International Journal of Latest Research in Science and Technology **ISSN (Online):2278-5299**
Volume 3, Issue 4: Page No.31-36. July-August 2014
https://www.mnkpublication.com/journal/ijlrst/index.php

INFLUENCE OF DIFFERENT EXCIPIENTS AND PROTEIN CHARACTERISTICS ON THEIR SUSCEPTIBILITY TO MICROENCAPSULATION PROCESS

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Abstract - This study evaluated the effects of microencapsulation process on different proteins. Insulin, albumin and casein were used as model proteins. Three methodologies to determine protein concentrations were compared: λ 280 nm, Lowry and Bradford colorimetric methods. Proteins were submitted to the first phase of double emulsion method, which is commonly used to produce polymeric delivery systems. Different excipient solutions were tested: NaCl, KSCN, $MgCl_2$ and NaH_2PO_4 all at 50 mM, PBS and H_2O were used as controls. Structural analysis of proteins was performed by SDS-PAGE and intrinsic fluorescence intensities. Results showed direct reading at 280 nm the most suitable for quantitative measures of the three proteins presenting linear coefficient (r^2) of 0.9849, 0.9992 and 0.9995 for insulin, albumin and casein, respectively. The excipient solution that allowed greater recovery of proteins was PBS, which recovered 45%, 44% and 90% of albumin, insulin and casein, respectively. For albumin, KSCN solution also enabled recovering 47% of processed protein. Electrophoretic pattern did not revealed fragmentation or aggregation of the three analyzed proteins. Structural analysis of proteins by measuring of intrinsic fluorescence has shown: PBS and NaH_2PO_4 solutions effectively maintained the integrity of protein structures for albumin, KSCN and $MgCl_2$ solutions for insulin, and PBS solution for casein. Taken all these results together, it was possible to conclude that phosphate-based solutions are best excipients to protect proteins with higher molecular weights or size, and chaotropic salts, such as $MgCl_2$, are the best one to low-molecular weight peptides as insulin.

Keywords: *Microspheres; PLGA; Protein stability; Protein encapsulation.*

I. Introduction

The advances and new techniques in the field of molecular biology and recombinant DNA during the last ten years have opened up the possibility of producing proteins and peptides for therapeutic usages in large-scale and well-controlled systems [1]. Proteins and peptides, especially those intended for human therapies, have some restrictions in their appliance due to many factors, of which poor oral bioavailability, no resistance to chemical conditions of the gastrointestinal environment or to tissue proteases, and reduced biological half-life [2-5]. These macromolecules commonly present complex conformational structures, which are very susceptible to physicochemical changes, affecting their shape and consequently their biological functions [6,7]. The maintenance of protein and peptide structural integrities is of crucial importance for therapeutic usages [8]. Encapsulating macromolecules within controlled delivery systems (CDS) could overcome these limitations.

The advantages of CDS include greater therapeutic efficacy with progressive and controlled release; decreased toxicity with increased retention time of bioactive molecules

Publication History
Manuscript Received : 30 July 2014
Manuscript Accepted : 9 August 2014
Revision Received : 11 August 2014
Manuscript Published : 31 August 2014

in the target tissue or blood; better patient compliance and convenience by simplifying the dosage for a singly or few doses [9]. Nevertheless, beside these beneficial effects, some biotechnological problems still arise from this attempt, for instance, strategies for preserving biological activity of these molecules along of microencapsulation process.

In this context, materials most commonly used for production of CDS are based on lipids (e.g. liposomes and micelles) and polymers [10, 11]. The copolymer PLGA or poly (lactic-co-glycolic acid) is one of the most studied to prepare CDS for human therapeutic purposes due to inherent factors such as biocompatibility and biodegradability, as well its approval by Food and Drug Administration (FDA) [12, 13].

There are many methods to prepare CDS using polymer materials, of which those based on emulsion techniques are well accepted for encapsulating several kinds of biomolecules because of its wide versatility. The double emulsion, i.e. water-in-oil-in-water ($W_1/O/W_2$) method followed by solvent evaporation/extraction is preferable to encapsulate proteins and peptides because it is a technique allowing their incorporation into aqueous-phase [14]. However, despite several advantages presenting by this methodology, it still has limiting drawbacks, which emerges from problems relating to low physicochemical stability of such macromolecules, mainly when they are submitted to first emulsion step (W_1/O). During this phase, proteins or peptides of interest are exposed to many physical and chemical forces leading to deleterious effects. The formation of organic solvent/water interfaces frequently results in damage to encapsulated proteins, for instance, adsorption, degradation, denaturation and precipitation are consequences commonly observed when this methodology is used [8,12,13]. The biotechnological rational to overcome these problems is to use biochemical and biophysical strategies to increase the stability of encapsulated proteins along of the process without using other heterologous molecules (e.g. PEG [14]), which could trigger adverse effects or reactions.

Considering all this background, the present study aimed to evaluate the effects of microencapsulation process by the double emulsion method on the structure of different proteins presenting distinctive physical chemical characteristics, such as size and molecular weights: insulin (~5,7kDa), albumin (~65 kDa) and casein (~90 kDa) were used as model proteins. Further, it was also evaluated possible influences of these characteristics and excipient solutions solubilizing these macromolecules, through structural analysis.

2. Materials and Methods

2.1. Chemicals

Human insulin (Humulin®) was acquired from commercial drug store; bovine serum albumin (BSA) and lyophilized casein were from Merck®, dichloromethane (CH_2Cl_2) and Folin-Ciacalteau were from Sigma-Aldrich Chemical® (Milwaukee/USA), sodium dodecyl sulphate (SDS) was from Nuclear®, polyacrylamide was from Ludwig-Biotec®. Salts NaCl, $MgCl_2$, NaH_2PO_4, KSCN and all other reagents were of analytical grade.

2.2. Calibration curve for quantitative protein measures

Serial dilutions from stock solutions of each studied protein were prepared at concentrations ranging from 0.03 mg/mL to 1.0 mg/mL to produce standard curves for protein quantifications using three different methods: direct reading at ʎ 280 nm, and colorimetric methods based on Lowry and Bradford's methodologies as described below. All measures were performed in triplicate, and results are expressed as mean and standard deviation (SD).

2.3. Direct reading at ʎ 280 nm

Serial dilutions of the three model proteins were analyzed by using of quartz cuvettes of optical path of 1 cm at 280 nm in a UV spectrophotometer (Shimadzu UV1800). PBS solution of pH 7.2 was used as blank control of scanning and reading.

2.4. Lowry's colorimetric method

120 µL of the solution containing different concentrations of proteins were added to 80 µL of NaOH solution (1M) and 800 µL of previously prepared reagent consisting of 1% CuSO4, 2% sodium potassium tartrate and 2% sodium carbonate (ratio 1:1:98). Further, 100 µL of Folin-Ciacalteau reagent diluted 1:2 in distilled water were added. The reaction was maintained protected from light for 30 minutes at room temperature and the absorbance read at λ 720 nm in a visible spectrophotometer (Biospectro sp220). Blank control was prepared only with PBS (pH 7.2).

2.5. Bradford's colorimetric method

It was added 100 mL of solution containing different protein concentrations to 100 µL of Bradford's solution (Coomassie Blue dye dissolved in ethanol and phosphoric acid). This reaction was maintained for 5 minutes at room temperature and absorbance read at λ 595 nm in a visible spectrophotometer (Biospectro sp220). Blank control was prepared only with PBS (pH 7.2).

2.6. First emulsion simulation (W_1/O)

It was simulated the first phase (W_1/O) of double emulsion ($W_1/O/W_2$) by submitting the three proteins solubilized in different excipients: PBS (control), H_20, and salt solutions (NaCl, NaH_2PO_4, $MgCl_2$, KSCN at 50 mM and pH 7.2) representing kosmotropics and chaotropics ions according to the Hofmeister's series [15]. Briefly, two milliliters of each solution containing the different proteins (1.0 mg/mL) were added to eight milliliters of CH_2Cl_2 (used as organic solvent) and this mixture was emulsified at 24000 rpm in an Ultraturrax® for two minutes. Subsequently, the resulting solutions were centrifuged at 5000 rpm for 10 minutes, and the supernatant was removed for protein recovering analysis.

2.7. Structural analysis by SDS-PAGE

Recovered proteins contained in the supernatant were submitted to SDS-PAGE for analyzing possible degradation or aggregation caused by the process. For this purpose, it was used a vertical polyacrylamide slab gel electrophoresis system (Hoefer Mighty Small II SE250/SE260) composed

of resolving gel at concentration of 12.5% and stacking gel at 5% in the presence of sodium dodecyl sulfate (SDS). Especially for insulin, which has a very low molecular weight of ~5.7 kDa, a resolving gel at concentration of 15% was used allowing adequate separation. The wells received 30 µL of each solution containing recovered proteins and electrophoresis was performed using following conditions: 50 mA, 120 V for 60 minutes. Native proteins (i.e. not submitted to the first emulsion process) were also applied as controls of integrity.

2.8. Structural analysis by intrinsic fluorescence intensity

Recovered proteins contained in the supernatant solutions were also analyzed in a PC1ISS spectrofluorimeter using quartz cuvettes with 1 cm optical length. The solutions were excited at λ 280 nm and the subsequent fluorescence emission intensities were measured at λ 300 to 400 nm. To study the relationship between "Fluorescence intensity X structure", casein and albumin, which has amino-acids tryptophan and tyrosine in their composition, were analyzed at 350/330 nm and insulin, which has only tyrosine, was analyzed at 330/300 nm. Native proteins (i.e. not submitted to the first emulsion process) were as control of integrity in fluorescence analysis.

2.9 Statistical Analysis

The results of protein measures are expressed as the mean ± standard deviation and they were statistically analyzed by variance analysis (ANOVA) followed by *Tukey*'s test. Differences statistically significant was considered when $p \leq 0.05$. Protein recovering is expressing in percentage.

3. Results and Discussion

3.1. Methods to quantify proteins

The linear coefficients obtained from three different methodologies to quantify the model proteins used in this study are presented in table 1.

Table 1. Coefficient of correlation (r^2) obtained from calibration curves for quantifying proteins.

Methods ► Proteins▼	λ 280 nm	Lowry	Bradford
Insulin	0.9849	0.9582	0.9675
Albumin	0.9992	0.9995	0.9866
Casein	0.9995	0.9947	0.9349

The direct reading at λ 280 nm was the method that showed higher linearity for the three analyzed proteins. This method is based on the absorbing capacity of the aromatic and hydrophobic amino acids, of which tryptophan and tyrosine are principal contributors at this wavelength. Amongst the three well-known experimental methodologies tested, the direct reading at λ 280 nm showed the highest correlation coefficients (r^2) as described 0.9849, 0.9992 and 0.9995 for insulin, albumin and casein, respectively. Although this method has been described to be subjected to many interferences, it also has been shown as a simple, adequate and rapid method to quantify protein recovered from stressing process as in this case. In addition, it allow the continuing use of the analyzed proteins by preserving them with no reactions with dyes, as occurs with Lowry's and Bradford methods [16-18]. Based on these results, this method was chosen to follow up the percentage of recovered proteins once submitted to the first phase of microencapsulation process.

3.2. Excipient solutions for protecting proteins submitted to W_1/O emulsion

The ability of different excipient solutions to maintain the three model proteins in aqueous phases protecting them from adsorption on organic solvent/water interface formed during microencapsulation process was also evaluated. For this, after centrifugation the percentage of recovered proteins from supernatant solutions or even the protein fractions that remained solubilized once submitted to the first step of the double emulsion of microencapsulation process was determined. The results are showed in the figure 1. In general, it was observed extensive losses of protein solubility after their submission to the microencapsulation process, commonly higher than 50 % of the added proteins. For albumin, KSCN and PBS solutions allowed the highest percentage of recovered proteins, 47% and 45%, respectively. The PBS solution was also the excipient from which it was possible to recovery the highest concentration of insulin and casein, 44% and 90%, respectively.

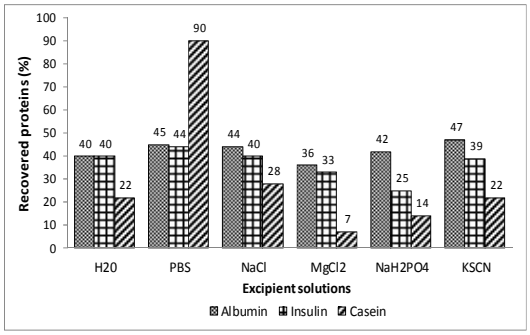

Figure 1. Percentage of proteins recovered after submitting to the first phase of double emulsion microencapsulation process.

The principal importance of determining the adequate excipient solutions for solubilizing proteins during microencapsulation process relies on to increase the protein's preference to the aqueous phase, protecting them from contact with solvent/water interfaces which may lead to deleterious effects [8, 19]. There are complex interactions between ions (i.e. cations and anions present in salts of the Hofmeister's series used along this study) and proteins in aqueous phases preventing macromolecule absorptions to hydrophobic/aqueous interfaces. This explanation is beyond the scope of this work, however this phenomenon may be better understood by reading ZHANG & CREMER (2006) [15]. Nevertheless, the deleterious effects of the first phase of double emulsion on the three model proteins used here was such strong to trigger adsorption of more than 50% of the added macromolecules. On the other hand, the use of proper saline solutions as protein solubilizing excipients, besides protecting them from adsorption on interfaces, also can minimize degradation during acid-dependent protein releasing from PLGA microspheres [20, 21]. At this stage, it is salutary to point out higher percentage of recovered

protein fractions as seen here does not means that all proteins have maintained their structures intact. This situation was further checked by performing protein integrity analysis as follows.

3.2. Structural analysis by SDS-PAGE

It was not observed formations of supramolecular aggregates and/or molecular fragmentation identified by SDS-PAGE (Figure 2). This was confirmed by total absence of other bands in the stained gel showing different molecular weight compared with the native protein (not processed protein used as control). SDS-PAGE is an electrophoretic method from which is possible to evaluate whether the protein underwent degradation or fragmentation throughout the process [22-24]. The results obtained for the three tested proteins are showed in the figure 2. For albumin and insulin, well-resolved bands were observed in stained gel (figure 2A and 2B), and for casein (figure 2C), three bands were formed representing the three different fractions (α, β and κ) contained in the casein structures.

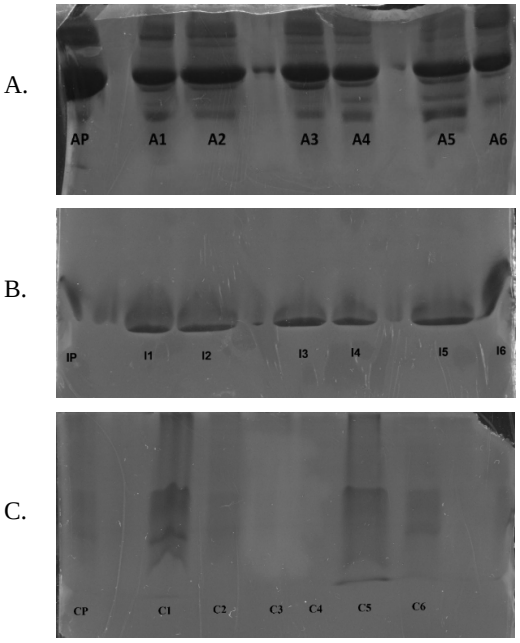

Figure 2. SDS-PAGE of the three proteins used as models. Letters followed by a numbers were used to identify each sample: A. Albumin; B. Insulin; C. Casein. Lane P=Native protein; 1=H_2O; 2=PBS; 3=NaCl; 4=$MgCl_2$; 5=NaH_2PO_4; 6=KSCN. *There was a low resolution of bands on lanes C3 and C4.

3.3. Structural analysis by intrinsic fluorescence

The ratio between fluorescence intensities measured at λ 350 nm/330 nm and 330nm/300 nm corresponds to the degree of tryptophan (W) and tyrosine (Y) exposition to the medium, respectively [25]. By comparing processed and native proteins according to intrinsic fluorescence intensities, it is possible to deduce the degree of exposure of hydrophobic amino acids (W and Y) moving from its inner region to outer surfaces, revealing even slight protein denaturation or conformational changes [25]. In this study, it was seen that insulin, which has I330 nm/I300 nm value of 0.37 for its native form, was more resistant to the stress caused by microencapsulation process, as it presented its conformation or fluorescence ratios similar or even less than native protein. Albumin and casein showed to be more susceptible to expositions of their hydrophobic amino acids (W) once processed under high rotation as shown in the figure 3. It also has been possible to observe the protein sizes seem to have some correlations with their susceptibility to denaturation or chain disarrangement leading to exposition of their hydrophobic amino acid residues. That affirmation is based on mean values obtained from differences among fluorescence intensity ratio of native and processed protein. For instance, mean differences of 0.04, 0.09 and 0.08 were obtained for insulin (mw ~5.7 kDa), albumin (mw ~65 kDa) and casein (~90 kDa), respectively. Moreover, based on similar analysis, the chaotropic salts $NaCl_2$ and KSCN solutions were the best excipients for insulin (lowest fluorescence intensity difference: 0.02); PBS and kosmotropic salt NaH_2PO_4 solutions were more adequate for albumin (lowest fluorescence intensity differences: 0.01 and 0.02 for each solution, respectively) and NaCl was more suitable for casein (fluorescence intensity difference: 0.02).

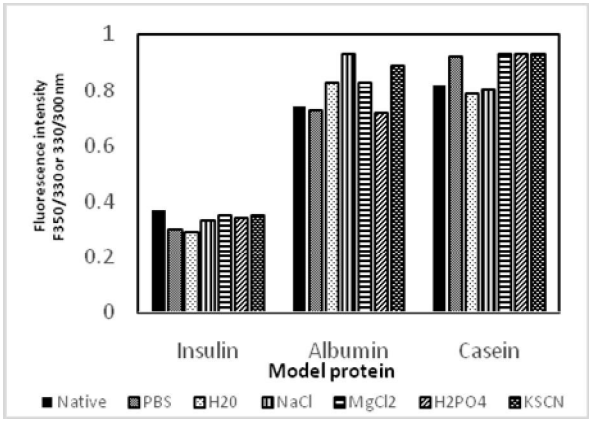

Figure 3. Analysis of protein structures by intrinsic fluorescence intensity. For albumin and casein the fluorescence intensity ratio was measured at λ 350/330nm and for insulin was at λ 330/300nm.

Again, interactions between ion solutions and macromolecules such proteins or peptides,which have many possibilities to form hydrogen bonds, are not trivial to explain, many other factors must be considered mainly when they are submitted to physical chemical forces as occurs during microencapsulation process or agitation at high rotations. Trindade et al (2012) [8] has found similar results with $MgCl_2$ showing to be best saline solution for solubilizing bee venom proteins before microencapsulation process without loss of biological or/and immunological recognizing. Corroborating the results found in this study, Namur et al (2007) [19] have also showed KSCN solution was most adequate excipient for encapsulating diphtheria toxoid (Dtxd) within PLGA microspheres.

Summarizing, it is important to emphasize the first thing that must be kept in mind when proteins or peptides microencapsulation is intended for therapeutic applications,

it is how to maintain the purity and integrity of these macromolecules. Even slight disturbances on their subunit chains, such as amino acids present in proteins may cause a loss of biological activity, and consequently the therapeutic objective may not be achieved. This ultimate purpose can be reached with simple solution as by varying the solubilizing excipient according to the type of macromolecules to guarantee minimal changes in their structures along of processing. Assays of protein recovery in terms of percentage, SDS-PAGE, and fluorescence spectrophotometry are rapid biochemical and biophysical methods that can be used to follow up protein changes.

4. Conclusion

Taken all results together, it was possible to conclude that direct reading at λ 280 nm is one of the most suitable methods to rapid quantify recovered proteins after their exposure to first phase of the double emulsion methodology commonly used for microencapsulation process. Electrophoresis gel pattern did not revealed degradation or fragmentation of the three studied proteins showing that size has no influence on their susceptibility. The best excipient maintaining conformational protein integrities during their contact with organic solvent/water interface was those based on phosphate salt as PBS or NaH_2PO_4 solution, but other salt solutions may also be used, for instance, KSCN and $MgCl_2$ for insulin and NaCl for casein, which have shown similar results. Therefore, by use of simple experiments, it is feasible to select better conditions for preventing conformational changes in the target protein structures, further avoiding loss of biological functions, maintaining their therapeutic efficacy.

Acknowledgments

Authors would like to thank to Federal University of Piauí (UFPI) for material and analytical supports; CNPq and FAPEPI for financial supports through approved projects (Universal CNPq - Proc. nº 474144/2012-7) and (FAPEPI-PPP - Proc. n°022/2012). UFPI, CNPq and CAPES by granting Scientific Initiation Scholarships (PIBIC) to the students: A.C.M. Lima-Filho; M.B. Reis; L.R. Carvalho; and P.H.H. Veloso-Júnior.

Conflict of interest

The authors have declared no conflict of interests in this study.

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