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

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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 6.6 mg/g of protein possessed ideal characteristics and used for *in vitro* dissolution studies. Protein release was rapid initially and sustained over 72 h 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.

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^{TM}) which form flexible gels upon hydration or those rich in guluronic acid residues which form firmer gels upon exudate absorption (e.g. Kaltostat^{TM}). Alginate dressings are non-toxic and aid in hemostasis as part of the wound healing process [10–13]. In addition, they

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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 60,000), dextran (Mw 35,000–45,000), isopropylβ-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), trismethylamine 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 (1×) 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 4 L of cold 1× PBS (4 °C) overnight and changing the dialysis buffer every 2 h afterwards with a minimum of 4 changes of (1×) PBS. 15 μL each of purified proteins [GST-GFP (5 μg), GST (2 mg) and GFP (1 mg)] and controls [Spectra Multicolor broad range protein molecular weight ladder (Fermentas, Cambridgeshire, UK) and bovine serum albumin (BSA) standards (75 μg)] were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) apparatus using 6 M guanidine containing Laemmli buffer and 10% (v/v) beta-mercaptoethanol (BME), with a running buffer (1×) as per manufacturer's instructions. The loaded samples were resolved by applying 100 V of direct current for 80 min. The gel was then stained with Coomassie brilliant blue for 2 h and de-stained with Coomassie de-staining solution for another 2 h, further soaked in 5% (v/v) glycerol/PBS and dried overnight using a gel drying kit (Promega, Hampshire, UK). Western blotting and immunodetection 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 30.2 mg/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 h) 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 h. The model proteins were added to the optimized gel with gentle stirring and heating (45 °C) until a homogenous mix was obtained (1 h) and allowed to stand for 5 mins (to remove air bubbles). 30 g was poured into Petri dishes (90 mm diameter) and placed in a vacuum oven at 40 °C for 18 h.

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 (1×10^4 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 (100 U/mL), streptomycin (100 μg/mL) and glutamine (292 μg/mL) (all under sterile conditions in a laminar hood) and incubated at 37 °C in 5% (v/v) CO_2 for 24 h. After 24 h, the cells were exposed to either PEI, dextran, GST-GFP, GST and GFP (0–3 mg/mL) in cell culture medium and incubated for 68 h. 10 μL (50 μg) of MTT from stock solution (5 mg/mL) was added to each well and the plate incubated for a further 4 h bringing the total incubation time to 72 h. The contents of the plate were decanted and 100 μL of DMSO was added to each well, incubated at room temperature for 30 min and the absorbance read on a Multi-scan EX Micro-plate photometer (Thermo Scientific, Essex, UK) at optical density (OD) 540 nm. For SA however, adherent cells (Vero, 1×10^4) were exposed to SA gel after 24 h. Data obtained was expressed as percentage cell viability (mean ± standard deviation of the mean).