

RESEARCH PAPER

**DEVELOPMENT AND EVALUATION OF LYOPHILIZED
THIOLATED-CHITOSAN WAFERS FOR BUCCAL DELIVERY
OF PROTEIN**

I. Ayensu^{1,2} and J. S. Boateng²

¹*Department of Pharmaceutical, Chemical and Environmental Sciences, School of Science,
University of Greenwich at Medway, Chatham Maritime, Kent, UK, ME4 4TB
i.ayensu@gre.ac.uk*

²*Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences,
KNUST, Kumasi
j.s.boateng@gre.ac.uk*

ABSTRACT

Annealed and non-annealed freeze-dried wafers from synthesised thiolated-chitosan have been developed and evaluated. Wafers were obtained by freeze-drying aqueous gels of the thiolated polymer incorporating per polymer weight, 10 % each of glycerol as plasticizer, D-mannitol as cryoprotectant and 50% BSA as model protein drug. The formulation was freeze-dried with or without the process of annealing. Texture analyzer was employed to investigate the in vitro mucoadhesive properties in tensile mode, residual moisture content by thermo-gravimetric analysis (TGA) while hydration capacity and drug release studies were performed in 0.1M PBS. Microscopic architecture was examined using scanning electron microscopy (SEM). Differential scanning calorimetry (DSC) was employed for protein phase separation studies and conformational stability by attenuated total reflectance-Fourier transform infra-red spectroscopy (ATR-FTIR) and confirmed with Circular dichroism (CD). The annealing process led to wafers with increased ease of hydration, improved in vitro mucoadhesive characteristics and enhanced BSA release, without affecting the conformational stability of the protein due to the presence of a cryoprotectant. These results show the potential application of annealed freeze-dried thiolated-chitosan wafers for buccal mucosa delivery of protein based drugs.

Keywords: *Thiolated-chitosan, freeze-drying, annealing, buccal delivery, protein*

INTRODUCTION

Chitosan (CS) (poly[-(1,4)-2-amino-2-deoxy-D-glucopyranose]) is a natural polymer obtained by deacetylation of chitin (Wu *et al.*, 2009). Due to its biocompatibility and biodegradation properties (VandeVord *et al.*, 2002), chitosan has been used primarily for the purpose of improved mucoadhesion, permeation enhancement and drug delivery (Agnihotri *et al.*, 2004)

via mucosal routes such as GI mucosa (Van Der Lubben *et al.*, 2001; Read *et al.*, 2005), buccal (Giunchedi *et al.*, 2002), and ocular (De Campos *et al.*, 2004).

The mucoadhesive properties exhibited by chitosan have been further improved by derivatization to generate thiolated-chitosans. Factors that can alter the interaction between the polymer

and the mucosal layer can affect the mucoadhesive property. These include polymer molecular weight (Andrew *et al.*, 2009), polymer concentration (Solomonidou *et al.*, 2001) and drying method. Freeze-dried formulations offer stable products, extend shelf-life and allows storage of products at room temperature (Bunte *et al.*, 2010). However, during the freezing stage of freeze-drying, low molecular weight components tend to undergo incomplete crystallization resulting in the formation of mixtures of different polymorphs (Liao *et al.*, 2007). Such processes may cause reproducibility and product characterization problems. Annealing may be employed to foster crystallization of the ingredients and to improve pore size of ice crystals (Searles *et al.*, 2001a).

This report describes the development and evaluation of the functional characteristics of freeze-dried annealed and non-annealed thiolated-chitosan wafers, as potential drug delivery systems via the buccal mucosa. The wafers were characterised for moisture content, mucoadhesion properties and microscopic structure using thermo-gravimetric analysis (TGA), texture analysis (TA) and scanning electron microscopy (SEM) respectively. The conformational stability of bovine serum albumin (BSA) was studied with attenuated total reflectance-Fourier transform infra-red spectroscopy (ATR-FTIR) and circular dichroism (CD). The effect of annealing on hydration, mucoadhesion and the release of BSA from the formulations were also evaluated.

MATERIALS AND METHODS

Materials

Chitosan (medium molecular weight, 190-310 kDa, 75-85 % deacetylated), BSA (mol wt ~ 66 kDa), glycerol, D-mannitol, Bradford reagent, thioglycolic acid (TGA), N-(3-dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDAC), L-cysteine hydrochloride, 5,5'-dithiobis (2-nitrobenzoic acid) (Ellman's reagent) and mucin (from bovine submaxillary gland, Type I-S) were all obtained from Sigma (Gillingham, UK). Pulverised gela-

tine was purchased from Fischer Scientific (Loughborough, UK). All other reagents were of analytical grade and were used without further purification.

Chitosan-4-thioglycolic acid (CS-TGA) synthesis and purification

Synthesis of CS-TGA was by method previously described (Saboktakin *et al.*, 2011). 500 mg of CS was dissolved in 50 mL of 0.1 M HCl. An equivalent of 500 mg of thioglycolic acid (TGA) was added followed by 50 mM EDAC in order to activate the carboxylic acid moieties of TGA, the pH adjusted to 5 with 1M NaOH at room temperature and stirred for 3 hours. Purification to remove unconjugated TGA and sulfhydryl immobilization determination of CS-TGA was by the method reported in literature (Bernkop-Schnurch *et al.*, 2004), using Float-A-Lyzer G2 dialysis device (8-10 kDa Molecular weight cut-off, Sigma Gillingham, UK) and Ellman's reaction with standard cysteine HCl curve ($r^2 > 0.99$) respectively.

Gel formulation and freeze-drying

Ten percent (per polymer weight) each of glycerol and D-mannitol as plasticizer and cryoprotectant respectively were added to the recovered dialysed gel and loaded with BSA as a model protein drug in a 2:1 CS-TGA: BSA ratio. The final formulated gel was stirred continuously for 30 minutes at room temperature to obtain a uniform gel which was kept under ambient conditions to remove all air bubbles.

Six grammes (6.0g) of the homogeneous gel was transferred into a mould (diameter 35mm) and freeze-dried using a novel freeze-drying cycle developed with differential scanning calorimetry (DSC) (Ayensu *et al.*, 2011) on a Virtis AdVantage XL 70 freeze dryer (Biopharma Process Systems, Winchester, UK). An annealing temperature of -25°C (3 hours) was applied during the freezing phase (-55°C). Primary and secondary drying were at -30°C and 20°C respectively, with a vacuum of 20 mtorr for 34 hours. The effect of annealing on the physico-mechanical properties of the

drug loaded formulations was determined by freeze-drying with or without the process of annealing. Resultant freeze-dried wafers were stored over anhydrous silica gel till ready for characterisation.

Physico-mechanical characterisation of wafers

SEM

The external surfaces of the freeze-dried wafers were placed on the exposed side of a double sided carbon adhesive tape on labelled stainless steel stubbs and sputter coated with gold for 120 seconds at 1 kV and 30mA (Edwards Sputter Coater S150B). They were then placed in the chamber of a Cambridge Stereoscan S-360 SEM (Class one equipment, London UK). Images were processed with i-scan2000 software after acquisition at an accelerating voltage of 20KV and a working distance of 15 mm.

Swelling capacity

The wafers were initially weighed and the swelling behaviour observed at predetermined time intervals (Wu *et al.*, 2009) by incubation at $37 \pm 0.1^\circ\text{C}$ in 25mL of 0.1M PBS solution (pH 6.8 ± 0.1 simulating salivary pH). The samples were removed and blotted off carefully between tissue papers to remove the surface-adhered liquid droplets and reweighed on an electronic balance to a constant weight. The percentage of water uptake was calculated as follows:

$$\text{Swelling capacity (\%)} = 100 \times (P_i - P) / P.. \quad (1)$$

where P_i is the weight of the hydrated wafer, and P is the initial weight of wafer.

Moisture content

The estimation of percentage residual moisture of the freeze-dried wafers was by Thermal Advantage TGA 2950 (TA Instruments, Crawley UK) (Wang, W. 2000). Wafers weighing between 3 – 6mg were placed in a previously tared 70 μ l aluminium crucible and a constant phase of heating at $15^\circ\text{C}/\text{min}$ from ambient temperature to a maximum of 150°C

was applied with a constant stream of dry nitrogen. The percentage weight loss from a second derivative plot was determined using TA Universal Analysis 2000 programme.

In vitro mucoadhesion studies

Mucoadhesive measurements were performed on the freeze-dried annealed and non-annealed CS-TGA-BSA wafers using our previously reported method (Ayensu *et al.* 2011). Briefly, samples ($n = 4$) were attached to a 75mm diameter adhesive rig probe with a double sided adhesive tape on a TA.HD.plus Texture Analyser (Stable Micro Systems, Surrey, UK) fitted with a 5kg load cell in tension mode. A set gelatine gel in a Petri-dish (diameter 88mm) was used to represent the buccal mucosal surface for the *in vitro* measurement. The model mucosal substrate was equilibrated with 2% mucin solution (adjusted to pH of 6.8 ± 0.1) for 60 seconds and placed on the instrument platform. The probe, lined with wafer was set to approach the model mucosal surface with pre-test speed of 0.5mm/s, test speed of 0.5mm/s and post speed of 1.00mm/s. The Texture Exponent 32 software was employed to record and process the data. The maximum force required to detach the wafer on the upper probe from the mucosal surface, known as the peak adhesive (PAF) was determined. The area under the curve (AUC) representing total work of adhesion (TWA) of the wafers was estimated from the force-distance plot whiles the cohesiveness of the samples was determined by the distance of travel. The influence of annealing on muco-adhesive strength of the thiolated wafers was investigated.

In vitro BSA release profiles

The freeze-dried wafers were immersed in beakers containing 50ml of 0.1M PBS (pH 6.8 ± 0.1) solution as release medium at $37 \pm 0.1^\circ\text{C}$, covered (Nochos *et al.*, 2008) and stirred at 150rpm with a magnetic stirrer. Fifty μL of each sample was withdrawn at predetermined time intervals, replaced with the same amount of the release medium to maintain a constant volume for 7 hours and treated with 1mL Brad-

ford's reagent and the absorbance measured at 595nm and 450nm (Ernst and Zor, 2010). A linearised calibration curve ($r^2 > 0.99$) was used to determine the concentration of the BSA released and the corresponding cumulative percentage drug release profiles of BSA were then plotted.

Stability of protein in freeze-dried wafers

DSC

The freeze-dried CS-TGA wafers were studied for possible phase separation after freeze-drying on the DSC. This involved the initial cooling on the DSC at 10 °C/min from 25 to -50 °C. It was then heated at a rate of 20 °C/min to 200 °C and the cycle repeated. An empty aluminium pan was used as reference. Mettler STARe software was used to analyse and evaluate the DSC thermograms.

ATR- FTIR analysis

ATR-FTIR spectra of the samples were acquired on an Excalibur series FTS 3500 ARX FTIR, equipped with Specac Golden gate (Varian, Oxford UK). Win IR PRO software of wave number range 600 – 4000 cm^{-1} was used for the analysis. The spectra were collected at a resolution of 1.5 cm^{-1} with 16 scans per spectrum. A background spectrum was acquired and assigned for use on subsequent spectral acquisitions for each sample.

Conformational stability of BSA- CD

The secondary structure of BSA in the freeze-dried wafers was examined using far-UV CD. Spectrum of a 0.5mg/mL solution of native BSA dissolved in 0.1M PBS (pH 6.8) as the negative control or BSA released from the freeze-dried wafers was recorded at 25 °C from wavelength range $240 \geq \lambda \geq 190$ with bandwidth 1nm, time-per-point 0.5 s, and cell path-length of 0.1 mm using CD spectroscopy (Chirascan, Applied Photophysis, Surrey, UK). The mean residue ellipticity $[\theta]_{\text{mrw}, k}$ was determined by:

$$[\theta]_{\text{mrw}, k} = \text{MRW} \times \theta_{\text{c}} / 10 \times d \times c \quad (2)$$

where θ_k is the observed ellipticity (degrees) at wavelength k , d is the pathlength (cm), and c is the concentration (g/mL) (Kelly *et al.*, 2005).

Statistical evaluation

Statistical evaluation of data was carried out using two tailed student t-test with 95 % confidence interval as the minimal level of significance.

RESULTS AND DISCUSSION

Chitosan-4-thioglycolic acid (CS-TGA) synthesis and purification

In the present study, TGA was bound to CS via amidation between the carboxylic group of TGA and the primary amino groups of CS. The reactivity of the primary amino group at the 2-position of glucosamine subunit of chitosan is exploited for the immobilization of thiol groups. The process was performed at a $\text{pH} \leq 5$ to prevent the accidental formation of disulphide bonds by air oxidation during synthesis. The reactive thiolate anion concentration for the oxidation of thiol groups is low at this pH, which prevents the formation of disulphide bonds. Ellman's reagent was employed in the determination of the amount of reduced and oxidized thiol groups immobilized (Hornof *et al.*, 2003) without previous quantitative reduction of disulfide bonds with borohydride. The CS-TGA conjugate exhibited 236 ± 26 μmol thiol groups per gram of polymer. A degree of modification of 25–250 mmol thiol groups per gram of chitosan leads to the highest improvement in the mucoadhesive and permeation enhancing properties of these thiolated chitosans (Sreenivas and Pai, 2009).

Gel formulation and freeze-drying

Flexible, tough, non-brittle and porous freeze-dried annealed and non-annealed CS-TGA-BSA wafers were produced with medium molecular weight chitosan as the base matrix. The primary drying phase of the freeze-drying cycle was deemed complete when the product temperature measured by means of probes equalled the shelf temperature of -30 °C after 14 hours.

Annealing as a process step maintains samples at a specified temperature below the equilibrium freezing point but above the glass transition temperature. The incorporation of an annealing process helps to achieve faster water vapour transport, shorter primary drying time and elegant porous wafers (Searles *et al.*, 2001b). This allows for the formation of larger ice crystals resulting in increased sublimation rate during the primary drying stage, creating larger pores in the process. In addition, the process of annealing facilitates crystallisation of active ingredients and the cryoprotectant (mannitol). This promotes solute and ice crystal growth and prevents premature crystallisation of metastable glass such as D-mannitol.

Physico-mechanical characterisation of wafers

SEM

SEM was used to study the pore size and structure of the freeze-dried wafers (morphology). Figure 1 shows representative micrographs of the freeze-dried wafers. The micrographs from the SEM show the effect of annealing on the pore size. Annealing promotes the merging of smaller ice crystals to form larger ones, the sublimation of which creates larger pores. The wafers formed an interconnecting network of polymeric circular pores, with comparable pore size distribution. The annealed CS-TGA-BSA wafers however, formed more fibrous spongy-

like porous network.

Swelling capacity

The difference in the swelling capacities of the annealed and the non-annealed CS-TGA-BSA wafers (Table 1) was statistically significant ($p < 0.003$). It is worth noting that the annealed wafers maintained their structural integrity during the incubation period (4 hours) due to the stronger mechanical structure. This was not the case for the non-annealed wafers which formed loose jelly-like and rather unstable swollen structures that consequently disintegrated upon removal from the hydration medium after two (2) hours of incubation at 37 °C.

Moisture content

The residual moisture contents of the freeze-dried wafers are shown in Table 1. The annealed wafers had a slightly lower residual moisture content compared to the non-annealed products; however, no significant statistical difference was observed ($p > 0.3$). The loss in weight observed for all the samples during the constant heating stage occurred between 60 °C and 120 °C, an indication of the fact that weight loss was due to bound water. Attaining adequate residual moisture content for wafers is vital as lower water content reduces molecular mobility and increases shelf-life by avoiding premature hydration of the active protein drug (Bunte *et al.*, 2010).

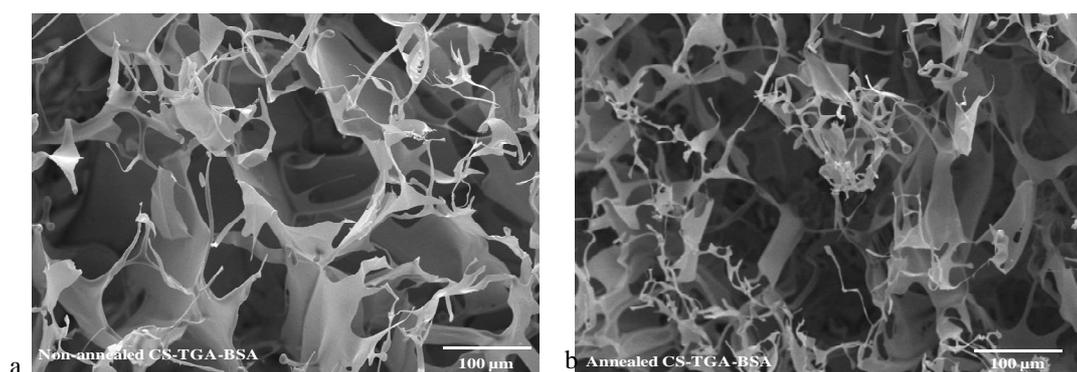


Fig. 1: Representative SEM micrographs showing annealed and non-annealed CS-TGA-BSA wafers ((a) Non-annealed CS-TGA-BSA (b) Annealed CS-TGA-BSA)

Table 1: Percentage drug loading capacity, moisture content and hydration capacity of wafers

Sample	% Moisture content (<i>n=4, mean ± SD</i>)	% swelling capacity (<i>n=4, mean ± SD</i>)
Annealed CS-TGA-BSA	1.7 ± 0.1	482 ± 18.2
Non Annealed CS-TGA-BSA	1.9 ± 0.4	434.0 ± 3.1

In vitro mucoadhesion studies

The process of annealing led to significant differences in the mucoadhesive properties of the CS-TGA wafers. The annealed CS-TGA-BSA wafers showed significant improvement in mucoadhesive properties (Fig. 2) compared to the non-annealed CS-TGA-BSA wafers. The annealed CSTGA-BSA wafers with enhanced swelling capacity had higher PAF and cohesiveness compared to the non-annealed CS-TGA-BSA wafers. The total work of adhesion (TWA) further showed a statistically significant difference ($p < 0.0001$) between the annealed and non annealed CS-TGA-BSA wafers, with an approximately 4-fold increase in mucoadhesive strength. The major differences observed in the PAF, TWA and cohesiveness of the wafers were largely due to the increased mucoadhesive strength impacted by the mechanically strong and fibrous porous structure of the annealed wafers.

In vitro BSA release profiles

The dissolution profiles of BSA from the freeze-dried wafers in 0.1M PBS is shown in Fig. 3. The total cumulative percent BSA release from the annealed and non annealed CS-TGA-BSA CS-BSA were 95.2 ± 7.3 % and 84.0 ± 4.7 % respectively. This difference is considered to be statistically significant ($p < 0.05$) with both formulations exhibiting sustained release profiles, typical of chitosan based formulations. The drug release may have been facilitated by the porous network of freeze-dried wafers as a result of increased surface of the dispersed drug in the porous cake which accelerates dissolu-

tion significantly (Bunte *et al.*, 2010). The total percent release was higher from the annealed CSTGA than the non annealed CS-TGA wafers due to its higher rate of hydration and enhanced cohesiveness. The cohesion and stability of a drug delivery system over the intended duration of drug release is often a requirement for controlled release (Peppas and Bury, 1985). The lower total percent release of BSA could be attributed to the reduced cohesiveness of the non-annealed wafers.

Stability of protein in freeze-dried wafers***DSC***

Multiple glass-transitions detected by DSC after freeze-drying indicate possible phase separation of protein in the freeze-dried wafer and/or presence of metastable crystalline structures. For phase separation studies after freeze-drying, no glass-transition temperatures were detected in the freeze-dried wafers, an indication of product stability during storage.

ATR- FTIR analysis

To further investigate the secondary structure of BSA in the drug loaded wafers, ATR-FTIR spectroscopy was employed. Structural information was obtained for the protein loaded freeze-dried wafers through the analysis of the conformationally sensitive amide I band, which is located between 1600 and 1700 cm^{-1} (Fig. 4). 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

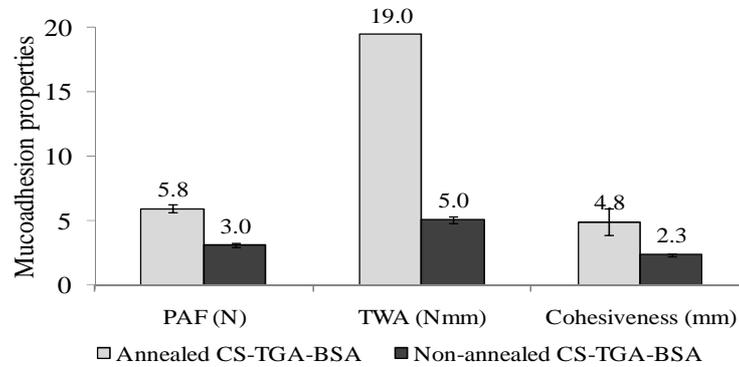


Fig. 2: *In vitro* mucoadhesion force measurements of wafers showing the PAF, TWA and cohesiveness ($n=4$ mean \pm sd)

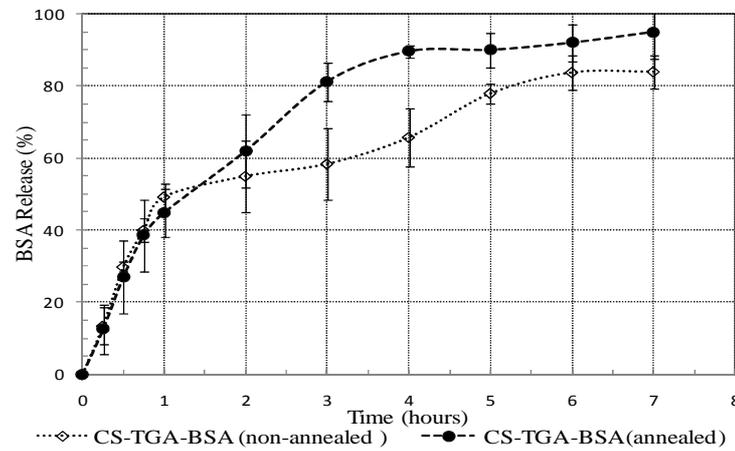


Fig. 3: Cumulative percent BSA release from annealed and non-annealed CS-TGA-BSA wafers ($n=4$ mean \pm sd)

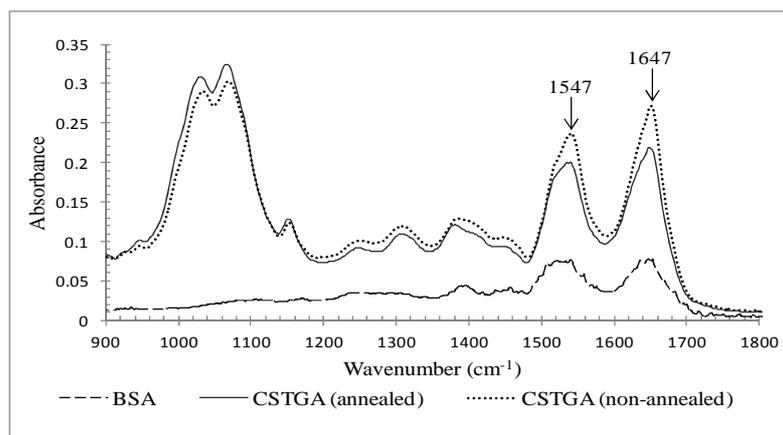


Fig. 4: ATR-FTIR Spectra of native BSA, annealed and non-annealed CS-TGA-BSA wafers

frequency which has a characteristic band position. The ATR-FTIR spectra show that the absorption peaks of annealed and non annealed CS-TGA-BSA at 1647cm^{-1} (amide I band) were stronger than those of native BSA due to the presence of chitosan. The absorption peak at 1574cm^{-1} is usually attributed to amide II band (C-N stretching and N-H bend) present in both BSA and chitosan.

In all FT-IR patterns, the main peaks corresponding to α -helical remained unchanged, suggesting that the protein structure maintained its folded conformation, and was neither degraded nor hydrolyzed during the production of freeze-dried CSTGA- BSA wafers irrespective of annealing process.

Physical stability of BSA- CD

The conformational stability of *in vitro* released BSA from the freeze-dried wafers was assessed by CD. The CD profile of native BSA and BSA released from the wafers, both in PBS at pH 6.8 in far UV range are shown in Fig. 5. As is evident from this figure, the native BSA had two minima around 208 and 222nm; the first band was assigned to α -helical structure, while the second band was assigned to the β -structure.

These results are consistent with the crystal structure of BSA (Quiming *et al.*, 2005). The ratios between the mean residue ellipticity at 208 and 222nm ($[\theta]_{208}/[\theta]_{222}$) were 1.10, 1.11 and 1.11 for native BSA and BSA released from the annealed and non annealed CSTGA-BSA respectively.

The similarity of the far-UV CD spectra and the mean residue ellipticity obtained confirmed the conformational stability of BSA in the freeze-dried wafers.

CONCLUSION

The various techniques used to characterise the freeze-dried annealed and non annealed thiolated chitosan wafers have indicated that the hydration capacity, mucoadhesion properties and release characteristics are influenced by the annealing process. The annealing step in the freeze-drying cycle helped to obtain wafers with the advantage of enhanced mucoadhesion properties, hydration capacity and consequently drug release property. The final freeze-dried product was an elegant, mechanically strong cake and is expected to exhibit long-term storage stability as the secondary structure of BSA was found to be conformationally stable. The principal implication of these findings is the

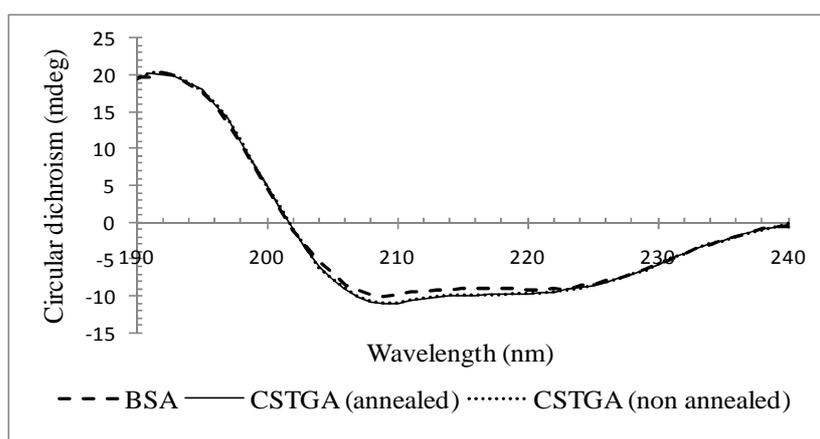


Fig. 5: CD Spectra of native BSA, annealed and non-annealed CSTGA-BSA wafers

potential application of the novel freeze-dried system for the delivery of proteins via the buccal mucosa.

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