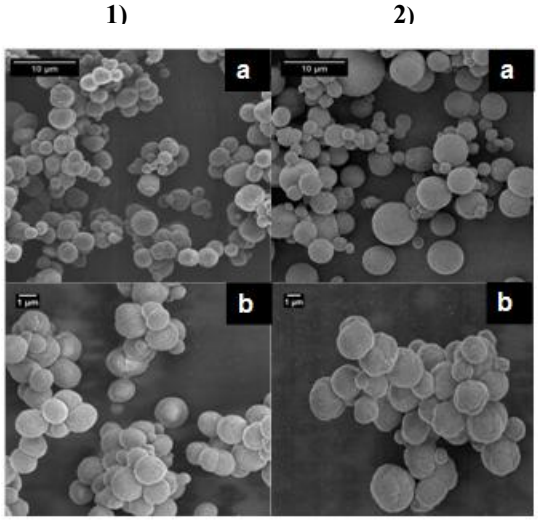

Figure II. Morphologies of unloaded (1 a and 2 a) and lysozyme-loaded $CaCO_3$ microspheres (1 b and 2 b) under SEM

The highest encapsulation yield we could obtain was about 47.5% at the starting protein concentration of 1.0 g/L, which is much higher than results obtained by interfacial reaction (Fujiwara 2008) or phase transition method (Fujiwara 2010). This result is expected to be enhanced by optimizing process parameters and experimental setup.

We successfully obtained $CaCO_3$ microspheres in supercritical CO_2 with high lysozyme encapsulation efficiency 47.5 ± 0.13 %, and lysozyme loading of 4.19 ± 1.13 % respectively (Table I). Lysozyme was scarcely encapsulated when co-precipitation process was used. We obtained a lysozyme loading of 0.12± 0.03 % and encapsulation efficiency of 2.26 ± 0.41 %. The first process allowed higher encapsulation efficiency and higher conservation of lysozyme activity.

Table I. Lysozyme encapsulation using supercritical process and $CaCO_3$ co-precipitation

Encapsulation mode	SC-CO_2	Co-precipitation
Lysozyme concentration (g/L)	1.0	1.0
Active lysozyme loading (%)	4.19 ± 1.13	0.12 ± 0.03
Encapsulation yield (%)	47.5 ± 0.13	2.26 ± 0.41
Average size (µm)	4.9 ± 0.30	1.86 ± 0.04

CONCLUSION

In the present research, we compared two solvent free processes to obtain calcium carbonate microparticles encapsulating proteins. We showed that SC-CO_2 plays an important role in encapsulating higher amount of protein. In addition SC-CO_2 process offers optimal conditions (Winters 1996) in terms of protein stability compared to the adsorption loading mode described by (Ueno 2005) or co-precipitation.

Therefore, further efforts will be needed for process optimization, particle characterization, and encapsulation of therapeutic protein, *in-vitro* release, and particle surface modification as the necessary steps for the development of the final product. Results obtained from this work may be useful as well for other applications where protein encapsulation is required.

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ACKNOWLEDGMENTS

We thank the SCIAM ("Service Commun d'Imagerie et d'Analyse Microscopique") of Angers for scanning electron microscopy as well as the LPEC ("Laboratoire de physique et de l'état condensé") of Le Mans for X-ray analysis. We finally thank the "Région Pays de la Loire" and the ANR ("Agence Nationale pour la Recherche", grant ANR-09-PIRI-0004-01) for the financial support.