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DESIGN AND EVALUATION OF CHITOSAN-BASED MICROPARTICLES AS MODELS OF PROTEIN DELIVERY SYSTEMS

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ABSTRACT: Controlled release systems for protein and peptide vaccines based polymers have been considered for many applications in medical therapies. Chitosan is non-toxic and easily absorbed biopolymers and is often used in many researches to sustained release drugs and proteic vaccines. This study investigates the kinetics of release *in vitro* of bovine serum albumin (BSA) from chitosan microparticles and the effect of the preparation process of the microparticles in the release rate. Chitosan microparticles were design by a coacervation / precipitation method under different time's sonication and different protein / chitosan ratio were used (0, 5 to 2 g. g^{-1}). To the *in vitro* study of the protein release, the microparticles were resuspended in PBS pH 7.4 at 37 °C under stirring and the samples in different intervals were aliquoted to dosage of BSA release. The morphology of the chitosan microparticles was examined with Electron Microscopy, which showed particle size between 10 - 30 µm and porous structures. Studies were conducted to investigate the BSA stability during the process of BSA loaded, no BSA degradation, through electrophoresis in polyacrylamide gel, were observed the microparticles prepared with 20 minutes of sonication had a higher speed BSA release, in relation to the other sonication times, being 0.86 $\mu g/mL^{-1}/ min^{-1}$, with BSA/chitosan ratio of 1, 5g. g^{-1}. These data showed that the preparation process is an important factor in controlling release of protein from chitosan microparticles, and this can become a potential for carrier vaccines in mucosal delivery systems.

INTRODUCTION: Currently there is a growing interest in the development of therapeutic vaccines in the field of cancer, autoimmune diseases and infections therapy's [1, 2].

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To date, vaccines are primarily administered parenterally, which limits the variety of dosage forms of sterile injectable liquids [3].

These drawbacks give rise to the need for new formulations of vaccine preparation that are easy and safe to administer. Accordingly, particular interest has been given to the use of delivery systems such as microparticle for the carried out and protein, allow controlled release and additionally can be functionalized on the surface to increase uptake and immune recognition [3, 4].

This technology is one of the current trends in the pharmaceutical area, with impact on the field of vaccine formulation. Most pathogens invade the body through the mucosae, being the mucosal immune system is the first line of defense against infections [5, 6]. Unfortunately, injectable vaccination cannot immunological protect the mucosal [7]. Needleless vaccination offers immense potential in terms of patient compliance and easy administration. The importance of the mucosal immune system and its potential for improving global health has inspired investigations, demonstrating that mucosal vaccines could be better alternative than the parental route [8, 9]. The current state of the art, micro / nanoparticle manufacturing technology is one of the options for achieving better antigen stability at different pH, and the possibility of immunological tolerance when using high doses of antigen [10, 11].

Compared with traditional delivery routes, such as oral and nasal vaccine delivery systems offer several attractive advantages, such as lower cost, ease of administration, increased patient compliance, reducing the need for personnel trained, as well as greater capacity for mass immunizations [12]. Recently, microencapsulation of vaccines, as in ovalbumin loaded liposomes, is receiving attention due to their ability to co-encapsulate multiple antigens, satisfactory immune responses and stable enough to be administered orally or nasally [3, 13].

Microparticulate carriers offer several attributes for use as protein antigens delivery systems; these include protection of antigens encapsulated in microparticles from extracellular enzymatic degradation and controlled release, allowing a possible single-injection vaccine formulation [14]. Among these strategies, microparticles made of biodegradable natural polymer have gained considerable interest in the past decades [6, 15, 16].

Chitosan, a natural cationic polysaccharide has showed to be suitable for drug and vaccine delivery, being extensively used as a carrier because of its stability, bioadhesive property, biodegradability, non-toxicity and enhanced penetration capacity across the mucosal barriers, besides has soft tissue compatibility. This polymer is a valuable excipient for delivers therapeutic of peptide, protein, antigen, oligonucleotide and gene [2, 17]. Chitosan-based microparticles, as drug delivery systems for proteins or peptides have been extensively investigated for decades, it's find application in a wide range of biomedical uses and was proposed for preparing protein stabilizing matrixes [18], promotes opening of tight junctions, has strong mucoadhesive properties [15, 19] and has been used as mucosal immunoadjuvant and immunopromoter [9, 20]. The positively charged amino groups on chitosan interact with the negatively charged cell surface, facilitating paracellular penetration of hydrophilic macro-molecules due to tight junction opening.

In this study chitosan was used because of the non-toxic features and antigen binding properties. Our goal was investigated the kinetics of release *in vitro* of bovine serum albumin (BSA), as model protein, from chitosan microparticles and the effect of the preparation process of the microparticles, in the release rate, ensuring that a protein in the end of the process could be intact, as a preliminary step for future application to mucosal delivery of protein vaccine.

MATERIAL AND METHODS: Chitosan (MW 80.000, degree of deacetylation 80%), bovine serum albumin and Polyacrylamide were obtained from Sigma Chemical Company Ltd., Tween® 80; sodium sulfate and other reagents were of analytical grade and used as received.

Preparation and Characterization of Chitosan Microparticles: The microparticles were prepared as described in Van Der Lubben *et al*., [21] with some modification. A 0.25% (w/v) chitosan solution was prepared in a mixture of 2% (v/v) acetic acid and 1% (w/v) Tween® 80 in MiliQ water. Then 2 ml of 10% (w/v) sodium sulfate was added dropwise (about 1 ml/min) to 200 ml chitosan solution under magnetic stirring and continuous sonication 600 W (5, 10 and 20 min) in ice. In the process, of ultrasonic pulses causes the dispersion of the microparticles while they are being formed. The microparticles suspension was subsequently centrifuged for 15 min (10.000 rpm). The pellet was resuspended in MiliQ water and this washing procedure was repeated twice before freeze-drying of microparticles overnight.

Initial visualization by Optical microscopy (OM) was performed for of chitosan microparticles, at different times of sonication, analyzing parameters such as approximate size, shape and uniformity. Through scanning electron microscopy (SEM), the chitosan microparticles were studied, analyzing the surface and porosity of the microparticles. For analysis, lyophilized chitosan microparticles were adhered to double-sided tape and sputter coated with 5 nm gold in a preparation chamber. Then the sputtered chitosan microparticles were observed with a JEOL 200T field emission SEM.

Determination of BSA Encapsulation Efficiency: The BSA loading of microparticles was performed by incubating 1% (w/v) chitosan microparticles and different concentrations, 0.5 - 2% (w/v) of BSA in PBS pH 7.3 under shaking (100 rpm) at 25 °C for 180 min. After incubation, the suspension was centrifuged (10.000 rpm for 10 min) then washed three times with MilliQ water and centrifugation at 10.000 rpm for 5 min to remove the unloaded BSA in surface of microparticles.

The loading efficiency was determined by quantifying the non-bound ovalbumin in the first supernatant with the protein assay method [22]. Therefore, loading capacity (LC) and loading efficacy (LE) were determined:

LC" [(total amount ovalbumin) - (free ovalbumin)] / weight microparticles,

LE" [(total amount ovalbumin) - (free ovalbumin)] / total ovalbumin.

BSA Release from Microparticles *in vitro*: The BSA release from chitosan microparticles was determined in PBS (pH 7.3). The 1% (w/v) chitosan microparticle suspension containing 0.5 - 2% (w/v) of BSA were incubated at 37 °C under shaking (100 rpm). After 10, 20, 40, 60, 80 and 100 min, the tubes were given a spin-off and samples of 500 µL were taken and centrifuged at 10.000 rpm for 10 min, the same volumes were replaced by PBS (pH 7.3). The released BSA in PBS was determined with the protein method [22], using a spectrophotometer HITACHI U-3200 at 610 nm.

Degradation of BSA - loaded Chitosan Microparticles: To confirm if the BSA loaded microparticles, prepared with 20 min sonication still contained BSA after release studies, the chitosan microparticles were dissolved. This was done by incubating BSA without microparticles, non-loaded microparticles and BSA loaded microparticles in acetic acid at 20% (w/v) pH 2.0. Thereafter, the degraded microparticles were separated from the digestion solution by centrifuging (1400 rpm for 20 min). The BSA in the supernatant was detected with protein method [22], using a spectrophotometer HITACHI U-3200 at 610 nm.

Gel Electrophoresis of Released BSA: Released BSA from loaded microparticles were analyzed by gel electrophoresis to determine possible protein degradation by loading and release processes. As controls, both non-loaded microparticles and free BSA was used. The forced release was also applied, through the loading the microparticles in H_2O MiliQ water with BSA and subsequently performing release studies in PBS (pH 7.3). Were utilized 1% (w / v) of the chitosan microparticles in PBS loaded with BSA at different concentrations (0.5%, 1%, 1.5% and 2%). Approximately 50 µg of protein per lane was applied. Gel electrophoresis was performed on an 8 - 25 gradient gel and run for 87Vh.

RESULTS:

Morphological Characterization of Micro-Particles: Observation by OM were performed with non-loaded chitosan microparticles, they showed a heterogeneity in the form and size, in function of different times of sonications, during the microparticles formulations, where it was observed a variation in size from 10 to 30 µm. Additionally, the chitosan microparticles development with 20 min of sonication time showed smaller size microparticles. The SEM in **Fig. 1**, showed that chitosan microparticles have very porous structures and rough surface, highest sonication time caused most porosity than the microparticles development with 5 and 10 min sonication time, this fact, as well as the size of the microparticles influence the release kinetics of BSA. Therefore, was also confirmed that the higher the sonication time, causes the smaller the size of the microparticles.

Bovine Serum Albumin Loading of Chitosan Microparticles: Both the loading capacity (LC)

and the loading efficacy (LE) were determined for chitosan microparticles prepared with different sonication times and different amounts of BSA 0.5 - 2% (w/v). The **Fig. 2** shows that the LC of chitosan microparticles with 5 min sonication time is not substantially influenced by the amount of the protein solution, 0.5 and 1.0% BSA were approximately 34%, where as 1.5% and 2% BSA, showed a slight increase of its LC, up to 40%, the same was observed with microparticles time sonication of 10 min. However, the microparticles with 20 min of sonication, the LC was about 50%, with 1.5% BSA, not be observed any alteration with 2.0 % BSA. The LE of the chitosan microparticles with 5 min sonication time was higher with 0.5% BSA and inversely proportional to the BSA highest concentrations, same was observed with 10 min time sonication. The LE percentages obtained with 0.5 % BSA were of 68, 73 e 81%, using chitosan microparticles with 5, 10 and 20 min sonications times, respectively. In general, the use of highest concentration of BSA as 1 to 2.0% to load microparticles formulated in 20 min sonication time, resulted in highest LE.

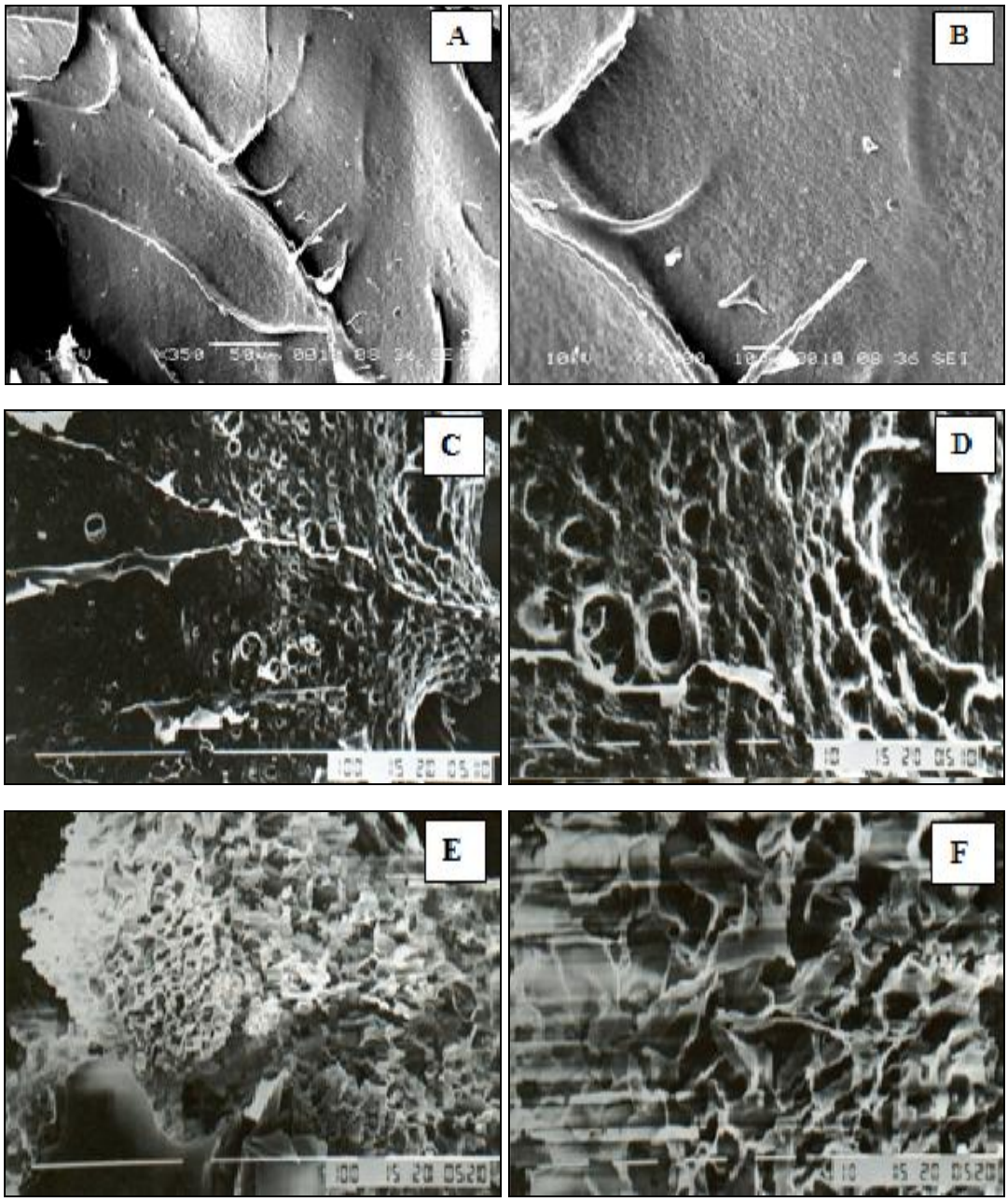

FIG. 1: SCANNING ELECTRON PHOTOMICROGRAPHS OF CHITOSAN MICROPARTICLES IN DIFFERENT TIME SONICATIONS, DETAIL OF THE SURFACE (A) MAGNIFICATION 350X; (B) 1000X (5 MINUTES); (C) 350X; (D) 1000X (10 MINUTES); (E) 350X AND (F) 1000X (20 MINUTES)

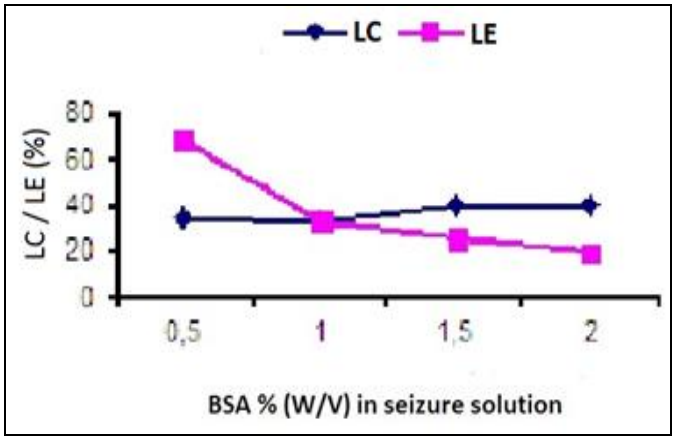

FIG. 2: DIFFERENT CONCENTRATIONS OF BSA LOADED BY CHITOSAN MICROPARTICLES 5 MIN SONICATIONS ACCORDING TO LC AND LE

***In vitro* Release of BSA from Chitosan Microparticles:** The *in vitro* release of BSA from chitosan microparticles were performed at neutral pH over a time span of 100 min, with chitosan microparticles formulated with 5 to 20 min of sonication time and loaded with different concentration of BSA to observe the influence of these conditions on the protein release. In the **Fig. 3** the protein release from microparticles formulated with 5 min of time sonication. In general, there was a low rate on BSA release and more noticeable with microparticles loaded with 0.5% BSA and that from 40 min happened a stabilization of release.

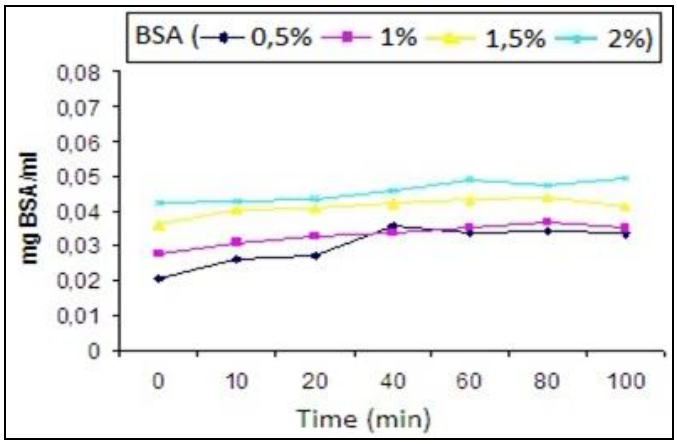

FIG. 3: BSA RELEASE FROM CHITOSAN MICRO-PARTICLES 5 MIN SONICATIONS TIME WITH GROWING AMOUNT OF BSA IMMOBILIZED

In **Fig. 4**, with microparticles formulated with a 10 min of sonication time, a higher release rate was observed in the BSA than that observed with 5 min **Fig. 3**, thus more pronounced with 0.5 and 1% of BSA. Therefore, with microparticles developed with 20 min sonication time, differently the other time sonication conditions, when was used the higher concentrations of BSA (1.5 and 2.0%) resulted in the highest rate of BSA release and we can be observed a stabilization in the BSA release with 60 min of assay in all the concentrations tested **Fig. 5**.

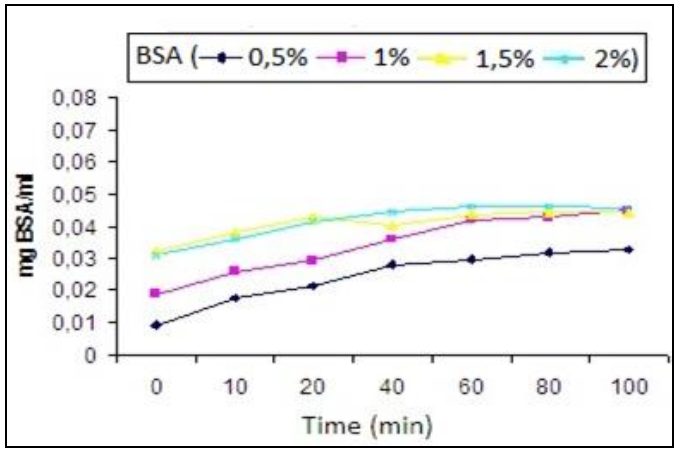

FIG. 4: BSA RELEASE FROM CHITOSAN MICRO-PARTICLES 10 MIN SONICATIONS TIME WITH GROWING AMOUNT OF BSA IMMOBILIZED

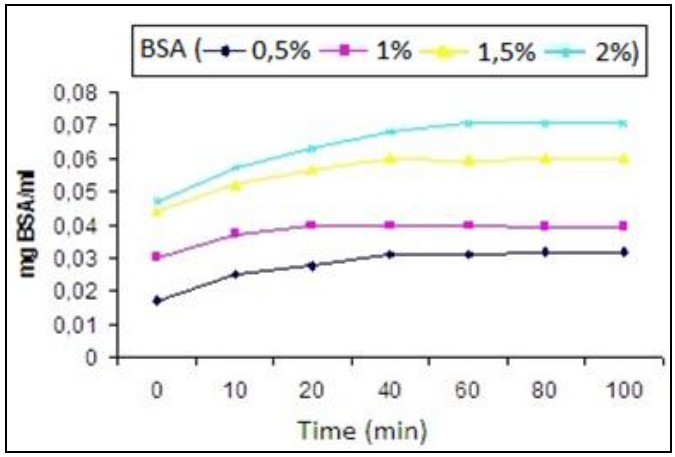

FIG. 5: BSA RELEASE FROM CHITOSAN MICRO-PARTICLES 20 MIN SONICATIONS TIME WITH GROWING AMOUNT OF BSA IMMOBILIZED

In the kinetics studies of BSA release **Fig. 6** we can observe the influence of different conditions assayed to microparticles formulations. In the microparticles with 20 min time sonication, all concentrations of BSA used for load the microparticles, except 1.0% showed the highest release speed. However, in the 5 and 10 min time sonication's, the BSA release rate were larger when was used 0.5% BSA to loaded the microparticles, and inversely proportional to the BSA highest concentrations used. Being that this reduction of rate release was greater when used microparticles with 5 min time sonication.

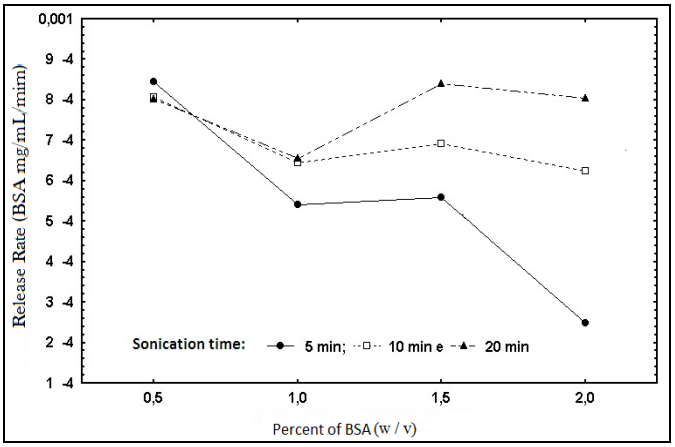

FIG. 6: INFLUENCE OF BSA CONCENTRATIONS AND SONICATION TIME OF THE PREPARATION OF CHITOSAN MICROPARTICLES ON THE RELEASE RATE OF THE PROTEIN AS A FUNCTION OF TIME

To verify if the BSA was still present in the chitosan microparticles after the loaded process and subsequent release studies, the chitosan micro-particles were dissolved. After 2 hours of incubation in acetic acid and pH 2, the chitosan microparticles were completely disintegrated and still about 33% BSA was determined in the suspension.

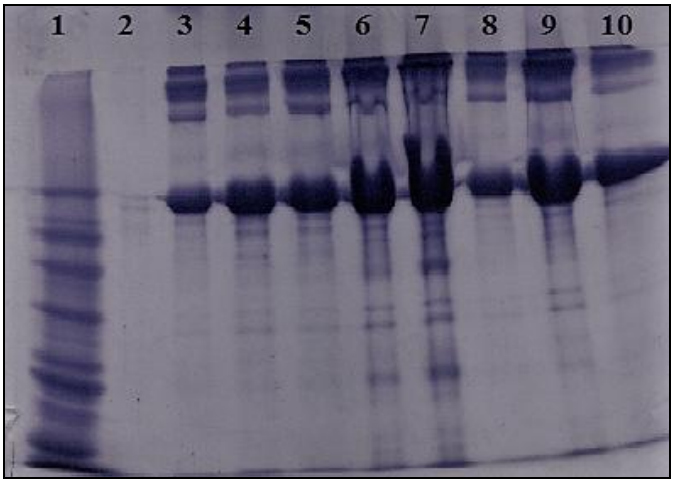

FIG. 7: GEL ELECTROPHORESIS FOR THE RELEASE OF BOVINE SERUM ALBUMIN:
1- Molecular Weight Labeled, 2- Empty Chitosan Microparticles, 3 - 0.5% BSA Encapsulated and Released In Pbs; 4 - 0.5% BSA Encapsulated in Ultrapure H_2O and Liberated in Pbs, 5 – 0.5% BSA Encapsulated in Pbs and Released in Ultrapure H_2O, 6 - 1% BSA in Pbs, 7- 1% BSA in Ultrapure H_2O, 8- Encapsulated 1.5% BSA and Released in Pbs, 9 - 1.5% BSA Encapsulated in Ultrapure H_2O and Released in Pbs, 10 - 1.5% BSA Encapsulated in Pbs and Released into Ultrapure H_2O.

Characterization of BSA Released from Chitosan Microparticles: To determine possible protein degradation during the encapsulation and release process, the polyacrylamide electrophoresis gel **Fig. 7** was performed to characterize the BSA released by microparticles that were loaded at different concentrations, assayed in PBS and MiliQ water medium. No difference could be observed between BSA released from the microparticles and BSA free and no protein was observed in line with empty microparticles. Since these results did not show any differences in molecular weight, it is assumed that no substantial degradation of BSA occurred during loading and release processes.

DISCUSSION: Mucosal vaccination is very attractive because offers several advantages over the parenteral strategy, such as no requirement for specialized medical personnel for the vaccine administration, the higher patient compliance and the ability to induce both systemic and mucosal immune responses [13]. This can be modulated by the morphological and physicochemical characteristics of microparticles, as size and porosity of the particle loaded with the immunologic agent [24, 25].

Van der Lubben *et al*., [21], studying the morphology of the chitosan microparticles formulated under sonication time of 20 min, observed a reasonable uniformity in size and morphology and the field emission (SEM) showed that the microparticles have porous structures and rough surface, similar results were founded here. Here we verify the influence of time sonication on chitosan microparticles formation and in our studies the chitosan microparticles also found results similar those of Van der Lubben on the sonicated particles for 20 min, which were observed to have smaller sizes and highest porosities than the sonicated particles for 5 and 10 min.

Microparticles with appropriate morphological characteristics, such as size and shape, and good encapsulation efficiency of protein for mucosal delivery purposes were obtained with addition of sodium deoxycholate and in the absence of surfactant [18]. Moura *et al*., [26] formulated chitosan microparticles crosslinked with genipin observed that the increase of biopolymer concentration and cross-linker agent concentrations provokes an increase in microparticles size, and the increase of the stirring rate has the opposite effect.

The control of size and size distribution of particles are necessary for obtaining reproducible controlled release behavior. Therefore, the preparation of uniform particles and optimal mean particle size are important for drug controlled release [27, 28]. XU, Y. *et al*., [18] observed that the degree of deacetylation and the size of the chitosan microparticles have influence on the BSA release rate. On the other hand, Cui - Yun Yu *et al*., [27], worked with drug delivery systems based on chitosan, alginate and pectin as pH sensitivity microparticles for oral delivery of protein drugs, the *in vitro* drug release study showed that the microparticles have high pH sensitivity, releases of BSA at acid pH are slow, while release at pH 7.4 is much faster [29].

In the release profiles, we observed that the release of BSA from chitosan microparticles at different sonication times and BSA concentrations in almost all tested conditions resulted in a burst effect in the early internships of the release. This suggests that proteins when loaded at higher concentrations, some of them remain poorly associated on the surface of the microparticles. Considering the pKa of chitosan is 6.5, and that the BSA under physiological conditions is a negatively charged protein, it can interact electrostatically with amino groups in chitosan more easily [18]. However, in pH 7.4, the initial bursting release would be due to less ionic interaction between the chitosan surface and BSA, this interaction could be weakened by deionization of the amine groups, leading to protein diffusion.

According Van Der Lubben *et al*., [21] through studies to determine both the loading capacities (LC) as the loading efficiency (LE) of chitosan microparticles, prepared similarly this work, with 20 min sonication time, obtained a LC around 33% for different BSA concentrations and concluded that the more ovalbumin is offered to the microparticles, the more BSA remains unbound during the loading process. Therefore, under same time conditions, with 0.5% BSA the LE of the chitosan microparticles were up to 85%, indicating that only a small amount of ovalbumin was lost during the loading process. Walke *et al*., [9, 29], showed that the protein release *in vitro*, in neutral pH with physiological saline, revealed that the cumulative release of monovalent tetanus toxoid from the microspheres was approximately up to 78%, which was started with a slight initial burst effect.

There was a direct relationship between the amount of BSA release and sonication time, when the sonication time was higher and more amounts of BSA used to load the microparticles, resulted the higher release rate. Therefore, with 20 min sonication, the LE of BSA loaded were highest using a concentration of BSA at 1.5 and 2.0% and a LC observed in these concentrations was bigger than with 5 and 10 min of sonication, which may be justified due to the higher porosity of these microparticles. XU Y *et al*., [18], worked with chitosan nanoparticles observed that increasing the BSA concentration of 0.2 - 2 mg/ml and decreasing the concentration of the chitosan nanoparticles 3 - 1 mg/ml there was a significant increase in loaded capacity of nanoparticles to BSA and observed that the degree of deacetylation and the size of the chitosan nanoparticles have a greater influence on the loaded capacity.

The addition of dichloroacetic acid to a chitosan solution was highly effective in the formation of microparticles with good protein encapsulation efficiency and appropriate morphological characteristics [11]. Another aspect was observed by the use of dual cross linkers using vanillin / TPP as a non-toxic co-cross linkers to prepare chitosan microspheres, which have good percentage of encapsulation efficiency, found to be 80% for monovalent tetanus toxoid, 76% for diphtheria toxoids and 79% for BSA [29].

L. Illum *et al*., [17] found that chitosan-based influenza antigen produced much higher antibody level than other polymer-based antigen system. Modulation of chitosan microparticles formulation can greatly improve and prolonger of stay in active form of drug and protein, on tissues and cells for release the drug sustainably there, as a result, the bio-availability of drug can be improved and the administration frequency of drug can be reduced, as a vaccine delivery system, chitosan can stimulate immunity system and works as an adjuvant.

According to reported by Van Der Lubben *et al*., [21] chitosan microparticles, elaborated with same methodology used in this work, presented good

environment to protein encapsulation. They confirmed, BSA stability, during the loading and release processes, confirmed by electrophoretic studies, that none degradation of this protein occurred. In this work, BSA releases from load microparticles were analyzed by gel electrophoresis polyacrylamide to investigate the possibility of degradation of BSA. We can observed that no protein degradation occurred during the loading and release processes, which is shown to be very important for the vaccine delivery systems. Similarly to the study by Kusonwiriyawong *et al*., [14], where integrity of BSA was observed in all bands in agarose gel after encapsulated process and releasing from the chitosan nanoparticles.

Ulker Guliyeva *et al*., [20] used chitosan microparticles as a gene carrier for oral administration, formulated by complex coacervation process and the stability of plasmid DNA was investigated. It showed that these microparticles can protect the encapsulated plasmid DNA, from nuclease degradation. On the other hand, the integrity of BSA was confirmed by Lameiro *et al*., [11] along the encapsulation process and the analysis showed that in all stages of protein micro-encapsulation remains intact.

CONCLUSION: The polymeric microparticles, intended for delivery of proteins, were successfully prepared. The chitosan microparticles with 5 min time sonication showed a minimum rate of BSA release, than other times assayed. In this way, the microparticles with 20 min time sonication got smaller size, highest porosity and thus a sustained release profile with increased availability of the protein.

These data showed that the preparation process is an important factor in controlling release of protein from chitosan microparticles, and this can become a potential for carrier vaccines in mucosal delivery systems.

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CONFLICT OF INTEREST: Nil

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