PREPARATION AND CHARACTERIZATION OF LIPOSOMES ENTRAPPING ALLERGENIC PROTEINS

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Abstract - This work presents results of the preparation and characterization of small unilamellar liposomes for entrapping allergenic proteins extracted from the biomass of *Dreschlera (Helminthosporium) monoceras* cultivated by solid fermentation. Protein was entrapped by the dehydration-rehydration method, using lyophilization of preformed liposomes in order to prevent their degradation The reconstitution of lyophilized liposomes by hydration, their capacity for entrapping allergenic proteins and their stability in plasma were analyzed. Liposomes were reconstituted in size by including trehalose sugar in the formulation. The protection of the liposomal membrane by trehalose was characterized by differential scanning calorimetry and X-ray diffraction. The reconstituted membrane had an osmotic behavior similar to that of nondehydrated ones. Allergenic proteins ranging in molecular weight from 14 to 170 kDa were entrapped in the lipid matrix with an efficiency of approximately 80%. These results are promising for producing liposomes by entrapping allergenic proteins from mold extracts, which can be useful for allergy therapy.
Keywords: liposomes, trehalose, allergenic proteins, allergy.

INTRODUCTION

Respiratory allergic disorders affect a great number of individuals throughout the world. Conventional immunotherapy uses free allergens which usually produce adverse systemic reactions of varying intensity and frequency. In order to circumvent these problems, some new strategies have been proposed, such as to produce a greater tolerance in patients by the slow release of allergens, reducing the frequency of injection. Liposomes are lipid vesicles used to encapsulate drugs and to act as adjuvants in vaccines. The capacity of these vehicles for controlled delivery of their contents can be used to produce formulations useful for allergy therapy by desensitization.

Allergens are mainly foreign proteins or glycoproteins with a molecular mass usually ranging between 5,000 and 170,000 Da. Spores and mycelium of molds are considered to be one of the main aeroallergens (Yunginger, 1988). Several species of allergenic molds have been isolated from atmospheric air, characterized and standardized for applications in diagnosis and immunotherapy. Previous studies showed that proteins extracted from the biomass of *Dreschlera (Helminthosporium) monoceras* were highly positive in cutaneous allergic reactions. This is one of the most frequent species found in several areas of Brazil, including the state of São Paulo. (Gambale et al., 1983; Mohovic et. al., 1988).

Desensitization immunotherapy has become the mainstay of allergy treatment in many places. It involves a serie of injections of the allergens to which the patient is sensitive, until reaching a maintenance level at which symptoms are effectively controlled. However, using free allergen in its conventional form, the risk of anaphylactic shock following allergen administration remains a serious drawback and has restricted the use of this potentially valuable procedure (Walls, 1992). In an attempt to increase the safety and effectiveness of desensitization treatments, new forms of presentation of allergens have been sought. The two major approaches to producing greater tolerance in patients are to reduce the frequency of allergen injection by slow release of the allergen and to reduce the allergenicity of the proteins while retaining their immunogenicity (Arora and Gangal., 1990).

Liposomes mimic cells and are used for encapsulation and sustained release of drugs in modern therapies. They are colloidal aggregates containing a continuous bilayer of phospholipids around an aqueous space. There has been much interest in liposomes as a drug carrier, not only because a wide range of biologically active substances can be encapsulated, but also because they can be injected into man or animals without adverse effects. Incorporation into liposomes can reduce the toxicity of a number of drugs, reduce general anaphylactic reactions and produce sustained release (Genin et al., 1994). In spite of the scarcity of data in the literature about allergenic responses and IgG and IgE titer, some studies on liposome-associated allergens have already yielded promising results. Suppression of IgE formation and an increase in IgG titer were verified for mite allergens entrapped in liposomes (Stewart et al., 1988). A more complete set of data on IgE and IgG after repeated injections in mice of allergenic proteins from *Artemisia scoparia* pollen entrapped in multilamellar liposomes were reported by Arora and Gangal (1990).

The preparation and characterization of allergenic extracts containing proteins from biomass of *Dreschlera (Helminthosporium) monoceras* cultured by solid fermentation had been previously studied. The molecular weight of proteins in the extract ranged approximately from 14 to 170 Kda, as determined by SDS-PAGE eletrophoresis. The allergenic extracts were reactive as characterized by *in vivo* cutaneous tests (positive in 40% of the atopic patients) and dot blotting (Hasan et al., 2002).

In this work, we describe the preparation and characterization of liposomes using the dehydration-rehydration process developed by Gregoriadis and Kirby (1984). This method employs mild conditions and is capable of efficient entrapment of a wide range of macromolecules, such as proteins. It uses lyophilization and is scalable in both steps of dehydration-rehydration. Small unilamellar conventional liposomes were produced using commercial phospholipid. The feasibility of producing a liposomal formulation for entrapping proteins from allergenic extracts of *Dreschlera (Helminthosporium) monoceras* molds and the potentiality for applications in allergy therapy by desensitization were evaluated.

MATERIALS AND METHODS

Chemicals

Soy phosphatidylcholine (Epikuron 200SH, PC) was obtained from Lucas Meyer. Cholesterol (Chol) was purchased from Avanti. Trehalose was obtained from Sigma. Reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Pharmacia. Other reagents and organic solvents were analytical grade. Deionized water was used throughout.

Preparation and Characterization of Allergenic Extract

Crude extracts of *Deschlera (Helminthosporium) monoceras* cultured by solid fermentation using wheat bran as substrate were obtained by extraction using deionized water. Allergenic extracts were prepared from the crude extract, precipitating proteins with ethanol and removing polyphenols and salts by dialysis, according to procedures developed by Hasan et al.,(2002) e Saraiva (2001). The molecular weight of proteins in allergenic extracts was determined by electrophoresis SDS-PAGE. The isoelectric point of proteins was determined by isoelectric focusing analysis (IEF), using a Phastsystem (Pharmacia), according to the manufacturer's preparation protocol. Phastgel 3-9 and IEF 3-10 markers were used. SDS-PAGE was done on 15 and 10% polyacrylamide with a Multiphor II (Pharmacia) at 500V and 10ºC for 45 minutes.

Preparation of Liposomes

Liposomes were prepared by the dehydration-rehydration process as described by New (1990) and

Gregoriadis and Kirby (1984). Briefly, a lipid mixture (PC:Chol 8:1 w/w) was dissolved in chloroform:methanol (9:1 v/v) and dried to a film in a round-bottom flask using a rotatory evaporator at 55ºC. Aqueous solution containing trehalose at a total lipid:trehalose 1:4 mass ratio was added to the film. Liposomes prepared at various concentrations of phospholipid (3.7 to 22.5 mg/mL) were sized by multiple extrusion through two stacked polycarbonate membranes (pore sizes 100nm) with a high-pressure extruder (Lipex Biomembranes, Vancouver, BC). The preformed liposomes were dehydrated by freeze-drying using a Labconco freezone lyophilizator, model 4.5 at -45ºC during twenty-six hours. Then they were removed, sealed and stored in a refrigerator at 4ºC. Dehydrated liposomes were allowed to reach room temperature and were reconstituted by adding 1/10 original volume of PBS buffer (10mM phosphate, 120mM NaCl, pH 7.4). The dispersion was kept at 55ºC for 1 hour and diluted with a 9/10 PBS buffer volume.

Characterization of Liposomes

The liposomes prepared were characterized by their phospholipid contents by quantification of the total phosphate in the samples, according to the methodology developed by Chen et al. (1956). The morphology of the dried vesicles was characterized by scanning electronic microscopy (LEICA, model LEO 440i) using an acceleration voltage of approximately 5-6KV. The crystallinity of lyophilized particles was evaluated by X-ray diffraction (Philips, model XPERT). The infrared spectra of the dried samples were recorded using a Boomem MB spectrometer (series Hartmann & Braun – Michelson) in order to confirm the interaction between trehalose and phospholipid. The samples were prepared in KBr and the infrared spectra were collected from 4000 to 400nm.

Entrapment of Allergenic Proteins

Allergenic proteins were incorporated during dehydration-rehydration of preformed liposomes. Equal volumes of allergenic extract (1.32mg/mL) and preformed liposomes at various concentrations were mixed and dehydrated by lyophilization. The particles obtained were rehydrated at 55ºC with PBS buffer for liposome reconstitution in a reciprocal shaker at a low agitation velocity during 1 hour. The liposomal dispersion entrapping proteins was diluted with PBS buffer until obtaining the concentration required for use and stored at 4ºC.

Measurement of Osmotically Induced Turbidity

Unilamellar liposomes encapsulating glucose solution (50mM) and 2mM EDTA in 10mM phosphate buffer (pH 7.4) had been prepared previously. A 0.5mL volume of dispersed liposomes was mixed with glucose solution at various concentrations to get a desired concentration gradient across the lipid bilayer membranes. Samples were incubated for 1h and then the turbidity of the mixture was measured at 450nm. Liposomes respond to osmotic gradient as ideal osmometers. Based on this behavior, a linear relation can be derived as follows:

$$(1/A)^{1.5} = \{V_{act}(C_{in}/C_{out}) + V_{dead}\}1/k$$

where A is the absorbance at a given wavelength, k represents a constant, V_{act} and V_{dead} denote the osmotically active and inactive volume of liposomes, respectively, and C_{in}/C_{out} is the ratio of solute concentrations in the inner to those in the outer parts of liposomes (Kim et al., 1999). Experimental data were analyzed by plotting $(1/A)^{1.5}$ and C_{in}/C_{out} in order to verify through the osmotic behavior the reconstitution of the liposomal structure after dehydration and rehydration.

Mean Diameter and Size Distribution

The mean diameter and particle size distribution of hydrated liposomes were determined using a dynamic light scattering system (Malvern 4700, Malvern, UK) at 90º with a He-Ne laser. The results were corrected for effect of viscosity of the medium using 1.348 as the refractive index. The size distribution of dehydrated liposomes (dried particles) as obtained with an optic microscope (Olympus BX60) coupled to the computer with Image Pro-plus software.

Differential Scanning Microcalorimetry

Calorimetric studies were carried out in a VP-DSC (MicroCal Inc) differential scanning calorimeter assisted by an origin data station. Scans in a range of 20 to 110ºC were recorded at a rate of 30ºC/hour. Each scan included a baseline subtraction of a scan made with the reference sample (buffer or water).

Liposomes Stability in Plasma

The stability of liposomes in human plasma was evaluated by incubating 200μL of empty liposomes (3.7mg/mL of total lipid) in 1 mL of plasma at 37ºC. Scans from 280 to 600nm were recorded in a spectrophotometer (Hitachi, model 2001) every 2 hours, during a total time of 28 hours.

RESULTS AND DISCUSSION

Characterization of Proteins in Allergenic Extract

Proteins in the allergenic extract were characterized by their molecular weight and isoelectric point. These are important variables for the entrapping process in liposomes. Figure 1 shows a SDS-PAGE of the proteins produced during 240h fermentation. It can be observed that the total spectrum composed of proteins ranging from 14 to 170 kDa was obtained only after 96 h. The assays for entrapment of proteins in liposomes were carried out using the allergenic extract from 144h of fermentation. Isoelectrofocusing results did not show a well-defined isoelectric point (PI) due to the presence of various proteins in the samples and the interference of other components in the extract. In spite of this, the PI´s of the majority of proteins can be assumed to be in the range of 5.85 to 7.35.

Osmotic Behaviour of Liposomes

Figure 2 (a and b) shows the relation between C_{in}/C_{out} and $(1/A)^{1.5}$ data for hydrated liposomes before and after lyophilization. In both cases a linear relationship could be obtained in the range of a 0.6 to 1.5 glucose concentration ratio, demonstrating that the liposomal membrane behaves as an osmometer and has a barrier function. Under more hypotonic conditions, the plot deviated from the linear relationship since the liposomes were lyzed, the solute leaked, and the liposomal membrane lost its barrier function. Under hypertonic conditions with C_{in}/C_{out} less than 0.6, the liposomes might have shrunk and the absorption date deviated from linearity. The similarity between the linear relationships in a and b graphics shows that the lipid aggregates preserved their membrane properties when liposomes were lyophilized and reconstituted by hydration.

Mean Diameter and Size Distribution

The liposome dispersions had a mean diameter of 140 ± 20nm and a size distribution of 100 to 300nm before lyophylization, The system was monodisperse with a polydispersity index of less than 0.1, as shown in Figure 3 (a). Rehydrated liposomes including trehalose showed a bimodal distribution with a mean diameter for the main population 30-40nm higher than that of the initial hydrated liposomes (Figure 3 (b)). The polydispersity index in this case was between 0.2 and 0.4. The rehydrated liposomes without trehalose were larger than those with trehalose (Figure 3 (c)), indicating fusion and aggregation. The population was heterogeneous with a polydispersity index of 1.0.

These results indicate that trehalose plays an important role in the reconstitution of liposomes. Trehalose is effective in preventing fusion and dehydration damage in phospholipids vesicles. In general, disaccharides are able to provide a fair measure of stabilization of unilamellar liposomes against leakage and fusion during freeze-drying processes. Stabilization occurs due to interaction between OH groups of trehalose and the phosphate group of phospholipids. We recorded infrared spectra of dry liposomes with and without trehalose (data not shown). The most striking changes were in the bands that were assigned to vibrations of the phosphate head group ($1236cm^{-1}$), which disappeared with trehalose. The bands assigned to OH deformations of the trehalose decreased when dispersing liposomes. These results confirm an interaction between the OH of the trehalose and the phosphate head group, probably by hydrogen bonding according to the work of Crowe (1988).

Dried liposomes had mean diameter of 6 ± 1.5μm, as measured by optical microscopy and image analysis.

Differential Scanning Microcalorimetry

We measured the phase transition temperature, Tc, for rehydrated and hydrated liposomes and dried powder particles. Figure 4 shows the thermograms obtained. It can be observed that hydrated and dehydrated liposomes showed similar Tc's: 51.61 and 52.51ºC, respectively. While significant differences in main phase transition temperatures were not observed, the enthalpy change in the rehydrated liposomes was reduced (Figures 4 (a) and (b)). This phenomenon could be attributed to packing differences in the bilayer after lyophylization.

When the liposomes are dehydrated, the packing density of the head groups increases, thereby increasing opportunities for van der Waals interactions between the hydrocarbon chains. As a result, dried liposomes have a higher transition temperature, Tc, and enthalpy changes than hydrated liposomes. However, due to the trehalose in the dried liposomes (lipid:trehalose 1:4 w/w), the results in Figure 4 (c) do not show a large difference in Tc (59. 42ºC) from that in hydrated liposomes. These results indicate that the addition of trehalose to dry liposomes in some respects mimics the addition of water, confirming the interaction between the trehalose and phosphate groups.

From the size and phase transition results, it can be concluded that trehalose protects the liposomal membrane during dehydration, but some fusion still occurs, producing slight differences in the mean diameter, size distribution and phase transition properties.
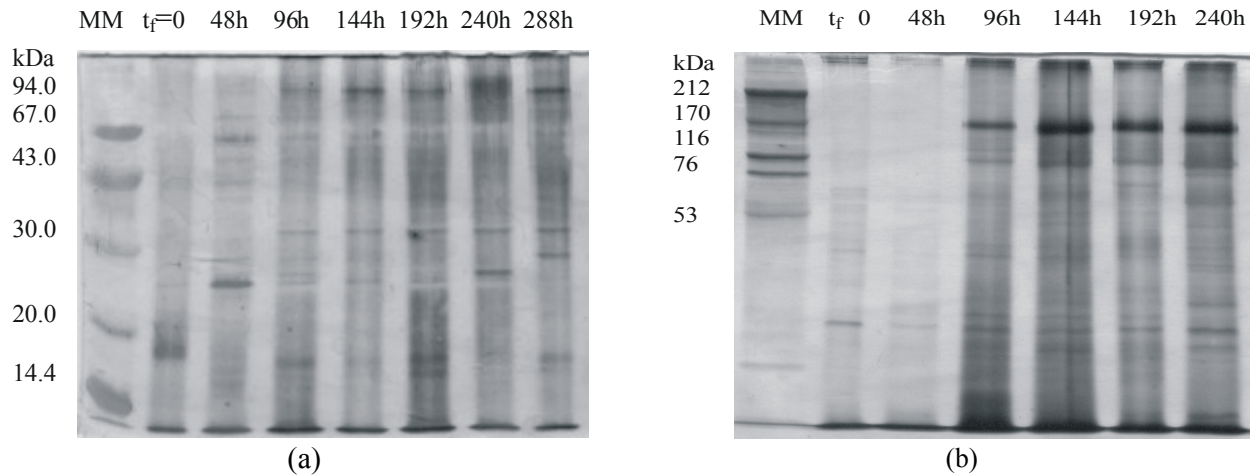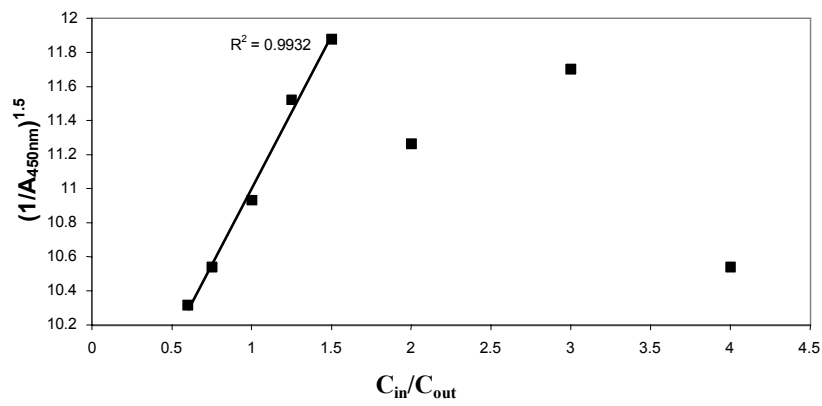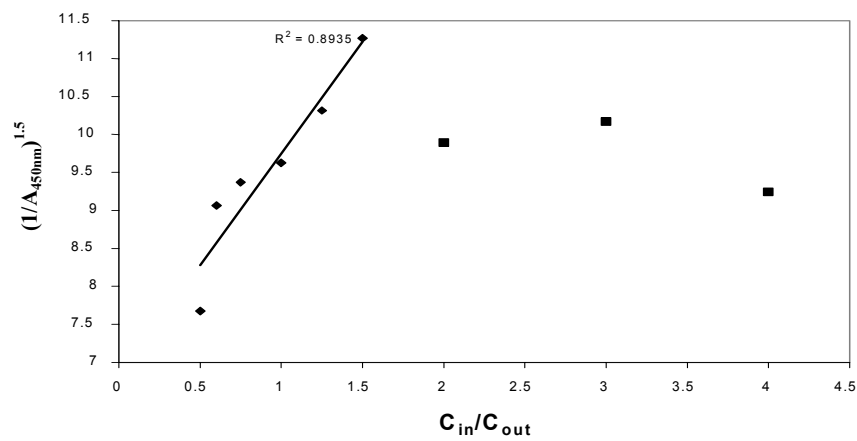
Figure 1: SDS-PAGE electrophoresis of allergenic proteins from *Dreschlera monoceras* (15% polyacrylamide gel), revealed with silver stain; MM: Molecular Marker and t_f = fermentation time (h). (a) proteins: low molecular weight (b) proteins: high molecular weight.

(a)

(b)

Figure 2: Osmotic behavior of liposomes. C_{in}/C_{out} is the ratio of solute concentrations in the inner to those in the outer parts of liposomes. (a) liposomes before lyophilization. (b) liposomes reconstituted by hydration.
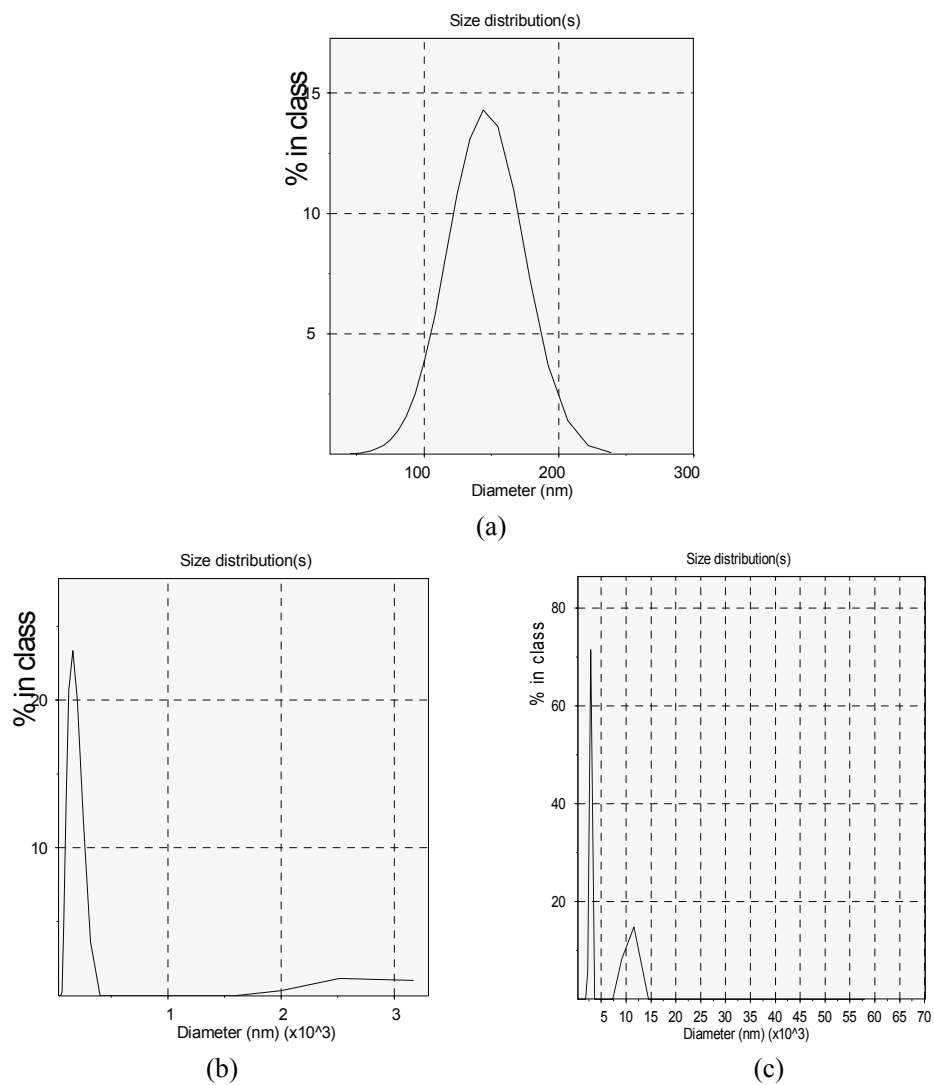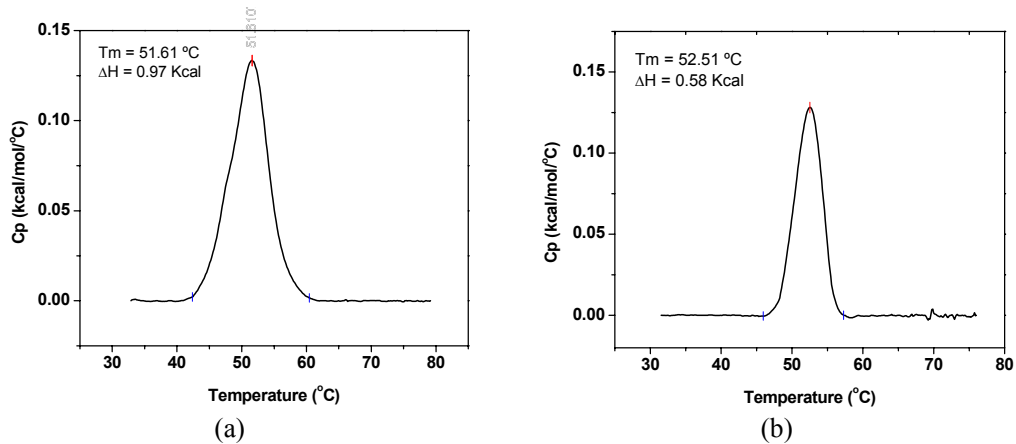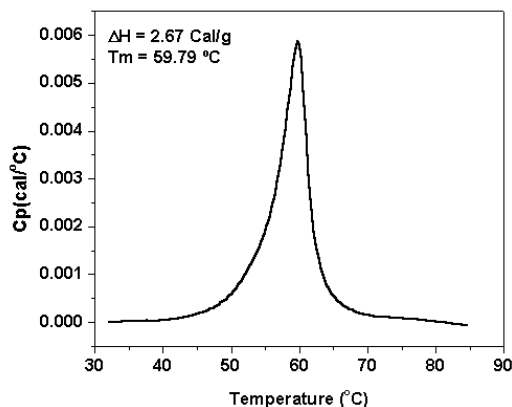
(a)

(b)

(c)

Figure 3: Size distribution of liposomes before and after lyophilization. (a) before and (b) after, with trehalose (lipid:trehalose 1:4) (c) after, without trehalose.

(a)

(b)

(c)

Figure 4: Differential scanning microcalorimetric traces of dehydrated and rehydrated liposomes composed of PC:Chol at a mass ratio of 8:1 including trehalose (lipid:trehalose 1:4 w/w). (a) hydrated liposomes before lyophilization. (b) liposomes reconstituted by hydration. (c) dried liposomes.
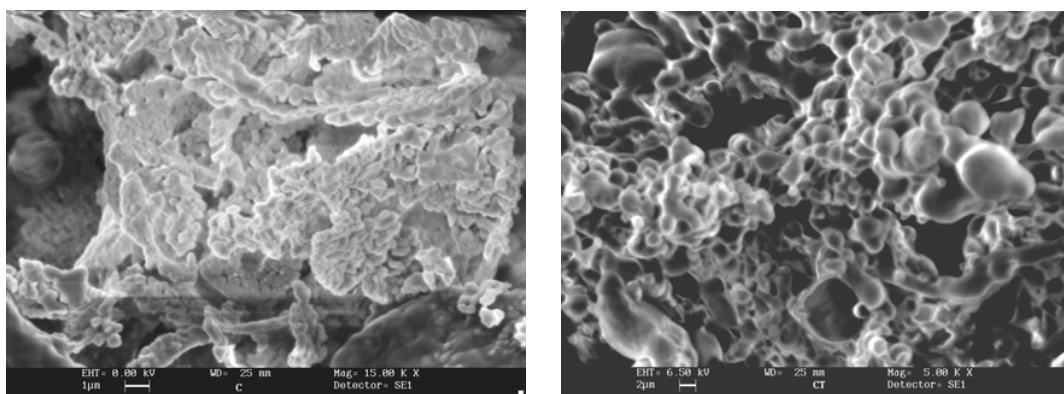
(a)   (b)

Figure 5: Scanning electronic microscopy of dried liposomes (PC:Chol 8:1 w/w).
(a) without trehalose. (b) with trehalose (lipid:trehalose 1:4).

Scanning Electronic Microscopy

Scanning electronic microscopies presented in Figure 5 show the morphology and size of dried liposomes with and without trehalose.

It can be observed that trehalose prevents strong aggregation of powder particles. In the first case, the fluffy aspect of the powder allows visualization of fine spherical structures ranging between approximately 2 to 3μm, and 6μm aggregates, as determined previously by optical microscopy. None of the sizes of aggregated particles are adequate for pulmonary administration of drugs.

Analysis of Crystallinity

The X-ray diffraction patterns for pure trehalose and dried liposomes with or without trehalose in their formulation are shown in Figure 6.

While the diffraction pattern for pure trehalose shows various sharp peaks, diffraction patterns for dried liposomes did not show any peaks in the same region of the spectrum, indicating the amorphous characteristic of dried liposomes. The peaks observed in those liposome diffraction patterns are attributed to NaCl in the buffer. The similar characteristics of the diffraction patterns for liposomes with and without trehalose demonstrate the inclusion of trehalose in the liposomal structure after lyophilization. The amorphous characteristics of lyophilized particles allow easy hydration and play an important role in the rehydration of liposomes and entrapment of hydrophilic drugs.

Storage Stability

To test their stability, liposomal dispersions were stored at 2 to 8ºC. After 5 months, the hydrated preparation did not show visible inhomogeneities (sedimentation). However, rehydrated preparation were stable for 3 months only. During that time, no

significant differences in size distribution and mean diameter occurred.

Plasma Stability

The results obtained on plasma stability when the liposomes were exposed to human plasma and the albumin solutions are shown in Figure 7 (a and b). Comparing the curves for pure albumin and albumin plus liposomes (Figure 7 (a)), the absorbance profiles show that liposomes with trehalose were quite stable in human albumin (40mg/mL) for 26 hours. However, when liposome dispersions with trehalose were exposed to human plasma, large changes in absorbance indicated instabilities due to fusion and disruption of aggregates after 10 hours (b).

Although conventional liposomes were stable during 8 hours in plasma, this length of time is not adequate for allergy therapy by desensitization. The presence of antigens in the blood stream during a prolonged period is necessary for desensitization. Modifications on the surface of liposomes must be introduced in order to reduce interactions with plasma components, producing more stable liposomes and the controlled release of antigens.

Entrapping of Allergenic Proteins in Liposomes

The efficiency of entrapping allergenic proteins in liposomes was approximately 80%. Figure 8 shows the profile for incorporation efficiency at various lipid concentrations for 1.32mg/mL protein in the samples.

These results confirm the efficacy of the dehydration-rehydration process in incorporation of hydrophilic drugs including proteins, as described by Gregoriadis and Kirby (1984). In spite of the large range of molecular weights of proteins in the allergenic extracts, electrophoresic analysis showed no exclusion of proteins entrapped in liposomes.
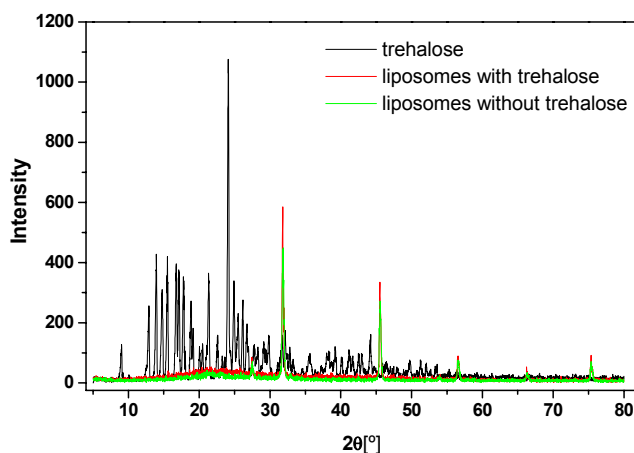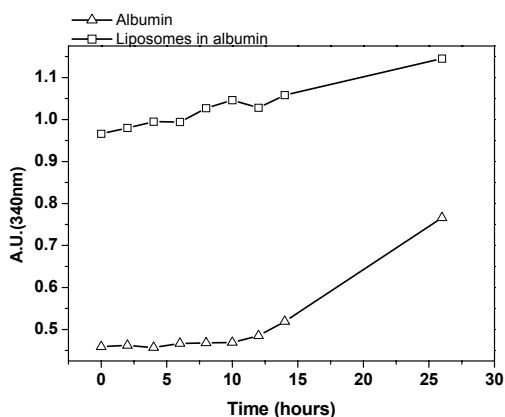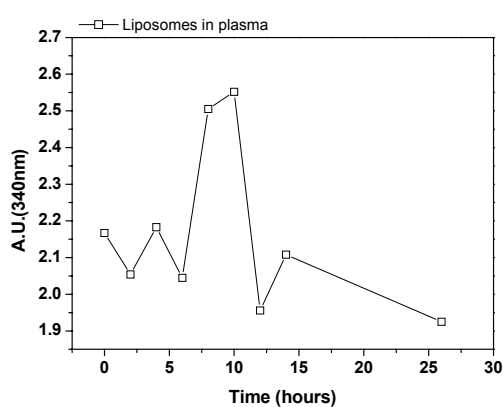

Figure 6: Ray-X spectra obtained for different samples: pure trehalose, liposomes without and with trehalose. The lipid matrix of liposomes was composed of PC:Chol at a mass ratio of 8:1 and added trehalose (1:4 lipid:trehalose).

(a)   (b)

Figure 7: Stability of liposomes in human albumin (a) and plasma (b). Liposome composition was PC:Chol (8:1w/w) and trehalose (lipid:trehalose 1:4 w/w).
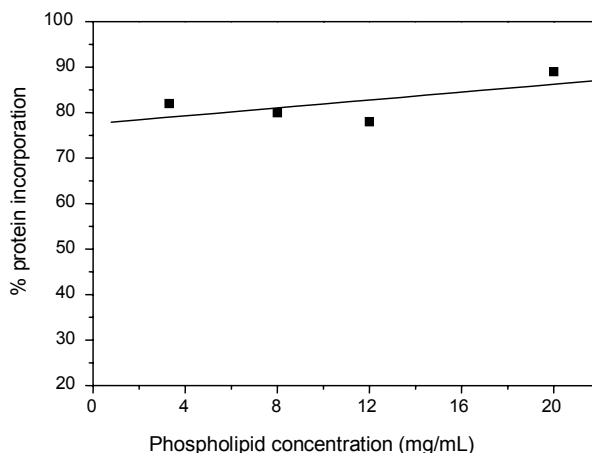
Figure 8: Efficiency entrapping of allergenic proteins. Liposomes were composed of PC:Chol 8:1 and total lipid:trehalose 1:4 mass ratio. The protein concentration was constant (1.32m/mL) in the assays.

CONCLUSIONS

The experimental results show that proteins from allergenic extracts can be efficiently entrapped in conventional liposomes by the dehydration-rehydration process. Reconstitution of the liposomal membrane structure and particle size was assured by inclusion of trehalose in the formulation. From a technological point of view these results are also valuable, due to the possibility of liposome production for entrapping protein antigens using a scalable and sterile process. These results are promising for applications in allergy treatments and other therapies.

ACKNOWLEDGMENTS

NOMENCLATURE

PC	Soy Phosphatidylcholine (Epikuron 200SH)
Chol	Cholesterol
SEM	Scanning electron microscopy
DSC	Differential scanning calorimetry
C_{in}/C_{out}	Ratio of solute concentrations in the inner to those in the outer parts of liposomes.

REFERENCES

Arora, N. and Gangal, S.V., Allergen Entrapped in Liposomes Reduce Allergenicity and Induce Immunogenicity on Repeated Injections in Mice. International Archives of Allergy and Applied Immunology, vol. 91, pp. 22-29 (1990).

Cevc, G., Phospholipid Handbook. Marcel Dekker: New York (1993).

Chen, P.S., Toribara, T.Y. and Warner, H., Microdetermination of Phosphorus. Analytical chemistry, vol. 28, pp. 1756-1758 (1956).

Crowe, J.H. and Crowe, L.M., Factors Affecting the Stability of Dry Liposomes. Biochimica et Biophysica Acta, vol. 939, pp. 327-334 (1988).

Gambale, W., Purchio, A. and Paula, C.R., .Influência de fatores abióticos na dispersão áerea de fungos na cidade de São Paulo, Brasil. Revista de Microbiologia, vol. 14, pp. 204-14 (1983).

Genin, I., Barrat, G., Xuan Thao, T., Delattre, J. and Puisieux, F., Otimization and Characterization of Freeze-dried Multilamellar Liposomes Incorporating Different Standardized Allergen Extracts. Allergy, vol. 49, pp. 645-652 (1994).

Gregoriadis, G. and Kirby, C., Dehydration-rehydration Vesicles: a Simple Method for High Yield Drug Entrapment in Liposomes. Biotechnology, November, pp. 979-984 (1984).

Hasan, S.D.M, Gambale, W., Zollner, R.L and Santana M.H.A., Fungi Allergens Produced by Solid-State Fermentation - Process Optimization and Allergen Characterization, Applied Biochemistry and Biotechnology, 2002 (submitted).

Kim, C.K., Chung, H.S., Lee, M.K., Choi, L.N. and Kim, M.H., Development of Dried Liposomes Contaning β-galactosidase for the Digestion of Lactose in Milk. International Journal of Pharmaceutics, vol. 183, pp. 185-193 (1999).

Mohovic, J., Gambale, W. and Croce J., Cutaneous Positively in Patients with Respiratory Allergens to 42 Allergenic Extracts of Air Bone Fungi Isolated in São Paulo, Brasil. Allergologia et Immunopathologia., vol. 16, pp. 397-40 (1988).

New, R.R.C., Liposomes: A Practical Approach. New York: IRL Press (1990).

Saraiva, C.P., Extração de proteínas com potencial alergênico da biomassa de *Drechslera (Helminthosporium) monoceras* produzida por fermentação em estado sólido. Master's thesis – School of Chemical Engineering, State University of Campinas, Campinas (2001).

Stewart, G.A., Thompson, P.J., Doyle, C. and Signor, C., Immunoglobulin Responses to Allergens for the House Dust Mite in Mite Allergic Adult Asthmatic-patients. Australian and New Zealand Journal of Medicine, vol. 18, pp. 544-544 (1988).

Yunginger, J.W., Allergens: Recent Advances. Pediatric Clinics of North America, vol. 35, pp. 981-93 (1988).

Walls, AF., Liposomes for Allergy Immunotherapy? Clinical Experimental Allergy, vol. 22, pp.1-2 (1992).