DESIGN AND ENGINEERING OF INJECTABLE MICROPARTICLES FOR SUSTAINED RELEASE OF THERAPEUTIC PROTEINS USING A SUPERCRITICAL FLUID COATING PROCESS

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INTRODUCTION

Therapeutic proteins are fragile biomolecules, which display a short plasma half-life after injection and cannot be administered by the oral route due to their poor stability in the gastro-intestinal (GI) tract and their poor oral bioavailability. They actually need an appropriate injectable delivery system to maintain therapeutic levels in the blood stream over long periods of time and hence avoid daily injections. Microparticulate systems with specific properties are currently developed and used for drug delivery. Current methods for the preparation of these microparticles are mainly based on emulsion-solvent extraction, phase separation or spray-drying. The main drawback of these techniques is the extensive use of organic solvents to either dissolve the polymer or induce phase separation and particle hardening. These solvents are suspected to be partly responsible for biological inactivation of the proteins incorporated in polymer microspheres. Protein instability in polymer microparticles has been recognized as a major problem, resulting in incomplete release of native protein which is denaturated or aggregated. Upon denaturation or aggregation, protein species become therapeutically inactive and may also induce side effects, related to immunogenicity or toxicity. Thus, development of new protein-loaded microparticle production processes should be focused on full preservation of structure and biological activity of the native protein during preparation, storage and release.
The use of supercritical fluids (SCF) for engineering of drug-loaded microparticles is a recent development. Many different approaches have been reported in the literature for the production of pure drug or drug-loaded microparticles, using an SCF either as a solvent, an anti-solvent, or even a swelling and plasticizing agent of the polymer and/or the drug[1,2]. Coating processes of preformed solid drug microparticles have been recently developed in our lab to produce sustained-release microparticles of fragile biomolecules [3]. They use SC or liquid CO_2 either as a solvent or a non solvent of the coating material, which can be a lipid compound or a polymer [4,5].

In the present work, recent results obtained by coating a therapeutic protein with a CO_2-soluble lipid material are shown and discussed. The optimization of the coating process carried out by properly choosing the coating material and characterizing its phase behavior in CO_2 will be presented. This optimized process makes it possible to produce well-controlled, highly reproducible matrix microparticles containing the therapeutic protein, whose *in vivo* evaluation in an appropriate animal model clearly shows a sustained-release profile without alteration of the protein.

I – MATERIALS AND METHODS

Interferon a-2b (IFNa-2b) particles to be coated were produced by milling and sieving (< 25 µm) a commercially available freeze-dried powder of human recombinant interferon α-2b (INF®, Gautier Cassara, Argentina). The mean particle diameter ($D_{4,3}$) of milled and sieved IFNa-2b particles is ~ 15 µm. The IFNa-2b titre of these particles was measured using an ELISA method, and found to be 2.866 x 10^6 pg per mg of particles.
A blend of glycerides, provided as a fine, white, free-flowing powder of glyceryl palmito-stearate, was used as the coating material. The melting point of this material was found to be 56.2 °C.

The phase behaviour of coating material/CO_2 systems was studied using a high-pressure variable volume view cell (SITEC, Maur/Zurich, Switzerland) equipped with a magnetic stirrer. A known amount of coating material was added to the view cell chamber. The chamber was then sealed with a pressure balance piston and CO_2 was pumped into the chamber until a clear, single-phase solution is obtained. At a given temperature (T), the cloud point was determined by slowly lowering the pressure (P) upon moving the pressure balance piston, until the solution becomes cloudy. The phase behaviour of coating material/CO_2 systems under the conditions of the coating process was studied by loading the view cell with a known amount of coating material and CO_2 until defined T/P conditions are reached (typically 45°C/200 bar), then modifying the T/P conditions in order to follow the T/P pathway of the coating process.
Protein particles were coated using an apparatus described elsewhere [3,6]. Known amounts of protein particles and coating material were introduced in a high-pressure stirred 1L vessel. The vessel was closed, and CO_2 was pumped in the vessel until the T/P conditions for solubilization of coating material are reached. After a 1 h agitation, an isochoric cooling was carried out, typically until a temperature of 20°C is reached. CO_2 was then slowly vented. Microparticles were harvested, sieved with a 150 µm sieving plate and kept under a nitrogen atmosphere.

In vivo evaluation of coated IFNa-2b microparticles was carried out using a series of 70 male, Swiss mice, 8 to 10 week old. The series of animals was divided into 7 sets of 10 mice (A to G). Prior to subcutaneous injection, the protein titre in the microparticles was assayed using a commercially available immunoenzymatic (ELISA) kit (R&D Systems, France). Then, the microparticles were redispersed in an aqueous vehicle for injection, containing carboxymethylcellulose. The particle size distribution and physical stability of the dispersions were characterized using a laser beam-scattering size analyzer (Mastersizer-S, Malvern, France). Dose uniformity was checked by assaying protein content in 8 samplings of the dispersion. Each animal received a dose of 500,000 IU IFNa-2b, that is ~ 3,414,000 pg protein, administered as a subcutaneous injection of a 0.9 mL suspension of microspheres. A standard 0.9 mL solution of non coated IFNa-2b containing the same dose was administered to the reference group for comparison. A 400 µL blood sample was collected from the retro-orbital venous sinus of animals, alternatively in set A to G of the series. The collected blood was centrifuged immediately to harvest the serum, which was then frozen and stored at – 20 °C until analysis. Circulating IFNa-2b concentration was measured using the ELISA method, and pharmacokinetic study was carried out over a period of 6 days. The limit of quantification of the ELISA method was determined and found to be 12.5 IU/mL.

II – RESULTS AND DISCUSSION

The encapsulation process involves the 3 steps as follows:

(i) dissolving the coating material in SC CO_2 under defined T/P conditions and dispersing insoluble protein-loaded particles in this SC solution under stirring.
(ii) inducing a precipitation of the coating agent onto the dispersed particles by a gradual and controlled variation of T/P conditions in the reactor. A coating is formed onto the microparticles. Deposition occurs progressively as the solubility of the coating material in CO_2 decreases and the coating material desolvates.
(iii) harvesting the composite microparticles after depressurization of the coating vessel.

The process can be described as a coating/granulation process. The coating of individual particles (e.g. protein-loaded powders) first results in the production of core-shell microcapsules. The granulation of the primary coated particles then results in the formation of microspheres where protein particles are embedded in the lipid matrix (Figure 1).

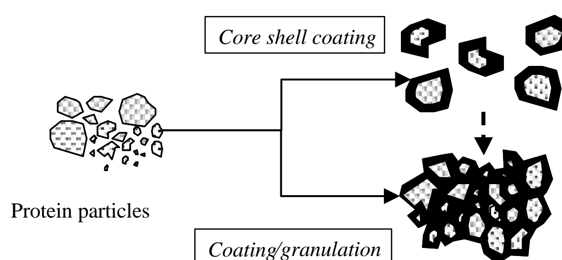

Figure 1 : Mechanism of protein encapsulation

The structure (microcapsule or microsphere), size distribution and release properties of coated particles were found to be dramatically influenced by:

- the operating conditions of the process (starting T/P conditions, weight fraction of coating material in CO_2/coating material system, T/P pathway and kinetics of T/P alteration, stirring conditions),
- the particle size distribution of initial protein particles,
- the loading ratio of lipid/protein particles (protein /coating material weight ratio),
- the coating material composition.

For given protein particles, coating material and loading ratio, the process has to be optimised as regards the structure, size and release properties of protein-loaded microspheres. The process optimisation relies on an in-depth study of the phase behaviour of coating material/CO_2 systems, performed using the high-pressure variable volume view cell. The cloud point curve of the coating material in CO_2 was recorded (Figure 2A). Then, an experimental range of weight fractions of coating material in CO_2 and starting T/P conditions could be selected. The phase equilibrium apparatus was also used to mimic the whole process without protein particles, in order to clear the question : “When and how does the coating material deposit onto host particles?”. The weight fraction of coating material in the coating material/CO_2 system and the starting T/P conditions were found to be key parameters for the coating process. These two parameters govern the T/P conditions under which the phase separation of the coating material/CO_2 system occurs, and the way the coating material deposits onto the host particles. Depending on these starting conditions, the phase separation of the coating material from the SC solution can lead to fine solid particles of coating material dispersed in SC CO_2 or to fine liquid droplets. An efficient coating, leading to a sustained-

release behaviour, is obtained by selecting an appropriate weight fraction of coating material in CO_2 and starting T/P conditions to obtain liquid droplets of coating material upon phase separation, which further solidify during the isochoric cooling step. The experimental phase behaviour for an optimised process pathway is presented in Figure 2B. Starting from a SC solution of coating material (0.37% weight fraction of coating material) at 45°C and 200 bar, the isochoric cooling resulted in the formation of fine liquid droplets of coating material at 42.4°C, then to a solid coating at 37.5°C.

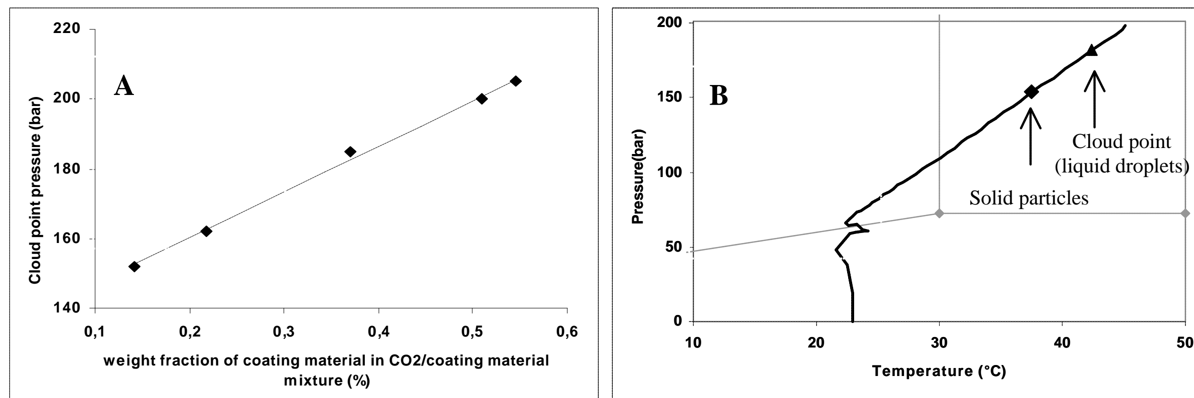

Figure 2: Phase behaviour of coating material/CO_2 system obtained using the high-pressure view cell. (A) Cloud point curve at 45°C (B) Phase behaviour during an optimised thermodynamic pathway of the coating process (starting T/P conditions : 45°C, 200 bar; weight fraction of coating material : 0.37% ; the system is cooled down to ~ 22°C, then CO_2 is vented).

These optimised operating conditions were then used to produce microspheres loaded with IFNa-2b particles. 3 g of coating material and 157.45 mg of IFNa-2b particles were added to a high-pressure 1L vessel. Initial conditions were 45°C and 200 bar and the weight fraction of coating material was 0.37%. The temperature was decreased down to 17°C during 31 min. CO_2 was then vented during 45 min. During the venting operation, the temperature was kept close to ~ 20°C. Composite microparticles were then harvested and sieved using a 150 µm sieve plate. The sieving yield was 91 wt%.

The IFNa-2b content of coated microparticles, as determined by ELISA, was 146.10^3 pg of IFNα-2b per mg of microparticles. Thus, composite microparticles contained 4.1 wt% protein lyophilizate. The matrix structure of coated microparticles is evidenced using optical microscopy. Figure 3A shows the microparticles at ambient temperature. By using a heating plate set at 70°C, the coating material of the microparticles was melted *in situ*. After melting of the coating material, it clearly appears that each individual coated lipid microparticle contains several protein particles embedded in the coating material matrix (Figure 3B).

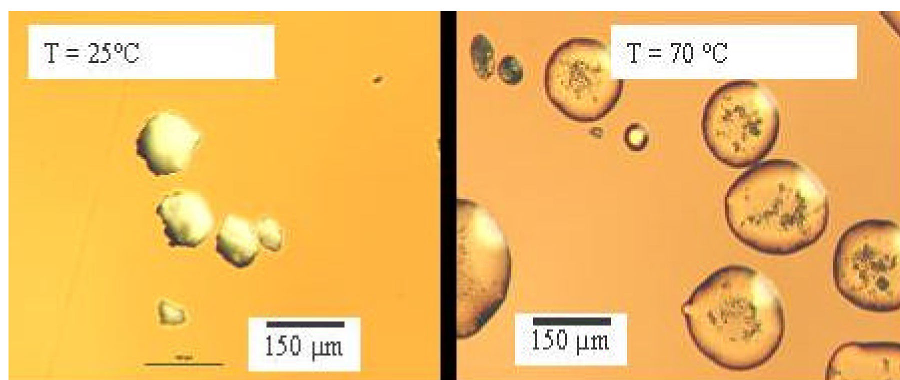

Figure 3: Optical microscopy. Coated INF® particles. Left: observation at ambient temperature. Right: observation at ~ 70°C (heating plate); protein particles appear in the

melted coating material matrix.
The particle size distribution of protein-loaded microspheres is shown in Figure 4. A narrow and unimodal particle size distribution is noticed, with a mean particle diameter ($D_{4,3}$) equal to 101.5 µm.

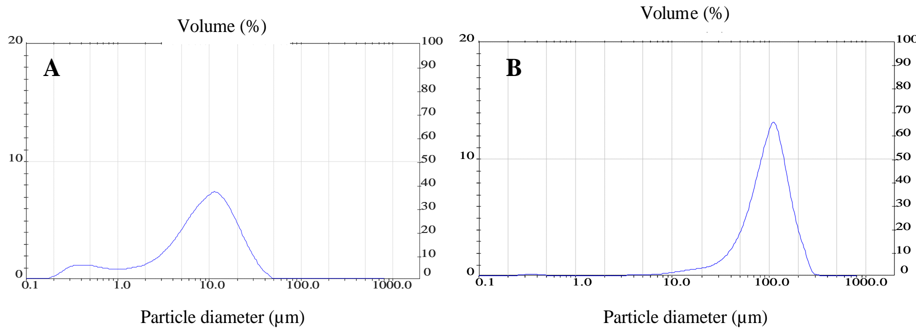

Figure 4: Particle size distribution of: (A) protein particles before coating, (B) microspheres loaded with protein particles.

The concentration of circulating IFNa-2b in the blood of the mice is shown versus time in Figure 5 (A to C), for animals receiving either IFNa-2b-containing microspheres or the reference solution of uncoated IFNa-2b. They clearly show that microspheres make it possible to strongly reduce the plasma peak concentration ("burst effect") and to get a sustained release of the protein in the blood of the mice. More precisely, the concentration of circulating protein remains significant and close to ~ 40 pg/mL, 77 h after the injection of the IFNa-2b-containing microspheres, whereas it reaches the limit of quantification at the same time in animals receiving the uncoated protein. From this experiment, it turns out that the time needed to release all the protein in the animal blood from these glyceride-based microspheres is ranging between 4 and 6 days.

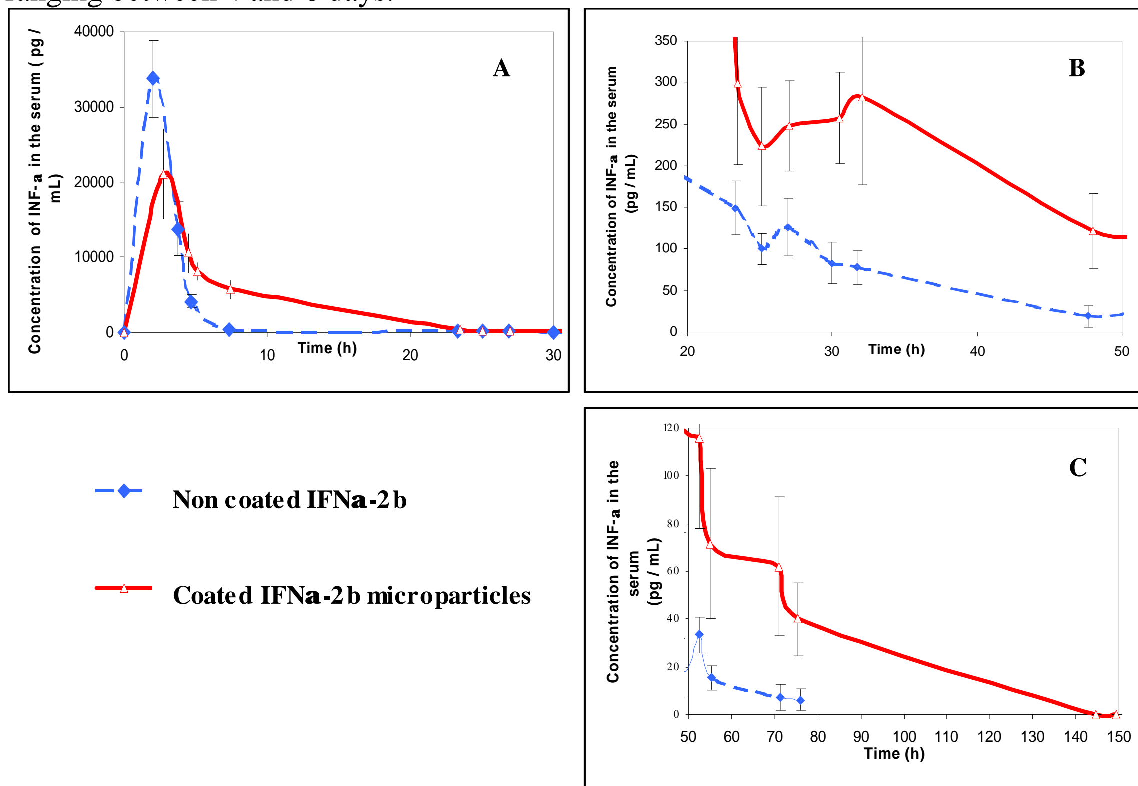

Figure 5: Concentration of circulating IFNa-2b in the blood of the mice versus time for animals receiving either IFNa-2b-containing microspheres (steady line) or reference solution of uncoated

IFNa-2b (dotted line) between : (A) 0 and 30 h, (B) 20 h and 50 h, (C)50 h and 150 h. The dose is 314,414 pg (500,000 IU)/mouse.

CONCLUSION

The SC CO_2-based coating process has been optimised by properly selecting the appropriate weight fraction of coating material in CO_2 and starting T/P conditions to obtain liquid droplets of coating material upon phase separation, that further solidify during the isochoric cooling step. The optimisation step has been carried out through an in-depth study of the phase behaviour of coating material/CO_2 systems, performed using a high-pressure variable volume view cell. IFNa-2b-containing microparticles have been prepared using this optimised process and shown to display a matrix structure, the lyophilised protein being dispersed in a solid lipid matrix enabling sustained release. *In vivo* experiments in mice confirm the prolonged release of the therapeutic protein over 4 to 6 days after a subcutaneous injection of the microspheres, together with a strong limitation of the burst effect and plasma peak concentration. These results are highly promising as regards the clinical development of new sustained-release formulations of therapeutic proteins, that will be able to fulfill still unmet medical needs related to high peak concentration and high injection frequency.

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