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Functionalization of lactose as a biological carrier for bovine serum albumin by electrospraying

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ABSTRACT

Electrohydrodynamic atomization (EHDA) is an attractive technique to make new types of composite particles for pharmaceutical use. The aim of this work is to prove that EHDA can be successfully used to attach nano/micro-particles of protein to lactose, the commonly used excipient for pulmonary delivery, keeping all the biological properties of the protein after dissolution of the complex. Bovine serum albumin (BSA) was used as a model protein. The atomization of BSA was tested with two different solvents, dimethyl sulfoxide (DMSO) and ethanol. The process using DMSO resulted in the formation of a thin layer of protein while the tests using ethanol resulted in the formation of spherical particles with mean diameters around 700 nm. Ethanol as solvent was also used to produce a composite formed by BSA adsorbed at the surface of lactose by electrostatic forces. No denaturation or significant conformational changes of the protein were observed, although an increase in the exposition of the lactose to the jet of the solution decreases the reproducibility of the method. Due to the absence of denaturation in the model protein, this new approach can be tested for the production of new formulations for dry powders for drug delivery systems.

1. Introduction

Particle technology (the science and technology related to the handling and processing of particles and powders; Rhodes, 2008) plays a key role in the development of new formulations for drug delivery systems (DDS). The small quantities of the active principle needed for each dosage are very difficult to evenly distribute in the solid state (Islam and Gladki, 2008). In the case of pulmonary delivery, an additional problem is that only a small fraction of the drug will reach the lungs and most of it will remain in the throat of the patient. This is particularly important when dealing with expensive molecules like proteins and peptides (Service, 1997; Wurm, 2004; Demain and Vaishnav, 2009). The most common drug delivery route for proteins is direct parenteral (intravenous, intramuscular, subcutaneous) which is inconvenient, painful and unsafe. Ideally, the use of non-invasive delivery method such as oral administration is suitable, however, proteins show a poor adsorption due to their large size and hydrophilicity and the gastro-intestinal tract environment promotes their extensive degradation. Thus, the development of new formulations based on particle technology, aims new DDS able to cross particular physical barriers, in order to better target the drug and improve its effectiveness, or on finding alternative and acceptable routes for the delivery of proteins drug (Rao et al., 2008). For these molecules and despite the restrictions associated with the use of pressurized metered dose inhalers (pMDIs) (Newman and Busse, 2002), the preferred option is still the use of dispersed aqueous solutions to deliver the proteins into the lungs (Service, 1997). The reasons for this are the difficulties associated with the use of the conventional techniques for particle size reduction like milling, recrystallization from liquid antisolvents, freeze-drying or spray drying (Subramaniam et al., 1997). Although these micronization techniques are very efficient in the processing of small molecules with highly crystalline morphologies, the disruptive nature of these processes make them inadequate to work with labile biological molecules, with complex structures, such as porous/hollow particles, non-spherical particles, composites, nano-aggregates and surface-modified materials (coated or encapsulated) (Chow et al., 2007). A good alternative for the production of nano- and micron-sized particles of biological active molecules is electrohydrodynamic atomization (EHDA), a process in which a liquid jet breaks up into droplets under the influence of electrical forces (Ciach, 2006; Grace and Marijnissen, 1994; Cloupeau

Abbreviations: EHDA, Electrohydrodynamic atomization; BSA, Bovine serum albumin; DMSO, Dimethyl sulfoxide; SDS-PAGE, Sodium dodecylsulphate polyacrylamide gel electrophoresis; CD, Circular dichroism; DDS, Drug delivery system; pMDI, Pressurized metered dose inhalers.

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and Prunet-Foch, 1994). A liquid is pumped through a nozzle at a low flow rate (10^{-6} L/h–10^{-3} L/h). An electric field is applied over the liquid by applying a potential difference between the nozzle and a counter electrode. When the electric stresses overcome the surface tension stresses, the emerging liquid meniscus from the tip of the nozzle is transformed into a conical shape. From the cone apex a jet emerges which breaks up into quasi-monodisperse droplets. Unipolarity of the droplets prevents their coagulation and dispersion is enhanced (Jayasinghe et al., 2002). Droplets of a few micrometers, or even smaller, can be obtained, which results in particles with a very narrow particle size distribution in the desirable range (Jayasinghe and Edirisinghe, 2002; Gomez et al., 1998; Yurteri et al., 2010). This process was already successfully applied to some proteins like insulin (Pareta et al., 2005) and bovine serum albumin (BSA), both pure (Xu and Hanna, 2007), encapsulated with chitosan (Elversson et al., 2002) or just in the production of chitosan solid micro and nanoparticles (Zhang and Kawakami, 2010). These last examples show how the versatility of this technique allows developing more complex formulations for biological active compounds. On the other hand, the properties of an excipient can improve the formulation by reducing the risk of local overdose, by allowing for more accurate dosage and by giving an additional control over the pharmacokinetics. However, simply mixing active ingredient with the excipient may lead to mixing and sub-optimal dosing. If we use instead a composite where the active molecule is physically attached to the excipient, it presents mainly the physical properties of the excipient. Only when the excipient is dissolved in the mucus, the protein is released and can act. This simple concept opens the door to several applications of these composites. In this work, a new composite was developed where the lactose, a commonly used excipient for pulmonary delivery (Zijlstra et al., 2004; Sambrook, 2001) was coated with BSA by electrohydrodynamic atomization (van Ommen et al., 2010). The obtained powder was analysed for concentration, morphology, particle size and biological activity to access the possibility of using this new formulation for pharmaceutical and biological applications.

2. Materials and methods

2.1. Materials

The lactose used in this work consisted of the commercial formulation LactoHall Crystals from DOMO, Friesland Foods (The Netherlands) ($d(10) = \pm 77 \times 10^{-6}$ m; $d(50) = \pm 123 \times 10^{-6}$ m and $d(90) = \pm 175 \times 10^{-6}$ m). The BSA (≥96%) and the acetic acid (>98%) were purchased from Sigma–Aldrich (The Netherlands). The dimethyl sulfoxide, DMSO, (≥99.9%) was supplied by Merck (Germany) and the ethanol (≥96%) was obtained from Brentag Nederland BV (The Netherlands).

2.2. Electrohydrodynamic atomization (EHDA)

The EHDA experiments were carried out in a setup schematically represented in Fig. 1. The solution of BSA was loaded into a syringe to be pumped using a syringe pump Aitecs, model SPE 10-S (Lithuania) which allows to work with very low flow rates (10^{-6} L/h–10^{-3} L/h). The solution was then sprayed through a restrictor with an internal diameter of 0.41×10^{-3} m which is connected to a high voltage power supply Heinzinger, model LCU 20000-05 pos (Germany). An electric field was applied over the liquid by applying a potential difference between the nozzle and a counter electrode connected to the support where the lactose particles were placed. The preliminary EHDA tests were planned to optimize the electrospraying process in terms of solvent and conditions to be used. In this case, no lactose was placed in the support

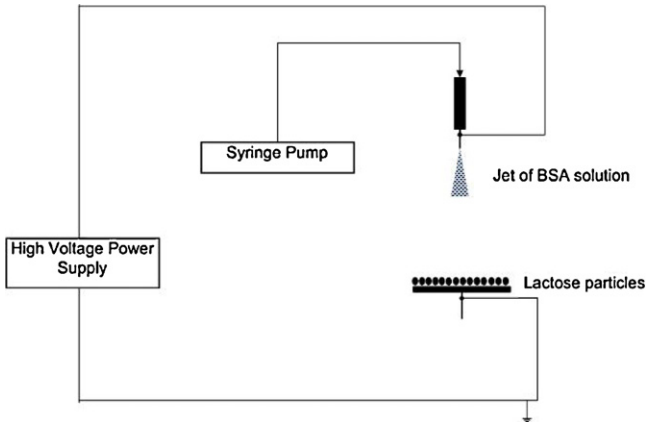

Fig. 1. Simplified scheme of the setup used for the EHDA experiments.

Table 1
Conditions used in the preliminary EHDA tests.

Parameter	Solvent: ethanol/acetic acid (96%/4%)	Solvent: DMSO/acetic acid (96%/4%)
Distance between the nozzle and the support where the particles where collected	5×10^{-2} m	5×10^{-2} m
Potential difference	4.8 and 5.4 kV	7.0 and 8.0 kV
Flow rate	8.33×10^{-8} L s^{-1}	2.78×10^{-8} and 5.56×10^{-8} L s^{-1}
Concentration of BSA	2.1×10^{-3} kg L^{-1}	4.3×10^{-3} and 6.8×10^{-8} kg L^{-1}
Time of exposition to jet	90, 300 and 600 s	60, 300 and 900 s

and only particles of pure BSA were produced. These experiments were performed using DMSO and ethanol as solvents. In both cases, the solutions consisted in a mixture of the solvent with acetic acid 96%/4% (V/V), respectively. The conditions of the trials are listed in Table 1. For the second set of experiments, lactose powder was used as host where the BSA was deposited after the electrospraying of the solution. For these tests, the conditions are listed in Table 2.

2.3. Powder characterisation

Images of all the powders were obtained using a scanning electron microscope Philips, model XL20 (The Netherlands). The samples were precoated with ultra thin gold layer using a sputter coater Edwards High Vacuum Co. International, model S-150 (USA).

2.4. Biological characterisation

The concentration of the protein in the powder was determined indirectly using a spectrophotometer from Hitachi, model U-2000 (Japan). For these measurements, the powder produced was dissolved in deionised water and the absorbance of the solution was measured at 280×10^{-9} m. Circular dichroism (CD) was used to

Table 2
Conditions used in the second set of EHDA experiments.

Parameter	Solvent: ethanol/acetic acid (96%/4%)
Distance between the nozzle and the support where the particles where collected	5×10^{-2}
Potential difference	4.8 and 5.4 kV
Flow rate	8.33×10^{-8} L s^{-1}
Concentration of BSA	2.1×10^{-3} kg L^{-1}
Lactose exposition to jet	90, 300 and 600 s

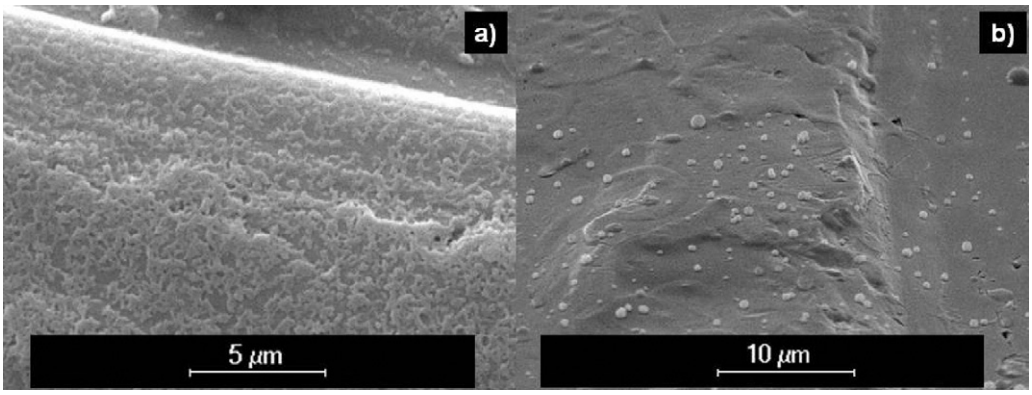

Fig. 2. SEM images of the powders obtained by EHDA using DMSO, (a), and ethanol, (b), in the conditions described in Table 1.

access conformational changes in the structure of the protein after dissolving the obtained formulation in water. The CD spectra were recorded on an Applied Photophysics Limited CD spectrometer, model PiStar 180 (United Kingdom). The spectra were scanned between 185×10^{-9} and 300×10^{-9} m with 1×10^{-9} m interval and averaging for 1 s. Five scans were recorded for each sample using a quartz cell of 1×10^{-3} m path length. Protein samples were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (stacking gel-4%, pH 6.8; separating gel-12%, pH 8.8) in SDS-PAGE running buffer according with a procedure described elsewhere (Sambrook, 2001).

3. Results and discussion

3.1. Influence of the solvent in EHDA

The first choice regarding solvents for this type of molecules should always be water, since these are biological systems and if one thinks about industrial applications, water is always easier and less expensive to deal with. Although water was already used as solvent for the electrohydrodynamic atomization (EHDA) of BSA (Pareta et al., 2005), its use in this case is not suitable since the goal of this work was the application of the technique coupled with BSA deposition on the surface of lactose particles. Since lactose is highly hygroscopic, one must be sure no water will reach the surface of the particles, otherwise lactose will no longer be a dry powder and the surface properties will change. Moreover, the distance between the tip of the injector and the surface of the lactose has to be long enough to ensure complete evaporation of the water, which would result in a very high dispersion of the BSA particles, with the consequent loss of protein. To avoid these effects, a decision was made to test more suitable solvents, namely DMSO and ethanol, commonly used in pharmaceutical industry and classified as class 3 solvents (Food and Drug Administration, 2003) (solvents with low toxic potential – no health-based exposure limit is needed). For both cases, it was necessary to add acetic acid to lower the pH of the solution and to increase the solubility of BSA. The preliminary tests (electrospraying BSA directly on the SEM support – Table 1) are presented in Fig. 2. As it can be seen, the product of the EHDA of BSA using DMSO as solvent, Fig. 2(a), results in the formation of a layer of protein on the surface of the SEM support. Although interesting and with several potential applications, this formulation is not suitable for the goal of this work and a choice was made to continue the study using the formulation obtained from the EHDA of BSA using ethanol as solvent, Fig. 2(b). In this case, discrete particles of BSA with a narrow particle size distribution and an average diameter around 700×10^{-9} m where obtained. The conditions used for the formation of the composite lactose – BSA are listed in Table 2. Figs. 3–5 show SEM images with different magnifications for the composite particles resulting from this second set of experiments. Fig. 3 shows a single lactose crystal where the BSA particles can be identified at the surface of the crystal but also on the support where the lactose was placed. A closer look to the lactose crystal, Fig. 4, shows that despite the irregularities in the surface, was possible to attach the BSA in a very consistent and uniform mode. Fig. 5 gives

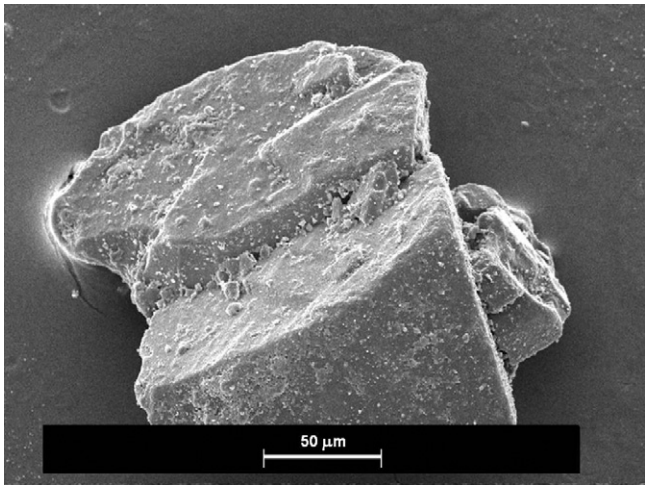

Fig. 3. SEM image of the composite lactose plus BSA obtained by EHDA (magnification: 350 times).

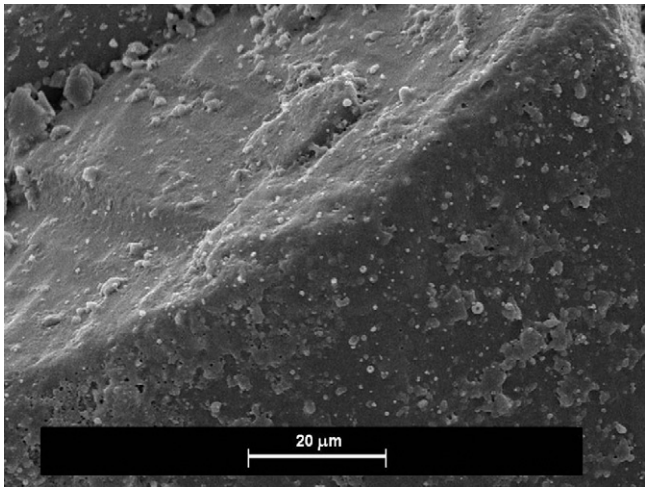

Fig. 4. SEM image of the composite lactose plus BSA obtained by EHDA (magnification: 1000 times).

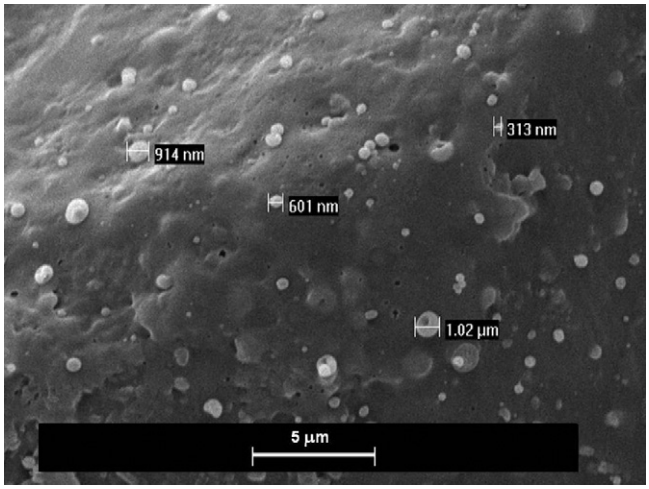

Fig. 5. SEM image of the composite lactose plus BSA obtained by EHDA (magnification: 3500 times).

more detailed information regarding morphology, shape and size of the BSA particles.

3.2. Biological evaluation of the composite lactose–BSA

To accurately determine the biological activity of the composite it was necessary to prepare new samples with a higher BSA concentration. These samples were prepared using the conditions described in Table 2, except for the exposition time of lactose to the jet. This parameter was increased to 1200 and 3600s. The obtained samples were dissolved in water and analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and by circular dichroism. An image of the SDS-PAGE gel is presented in Fig. 6. The figure indicates that the time of exposition to the jet influences the concentration of the protein in the composite, as expected. This tendency was also observed in the concentration determinations carried out by absorbance measurements. The absence of impurities in the lanes containing the composite, even in the lane 3 where the intensity of the BSA band is higher than the others, is an effect that has to be studied in more detail since it is not possible to establish purification of the protein or impurities concentration under the detection limit. Nevertheless, the absence

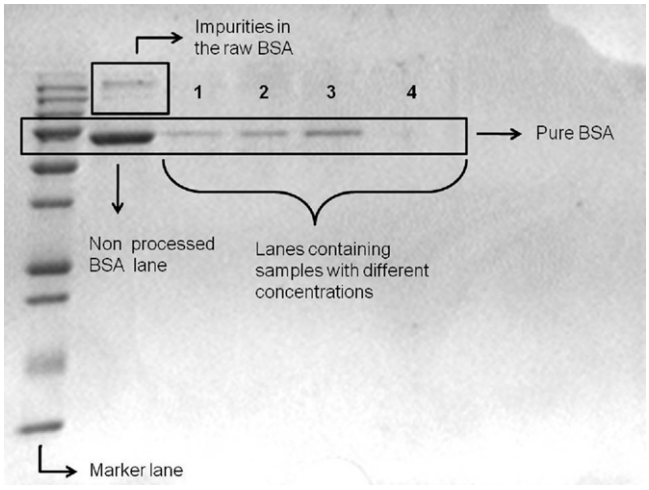

Fig. 6. SDS-PAGE of the BSA not processed and of 4 different samples of the composite lactose–BSA. Lanes 1, 2 and 4 – Lactose exposed to the jet for 1200s. Lane 3 – Lactose exposed to the jet for 3600s.

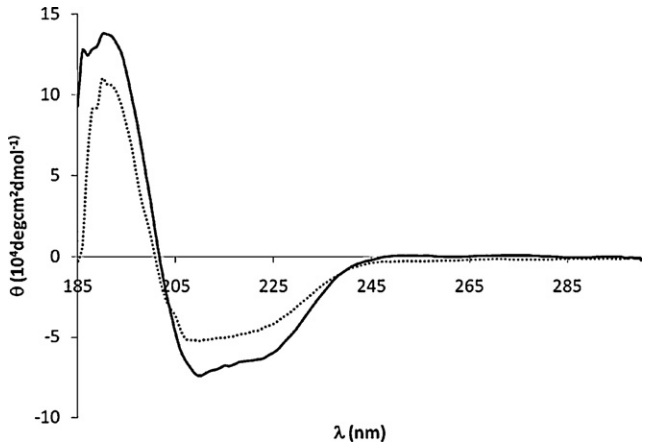

Fig. 7. CD spectra of BSA. _ Pure BSA; . . . BSA mixed with lactose and corrected for its effect in the spectra.

of impurities in this gel indicates that BSA did not suffer degradation with the electrodeposition process. For the conformational analysis by circular dichroism, it was necessary to take into account the presence of the lactose in solution, since it also absorbs in this wavelength region. The CD spectra of BSA are represented in Fig. 7, both pure and mixed with lactose (without being processed). For this last case, it was necessary to subtract the spectrum of pure lactose. Although this treatment clearly improves the quality of the spectrum, Fig. 7 shows that the resolution of the smaller features is not possible, most probably due to interactions between the lactose and BSA in solution. The difference between the amplitude of the spectra is due to differences in the concentration of both solutions. The CD spectra of the composite samples exposed to the jet for 1200s are shown in Fig. 8, while the samples exposed to the jet for 3600s are presented in Fig. 9. These two figures show that BSA does not suffer significant conformational changes in all the samples analysed and that the EHDA process allows the protein to conserve the main structure. On the other hand, the comparison between the two figures indicates that an increase in the exposition time to the jet reduces the reproducibility of the method and induces some slight conformational changes in BSA. This effect can be due to an excessive time subjecting the protein to the electrical field. Also, by increasing the exposition time, the probabilities for jet instabilities increase. Nevertheless, these issues are not impeditive

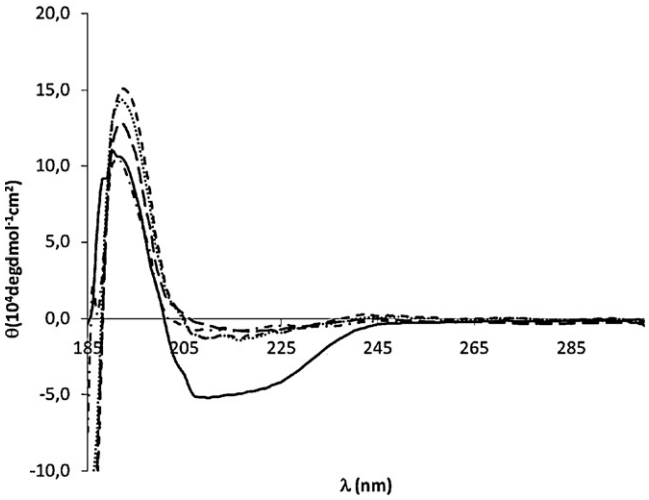

Fig. 8. CD spectra of BSA. _ Pure BSA; the other 4 spectra are from samples exposed to the jet for 1200s.

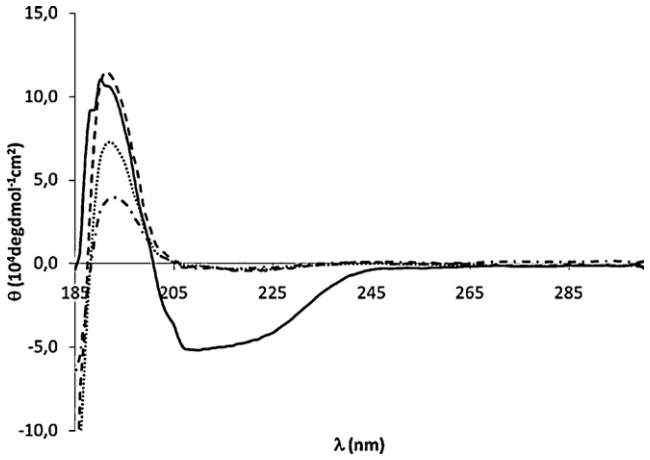

Fig. 9. CD spectra of BSA. _ Pure BSA; the other 3 spectra are from samples exposed to the jet for 3600s.

for the continuous processing of BSA via EHDA and the formation of the composite lactose–BSA.

4. Conclusion

A new formulation consisting on a composite of lactose and BSA was successfully produced by electrohydrodynamic atomization (EHDA), where BSA particles with mean diameters around 700×10^{-9} m were adsorbed at the surface of lactose crystals by means of electrostatic forces. Biological tests have shown that the protein does not suffer degradation or significant conformational changes with the process. This new formulation can be tested for pulmonary delivery of proteins, giving an additional control over the pharmacokinetics, since the protein is only released after dissolution of lactose. Until the dissolution of the composite, the dominant properties of this formulation are the properties of the lactose and the protein cannot be identified by the organism of the patient while attached to the lactose. This opens several possibilities for new formulations.

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