Development functional characterization of alginate dressing as potential protein delivery system for wound healing

Frederick U. Momoh\textsuperscript{a,1}, Joshua S. Boateng\textsuperscript{a\#,1}, Simon C.W. Richardson\textsuperscript{b}, Babur Z. Chowdhry\textsuperscript{b}, John C. Mitchell\textsuperscript{a}

\textsuperscript{a} Department of Pharmaceutical Chemical and Environmental Sciences, Faculty of Engineering and Science, University of Greenwich at Medway, Central Avenue, Chatham Maritime, ME4 4TB, Kent, UK.

\textsuperscript{b} Department of Life and Sports Sciences, Faculty of Engineering and Science, University of Greenwich at Medway, Central Avenue, Chatham Maritime, ME4 4TB, Kent, UK.

\# Correspondence: Dr Joshua Boateng J.S.Boateng@gre.ac.uk joshboat40@gmail.com

\textsuperscript{1}Joint First Authors
ABSTRACT

This study aimed to develop and characterize stable films as potential protein delivery dressings to wounds. Films were prepared from aqueous gels of sodium alginate (SA) and glycerol (GLY) (SA:GLY 1:0, 1:1, 1:2, 2:3, 2:1, 4:3). Purified recombinant glutathione-s-transferase (GST), green fluorescent protein (GFP) and GST fused in frame to GFP (GST-GFP) (model proteins) were characterized (SDS PAGE, Western blotting, immune-detection, and high sensitivity differential scanning calorimetry) and loaded (3.3, 6.6 and 30.2 mg/g of film) into SA:GLY 1:2 film. These were characterized using texture analysis, differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), scanning electron microscopy, swelling, adhesion, dissolution and circular dichroism (CD). The protein loaded dressings were uniform, with a good balance between flexibility and toughness. The films showed ideal moisture content required for protein conformation (TGA), interactions between proteins and film components (DSC), indicating stability which was confirmed by CD. Swelling and adhesion showed that formulations containing 6.6mg/g of protein possessed ideal characteristics and used for in vitro dissolution studies. Protein release was rapid initially and sustained over 72 hours and data fitted to various kinetic equations showed release followed zero-order and Fickian diffusion. The results demonstrate the potential of SA dressings for delivering therapeutic proteins to wounds.

Key words: Alginate dressing, GST-GFP Proteins, Wound healing.
1. Introduction

A wound is defined as a disruption of normal anatomic structure and physiology [1] of a tissue and represents damage of natural defense barriers which encourages invasion by microorganisms [2]. The process of wound regeneration is a complex combination of matrix destruction and reorganization [3] which requires well-orchestrated processes that lead to the repair of injured tissues [4]. These processes are integrations of complex biological and molecular events culminating in cell migration, proliferation, extracellular matrix deposition and the remodeling of scar tissues [5]. This process is driven by numerous cellular mediators including cytokines, nitric oxide, and various growth factors [6] (most of them proteins) which stimulate cell division, migration, differentiation, protein expression and enzyme production. Their wound healing properties are mediated through the stimulation of angiogenesis and cellular proliferation [7] which affects the production and degradation of the extracellular matrix and also plays a role in cell inflammation and fibroblast activity [8]. The field of biologic wound products aims to accelerate healing by augmenting or modulating these inflammatory mediators. These products have experienced remarkable growth as our understanding of the wound healing response has increased [6], coupled with the large number of recombinant proteins being investigated for therapeutic applications.

Alginate dressings are bioactive formulations composed of a polysaccharide polymer called alginic acid which contains guluronic and mannuronic acid units [9]. These dressings can occur in the form of fibers rich in mannuronic acid (e.g. Sorbsan™) which form flexible gels upon hydration or those rich in guluronic acid residues which form firmer gels upon exudate absorption (e.g. Kaltostat™). Alginate dressings are non-toxic and aid in hemostasis as part of the wound healing process [10-13]. In addition, they activate human macrophages to produce tumor necrosis factor-α (TNFα) which initiates inflammatory signals [14].
The therapeutic effects of large macromolecules such as proteins and growth factors are limited by their low bioavailability and poor stability, whilst multiple injections can result in poor patient compliance. Therefore, drug delivery systems such as adhesive film dressings present a valid approach to overcome these limitations since films are simple, easy to prepare and characterize. Further, being in the dry state, it’s easy to incorporate and stabilize labile proteins without the need for more expensive drying approaches such as freeze-drying, however, this depends on the type of protein and the temperature of drying. It has been proposed that films have potential to be used to deliver genetic and protein based molecules to wound sites [15]. Alginate film dressings are easily biodegradable and painlessly removed via saline irrigation when trapped in the wound thus preventing damage to newly formed granulation tissue [16, 17].

The requirement of wound management products with ideal characteristics has necessitated the need for advanced formulations such as alginate having improved physico-mechanical properties and general functional performance such as bioadhesion, but which are also able to actively take part in the wound healing process [2, 18]. In this study, we report on the use of film dressings formulated from two readily biodegradable materials; SA (film forming polymer) and GLY (plasticizer), loaded with recombinant proteins (GST, GFP and GST-GFP) as model protein drugs for potential wound healing. Films were prepared from aqueous gels of SA by solvent casting and characterized for functional characteristics expected for wound dressings.

2. Experimental

2.1 Materials
Nitrocellulose membrane, thiazolyl blue tetrazolium bromide, polyethyleneimine (branched, Mn 60000), dextran (Mw 35000-45000), isopropyl-β-D-1-thiogalactopyranoside (IPTG), L-glutathione, guanidine hydrochloride, MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma (Gillingham, UK). Tryptone was obtained from Oxoid, (Hampshire, UK). Yeast extract, dimethyl sulfoxide (DMSO), tris(methylamine and sodium chloride were obtained from Fisher Scientific, (Leicestershire, UK). Glutathione sepharose 4B, ECL Western blotting detector reagents 1 and 2 were obtained from GE HealthCare, (Buckinghamshire, UK). Acrylamide/Bis 37.5:1 and Bradford reagent (1x) were obtained from Bio-Rad, (Hempstead, UK). Anti-rabbit immunoglobulin (IgG)-Horseradish peroxidase (HRP) conjugated and GFP were obtained from Invitrogen, (Paisley, UK). Anti-Rabbit IgG-HRP and GST were obtained from Abcam, (Cambridge, UK). Recombinant GST-GFP, GST and GFP were prepared in house (Richardson lab, University of Greenwich, UK). Sodium alginate [medium viscosity (≥2000 cps) grade; M/G ratio of 1.56], glycerol and bovine serum albumin were all obtained from Sigma-Aldrich, (Gillingham, UK). Dulbecco’s-modified eagle’s medium (D-MEM), PBS, penicillin, streptomycin and glutamine were all obtained from Gibco, (Paisley, UK). Gelatin was obtained from Fluka Analytical, (Steinheim, Germany) and calcium chloride from Sigma Aldrich, (Steinheim, Germany).

2.2 Recombinant protein preparation, purification and characterization

The protein production, purification, immuno-detection and characterization were performed according to that previously reported [19, 20]. The eluted proteins (GST-GFP, GST and GFP) were sealed in cellulose acetate dialysis membrane and dialyzed against 4L of cold 1x PBS (4°C) overnight and changing the dialysis buffer every 2 hours afterwards with a minimum of 4 changes of (1x) PBS. 15μL each of purified proteins [GST-GFP (5μg), GST (2mg) and GFP (1mg)] and controls [Spectra Multicolor broad range protein molecular weight ladder
(Fermentas, Cambridgeshire, UK) and bovine serum albumin (BSA) standards (75µg)] were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) apparatus using 6M guanidine containing Laemmli buffer and 10% (v/v) beta-mercaptoethanol (BME), with a running buffer (1x) as per manufacturer’s instructions. The loaded samples were resolved by applying 100V of direct current for 80 minutes. The gel was then stained with Coomassie brilliant blue for 2 hours and de-stained with Coomassie de-staining solution for another 2 hours, further soaked in 5% (v/v) glycerol / PBS and dried overnight using a gel drying kit (Promega, Hampshire, UK). Western blotting and immuno-detection was used to detect GFP-GST after separation and its immobilization on a solid phase-support. The experiment was performed in accordance with the manufacturer’s instructions and as previously reported [19]. The specific protein bands were identified by superimposing the developed X-ray film onto the membrane in the cassette.

2.3 Preparation of film dressings
Various sodium alginate (SA) gels (1% w/w) with and without plasticizer (GLY) were employed to determine the best SA:GLY ratio (SA:GLY – 1:0, 1:1, 1:2, 2:3, 2:1, 4:3) for the preparation of uniform and homogeneous films. Drug loading was achieved by formulating the selected optimized film prepared above, with increasing drug concentrations (3.3, 6.6 and 30.2mg/g of film) for all three proteins. SA was added gently and in small quantities (so as to avoid formation of lumps) to warm PBS (45°C) in a beaker and magnetically stirred until SA was completely dissolved (2 hours) to yield a clear homogeneous gel. The required amount of GLY was added to the gels with continuous stirring and heating for a further 1 hour. The model proteins were added to the optimized gel with gentle stirring and heating (45°C) until a homogenous mix was obtained (1 hour) and allowed to stand for 5 minutes (to remove air
bubbles). 30g was poured into Petri dishes (90mm diameter) and placed in a vacuum oven at 40°C for 18 hours.

2.4 MTT cytotoxicity assay

MTT assay was used to evaluate the cytotoxicity of the proteins and SA using dextran (Mw 35,000-45,000) and polyethyleneimine PEI (branched, Mn ~ 60,000), as negative and positive controls respectively. Adherent Vero cells (1x10^4 cells/well) were used to seed a sterile, flat-bottom 96-well tissue culture plate containing Dulbecco’s modified eagles medium (D-MEM) plus 10% (v/v) PBS, penicillin (100U/mL), streptomycin (100µg/mL) and glutamine (292µg/mL) (all under sterile conditions in a laminar hood) and incubated at 37°C in 5% (v/v) CO₂ for 24 hours. After 24 hours, the cells were exposed to either PEI, dextran, GST-GFP, GST and GFP (0-3mg/mL) in cell culture medium and incubated for 68 hours. 10µL (50µg) of MTT from stock solution (5mg/mL) was added to each well and the plate incubated for a further 4 hours bringing the total incubation time to 72 hours. The contents of the plate were decanted and 100µL of DMSO was added to each well, incubated at room temperature for 30 minutes and the absorbance read on a Multi-scan EX Micro-plate photometer (Thermo Scientific, Essex, UK) at optical density (OD) 540nm. For SA however, adherent cells (Vero, 1x 10^4) were exposed to SA gel after 24 hours. Data obtained was expressed as percentage cell viability (mean ± standard deviation of the mean).

2.5 Thermal analysis

2.5.1 High sensitivity differential scanning calorimetry (HSDSC)

Preliminary characterization of the three model proteins were investigated using HSDSC determining the effect of pH (6.0, 7.5, 8.0 and 10.0), scan rate (0.5, 1.0 and 2.0°C/minute), protein concentrations (1.0, 2.5 and 5.0mg/mL) and reversibility. Degassed buffer and protein
solutions (800µL) were loaded into the reference and sample capillary cells using a calibrated automatic pipette. The cells were covered using rubber caps on same sides. The entire cell chamber was then tightly covered with the chamber lid to maintain constant pressure and samples analyzed with a pressure of 3 atmospheres, equilibration for 600 seconds and heating from 10°C to 95°C at the scan rates above. Prior to sample analyses (both water and buffer scans were run using the same parameters described above for analyzing the samples and showed a flat baseline which was used as reference scans before analyzing the samples.

2.5.2 Differential scanning calorimetry (DSC)

Before analyzing of the samples, the DSC instrument was calibrated. Two different calibration experiments of the DSC machine (Q2000 TA instrument. The first experiment was performed in two stages i.e. determination of the cell resistance and capacitance. The determination of the cell resistance was performed with an empty cell. During this experiment, the cell was equilibrated at -90°C and held at this temperature (isothermal) for 5 minutes, followed by a heating ramp from -90 to 400°C at a rate of 20°C/min. The determination of the cell capacitance, involved a similar experimental procedure as the cell resistance but sapphire discs of known weight and heat capacity were placed on the reference and sample cells. The second calibration experiment involved the determination of the cell constant and temperature calibration, which were obtained from a single experiment. In this experiment 1-5 mg of indium standard was pre-heated to which is above its melting transition temperature and held isothermally (5 minutes). The sample was then cooled to 100°C, held isothermally for a further 5 minutes and subjected to a heating ramp (10°C/min) to a temperature above the melting transition. The enthalpy of fusion was determined by integration and compared with the known value (28.71 J/g). The cell constant was calculated as the ratio between the experimentally determined and expected value and expected to be
between 1 and 1.2. The melting temperature was determined using the extrapolated onset value, and this was also compared with the known value (56.6°C) and the difference calculated for temperature accuracy.

DSC analysis was carried out on the starting materials (SA, GLY, GST, GFP and recombinant GST-GFP), formulated gels, as well as blank (non-protein) and protein loaded films. About 19.0-20.0mg of GLY and gels, 3.3-8.0mg of SA, blank and protein loaded films were loaded into tarred Tzero aluminium pans which were crimped and hermetically sealed with one pin hole on the lid using a Tzero sample press (TA instruments, Crawley, UK). The analysis was performed using a Q2000 calorimeter (TA Instruments, UK), under inert nitrogen (N₂) gas at a flow rate 50mL/minute, equilibration at -90°C, isothermal for 5 minutes and finally dynamic heating to 400°C at a heating rate of 10°C/minute.

2.5.3 Thermogravimetric analysis (TGA)
Tests were carried out on the starting materials [(SA, GLY), recombinant GST-GFP, GST and GFP (proteins) and the blank and protein loaded films. Analysis was carried out using a Q5000-IR TGA instrument (TA Instruments, Crawley, UK) by loading about 8.0 - 10.0mg (SA, GLY), 9.5-10.0mg (proteins) and 3.0-3.6mg (film). The analysis was performed under inert nitrogen (N₂) gas at a flow rate of 50mL/minute and dynamic heating from ambient (~25°C) to 600°C at a heating rate of 10°C/minute.

2.6 Tensile characterization
The tensile properties of the films (thickness, 0.1mm) were evaluated using a TA HD Plus (Stable Micro Systems Ltd, Surrey, UK) texture analyzer equipped with a 5kg load cell and a Texture Exponent-32® software program. The films (n=3), free of any physical defects (cracks
or tears) were cut into dumb-bell shapes and stretched between two tensile grips at a speed of 6mm/s using a trigger force of 0.1N until films broke. The distance between the grips was 3mm whilst the width of the films was 1mm. Testing was first carried out on the blank (non-protein loaded) films with different plasticizer concentrations (SA:GLY, 1:0, 1:1, 1:2, 2:3, 2:1, 4:3) to determine the film with optimum mechanical (tensile) properties [15] for protein loading. Further to this, tests were carried out on protein loaded films. The tensile strength (brittleness), Young’s modulus (rigidity/stiffness) and elongation (elasticity and flexibility) at break were determined from the force-time profiles using equations 1, 2 and 3.

\[
\text{Tensile strength (N/mm}^2\text{)} = \frac{\text{Force at break (N)}}{\text{Initial cross sectional area (mm}^2\text{)}} \quad \text{Equation 1}
\]

\[
\text{Elastic Modulus (mPa)} = \frac{\text{Slope}}{\text{Initial cross sectional area (mm}^2\text{)} \times \text{cross–head speed (mm/s)}} \quad \text{Equation 2}
\]

\[
\text{Elongation at break (\%)} = \frac{\text{increase in length (mm) at break}}{\text{initial film length (mm)}} \times 100 \quad \text{Equation 3}
\]

2.7 Scanning electron microscopy (SEM)
This was used to evaluate the surface morphology and topography of the films with and without proteins. Films were cut into rectangular (3x5mm) pieces and placed on the exposed side of a double-sided carbon adhesive tape stuck onto aluminum stubs (Agar Scientific, Essex, UK). Images were acquired using a Hitachi SU 8030 FEG-SEM (Hitachi High-Technologies, Tokyo, Japan) by generating secondary electrons at an accelerating voltage of 2kV and working distance of 15mm and magnification of x50.

2.8 Hydration and swelling
The swelling capacity of the formulated blank and protein loaded films were determined in simulated wound fluid (SWF) containing 0.02M calcium chloride, 0.4M sodium chloride, 0.08M tris-methylamine and 2% (w/v) bovine serum albumin in deionized water. The pH was adjusted to 7.5 using 2M HCl, mimicking chronic wound with pH reported to be in the range of 7.2 to 8.9 [21]. Films were cut into 2x2 cm strips, weighed and immersed in SWF (10mL). The weight change of the hydrated films was determined every 15 minutes for 120 minutes. Hydrated films (n = 3) were blotted carefully with filter paper to remove excess SWF on the surface and reweighed immediately on an electronic balance. The percentage swelling index (%Is) was calculated from equation 4.

\[
\%Is = \frac{W_s - W_d}{W_d} \times 100
\]

(Equation 4)

Where \(W_s\) is the weight of films after hydration and \(W_d\) is the weight of films before hydration.

2.9 In vitro wound adhesion

In vitro wound adhesion test was carried out on the blank and protein loaded films using a TA HD plus Texture Analyzer (Stable Micro System, Surrey, UK) fitted with a 5kg load cell in tension mode. Films (n=4) were cut to square strips (2x2cm) and attached to a 75mm diameter probe using double sided adhesive tape. Prior to testing, 20g of 6.67% (w/v) gelatin was poured into a Petri dish (90mm in diameter) and allowed to set at 4°C overnight. 500µL of SWF (pH 7.5) was spread evenly using an agar plate spreader so as to simulate a wound surface. The films were kept in contact with the gelatin solution for 1 minute before detachment. The probe was set at a pre-test speed of 0.5mm/s, test speed of 0.5mm/s, a post-test speed of 1mm/s, and an applied force of 1N. The peak adhesive force (PAF) representing maximum force required to separate the films from the simulated wound surface, the area under the curve (AUC) representing the total work of adhesion (TWA) and the cohesiveness
representing the distance travelled (mm) before detaching from the simulated wound surface were determined.

2.10 In vitro protein dissolution and release studies

The in vitro protein dissolution and release studies were carried out as previously described \cite{22}. A modified Franz diffusion cell with a wire mesh washed by 8mL SWF (pH 7.5, 37°C) was used to simulate the natural wound environment. The protein (6.6mg/g) loaded film dressings (50mg, n=4) were placed on the wire mesh. Aliquots (200μL) of SWF was withdrawn at regular intervals and analyzed using Bradford assay and replaced with same volume of fresh SWF (pH 7.5) to maintain a constant volume and sink conditions. The absorbance of the sampled aliquot was measured using a Multi-scan EX Micro-plate photometer (Thermo Scientific, Essex, UK) at 595nm and 450nm and the ratio of the absorbance values determined (from linearization of the curve as described in \cite{23,24}. The cumulative percentage (%) drug release was plotted against time and the proteins release kinetics determined by finding the best fit of the % release against time data to Higuchi (equation 5), Korsmeyer-Peppas (equation 6), zero order (equation 7) and first order (equation 8) equations.

\begin{equation}
Q_t = k_H t^{1/2}
\end{equation}

Equation 5

\(Q_t\) is the amount of drug released at time \(t\), \(k_H\) is the (Higuchian) release rate constant.

\begin{equation}
\ell_0 \left( \frac{Q_t}{Q_\infty} \right) = \ell_0 k + n \ell_0 t
\end{equation}

Equation 6

\(Q_t\) is the amount of drug released at a given time \(t\), \(Q_\infty\) is the amount of drug present initially, \(k\) is a constant involving the geometry and structural characteristics of the film and \(n\) release exponent.

\begin{equation}
Q_t - Q_0 = k_0 t
\end{equation}

Equation 7

\(Q_t\) is the amount of drug released in time \(t\), \(Q_0\) is the amount of drug dissolved at time zero and \(k_0\) is the zero-order release rate constant.
\[ t_n \left( \frac{Q_\infty}{Q_1} \right) = k_1 t \]  
Equation 8

\[ Q_\infty \text{ is the initial total amount of drug present, } Q_1 \text{ is the amount of remaining drug at time } (t) \text{ and } k_1 \text{ is the first order release rate constant.} \]

2.11 Far-UV circular dichroism spectroscopy

The conformational (secondary) structures of the pure model proteins (GST-GFP, GST and GFP) and released protein from the films dressings were examined in the far-UV region of a circular dichroism (CD) instrument; wavelength range (190–260nm), band width (1nm), path length (0.01cm) and 10 seconds time per point, in 0.01M PBS (pH 7.5) at 20°C using a Chirascan CD spectrometer (Chirascan, Applied Photophysics, UK).

2.12 Statistical analysis

The various formulations and experimental variables used to characterize the films were compared by statistical data evaluation (Microsoft Excel, Office 2013 software) using a two tailed student t-test at 95% confidence interval (p-value < 0.05) as the minimal level of significance.

3 Results

3.1. Protein characterization

The molecular weights of the proteins observed on the gel were 52kDa, 27kDa and 28kDa confirming the proteins of interest i.e. GST-GFP, GFP and GST (pGEX3x and pGEX5x) respectively. The molecular weights observed from immune-blotting: GFP (27 kDa), GST (28 kDa) and GST-GFP (52kDa), shown in Fig. 1a, 1b and 1c respectively, correspond to that reported in the literature [19] and confirmed the Coomassie observations.
3.2 MTT cytotoxicity assay

Fig. 2 shows the toxicity profile for dextran and PEI, GST-GFP, GST, GFP and SA respectively (n = 6). The results showed 5-10% cell viability for PEI with cell death at 72 hours and 100% cell viability for dextran as was expected. Almost 100% cell viability was observed for GST-GFP, GST, GFP and SA after 72 hours, with negligible cell death noticed and therefore, all three proteins and SA were confirmed as non-toxic. The results (Fig. 2F) show a clear profile of the cytotoxicity of SA on adherent epithelial mammalian cells (Vero (ATCC® CCL-81™) confirming that SA is non-toxic under the conditions tested. This is not surprising since SA is approved for oral formulations and moist wound dressings and therefore the results here confirm its safety for use as a protein delivery dressing for wound healing.

3.3 Thermal analysis

3.3.1 High sensitivity differential scanning calorimetry (HSDSC)

Table 1 shows the HSDSC profiles of the three proteins obtained by varying three main experimental conditions (scan rate, pH and concentration). Detailed description of the results showing the effect of the three experimental variables on the HSDSC profiles are provided as supplementary data in appendix A1.

3.3.2 Differential scanning calorimetry (DSC)

All three proteins showed similar characteristics as observed in their thermograms (Fig. 3A). Detailed descriptions of the DSC results for the pure proteins are given in appendix A2. GLY showed two endothermic peaks at 136.54°C and 293.67°C attributed to water loss and boiling (Fig. 3B) whilst SA showed one endothermic peak at 109.23°C and an exothermic peak at 242.59°C (Fig. 3C) that can be attributed to dehydration and thermal degradation of intermolecular side chains respectively [26, 27]. Differences were observed between the DSC
thermograms of the blank and protein loaded films (Fig. 3D) which could be an indication of interaction between the polymer and proteins. The blank film was characterized by two endothermic transitions at 98.61°C and 250.05°C (Fig. 3D). However, the protein loaded films showed four endothermic transitions with multiple stages of polymer degradation with the exception of GST (30.2, 3.3mg/g) and GST-GFP (30.2mg/g) respectively, which showed two endothermic transitions (Fig. 3D). The high dehydration temperatures seen in both blank and protein loaded films with endset peak at 126.32°C can be attributed to bound water molecules within the polymeric film allowing for more hydrophobic interactions between protein molecules.

3.3.3 Thermogravimetric analysis (TGA)

Table 2 shows the different (1st – 4th) thermal events and the dynamic weight loss associated with those events. In all cases, the first dynamic weight loss observed can be attributed to desorption of water hydrogen bonded to the polymer structure [28]. SA powder had higher moisture content (18.24%) than the films (6.52-16.68%) which could be attributed to the drying process employed when formulating the films. The peak temperature at which the moisture content within the blank film matrix was lost was significantly lower (45.1°C) than those of the protein loaded films. This bonded water can be clearly seen in all protein loaded film temperatures ranging from 53.9°C to 112.2°C. The degradation temperatures decreased for all the films in comparison to the starting material (SA). This can be attributed to the effect of the formulation process in changing the physiochemical properties of the starting material due to interactions between the components of the formulation. SA showed a three stage degradation process (236.7°C, 257.6°C and 388.7°C) that can be attributed to the presence of carbonaceous residues [29]. However, GLY only showed one main thermal event above 200°C at a temperature of 220.4°C which might relate to boiling as observed in DSC, though the
temperatures are different. This shows that the starting materials (SA and GLY) are thermally stable up to temperatures above 200°C.

3.4 Mechanical tensile characterization

Table 3A shows that unplasticised films (SA:GLY 1:0) were highly brittle as evidenced by having the lowest % elongation (1.85 ± 0.19%) and highest values for both elastic modulus and tensile strength, implying these could cause trauma to newly formed skin cells on a healing wound [15]. However, addition of GLY caused a general increase in flexibility as evidenced by the increased % elongation (from 1.85 to 38.84%) and decrease in both Young’s modulus (rigidity) (from 20.77 to 0.40mPa) and tensile strength (brittleness) (from 51.34 to 6.12 N/mm²). This can be attributed to GLY interpolating itself between SA polymer chains resulting in reduced interaction and the intermolecular cohesive forces between the polymer chains [30, 31].

Table 3B shows the variations in tensile profiles based on the type and amount of protein for the optimized films (SA:GLY 1:2). The % elongation at break reduced from 38.84 ± 0.86% for blank films to between 23.31 ± 4.04 and 5.46 ± 0.92% depending on the type and amount of protein loaded. These values are below that considered ideal for wound dressing as it suggests lower elasticity. However, the elastic (Young’s) modulus and tensile strength values showed the films were not too brittle and this was confirmed during physical handling of the drug loaded films. Further, the three different protein loaded films possessed different levels of flexibility with GST-GFP films having the highest flexibility (highest % elongation) as opposed to GST and GFP loaded films. This could be as a result of GST-GFP being a construct of both proteins, therefore an increase in molecular weight.
From the results in Table 3, it can be seen that on the whole, Young’s modulus decreased with increasing concentrations of proteins with the exception of GFP where the value increased from 0.97 ± 0.40 mPa for 3.3 mg/g film to 2.14 ± 0.34 mPa for 6.6 mg/g film but then decreased to 0.88 ± 0.17 mPa for the 30.2 mg/g film. This suggests that the protein incorporated in the films improved the films toughness and ability to withstand mechanical pressure whilst maintaining enough flexibility. Generally, a decrease in tensile strength was observed for most of the protein loaded films (except GFP 6.6 mg/g and GST 3.3 mg/g films) in comparison to the blank films, implying a reduction in film brittleness. This suggests that the proteins possess some degree of plasticizing effect on the films, thereby imparting flexibility, elasticity and improved toughness.

3.5 Scanning electron microscopy (SEM)

Fig. 4 shows that increasing GLY (plasticizer) concentration had an effect on the film morphology. The unplasticised film showed a clear uniform morphology whilst films prepared from gels containing SA:GLY 2:1, 4:3, 1:1 showed a rough uneven topography. Furthermore, it can be seen from Fig. 4 that with further increase in the concentration of GLY in the original gel (SA:GLY 2:3, 1:2), the topography of the films smoothen out, therefore producing homogenous uniform films that will be suitable for protein loading. SA film containing GLY in ratio SA:GLY 1:2, was chosen as being the most uniform of the six formulated films (Fig. 4) and used for protein loading, which confirms the tensile results.

The proteins (GFP, GST and GST-GFP) had little impact on the film morphology and topography (Fig. 5) of the optimized films though slight differences could be observed between GFP, GST and GFP-GST loaded films based on the drug loading, GFP, GST and GST-GFP (Fig. 5 A, D and G) respectively.
3.6 Hydration and swelling

It can be observed from Fig. 6 that most of the films showed percentage swelling index values ranging from approximately 650 to 1000% which were not significant (p > 0.05) as evidenced by the positions of the standard deviation bars. However, two films with higher concentrations (30.2mg/g) of GST-GFP, and GFP) possessed significantly (p < 0.05) higher percentage swelling index values compared to the other drug loaded films. The higher percentage swelling index observed in the higher protein (30.2 mg/g) loaded films could be attributed to the high protein content attracting water molecules due to its increased solubility. Both blank and protein loaded films showed high percentage swelling index, indicating a high holding capacity for wound exudate while still maintaining their structural integrity which can be attributed to hydrogel properties of SA.

3.7 In-vitro wound adhesion

The peak adhesive force (PAF) representing maximum force required to separate the films from the simulated wound surface, the area under the curve (AUC) representing the total work of adhesion (TWA) and the cohesiveness representing the distance travelled (mm) before detaching from the simulated wound surface were determined. Fig. 7 showed that the blank films had the highest cohesiveness and TWA values with the latter indicating the strong interactions (hydrogen bond formation) between the polymeric chains of SA and the simulated wound surface. There was no statistically significant difference observed in PAF (stickiness) between the GFP loaded films and the blank film (p = 0.7132, 0.0610, 0.7703 respectively). However, there was significant differences observed in TWA between the blank and GFP loaded films (p = 0.0045, 0.0010, 0.0022 respectively). In addition, GFP loaded films containing 30.2mg/g, 6.6mg/g of the protein showed no significant difference in cohesiveness
with the blank films ($p = 0.0807, 0.1375$) while GFP loaded film containing 3.3mg/g of the protein was significantly different from the blank film in cohesiveness ($p = 0.0211$).

Generally, it was also noted (Fig. 7) that with decrease in protein concentration, an increase in adhesive strength (TWA and PAF) was observed for all protein loaded films. This could be the result of higher protein loading (30.2mg/g) impacting on the films, providing less free hydrogen bonding sites leading to higher hydration as seen in Fig. 7 and less adhesive strength.

3.8 In vitro protein dissolution and release studies

Fig. 8 shows that the film dressings appeared to show rapid initial release of protein followed by constant release over a longer period. However, GST loaded dressing showed higher total cumulative release (90%) than GFP (78%) and GST-GFP (67%) dressings. It can also be seen that 78%, 70% and 64% release from GST, GFP and GST-GFP loaded dressing films respectively occurred within the first 2 hours (Fig. 8 inset). According to Table 4, GST-GFP protein release was proportional to time which is a non-concentration dependent mechanism involving the swelling and dissolution of the polymeric matrix (zero order mechanism). GFP released was proportional to the square root of time ($t^{1/2}$) indicating a Fickian diffusion controlled mechanism. GST however, had identical $R^2$ values for both Higuchi and zero order mechanisms. Therefore, GST release data was further evaluated using the Korsmeyer-Peppas equation and the diffusional exponent ($n$) was determined to be less than 0.5 ($n < 0.5$) indicating a quasi-Fickian diffusion mechanism [32].

3.9 Structural stability of model proteins by far-UV CD spectroscopy

Fig. 9A, B and C show the far-UV spectra of GST-GFP, GST and GFP in their native state (control) and after release from the SA film dressings (post-formulation). The ratios of the mean residue ellipticity were calculated as previously described. The two maxima bands
observed at 209 and 222nm [33, 34] were respectively assigned to the α-helical and β-sheet structures of GST-GFP (Fig. 9A) and GST (Fig. 9B). GST-GFP and GST released from SA films and the native protein showed similar mean residue ellipticity ratios ($\theta_{209} / \theta_{222}$) of 1.0 (GST-GFP) and 1.2 (GST). Fig. 9C (GFP) shows that GFP predominantly consisted of β-sheet structures and has also been reported by Visser and co-workers [35]. The similarity in the far-UV spectra (Fig. 9A, B and C) and the mean residue ellipticity ratios obtained (GST-GFP and GST) pre and post-formulation confirmed the conformational stability of all three proteins within the film dressings.

4. Discussion

The model proteins (GST, GFP and GST-GFP) were chosen because they could be readily cultured using bacteria (in house), isolated and characterized with various physical and bio-analytical techniques. This was necessary due to the large amounts of proteins needed during the formulation development and optimization process. Coomassie staining was used to detect the molecular weights of the recombinant GST-GFP protein and BSA at concentrations 5µg and 75µg respectively. BSA was used as a control to validate that the gel was working optimally as its molecular weight is constant (~66kDa) and confirmed that the proteins were separated according to molecular weight.

All the materials used were generally considered as safe (GRAS). Dextran (synthesized by Leuconostoc bacteria) is a complex polysaccharide made of glucose molecules [36] and was used as a negative control due to its low toxicity. On the other hand, PEI is a commercially available polyamine [37] and a gene carrier with reasonable transfection efficiency and high cytotoxicity. It is reported in literature [38, 39] that SA is generally regarded as non-toxic and used in oral formulations as well as food substances, however, none of these literature
references show a clear profile on the absence of toxicity of SA against epithelial cells. In the current study, safe model proteins have been used but this test can also be used in determining toxicity levels of growth factors (which play an important role during wound healing) on live mammalian epithelial cells. This will help to investigate the effect of different dose levels of growth factors delivered directly to wound sites, to avoid excessive proliferation of cells and thus, preventing the risk of triggering cancerous cells.

The stability of the proteins under various conditions were investigated using various thermal analysis techniques. Though two related scanning calorimetry techniques (HSDSC and DSC) were used, this was necessary since the HSDSC is effective for analyzing sensitive biological samples such as proteins as well as liquid samples (solutions) whilst DSC is generally more useful for samples in the solid state and small molecules. The HSDSC data shows that the GFP is a more thermally stable protein than GST. Therefore high temperatures of up to 70°C (14°C less than the $T_{\text{max}}$ of GFP at pH 7.5) and temperatures of up to 45°C (14°C less than the $T_{\text{max}}$ of GST at pH 7.5) can be employed during formulation or processing. The ratio $T_m/T_{\text{max}}$ is an indicator of thermal stability and generally, the higher the $T_m/T_{\text{max}}$, the more thermodynamically stable the protein [25]. Generally, the variations observed in the HSDSC can be attributed to the influence of pH, causing aggregation and/or degradation of the proteins within the buffers at the various pH values especially at 6.0 and 10.0.

DSC was used to determine possible interactions between the various film components as well as stability of the proteins within the film matrix. The exothermic peak observed in SA was not seen in the formulated gels or in the films possibly due to interactions between the formulation components, and molecular dispersion of the protein drugs within the formulation [40]. This observation is similar to that previously reported in another study [41] where degradation
exotherm of pure SA was absent in corresponding drug loaded alginate beads but rather, an endotherm, corresponding to the interaction of alginate with calcium ions naturally present in SA was observed. The differences observed between the DSC profiles could be an indication of fewer interactions between the GST proteins (3.3 and 30.2mg/g) and the polymer network and further evidenced by the closeness of the dehydration peak temperatures and enthalpies for GST (3.3 and 30.2mg/g) loaded and blank films.

The TGA results demonstrate that the different films generally possessed similar water content. The higher temperature of complete water loss in protein loaded films could be related to intermolecular forces such as hydrogen-bonds, van der Waals force and hydrophobic interactions between the proteins, and the starting materials within the film matrix, resulting in well-ordered bound water compared to the free water in the blank films. It is reported that water molecules play a vital role in maintaining the structure, dynamics, stability and function of biological molecules as they are responsible for packing and stabilization of the protein structure particularly in forming H-bond networks and screening of electrostatic interactions \[42\]. Papoian et al., reported a substantial improvement in protein structure prediction by adding a water-based potential to a well-known Hamiltonian for protein structure prediction \[43\]. Wetting the Hamiltonian improved the predicted structures, particularly of large proteins (>115 amino acid residues) through long range interactions between charged or polar groups facilitated by water molecules. However, bulk free water allows for rotational freedom within proteins, causing flexibility and enzymatic activities, thus, increasing reactivity and therefore an increase in entropy (disorderliness) in the protein \[44\].

Overall, the thermal analysis data shows the impact of the dressing formulation on the properties of the protein and vice versa in terms of stability and mechanical integrity.
respectively. At the temperature of 45°C and 40°C used for gel preparation and oven drying respectively, it is feasible to undertake the formulation development of alginate based dressing incorporating therapeutically relevant macromolecules without causing degradation. However, this will need to be confirmed with actual therapeutic proteins such as growth factors.

Texture analysis was used to measure the tensile properties; first to determine the effect of GLY concentrations the film behavior and the resulting data used to select the most appropriate formulation for protein loading and determine effect of drug concentration on the film tensile properties. Generally for film dressings, a balance between toughness (rigidity) and elasticity (flexibility) is required [15]. Tough films allow ease of handling without being sticky and folding up, whilst being flexible enough to allow easy application to the wound site and enable applications to difficult areas of the body such as parts around the joints and under the foot. This is normally achieved by having a % elongation value between 30–60% [15, 40] and this was only satisfied by the SA:GLY 1:2 films with % elongation value of 38.84% and were therefore selected for drug loading and further testing.

The rough and uneven topography (SEM) observed in films prepared from gels containing SA:GLY 2:1, 4:3, 1:1, can be detrimental to protein loading as content uniformity cannot be achieved in these films due to their rough topography. Rather, loaded drugs could be trapped and non-uniformly dispersed across the rough surfaces of these films, thereby hindering dosage accuracy as well as consistent drug release.

For effective wound healing, an ideal dressings is expected to be able to absorb large quantities of exudate whilst maintaining its structural integrity over long periods as well as keeping the
wound environment moist to facilitate wound healing. SA dressings are good absorbents that gradually form hydrophilic gels upon contact with wound exudate, thereby promoting a moist wound environment, the formation of granulation tissue and wound healing. It is reported [2] that moderate to high exuding wounds produce approximately 3-5 mL of wound exudate / 10 cm² in 24 hours. Therefore, 0.6-1.0 mL wound exudate is produced per 2 cm² in 24 hours.

In this study, films (blank and protein loaded) absorbed 625-1732% of SWF which is an indication that these dressings can absorb high amounts of wound exudate and can be used for moderate to high exuding wounds. It is reported that excessive hydrations as seen in the higher protein loaded films (30.2mg/g) (Fig. 6) can lead to reduced bioadhesion due to the formation of a slippery surface between the films and the simulated wound surface [45]. Adhesivity in wound healing is important as wound dressing should be self-adhesive with the wound so as not to fall off but be easily removed and painless [7].

Furthermore, the higher swelling properties of the 30.2mg/g protein loaded films could have led to a reduction in flexibility, which is important as it determines the extent of entanglement and enhances interpenetration between polymer (SA) and the simulated wound surface. The comparison of the swelling and bio-adhesive properties of the different formulations was used to determine the film dressing with the ideal functional properties. Based on the observed profiles, the 6.6mg / g protein loaded film was concluded to be the dressing with the optimum swelling and bio-adhesive properties and was subsequently used for in vitro drug (protein) dissolution studies.

The differences observed in the overall % cumulative release might relate to the relative difference in solubility between the three proteins as well as their interactions with the polymer (SA). In addition, initial burst release may be attributed to the dissolution and rapid release of
the surface associated protein molecules coupled with initial hydration and swelling above 60% in the first hour. Generally for a polymeric matrix such as solvent cast films, swelling, and solute diffusion and matrix degradation are proposed as the main driving forces responsible for drug release [46, 47]. Overall, it can be seen (Fig. 8) that after the initial burst release, the protein release was sustained over a period of 72 hours for all three protein loaded films. This second phase could be attributed to diffusion from the hydrated and swollen gel. This will help prevent frequent changing of the dressings so as not to disrupt newly formed skin tissues, reduce side effects through extended dosing as well as for patient compliance [46].

5 Conclusions

Adhesive SA film dressings were successfully developed as potential protein delivery systems for wound healing. The blank (SA:GLY 1:2) film was determined to be the optimized formulation for protein drug loading and further development. The absence of free water molecules within the film matrix was advantageous to ensure protein stability in the film and was confirmed by CD. Overall, the formulations containing 6.6mg of protein per gram of film exhibited optimum hydration and adhesive properties required for wound dressings. Further, protein release from the dressing was sustained over 72 hours which is expected to allow good bioavailability of the model protein drug at the site of action.

6 Conflict of interest

The authors report no conflict of interest

7 References


Figure Legends

Fig. 1. (a) Developed X-ray film showing detection of affinity purified GFP by western immunoblotting (anti body dilutions, 1:3000, exposure time; 10 seconds); (b) developed X-ray film showing detection of affinity purified GST by western immunoblotting (anti body dilutions, 1:3000, exposure time; 10 seconds) and (c) developed X-ray film showing detection of affinity purified recombinant GST-GFP by western immunoblotting (anti body dilutions, 1:2000, exposure time; 1 second).

Fig. 2. Toxicity profiles of SA (starting material), dextran and PEI used as negative and positive controls respectively (n=6 ± SD), the three model protein drugs (GST, GFP and GST-GFP) (n=6 ± SD) against vero cell lines after 72 hours exposure time.

Fig. 3. DSC thermograms of (A) GLY, (B) SA and (C) the blank and protein loaded films.

Fig. 4. SEM micrographs (x50 magnification) showing the effect of increasing GLY concentrations on film topography and morphology.

Fig. 5. SEM micrographs (x200 magnification) showing the effect of protein loading on the surface morphological properties of the plasticized SA: GLY (1:2) films containing [GFP (A-C), GST, (D-F) and GST-GFP (G-I) loaded film from high (left) to low (right) concentrations (30.2, 6.6 and 3.3mg/g) respectively.

Fig. 6. Hydration and swelling profiles of the blank and protein loaded film dressings (n=3 ± SD).
Fig. 7. In vitro adhesive profiles for blank and drug loaded films (n=4 ± SD)

Fig. 8. Dissolution profiles of protein (6.6mg/g) loaded film dressings (n = 4, ± SD)

Fig. 9. CD spectra of (A), GST-GFP, (B), GST and (C), GFP in native state and post release from film dressing (0.96mg/mL solution used).
Thermal stability of proteins (GST-GFP, GST and GFP) as a function of scan rate, concentration and pH using HSDSC.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Scan rate (°C/min)</th>
<th>Concentration (mg/mL)</th>
<th>ΔH (KJ/mol)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (°C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>0.5</td>
<td>5.0</td>
<td>73.25</td>
<td>56.27</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>1.0</td>
<td>5.0</td>
<td>91.35</td>
<td>57.77</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>2.0</td>
<td>5.0</td>
<td>66.14</td>
<td>59.09</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>0.5</td>
<td>2.5</td>
<td>71.97</td>
<td>55.55</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>1.0</td>
<td>2.5</td>
<td>71.77</td>
<td>57.32</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>2.0</td>
<td>2.5</td>
<td>88.67</td>
<td>59.19</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>0.5</td>
<td>1.0</td>
<td>96.47</td>
<td>56.30</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>1.0</td>
<td>1.0</td>
<td>102.14</td>
<td>57.21</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>2.0</td>
<td>1.0</td>
<td>126.87</td>
<td>60.70</td>
<td>7.5</td>
</tr>
<tr>
<td>GST</td>
<td>1.0</td>
<td>1.0</td>
<td>6.27</td>
<td>55.32</td>
<td>6.0</td>
</tr>
<tr>
<td>GST</td>
<td>1.0</td>
<td>1.0</td>
<td>82.30</td>
<td>56.96</td>
<td>8.0</td>
</tr>
<tr>
<td>GST</td>
<td>1.0</td>
<td>1.0</td>
<td>53.71</td>
<td>51.69</td>
<td>10.0</td>
</tr>
<tr>
<td>GFP</td>
<td>0.5</td>
<td>5.0</td>
<td>88.24</td>
<td>81.58</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>1.0</td>
<td>5.0</td>
<td>67.61</td>
<td>83.03</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>2.0</td>
<td>5.0</td>
<td>90.62</td>
<td>84.32</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>0.5</td>
<td>2.5</td>
<td>90.99</td>
<td>81.57</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>1.0</td>
<td>2.5</td>
<td>69.89</td>
<td>83.05</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>2.0</td>
<td>2.5</td>
<td>95.71</td>
<td>84.46</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>0.5</td>
<td>1.0</td>
<td>95.15</td>
<td>82.14</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>1.0</td>
<td>1.0</td>
<td>68.35</td>
<td>83.36</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>2.0</td>
<td>1.0</td>
<td>93.75</td>
<td>84.49</td>
<td>7.5</td>
</tr>
<tr>
<td>GFP</td>
<td>1.0</td>
<td>1.0</td>
<td>43.06</td>
<td>81.22</td>
<td>6.0</td>
</tr>
<tr>
<td>GFP</td>
<td>1.0</td>
<td>1.0</td>
<td>78.48</td>
<td>82.99</td>
<td>8.0</td>
</tr>
<tr>
<td>GFP</td>
<td>1.0</td>
<td>1.0</td>
<td>51.71</td>
<td>76.83</td>
<td>10.0</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>0.5</td>
<td>5.0</td>
<td>86.18</td>
<td>112.61</td>
<td>55.44</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>1.0</td>
<td>5.0</td>
<td>91.55</td>
<td>125.87</td>
<td>56.51</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>2.0</td>
<td>5.0</td>
<td>72.69</td>
<td>104.63</td>
<td>57.96</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>0.5</td>
<td>2.5</td>
<td>46.96</td>
<td>61.08</td>
<td>54.44</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>1.0</td>
<td>2.5</td>
<td>95.37</td>
<td>127.35</td>
<td>56.08</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>2.0</td>
<td>2.5</td>
<td>67.80</td>
<td>91.54</td>
<td>57.52</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>0.5</td>
<td>1.0</td>
<td>66.98</td>
<td>112.14</td>
<td>54.10</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>1.0</td>
<td>1.0</td>
<td>72.43</td>
<td>78.94</td>
<td>55.49</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>2.0</td>
<td>1.0</td>
<td>79.86</td>
<td>105.12</td>
<td>57.24</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>1.0</td>
<td>1.0</td>
<td>60.22</td>
<td>67.45</td>
<td>55.17</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>1.0</td>
<td>1.0</td>
<td>50.06</td>
<td>207.21</td>
<td>55.34</td>
</tr>
<tr>
<td>GST-GFP</td>
<td>1.0</td>
<td>1.0</td>
<td>70.29</td>
<td>131.12</td>
<td>52.81</td>
</tr>
</tbody>
</table>
Table 2 Dynamic weight loss (%) and degradation temperatures (°C) of samples (n=3, mean ± SD). The 1st represents water loss the remaining refer to weight loss due to other events, mainly degradation.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Dynamic weight loss (%)</th>
<th>Degradation temperatures (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st (water loss)</td>
<td>2nd</td>
</tr>
<tr>
<td>SA</td>
<td>18.2±0.7</td>
<td>36.3±0.5</td>
</tr>
<tr>
<td>GLY</td>
<td>16.1±0.1</td>
<td>84.0±0.1</td>
</tr>
<tr>
<td>BLK films</td>
<td>13.6±0.2</td>
<td>57.1±0.4</td>
</tr>
<tr>
<td>GFP films (30.2mg/g)</td>
<td>9.5±0.0</td>
<td>11.4±1.2</td>
</tr>
<tr>
<td>GFP films (6.6mg/g)</td>
<td>6.5±0.0</td>
<td>15.1±0.2</td>
</tr>
<tr>
<td>GFP films (3.3mg/g)</td>
<td>5.5±0.1</td>
<td>16.2±0.8</td>
</tr>
<tr>
<td>GST films (30.2mg/g)</td>
<td>16.7±0.1</td>
<td>49.9±0.0</td>
</tr>
<tr>
<td>GST films (6.6mg/g)</td>
<td>13.8±0.5</td>
<td>52.8±0.5</td>
</tr>
<tr>
<td>GST films (3.3mg/g)</td>
<td>14.7±0.5</td>
<td>50.8±0.0</td>
</tr>
<tr>
<td>GST-GFP films (3.3mg/g)</td>
<td>15.1±0.2</td>
<td>54.5±0.2</td>
</tr>
<tr>
<td>GST-GFP films (6.6mg/g)</td>
<td>11.9±0.2</td>
<td>16.7±0.6</td>
</tr>
<tr>
<td>GST-GFP films (3.3mg/g)</td>
<td>12.6±0.0</td>
<td>58.4±0.4</td>
</tr>
</tbody>
</table>
Table 3

(A) The effect of increasing plasticizer (GLY) on the mechanical (tensile) properties of blank SA films (mean ± SD, n=3); (B) Mechanical (tensile) properties, % elongation at break, Young’s modulus and tensile strength of optimized films (SA:GLY 1:2) loaded with proteins at different concentrations [mean ± SD, (n = 3)].

<table>
<thead>
<tr>
<th>Films - Blank</th>
<th>% elongation at break (mean ± SD)</th>
<th>Young’s modulus (mPa) (mean ± SD)</th>
<th>Tensile strength (N/mm²) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA:GLY (1:0)</td>
<td>1.85 ± 0.19</td>
<td>20.77 ± 4.19</td>
<td>51.34 ± 6.76</td>
</tr>
<tr>
<td>SA:GLY (2:1)</td>
<td>5.37 ± 0.96</td>
<td>5.43 ± 2.00</td>
<td>21.26 ± 0.25</td>
</tr>
<tr>
<td>SA:GLY (4:3)</td>
<td>19.70 ± 1.77</td>
<td>3.21 ± 0.72</td>
<td>12.39 ± 0.43</td>
</tr>
<tr>
<td>SA:GLY (1:1)</td>
<td>7.43 ± 0.87</td>
<td>3.12 ± 2.62</td>
<td>9.04 ± 0.59</td>
</tr>
<tr>
<td>SA:GLY (2:3)</td>
<td>10.10 ± 2.12</td>
<td>0.80 ± 0.34</td>
<td>3.81 ± 0.51</td>
</tr>
<tr>
<td>SA:GLY (1:2)</td>
<td>38.84 ± 0.86</td>
<td>0.40 ± 0.08</td>
<td>6.12 ± 0.11</td>
</tr>
</tbody>
</table>

(B) Films – Drug loaded

<table>
<thead>
<tr>
<th>Films – Drug loaded</th>
<th>% elongation at break (mean ± SD)</th>
<th>Young’s modulus (mPa) (mean ± SD)</th>
<th>Tensile strength (N/mm²) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST films (30.2mg/g)</td>
<td>11.76 ± 2.55</td>
<td>0.42 ± 0.14</td>
<td>4.07 ± 1.19</td>
</tr>
<tr>
<td>GST films (6.6mg/g)</td>
<td>5.46 ± 0.92</td>
<td>0.49 ± 0.13</td>
<td>2.56 ± 0.52</td>
</tr>
<tr>
<td>GST films (3.3mg/g)</td>
<td>6.20 ± 1.04</td>
<td>2.44 ± 0.35</td>
<td>6.36 ± 1.82</td>
</tr>
<tr>
<td>GST-GFP films (30.2mg/g)</td>
<td>20.74 ± 3.25</td>
<td>0.79 ± 0.18</td>
<td>5.04 ± 0.88</td>
</tr>
<tr>
<td>GST-GFP films (6.6mg/g)</td>
<td>23.38 ± 7.61</td>
<td>0.54 ± 0.07</td>
<td>4.77 ± 0.70</td>
</tr>
<tr>
<td>GST-GFP films (3.3mg/g)</td>
<td>19.04 ± 2.46</td>
<td>0.87 ± 0.21</td>
<td>4.50 ± 0.43</td>
</tr>
<tr>
<td>GFP films (30.2mg/g)</td>
<td>9.33 ± 0.66</td>
<td>0.88 ± 0.17</td>
<td>3.77 ± 0.87</td>
</tr>
<tr>
<td>GFP films (6.6mg/g)</td>
<td>7.78 ± 1.86</td>
<td>2.14 ± 0.34</td>
<td>6.16 ± 1.32</td>
</tr>
<tr>
<td>GFP films (3.3mg/g)</td>
<td>23.31 ± 4.04</td>
<td>0.97 ± 0.40</td>
<td>5.05 ± 0.33</td>
</tr>
</tbody>
</table>
Table 4 Release parameters obtained from fitting the dissolution data into different kinetic equations for the protein loaded film dressings

<table>
<thead>
<tr>
<th>Protein loaded films</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
<th>Korsmeyer-Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_0$ (%) min$^{-1}$</td>
<td>$R^2$</td>
<td>$K_1$ (min$^{-1}$)</td>
<td>$R^2$</td>
</tr>
<tr>
<td>GST 6.6mg/g</td>
<td>0.242</td>
<td>0.906</td>
<td>-0.013</td>
<td>0.904</td>
</tr>
<tr>
<td>GFP 6.6mg/g</td>
<td>0.010</td>
<td>0.960</td>
<td>-0.001</td>
<td>0.977</td>
</tr>
<tr>
<td>GST-GFP 6.6mg/g</td>
<td>0.061</td>
<td>0.963</td>
<td>-0.002</td>
<td>0.961</td>
</tr>
</tbody>
</table>

$K_0$, $K_1$, $K_H$, $K_P$ are the release rate constant for zero order, first order, Higuchi and Korsmeyer-Peppas kinetic models respectively, $n$ is the release exponent and $R^2$ is the correlation coefficient.
Fig. 1
Fig. 2
(A)
(B) 

(C)
Fig. 3

(D)
Fig. 4

SA : GLY 1:0  
SA : GLY 2:1  
SA : GLY 4:3

SA : GLY 1:1  
SA : GLY 2:3  
SA : GLY 1:2
Fig. 6
Fig. 7
Fig. 8
Fig. 9

(A) GST-GFP preformulation
(B) GST-GFP post-formulation

(C) GST preformulation
(D) GST post-formulation

Fig. 9
APPENDIX - SUPPLEMENTARY DATA

High sensitivity differential scanning calorimetry (HSDSC)

Generally, the results show that in all three proteins, an increase in scan rate from 0.5 to 2.0°C/minute increased the $T_{\text{max}}$ at pH 7.5 for the same protein concentration. From Table 1, it can also be seen that the optimum pH for the three proteins was 7.5 due to the higher $T_{\text{max}}$ observed when compared to that of the other pH values (6.0, 8.0 and 10.0). Further, Table 1 also shows that the optimum pH for the three proteins was 7.5 due to the higher $T_{\text{max}}$ observed when compared to that of the other pH values (6.0, 8.0 and 10.0). Comparing the $T_{\text{max}}$ of the individual proteins (GST, GFP) to the $T_{\text{max}}$ of the proteins within the construct (GST-GFP) at 1mg/mL and pH 7.5, it can be seen (Table 1) that GST was thermally more stable on its own than in the presence of GFP in the construct protein (GST-GFP) at all three scan rates (0.5, 1.0 and 2.0°C / minute). However, the $T_{\text{max}}$ for GFP alone and within the construct were similar at scan rates (1 and 2°C/minute) and differing by about 1.0°C at a scan rate 0.5°C/minute. Therefore, it can be concluded that GFP influenced the thermal stability of GST.

Enthalpy change ($\Delta H$) fluctuated with scan rate for all three proteins which indicates that the rate of scanning influences the thermal denaturation process of the three proteins. Concentration also influenced $\Delta H$ for all three proteins though there was no direct correlation. However, concentration did not influence the $T_{\text{max}}$ significantly and therefore a concentration of 1mg/mL was used for all three proteins to evaluate the effect of pH on the proteins thermal stability.

From Table 1, it can be seen that the optimum pH for the three proteins was 7.5 due to the higher $T_{\text{max}}$ observed when compared to that of the other pH values (6.0, 8.0 and 10.0). For example, in the case of GST, the $T_{\text{max}}$ at the different pHs (7.5, 6.0, 8.0 and 10.0), decreased
from 57.21°C (pH 7.5), 56.96°C (pH 8.0), 55.32°C (pH 6.0) to 51.69°C for pH 10.0. In addition, ΔH decreased from 102.14, 82.30 kJ/mol, 53.71 kJ/mol and 6.27 kJ/mol for pH’s (7.5, 8.0, 10.0 and 6.0) respectively, significantly reducing the enthalpy of the reaction. Similar results were also observed for GFP. However, for the construct protein (GST-GFP), the differences in T\text{max} between pHs were not as high compared to the individual proteins (GST and GFP). The T\text{max} ranged from 55.49°C, 55.34°C, 55.17°C for pH 7.5, 8.0, 6.0 respectively with about 3°C difference for pH 10.0 (52.81 kJ/mol). However, the difference in ΔH was higher for all four pH values; 7.5 (72.43 kJ/mol), 10.0 (70.29 kJ/mol), 6.0 (60.22 kJ/mol) and 8.0 (50.06 kJ/mol). For GFP within the construct protein (GST-GFP), T\text{max} values observed were 83.19°C, 79.86°C, 76.15°C and 70.18°C, at pH values of 7.5, 6.0, 10.0 and 8.0 respectively. Comparing the T\text{max} of the individual proteins (GST, GFP) to the T\text{max} of the proteins within the construct (GST-GFP) at 1mg/mL and pH 7.5, it can be seen (Table 1) that GST was thermally more stable on its own than in the presence of GFP in the construct protein (GST-GFP) at all three scan rates (0.5, 1.0 and 2.0°C / minute) with the difference in T\text{max} between 2.0–3.0°C. However, the T\text{max} for GFP alone and within the construct were similar at scan rates (1 and 2°C/minute) and differing by about 1.0°C at a scan rate 0.5°C/minute. Therefore, it can be concluded that GFP influenced the thermal stability of GST.

A2 Differential scanning calorimetry (DSC)

The peaks around 100°C are associated with protein decomposition, however, at this temperature, all three protein would have denatured from their native state. This suggests that the peak at 100°C could be decomposition of denatured proteins but this may require further investigation. Peaks at around 0°C are due to thermal melting of the proteins as the temperature increased. The peak at approximately -20°C can be attributed to phase transition of the proteins in the crystal state prior to melt at 0°C. Both GST and GFP showed this phase transition at -
22.89°C and -22.09 respectively. However, GST-GFP produced two peaks at this phase that can be attributed to the presence of both GST and GFP in the recombinant GST-GFP.