Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 304 (2005) 124–134
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Effect of lyophilization on the structure and phase changes of PEGylated-bovine serum albumin

Virgilio Tattini Jr.[a], Duclerc F. Parra[b], Bronislaw Polakiewicz[a], Ronaldo N.M. Pitombo[a,*]

[a] *Department of Biochemical and Pharmaceutical Technology, Pharmaceutical Sciences School, University of São Paulo, Av. Prof. Lineu Prestes, 580, Bloco 16, CEP 05508-900 São Paulo, SP, Brazil*
[b] *Nuclear and Energetic Research Institute/University of São Paulo, São Paulo, Brazil*

Received 25 February 2005; received in revised form 30 June 2005; accepted 10 August 2005
Available online 26 September 2005

Abstract

Poly (ethylene glycol) (PEG) conjugation masks the protein's surface and increases the molecular size of the polypeptide, thus preventing the approach of antibodies or antigen processing cells and reducing the degradation by proteolytic enzymes. Proteins are readily denatured by numerous stresses arising in solution (e.g., heating, agitation, freezing and pH changes) or by chemical reactions (e.g., hydrolysis and deamidation), many of which are mediated by water. Lyophilization is most commonly used to prepare dehydrated proteins, which, theoretically, should have the desired long-term stability at ambient temperatures. Through Raman spectroscopy, differential scanning calorimetry (DSC) associated with the determination of water content by Karl Fisher titration, it was observed that after the modification of BSA–PEG in a ratio of 1:0.25 showed lower degree of structural alterations and consequently lower variation on the physical–chemical characteristics when it was compared to BSA–PEG (1:0.5). Moreover, the BSA–PEG (1:0.25) optimizes the conditions during the lyophilization process and storage of the protein.

Keywords: Freeze–drying; Lyophilization; PEGylation; Bovine serum albumin; Glass transition; Raman spectroscopy

1. Introduction

PEGylation is of interest in applied biotechnology because upon modification it masks the protein's surface, increases the molecular size of the polypeptide, conveys to molecules its physical–chemical properties and therefore also modifies biodistribution and solubility of peptide and non-peptide drugs. This property provides new techniques in biocatalysis and in pharmaceutical technology where many insoluble drugs are solubilized by poly (ethylene glycol) (PEG) conjugation and thus more easily administered (Herman et al., 1995).

* Corresponding author. Tel.: +55 11 3091 3665; fax: +55 11 3815 6386.
E-mail address: pitombo@usp.br (R.N.M. Pitombo).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.08.006

Castellanos et al. (2005) have demonstrated the effectiveness of the covalent modification of alpha-chymotrypsin with methoxy-PEG to afford its stabilization during encapsulation in poly (lactic-co-glycolic) acid (PLGA) microspheres by a solid-in-oil-in-water method. Alpha-chymotrypsin was chemically modified with PEG (M_w = 5000) using molar ratios of PEG-to-chymotrypsin ranging from 0.4 to 96. The results demonstrated that PEG modification was able to prevent chymotrypsin aggregation and activity loss upon solid-in-oil-in-water encapsulation in PLGA microspheres. It is demonstrated that it is essential to optimize the degree of protein modification to ascertain protein stability upon encapsulation.

The role of PEG in this study was acting as a modifier agent where a covalent bond is formed between the PEG polymer and polypeptide drug of choice. Studies of PEG in solution show that each ethylene glycol sub-unit is tightly associated with two or three water molecules. A binding process with water makes PEGylated compounds function as though they are 5–10 times larger than a corresponding soluble protein of similar molecular weight. Further, the PEG polymer with associated water molecules is very mobile, and acts like a shield to protect the attached drug from enzyme degradation, pH and temperature changes, interactions with cell surface proteins, and provides increased size to prevent rapid renal filtration and clearance. In general, a PEG polymer is first chemically activated in order to react with a polypeptide drug (Katre, 1993). The activated PEG derivative is then covalently linked to a reactive group on the polypeptide drug. Changes in the size, structure and molecular weight of PEG polymers can affect the biological activity of the attached drug. In general, PEGylation of a polypeptide lowers its renal clearance, increases its half-life and improves its biological activity (Harris et al., 2001).

Lyophilization is often used to stabilize protein products with limited shelf lives in solution. Therefore, it is not surprising that more than one quarter of the therapeutic protein products in the market are lyophiles. The determination of the lyophilization cycle is important because of physical changes that occur in the solution during freezing and drying phases of the process. Due to the amorphous nature of protein and stabilizer (most commonly sugars and polyols), lyophilized formulations often exhibit a glass–rubber transition that is an important parameter in the development of the freeze–drying cycle. Therefore, the glass transition of a lyophilized product can be studied and applied to improve processability, quality and stability of the product (Chen and Oakley, 1995).

However, infrared spectroscopic studies have documented that the acute freezing and dehydration stresses of lyophilization can induce protein unfolding (Dong et al., 1995). The application of Raman spectroscopy to characterize natively unfolded proteins has been underdeveloped, even though it has significant technical advantages. The structural changes as a result of freeze–drying have been investigated, especially by Raman spectroscopy. In general, drying results in a decrease of α-helix and random structure and an increase in β-sheet structure. Roy and Gupta (2004) studied the case of basic fibroblast growth factor and γ-interferon. The enhanced FTIR showed large conformational changes and aggregation during freeze–drying, which could be prevented by using sucrose as a lyoprotectant. In the presence of moisture, freeze–dried proteins can undergo disulphide interchange and other reactions, which lead to inactivation. Such molecular changes during storage have been described for human insulin, tetanus toxoid and interleukin-2.

The underlying assumption is that IR spectral changes in the amides I and III regions upon protein dehydration are caused by protein structural changes. However, it has been claimed that amide I IR spectral changes could be the result of water removal per se. Al-Azzam et al. (2002) have investigated the structure of horseradish peroxidase (HRP) and poly (ethylene glycol)-modified HRP (HRP–PEG) under various conditions (in aqueous solution, the amorphous dehydrated state and dissolved/suspended in toluene and benzene) by UV–visible (UV–vis), FTIR and resonance Raman spectroscopy. The resonance Raman and UV–vis spectra of dehydrated HRP–PEG dissolved in neat toluene or benzene were very similar to that of HRP in aqueous buffer, and thus the heme environment (heme iron spin, coordination and redox state) was essentially the same under both conditions. The amide I IR spectra of HRP–PEG in aqueous buffer and of dehydrated HRP–PEG dissolved in neat benzene and toluene were also very similar, and the secondary structure compositions (percentages of α-helices and β-sheets) were within the standard error the same.

The aim of the current study was to examine the effects of lyophilization on the structural and phase changes of PEGylated-BSA. The behavior of the modified protein during each stages of lyophilization process was evaluated through DSC thermal analysis and Raman spectroscopy.

2. Materials and methods

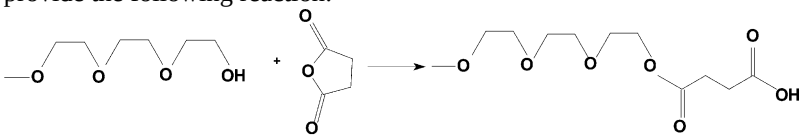
2.1. Materials

Crystallized bovine serum albumin was purchased from INLAB (Sao Paulo, Brazil).

Methoxy-polyethyleneglycol 5000 (mPEG), average molecular weight 5000, was obtained from Sigma Chemical Co. (St. Louis, EUA).

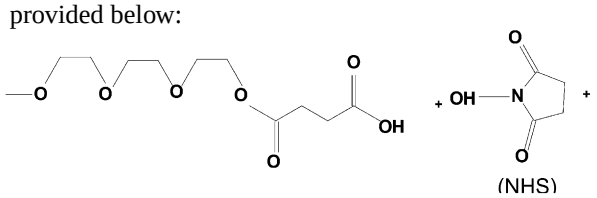
2.2. Methoxy-polyethyleneglycol 5000 synthesis

To obtain the succinyl derivative it was necessary to provide the following reaction:

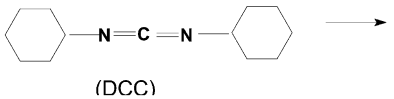


Methoxy-PEG 5000 succinic anhydride succinic carboxy derivative

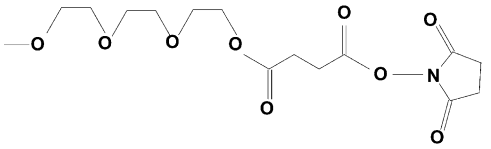
The reaction of methoxy-PEG activated complex is provided below:



(NHS)



(DCC)



Activated complex

Mw = 5000

Table 1
Amount spent in the methoxy-polyethyleneglycol activation reaction

Methoxy-PEG 5000 (g)	15
Ethyl acetate (mL)	150
N-Hydroxysuccinimide (NHS) (g)	0.86
Diciclohexylcarbodiimide (DCC) (g)	1.55

The amount spent in the methoxy-polyethyleneglycol activation reaction is mentioned in Table 1.

Dehydrated ethyl acetate (standard analytic ethyl acetate also fits), methoxy-PEG (already as a succinyl derivative), *N*-hydroxysuccinimide (NHS) and diciclohexylcarbodiimide (DCC) were poured into a glass reactor with magnetic agitation and controlled heat. The mix rapidly heated till complete reagents solution and resting for 24 h at $30 \pm 1\,^{\circ}C$. At the end of this period, the reaction mass was filtered and left resting for another 24 h at $5 \pm 1\,^{\circ}C$ and then filtered to isolate the solid product that was washed with ice cold ethyl acetate. Next, the product was vacuum-dried till reach a constant weight. The material was then dissolved three times its mass in benzene, and then, the same volume of petroleum ether was added, the material was cooled and filtered two more times (Clark et al., 1996).

The final methoxy-PEG succinimide yield (obtained following the above descriptions) was 7.47 g of dried base.

This material was kept in flask under a nitrogen blanket and used in the PEGylation reaction with bovine serum albumin.

2.3. Preparation of BSA–PEG solution

A stock solution of 100 mg/mL (150–300 μM) BSA was prepared in 5 mM phosphate buffer pH 7.4 and exhaustively dialyzed overnight against the same buffer at $4 \pm 1\,^{\circ}C$.

It was poured into three reaction vessels, respectively: 150 mL of an alkaline phosphate buffer and 100 mL of a BSA solution 40 mg/mL.

It was added to each vessel, respectively, 1 g (BSA–PEG, 1:0.25), 2 g (BSA–PEG, 1:0.5) and 4 g (BSA–PEG, 1:1) of activated PEG 5000.

The solutions were kept at 30 ± 1 °C and then transferred to a cold chamber at 8 ± 1 °C, where they stayed "overnight". The three samples were purified on a Sephadex column G150 calibrated with Tris buffer 100 mM, pH 8.6, and eluted with the same buffer (Clark et al., 1996). Then, the modified BSA solutions with PEG 5000 were taken into lyophilization.

2.4. Lyophilization of a BSA–PEG solution

The solutions were lyophilized in a FTS Systems, model TDS-00209-A, microprocessor controlled tray dryer (Dura-Stop, Dura-Dry-MP).

A 2 mL of an aqueous solution of BSA–PEG (1:0.25) and (1:0.5) was filled manually into 5 mL glass vials (10 R) and partially closed with rubber stoppers. The freezing step was performed by placing the vials onto ultra-freezer for 4 h with pre-cooled shelves at −70 ± 0.5 °C. Then, the vials were transferred onto the lyophilizer shelves pre-cooled at −25 ± 1 °C. The primary drying was conducted at −30 ± 1 °C (product temperature), chamber pressure at 140 ± 5 mTorr and condenser temperature at −90 ± 1 °C. The secondary drying was conducted at 25 ± 1 °C (product temperature), chamber pressure at 50 ± 5 mTorr and condenser temperature at −90 ± 1 °C (Fig. 4). The chamber and condenser pressures were evaluated by a MKS Baratron Type 122A Absolute Pressure Transducer. The chamber pressure was controlled by a vacuum bleed valve mounted on the inside of the cabinetry on the top of the chamber. It runs to the rear of the Tray Dryer to an easily accessible port. The port is equipped with a filter that is an unlaminate 0.2 μm PTFE (Teflon) membrane sealed between screen supports. The end of primary and secondary drying was determined by a Hygrometer Endress + Hauser Hygroguard 2550 microprocessor controlled trace moisture analyzer. All dried samples were stoppered under vacuum pressure at 50 ± 5 mTorr.

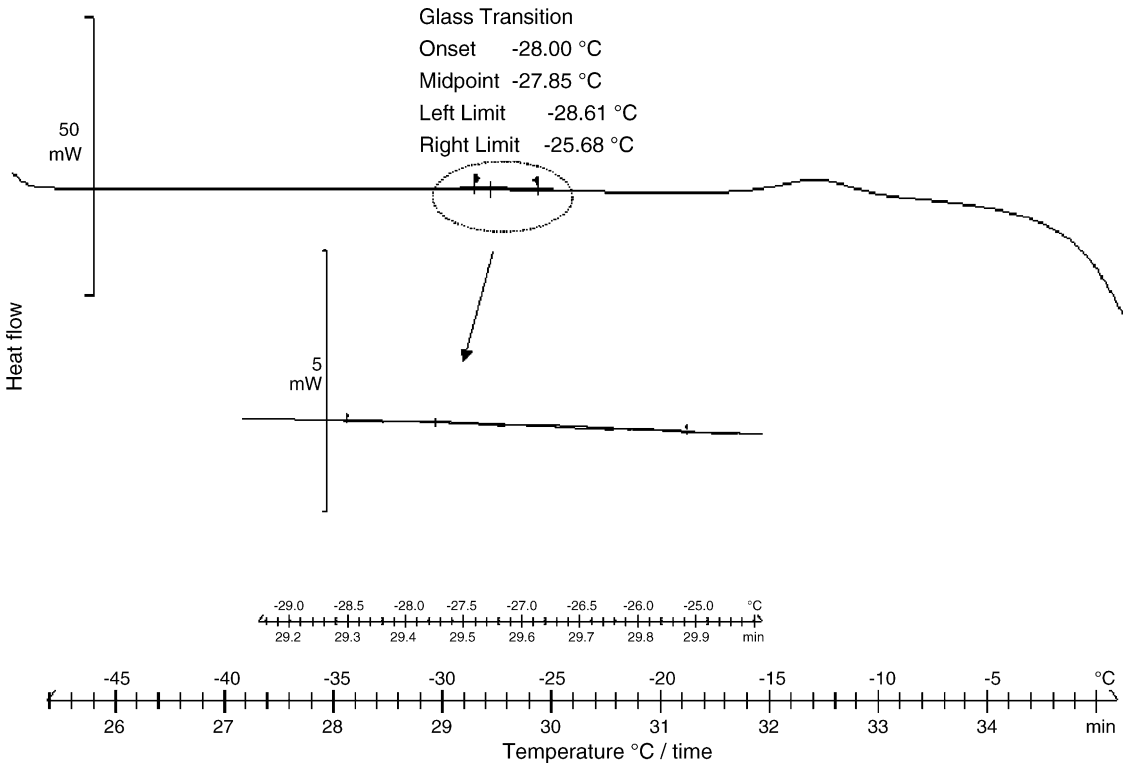


Fig. 1. DSC thermogram of BSA–PEG solution (1:0.25), illustrating the events occurred during the heating of frozen sample.

2.5. Determination of glass transition temperature

The glass transition temperature of a BSA–PEG solutions (T'_g) and dried samples (T_g) were determined by DSC using a METTLER TOLEDO DSC 822 unit. The DSC was calibrated using mercury and indium standards, with onset temperatures of −38.87 and 156.6 °C, respectively. For the frozen solutions the samples were frozen at −50 °C, and then warmed at 0 °C, both using a cooling/heat flow of 5 °C/min and Nitrogen flow rate of 50 mL/min. In order to prevent contamination of the dried samples by moisture in the surrounding air, the lyophilized samples were weighed into aluminium DSC pans in a dry box filled with air of equilibrated residual humidity (rh), achieved by the presence of anhydrous phosphorous pentoxide as a desiccant. A portable hygrometer monitored humidity in the dry box. The samples were frozen at 0 °C and then warmed at 60 °C, both using a cooling/heat flow of 5 °C/min. The T'_g and T_g values were measured through five analyses for each BSA–PEG ratio.

2.6. Structural analysis

The secondary structure of BSA–PEG solution and dried samples were determined by FT-Raman spectroscopy using a BRUKER FRA 106/S Raman module on an IFS 28/N. All spectra were the average of 200 scans for solid samples and 4000 scans for solutions at 4 cm^{-1} resolution. All experiments were performed at room temperature (25 °C).

The structural analysis was conducted on native solution, lyophilized powder and lyophilized material reconstituted.

For spectral acquisition tubes were filled with: 150 mL of solution sample; 30 mg of solid sample; 150 mL of rehydrated sample.

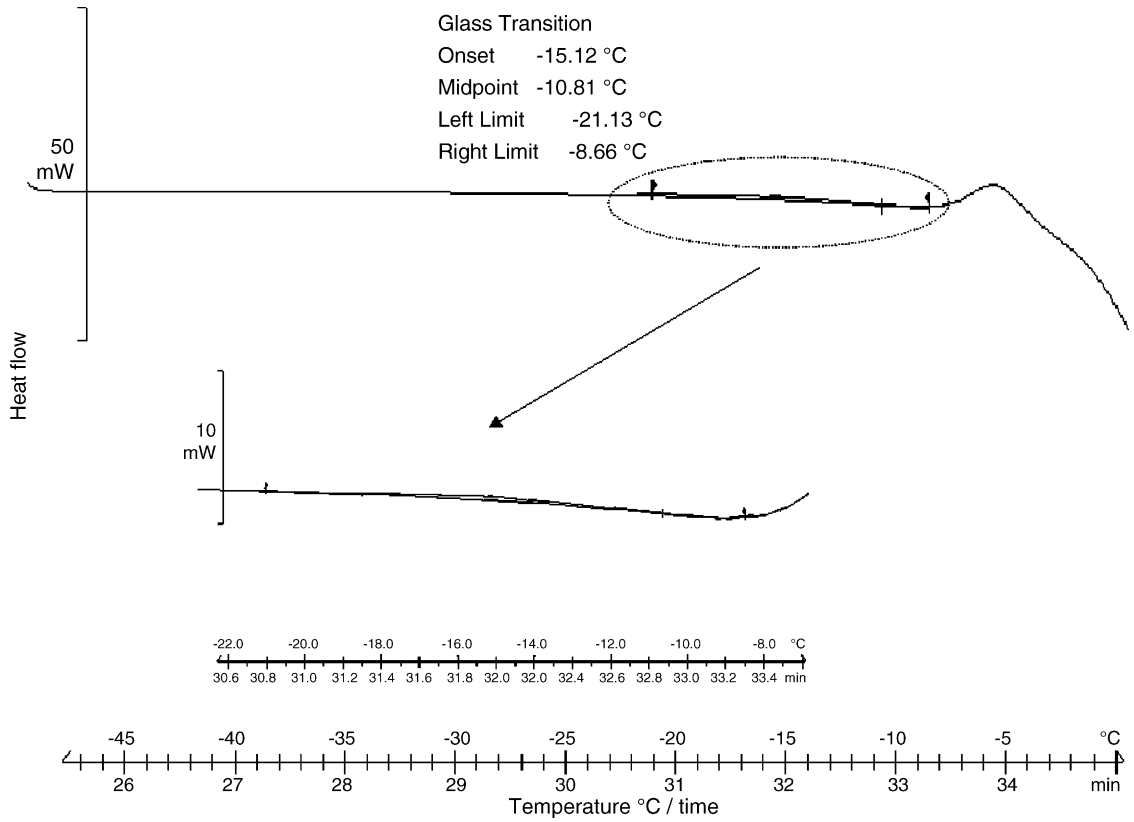


Fig. 2. DSC thermogram of BSA–PEG solution (1:0.5), illustrating the events occurred during the heating of frozen sample.

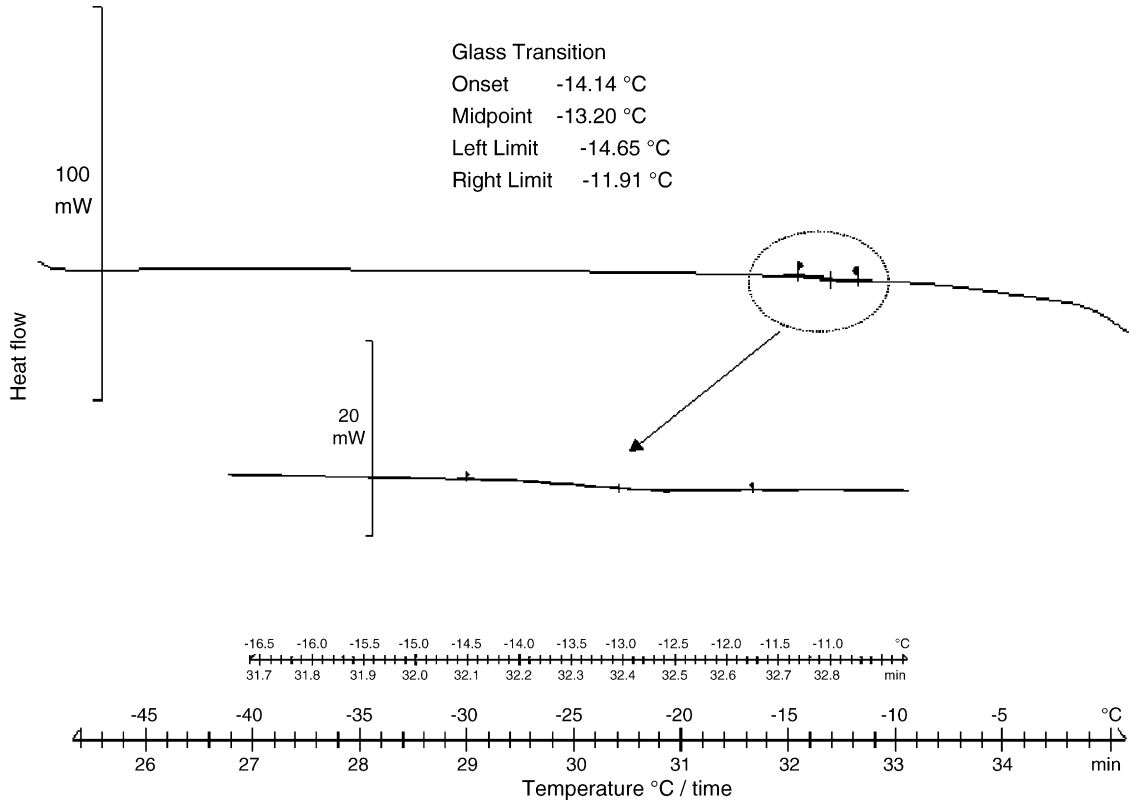


Fig. 3. DSC thermogram of BSA–PEG solution (1:1), illustrating the events occurred during the heating of frozen sample.

This procedure was conducted under controlled relative humidity (dry box) achieved by the presence of anhydrous phosphorous pentoxide as a desiccant. A portable hygrometer monitored humidity in the dry box.

2.7. Determination of water content

The water content of BSA–PEG freeze–dried samples was determined by Karl Fisher titration using a METTLER TOLEDO DL 31 Karl Fisher Titrator.

It was added a known amount of anhydrous methanol with a syringe to the lyophilized BSA–PEG container. The methanol has dissolved the sample. Known amounts of sample and methanol are withdrawn and added to the Karl Fisher titration vessel for moisture determination. The moisture content of the anhydrous methanol is determined as the blank (FDA, 1990).

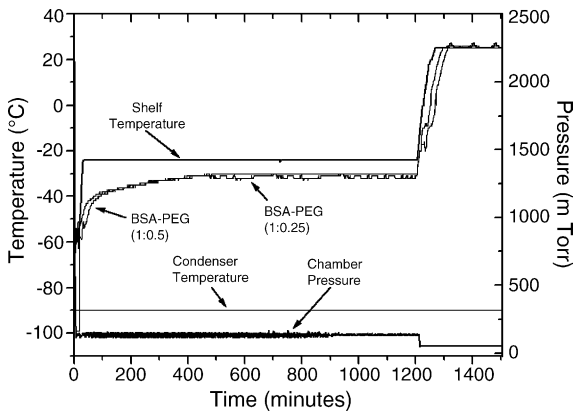


Fig. 4. Comparison of lyophilization process of BSA–PEG (1:0.25) and (1:0.5). The samples presented the same behavior during the process.

3. Results and discussion

Figs. 1–3 represent the DSC thermograms of the BSA–PEG (1:0.25), (1:0.5) and (1:1), respectively. During the rewarming step, all samples presented a change in the heat capacity in the baseline. This baseline shift is related to glass transition temperature (T_g'). The glass transition temperatures were −28, −11 and $-13 \pm 1\,^{\circ}C$ for the BSA–PEG (1:0.25), (1:0.5) and (1:1), respectively. After the T_g' an exothermic peak was observed for the BSA–PEG (1:0.25) and (1:0.5). This peak refers to the recrystallization of some BSA–PEG solution compounds, as for instance, the phosphate buffer into the solution and also the polyethylene glycol recrystallization.

According to Radaev and Sun (2002), during freezing PEG has the tendency to crystallize. However, this characteristic is dependent to size and concentration of PEG. On the BSA–PEG (1:1) the solution showed a well-defined glass transition temperature without crystallization peak due to the strongly amorphous characteristics during it's freezing.

Amin et al. (2004) have used the PEG as cosolvent system and they have observed that addition of PEG 400 to commonly used bulking agents, such as mannitol, sucrose or polyvinylpyrrolidone, caused a significant change in the thermal properties of the bulking agents as observed by modulated differential scanning calorimetry. In addition, PEG 8000 was evaluated as a bulking agent because it also can function as a cosolvent in solution and forms an acceptable cake after lyophilization. Addition of PEG 400 to PEG 8000 caused negligible changes in the thermogram of this bulking agent. Surprisingly, the combination of PEG 8000 and PEG 400 forms a solid lyophilized cake. The current system can be best described as the lyophilization of a miscible solution of PEG 8000 and PEG 400 resulting in a lyophile that has a crystalline structure of PEG 8000, which is able to support PEG 400.

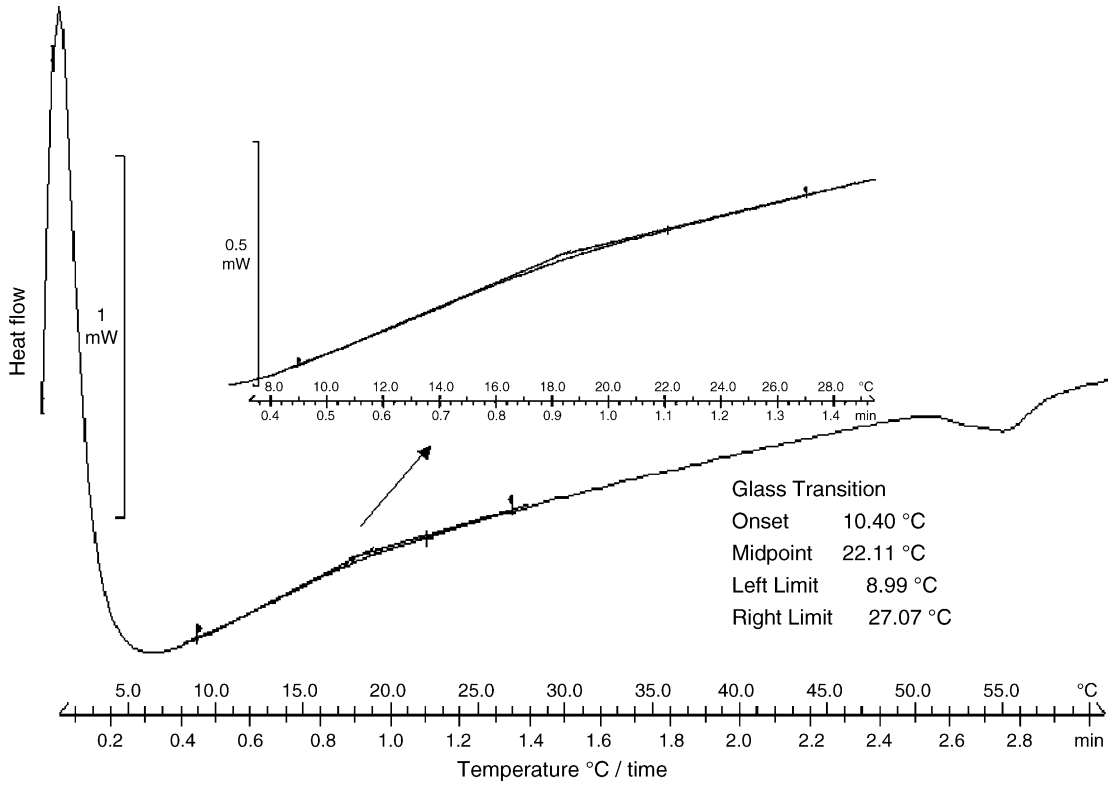


Fig. 5. DSC thermogram of BSA–PEG lyophilized (1:0.25) illustrating the T_g during the heating of the sample. Residual moisture (≅6.4%).

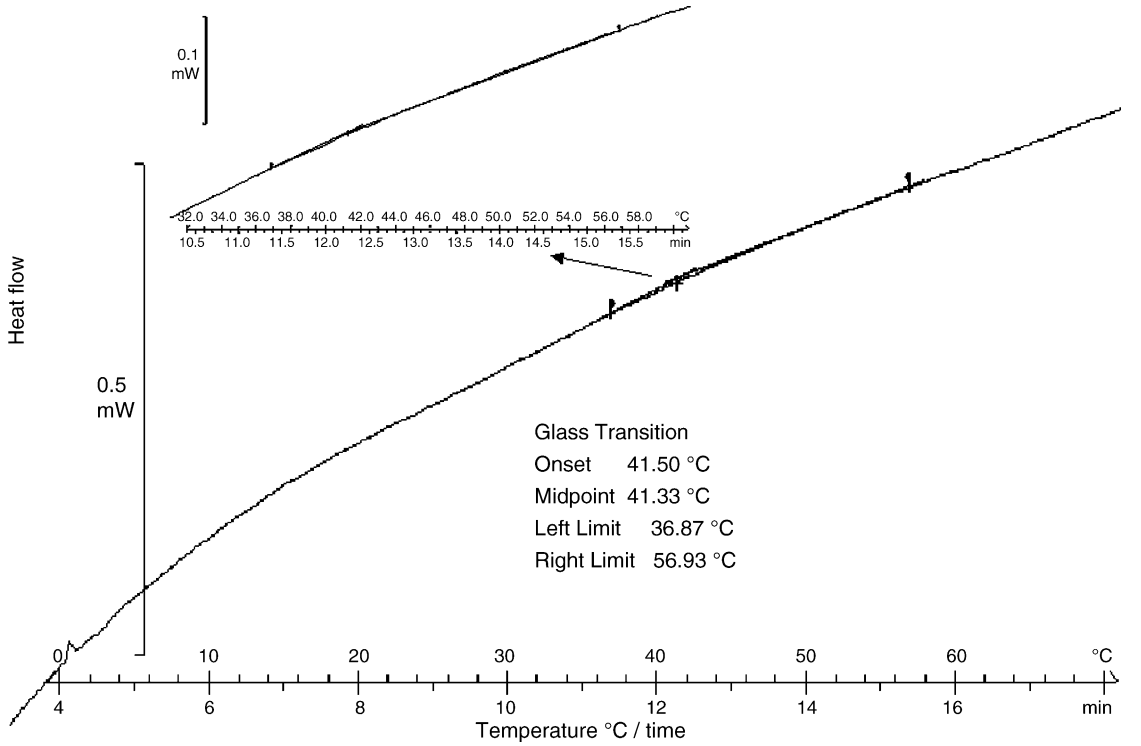


Fig. 6. DSC thermogram of BSA–PEG lyophilized (1:0.5) illustrating the T_g during the heating of the sample. Residual moisture (≅5.3%).

The BSA–PEG (1:0.25) and (1:0.5) lyophilization were conducted under the same conditions (temperature, pressure and time).

In Fig. 4, no difference can be seen related to the BSA–PEG (1:0.25) and (1:0.5) behavior during lyophilization. However, the continuity of BSA–PEG (1:1) studies was not possible because of the difficulties found during the lyophilization of the material. The BSA–PEG (1:1) presented a jelly-kind material preventing water from subliming during the primary drying. It takes more then 50 h to dry and even so at the end of the process the cake had showed collapse signs.

After the lyophilization process it was determined the residual moisture of the lyophilized powder by Karl Fisher titration. The BSA–PEG (1:0.25) showed residual moisture between 4.3 and 6.4% (w/w) and the BSA–PEG (1:0.5) between 2.3 and 5.3% (w/w). Even the lyophilization cycle were conducted under the same parameters for both BSA–PEG rates we could suppose that as higher the PEGylation rate higher the hydrophilic groups linked to BSA consequently smaller the water retention by the system.

Figs. 5 and 6 represent the DSC thermograms of the BSA–PEG (1:0.25) and (1:0.5) lyophilized powder, respectively. A raise on T_g value of the material can be noted. The glass transition temperature of lyophilized samples were 22 ± 1 and $41 \pm 1\,^{\circ}C$ for BSA–PEG (1:0.25) and (1:0.5), respectively. The BSA–PEG (1:0.25) presented an endothermic peak at $57 \pm 1\,^{\circ}C$ and it is related to the material decomposition during the sample heating. The same event was not observed during the BSA–PEG (1:0.5) heating.

Structural information is obtained through the analysis of the conformationally sensitive amide I band, which is located between 1600 and 1700 cm^{-1}. This band is due to the in-plane C=O stretching vibration, weakly coupled with C–N stretching and in-plane N–H bending. Each type of secondary structure (i.e., α-helix, β-turn and disordered) gives rise to a different C=O stretching frequency and, hence, has a characteristic band position, which is designated by wavenumber (cm^{-1}). Band positions are used to determine the secondary structural types present in a protein. An analysis of the infrared bands in the amide I region can pro-

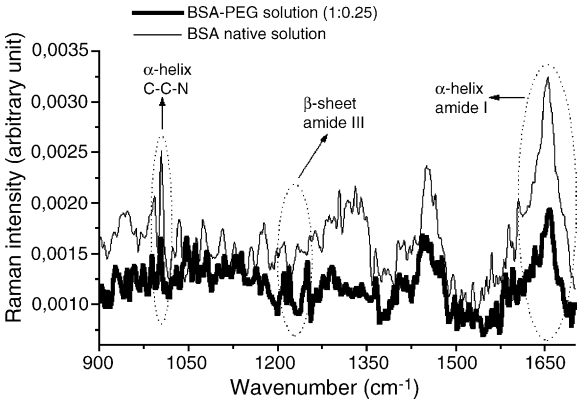


Fig. 7. Comparison of Raman spectra of BSA native solution (10%) and BSA–PEG solution (1:0.25).

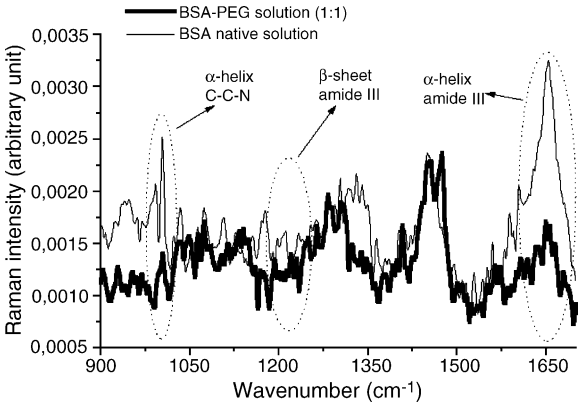


Fig. 9. Comparison of Raman spectra of BSA native solution (10%) and BSA–PEG solution (1:1).

vide quantitative as well qualitative information about the secondary structure of the protein (Carpenter et al., 1998).

Figs. 7–9 compare the Raman spectrum between native BSA solutions and different PEGylation rates of BSA–PEG solutions. It was observed that the modification of bovine albumin with methoxy-polyethyleneglycol induced structural alterations in the protein conformation. This fact is related to the different intensities and localization of the absorbance peaks in the Raman spectrum.

A α-helix structure loss was indicated by intensity diminution at 1654 cm^{-1} band. The BSA–PEG (1:0.25) presented less α-helix (1654 cm^{-1}) structural variation when compared to BSA–PEG (1:0.5) and (1:1). In the amide III region, the bands between 1230 and 1240 cm^{-1} were attributed to the fact that the β-sheet structures also presented spectral alterations, whenever in position as in the intensity, after PEG conjugation.

The intensity increase in the β-sheet structures related bands is a characteristic sign of protein aggregation, inducted by the raise of intermolecular interactions between the H^+ group, because those polarized groups need to satisfy the necessity of hydrogen links through intra or intermolecular interactions occasioned by water removal. The native bovine albumin has in its structure more quantity of hydrophilic groups not available in comparison with BSA–PEG. We can observe that the higher the

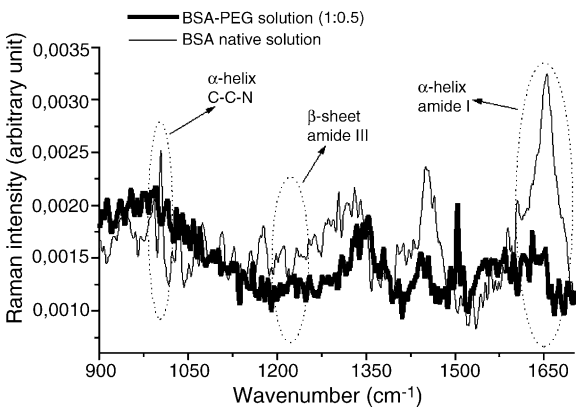


Fig. 8. Comparison of Raman spectra of BSA native solution (10%) and BSA–PEG solution (1:0.5).

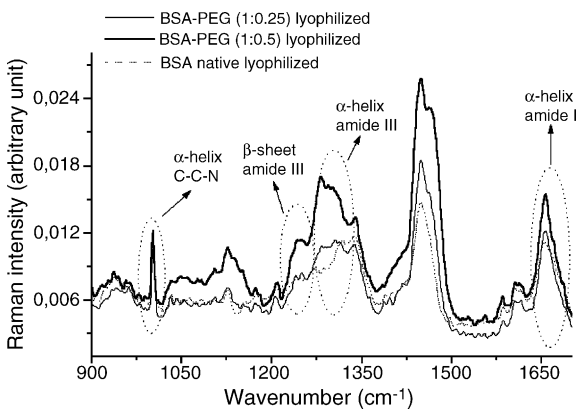


Fig. 10. Comparison of Raman spectra of BSA native lyophilized and BSA–PEG (1:0.25) and (1:0.5) lyophilized.

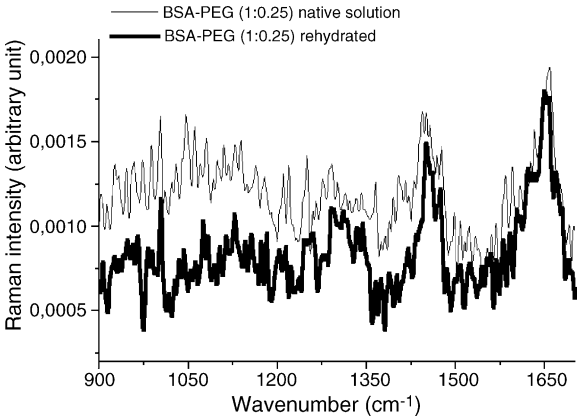


Fig. 11. Comparison of Raman spectra of BSA–PEG (1:0.25) native and rehydrated solution.

quantity of PEG linked to the BSA, the lower will be the maintenance of the secondary structure, occurred by the low water retention by hydrophilic groups.

In Fig. 10, we observed that the lyophilized BSA–PEG (1:0.25) presented a better maintenance of the secondary structure of the protein than BSA–PEG (1:0.5). This result can also be related to a lower alteration or ruptures of the hydrogen bridges and van der Waals forces.

Figs. 11 and 12 showed that the BSA–PEG (1:0.25) presented better results of structural maintenance, after lyophilization and rehydration when compared to BSA–PEG (1:0.5). Three bands (1620, 1630 and 1690 cm^{-1}) attributed to antiparallel β-sheet vibrations suffered different alterations levels. The amides I and

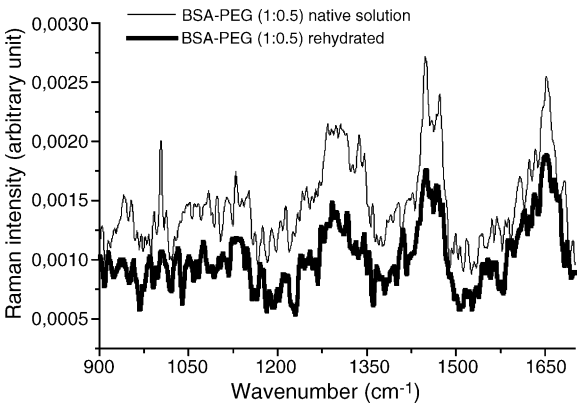


Fig. 12. Comparison of Raman spectra of BSA–PEG (1:0.5) native and rehydrated solution.

III regions presented structural alterations at different levels in relation to the different PEGylation rates.

The maintenance of bovine albumin structural conformation is directly related to water retention through the NH_2 groups linked to lysine. The 1000 cm^{-1} wavelength band related to the α-helix (C–C–N) practically disappeared after BSA modification with PEG at (1:0.5) ratio.

After rehydration of the lyophilized powder, the intensity distribution on band between 1320–1340 cm^{-1} related to the aliphatic lateral chains was higher for the BSA–PEG (1:0.25).

The lyophilization-induced spectral alterations in the conformationally sensitive amide I region are due to protein unfolding and not simply to the loss of water from the protein. The intrinsic effects of water removal on the vibrational properties of the peptide bond, and hence protein infrared spectra, were found to be insignificant by Prestrelski et al. (1993). If the direct vibrational effects of water removal were responsible for drying-induced spectral changes, then the infrared spectra of all proteins should be altered to the same degree in the dried solid, which is not the case (Carpenter et al., 1998).

It was observed two different behaviors of BSA–PEG lyophilized powder related with structural unfolding:

- the BSA–PEG (1:0.25) regains the native conformation upon rehydration (reversible unfolding);
- a significant fraction of BSA–PEG (1:0.5) aggregates upon rehydration (irreversible unfolding).

It has been documented through several proteins that prevention of aggregation and recovery of activity after rehydration correlate directly to retention of the native structure in the dried solid (Carpenter et al., 1998).

4. Conclusions

The present work allowed us to expose the following conclusions:

- After the bovine albumin conjugation with methoxy-PEG, the BSA–PEG (1:0.25) presented a lower degree of the structural alterations and also a lower variation in the physical–chemical characteristics of the protein when compared to BSA–PEG (1:0.5) and (1:1).

- The bovine albumin conjugation with methoxy-PEG has increased the glass transition temperature for the protein solution as for the lyophilized powder, optimizing the lyophilization process and also the storage conditions below the collapse temperature of the material.
- The critical parameters (T'_g, T_P, T_S, P_C and T_g) for the lyophilization process and storage conditions of the modified BSA–PEG for the (1:0.25), (1:0.5) and (1:1) rates were determined.
- The results obtained suggest a more profound study to determine the ideal rate of PEGylation, as the molecular modification of the BSA resulted in different levels of structural alterations, a major importance factor for the preservation of biological products.

Acknowledgements

The authors acknowledge the financial assistance of CNP_q, CAPES, FAPESP. The authors are grateful to Prof. Dr. Reinaldo Guidici and Dr. Marlon Martins dos Reis (Chemical Engineering Department, Polytechnic School, LSCP Group, University of São Paulo) for his valuable advice on the use of the Raman spectrometer. The authors wish to thank Prof. Dr. José Abrahão Neto (Department of Biochemical and Pharmaceutical Technology, Pharmaceutical Sciences School, University of São Paulo) for his assistance on the PEGylation reaction and Mr. Edson Ghilardi (CQMA-IPEN) for his assistance on the DSC analysis.

References

Al-Azzam, W., Pastrana, E.A., Ferrer, Y., Huang, Q., Schweitzer-Stenner, R., Griebenow, K., 2002. Structure of poly(ethylene glycol)-modified horseradish peroxidase in organic solvents: infrared amide I spectral changes upon protein dehydration are largely caused by protein structural changes and not by water removal per se. Biophys. J. 83, 3637–3651.

Amin, K., Dannenfelser, R.M., Zielinski, J., Wang, B., 2004. Lyophilization of polyethylene glycol mixtures. J. Pharm. Sci. 93, 2244–2249.

Carpenter, J.F., Prestrelski, S.J., Dong, A., 1998. Application of infrared spectroscopy to development of stable lyophilized protein formulations. Eur. J. Pharm. Biopharm. 45, 231–238.

Castellanos, I.J., Al-Azzam, W., Griebenow, K., 2005. Effect of the covalent modification with poly (ethylene glycol) on alpha-chymotrypsin stability upon encapsulation in poly (lactic-co-glycolic) microspheres. J. Pharm. Sci. 94, 327–340.

Chen, T., Oakley, D.M., 1995. Thermal analysis of proteins of pharmaceutical interest. Thermochim. Acta 248, 229–244.

Clark, R., Olson, K., Fuh, G., Marian, M., Mortensen, D., Teshima, G., Chang, S., Chu, H., Mukku, V., Canova-Davis, E., Somers, T., Cronin, M., Winkler, M., Wells, J.A., 1996. Long-acting growth hormones produced by conjugation with polyethylene glycol. J. Biol. Chem. 271, 21969–21977.

Dong, A., Prestrelski, S.J., Allison, S.D., Carpenter, J.F., 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J. Pharm. Sci. 84, 415–424.

FDA (Food and Drug Administration), 1990. Guideline for the Determination of Residual Moisture in Dried Biological Products. Center for Biologics Evaluation and Research (CBER).

Herman, S., Hooftman, G., Schacht, E., 1995. Poly(ethylene glycol) with reactive endgroups: I. Modification of proteins. J. Bioact. Compat. Polym. 10, 145–187.

Harris, J.M., Martin, N.E., Modi, M., 2001. Pegylation a novel process for modifying pharmacokinetics. Clin. Pharmcokinet. 40, 539–551.

Katre, N.V., 1993. The conjugation of proteins with polyethylene glycol and other polymers: altering properties of proteins to enhance their therapeutic potential. Adv. Drug Del. Rev. 10, 91–114.

Prestrelski, S.J., Tedeschi, N., Arakawa, T., Carpenter, J.F., 1993. Dehydration-induced conformational changes in proteins and their inhibition by stabilizers. Biophys. J. 65, 661–671.

Roy, I., Gupta, M.N., 2004. Freeze–drying of proteins: some emerging concerns. Biotechnol. Appl. Biochem. 39, 165–177.

Radaev, S., Sun, P.D., 2002. Crystallization of protein–protein complexes. J. Appl. Cryst. 35, 674–676.