Behaviour of different proteins exposed to microencapsulation process: influence of their structures, size and excipient solutions.

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INTRODUCTION AND OBJECTIVE

Nowadays, there are many attempts to become protein delivery system available to pharmaceutical industries. Many proteins and peptides, which have pharmacological applications on human health, have been under study. But, beside this fact, there are many inconveniences to be overcome yet, meanly those inherent to encapsulation process, such as, protein instability, degradation process, and loss of biological activity, among others. One of the most studied systems to encapsulate proteins is microspheres produced by double emulsion methodology followed by solvent evaporation using biodegradable and biocompatible polymers. In this case, PLGA (poly-lactide-glicolide-acids) is one of the most promisor polymers in this field. However, the applicability of this methodology is limited due to problems of poor physical and chemical stability of proteins, especially in the first emulsion, in which the formation of interfaces between organic solvent/water results in damage to the encapsulated molecules. During this process, protein or peptide of interest are exposed to many physical and chemical forces, which can lead to deleterious effects.
The biotechnological rational to overcome these problems is to use available biochemical and biophysical strategies to increase the stability of encapsulated proteins.
In this context, the present study aimed to evaluate the effects of microencapsulation process by double emulsion method on the structure of different proteins, examining the possible influence of their molecular characteristics and excipient solutions, by quantifying and measuring structural conformations.

MATERIAL AND METHODS

Calibration curves to measure protein
Subsequent serial dilutions from stock solutions of proteins (1 mg/mL in PBS buffer at pH 7.2) were made with concentrations ranging from 0.03 to 1.0 mg/mL in order to produce calibration curves for the determination of proteins using three different methods: spectrophotometric reading at λ 280 nm, and colorimetric methods of Lowry and Bradford.

Simulation of the phase W1 of double emulsion (W1/O/W2)
The simulation of the first emulsion was performed with proteins contained in different excipients: PBS (control), H_2O, and salt solutions (NaCl, $MgCl_2$, KSCN and NaH_2PO_4 - 50 mM - pH 7.2). Two millilitres of each solution containing proteins were added to eight millilitres of CH_2Cl_2 (organic solvent), which were emulsified in Ultraturrax® at 24000 rpm for two minutes. After, samples were centrifuged for 10 minutes at 5000 rpm, separating the supernatant for further analysis.

Structural analysis by SDS-PAGE
It was applied 30 µL of each recovered protein from the different solutions to wells formed in the polyacrylamide gel, and the electrophoresis was performed at 50 mA, 120 V for 1 hour.

Structural analysis by intrinsic fluorescence
Recovered protein solutions were excited at 280 nm and fluorescence emission measured from 300 to 400 nm in a spectrofluorimeter using quartz cuvettes with 1 cm optical length. However, to study the relationship "Fluorescence X Structure" albumin and casein was analysed at 350/330 nm and insulin at 330/300 nm.

RESULTS AND DISCUSSION

In table 1 are showed coefficients of correlations obtained from three different methodologies to quantify proteins. The absorbance reading at 280 nm was the method with the highest linearity, in terms of concentration and absorbance units. Thus, we decided to choose that method to follow up the processed protein.

Table 2: Coefficient of correlation (r^2) obtained from calibration curves for quantifying proteins.

Methods ▶ Proteins ▼	λ 280nm	Lowry	Bradford
Insulin	0,9849	0,9582	0,9675
Albumin	0,9992	0,9995	0,9866
Casein	0,9995	0,9947	0,9349

Once the three studied proteins were submitted to the first phase of the double emulsion, they were quantified in order to observe possible adsorption on the interfaces organic solvent/water or the presence of denatured molecules (Figure 1).

It was not observed formations of supramolecular aggregates and/or molecular fragmentation identified by SDS-PAGE (Figure 2). This was confirmed by

total absence in the gel of other bands showing different molecular weight compared with the standard protein (native protein).

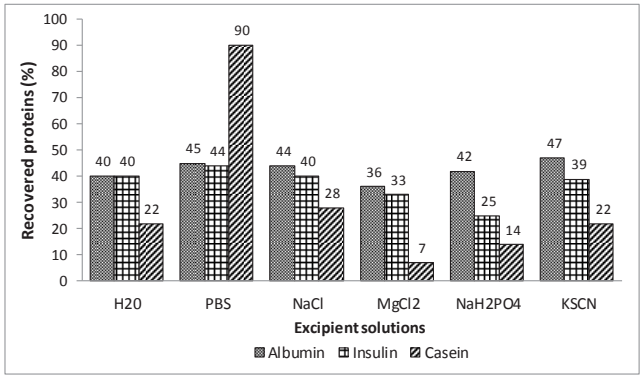

Figure 1: Percentage of proteins recovered after W_1 phase exposition.

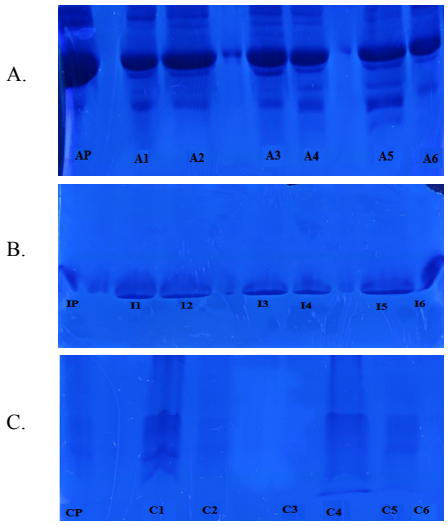

Figure 2: SDS-PAGE of model proteins. A. Albumin; B. Insulin; C. Casein. Lane P=Native protein; 1=H_2O; 2=PBS; 3=NaCl; 4=$MgCl_2$; 5=NaH_2PO_4; 6=KSCN

Overall, there were slight changes in the structures of all three proteins after their exposition to W_1 phase (Figure 3). This was observed by changes in the exposure of tryptophan in albumin and casein, and tyrosine in the insulin.

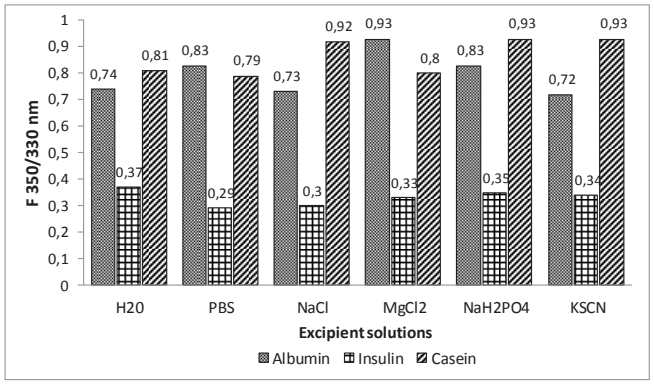

Figure 3: Intrinsic fluorescence of model proteins. For insulin the ratio used was F 330/300nm.

Analysis of fluorescence showed that for albumin, PBS and NaH_2PO_4 were excipients that better maintained its structural conformation nearest the native conformation; for insulin, KSCN and $MgCl_2$ were best ones; followed by NaCl for casein.

CONCLUSIONS

Taken together, all results of this study showed that the absorbance reading at 280 nm was the best method to measure protein recovering after exposition to the first phase of double emulsion of microencapsulation process.

The electrophoretic pattern showed no degradation or fragmentation of the three studied proteins.

The best excipient for maintaining protein conformation during processing was PBS, although other salt solutions also can be used, such as NaH_2PO_4 for albumin, KSCN and $MgCl_2$ for insulin and NaCl for casein producing similar results.

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