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DESIGN, PREPARATION, AND ACTIVITY OF COTTON-BASED WOUND DRESSINGS FOR CHRONIC WOUNDS

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Abstract

Chronic wounds are a major worldwide health problem. Cotton gauze is a standard of care in the management of chronic wounds and is still routinely employed in hospitals and nursing homes for long term wound care. We consider in this paper molecular modifications of cotton gauze to improve chronic wound healing. The presence of elevated levels of elastase in non-healing wounds has been associated with the degradation of important growth factors and fibronectin necessary for wound healing. In the healing wound a balance of elastase and antiproteases prevents this degradation from taking place. Cotton cellulose modified to release elastase inhibitors or selectively sequester elastase would provide a gauze wound dressing that decreases high levels of destructive elastase found in the chronic wound.

Introduction

Cotton gauze has been manufactured and utilized for the last two centuries as a standard wound dressing in the care of both acute and chronic wounds. Although it is still used in much the same manner as originally conceived there have been some fiber modifications that have improved its quality and versatility in medical applications. A variety of cotton-based, medicated tulle dressings have been employed with ointments that serve as carriers for a range of medicaments (1). Improvements in stretch gauze with slack mercerization replaced the need for rubber-embedded gauze (2). Cotton gauze is a cellulosic material and the molecular modification of cellulose to form bioactive analogs of potential use in wound healing is a route to developing intelligent cotton-based dressings (3).

The design of the gauzes employed in this study was based on structure function relationships of neutrophil elastase to peptide-substrate conjugates of cellulose (4). The goal of the work is the design of rational cellulose modifications that are safe, economical and effective to use clinically in promoting the healing of chronic wounds. The ability of chronic wound fluid to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of endogenous proteinase inhibitors in the chronic wound. To this end we have examined five cotton gauze modifications designed for sequestration of elastase from wound fluid. These modifications are structurally incorporated into the cellulose of the cotton fiber through economical finishing techniques. The structural modifications made to the cotton dressings were designed based on enzyme structure/function relationships between the cellulose fiber, elastase active site, and amino acid charge composition of elastase. This paper outlines the preparation and assay of five modified wound dressings based on this design approach. Initially we examined four types of gauzes having both electrophilic and anionic structural modifications that enhance elastase affinity for the dressing. These modifications were found to be effective in sequestering elastase. Subsequently a fifth cotton gauze modification was studied that combines both the electrophilic and anionic features of these wound dressings.

Material and Methods

Preparaton of Periodate-Oxidized, Sulfonylated, Carboxymethylated, and Phosphorylated Cotton

Type VII cotton gauze (12 Ply – 4in X 4in USP) was applied to the treatments outlined here. Cotton gauze was oxidized with 0.2M sodium metaperiodate (pH 5) at 40°C for 1 hour to give dialdehyde oxycellulose. The cotton gauze was sulfonated by washing the dialdehyde oxycellulose with 5% sodium bisulfite ($NaHSO_3$, under pH4.5, liquor ratio 1:60) for 3 hours (5).

Phosphorylation of cotton gauze was accomplished by a patent pending process. The process involved applying inorganic phosphate salt (sodium hexametaphosphate) to cotton gauze in 4–16% composition. Urea was included in the formulation on a 2:1 weight ratio of urea to phosphate. All formulations contained 0.1% Triton X-100 as a wetting agent. The cotton gauze was padded to 80–90% wet pickup and then dried at 60°C . The samples were cured at 160° C for 7 min.

Carboxymethylation was completed as outlined previously (6). A solution was made by mixing 25% monochloroacetic acid with 50% sodium hydroxide solution. This solution was used to pad a sample of cotton gauze to a wet pickup of 135%. The wet sample was then placed in an oven at 100° C, and dried/cured for 10 minutes.

Evaluation of Wound Dressing Products

Table 1 describes the functional group modifications of the anhydroglucose units in cellulose made on the cotton gauze. Structure I is of dialdehyde cotton gauze (DAG) and II of the resulting sulfonation of DAG which yields the bisulfite addition adduct designated as sulfonated cotton cellulose (SOC). Structures III and IV depict the carboxymethylated and phosphorylated anhydroglucose which result from carboxymethylation and phosphorylation.

Degree of Substitution

The degree of substitution (D.S.) was determined by a standard degree of substitution relationship (7) for cellulose (based on the per cent of total phosphorous and sulfur for the phosphorylated and sulfonated samples). Base titration of free carboxyls was employed to determine D.S. levels on CMC (6). The phosphorylated and sulfonated cotton cellulose D.S. levels were 0.035 and 0.011 respectively. This corresponds to one phosphate for every 28 anhydroglucose units and one sulfate for every 91 anhydroglucose units. The degree of substitution for the dialdehyde was also 0.011 since the bisulfite addition reaction is utilized to determine D.S. levels for dialdehyde cotton. The degree of substitution for carboxymethylated cotton cellulose was 1.4.

Results

Effect of Modified Gauzes on Elastase Activity

Initial experiments examined the ability of the modified cotton celluloses to absorb purified neutrophil elastase. Twenty-five, fifty and seventy-five milligram quantities of gauze were soaked to saturation for an hour in one milliliter of buffered solution containing 0.2 units of elastase. Unbound enzyme was removed by filtration followed by pressing under high pressure. The recovery of buffer from the filtration process was found to be 90%.

The assessment of elastase activity in solution exposed to the treated gauze was performed on the unbound enzyme. Extractable elastase activity was assayed in a 96-well format using MeOSuc-Ala-Ala-Pro-Val-pNa for substrate hydrolysis (8). The analysis of kinetics of elastase activity is based on the relative initial velocity (v_o) values for enzyme solutions exposed to cotton gauze. This is based on the classical enzyme substrate complex formation at equilibrium;

Where E, S and P are enzyme, substrate, and product, respectively. The comparative turnover number of elastase in solution is equal to the kinetic constant k_3 as shown above.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P$$

The initial velocity (v_o) is directly related to elastase activity as measured by product formation. Measurement of elastase activity remaining in solution upon treatment with the gauze was accomplished by monitoring the reaction rate within a thirty-minute time frame. A plot of v_o values shown in Figure 2 for the samples demonstrates this dose response relationship. The plot of v_o values was within the same range for the dialdehyde, sulfonated and phosphorylated cotton. A decrease in active enzyme sites was apparent from the decreasing dose response relation of the treated gauze samples with dialdehyde, sulfonated, carboxymethylated and phosphorylated cotton. The decreased rate reflects a decrease in units of elastase activity retained in the eluted buffer. A similar decrease in velocity was demonstrated with increasing weight of treated gauze. The lower v_o values for the treated samples when compared with the untreated cotton gauze suggests that the elastase activity is retained in the treated cotton gauze due to selected modifications on the gauze. Retention of elastase activity in treated gauze was found to be four-fold higher than in untreated gauze.

Elastase-Lowering Activity in Wound Fluid

The dialdehyde cotton gauze (DAG) was selected for further evaluation using human wound fluid (9). To assess the ability of the modified gauze to lower wound fluid-containing elastase activity in a dose response relationship DAG samples and untreated cotton gauze were placed in wound fluid ranging in concentration from 2.5 to 20 milligrams of gauze per microliter of patient wound fluid. The solutions of chronic wound fluid taken from soaked DAG samples were treated with an elastase recognition peptide sequence, which is a known substrate for measurement of elastase activity in wound fluid (11). The substrate was allowed to react in the wound fluid solutions. Wound fluid from soaked DAG samples possessed less elastase activity when compared with untreated cotton gauze. Based on the initial velocities at varying gauze concentrations a 2-5 fold decrease in the rate of elastase substrate hydrolysis corresponds to increasing dialdehyde cotton gauze concentrations. This reflects the ability of the DAG samples to remove elastase activity from wound fluid over untreated cotton gauze. DAG extracted 2-5 fold more elastase activity with increased gauze loading per volume of wound fluid when compared with untreated gauze with increasing concentrations of gauze (10). An increase in extracted elastase activity from wound fluid is demonstrated by the increase in ratio of elastase activity (V_{UT}/V_{DAG}) found in wound fluid exposed to UT gauze versus DAG as shown in Figure 3.

Measurement of protein levels remaining in the wound fluid following incubation with the gauzes was performed to compare the relative amounts of protein taken up by treated and untreated gauze. Lower levels of protein were found in the wound fluid soaked from DAG than from the untreated cotton. This is consistent with the lower activity of elastase found in the wound fluid soaked from DAG samples.

Optimization of Gauze Properties in a Single Treatment

Since the dialdehyde cotton gauze was shown to be the most effective in lowering elastase activity in solution it was studied in wound fluid for elastase-lowering activity as discussed above. However, an additional design was tested during the course of this work, and is based on the both the negatively charged and electrophilc properties conferred by the carboxymethylated and aldehyde cotton gauze. Thus, elastase-sequestering wound dressings were also prepared as the fructose and glucose citrate conjugates on the cotton fibers of gauze dressings (12). The design of these modified gauzes was based on anionic citrate-esterified cellulose and the open chain isomers of the hemiketal and hemiacetal of fructose and glucose, which possess partial ketone, and aldehyde functionality as is found in synthetic elastase inhibitors.

The monosaccharides were crosslinked to the cotton cellulose with an acid catalyzed citric acid reaction (9). The structures in Figure 4 demonstrate the bonding in which the monosaccharide may be linked through the citrate ester to cellulose and the manner in which the monosaccharide isomers may interconvert forming aldehyde or ketone functionalities. The aldehyde and ketone functionalities may interact with the active site of elastase to enhance uptake. The acid-catalyzed crosslinking esterification of cotton cellulose with citrate gives rise to an ester bond linking the citrate to cellulose, some free unesterified carboxyls, and monosaccharide conjugates linked to citrate. The reaction of a monosaccharide in the presence of citrate esterified cellulose provides two available carboxyls for cyclic anhydride-mediated esterification of the monosaccharide and yields a monosaccharide ester of citrate esterified cellulose. However, the preferred sites of esterification in cellulose and the monosaccharides are not known.

Characterization and Effect of Modified Gauze on Elastase Activity

The citrate glucose and fructose conjugates of cellulose were characterized through base hydrolysis of the monosaccharide ester linked to cellulose followed by high performance anion exchange (HPAE) chromatography with pulsed amperometric detection (PAD) of the hydrolysis products (12). Since the monosaccharides are attached to the cellulose fiber through an ester linkage to citrate-crosslinked cellulose, the ester bond may be hydrolyzed by base treatment of the modified cotton gauze to give release of fructose or glucose. The esterified glucose and fructose released from the cotton fiber by base hydrolysis of the citrate ester were measured quantitatively using HPAE-PAD. Quantitative measurement of the hydrolyzed glucose and fructose is shown in Table 2 and enabled determination of the amount of monosaccharide conjugate esterified on the cotton gauze. The amount of glucose and fructose found to be conjugated through citrate to the cellulose was 4 milligrams and 0.9 milligrams per gram of cotton, respectively.

The ability of the modified cotton gauze to absorb neutrophil elastase wound fluid was measured in the same manner as described above for the dialdehyde gauze. Quantities of conjugated cotton gauze were soaked to saturation for an hour in wound fluid solutions containing high titers of elastase, and the kinetics of elastase activity were measured from these solutions based on the relative initial velocity (v_o) values for enzyme solutions exposed to cotton gauze as described previously (12).

The measurement of elastase activity remaining in solution upon treatment with the gauze was accomplished by monitoring the reaction rate within a thirty-minute time frame. The reaction progress curves for treated samples are shown in Figure 5. Citric acid crosslinking of fructose and glucose to cellulose lowers elastase activity. A dose response relation within a range of sample gauze weights (10 – 100 mg) demonstrates a lowering of elastase activity with all of the cellulose conjugates. In comparison to the glucose-citrate conjugate (GAC), the fructose-citrate conjugate (FAC) lowered elastase activity and demonstrated a more potent dose response per gram of gauze.

Selection of Modified Gauze Samples For Chronic Wound Clinical Trials

This work demonstrates that fibers of cotton gauze modified with aldehydic and anionic functionalities remove elastase from wound fluid. Our conclusion from this work is that the rationally designed cotton gauze dressings having these structural modifications promoting elastase uptake from wound fluid are ideal candidates for further evaluation in patients with chronic wounds. Current efforts are being made to develop these cotton finishing processes on an industrial scale to produce cotton-based chronic wound dressings for clinical trials. We have selected two types of cotton gauze modificaitons discussed here for development, manufacturing and subsequent clinical studies. These two gauzes are the dialdehyde cotton gauze (Figure 1, structure I) and the fructose-citric acid cellulose conjugate (Figure 4, structure II). When compared in wound fluid for their elastase-lowering activity (as shown in Figure 6) both modified cotton wound dressings demonstrated a dose dependent lowering of elastase activity. However the fructose-citric acid cellulose conjugate demonstrate more robust activity in removing elastase from wound fluid. Comparison of the dialdehyde cotton gauze and the fructose citric acid cellulose conjugate in chronic wound clinical trials should be useful in further understanding the mechanism of how rationally designed wound dressings function in the wound environment.

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Table 1. Modifications of Cotton Gauze.

Structures (Figure 1)	Description of Cellulosic Gauze Finish	Abbreviation
I	Dialdehyde Cotton Gauze	DAG
II	Sulfonated Cotton Gauze	SOC
III	Carboxymethylated Cotton Gauze	CMC
IV	Phosphorylated Cotton Gauze	PSC
V	Cotton Gauze	COT

Table 2. Results of ion chromatography quantification of fructose and glucose hydrolysates taken from monosaccharide-citrate cellulose conjugates.

Gauze Sample	Gluc (ppm)	Fruc (ppm)	Gluc µg/g cotton	Fru µg/g cotton
Untreated gauze	0.74	0.16	17.0	3.6
Citric acid cellulose conjugate	1.84	0.11	40.6	2.5
Glucose-citric acid cellulose conjugate	231.2	9.2	4076.4	162.2
Fructose-citric acid cellulose conjugate	6.84	39.36	156.8	902.4

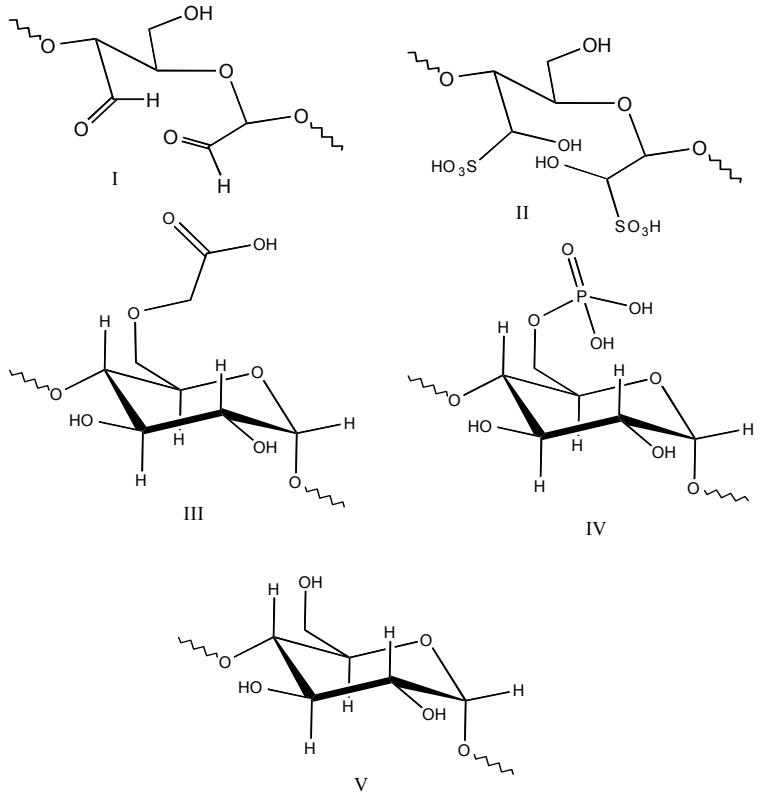

Figure 1. Representative structures of modified anhydroglucose monomer units in the cellulose chain upon treatment of the cotton gauze. I, structure of dialdehyde cotton cellulose; II, sulfonated cotton cellulose; III, carboxymethylated cotton cellulose; IV, phosphorylated cotton cellulose; V cotton cellulose.

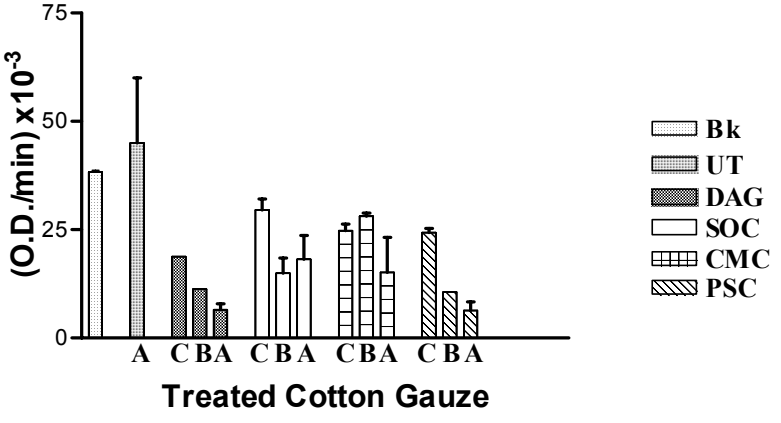

Figure 2. Initial velocities (v_o) are compared for solutions taken from treated gauze samples. Treated gauze were soaked in solutions of elastase (0.2 units/mL) and gauze were pressed dry and the unretained enzyme solutions assayed by spectrophotometric monitoring of substrated hydrolysis. Weights of gauze samples were 75 (A), 50 (B), and 25 (C) milligrams. The abbreviated designations for these samples are blank (BK), untreated (UT), dialdehyde cotton gauze (DAG), sulfonated cotton gauze (SOC), carboxymethylated cellulose cotton (CMC) and phosphorylated cotton (PSC).

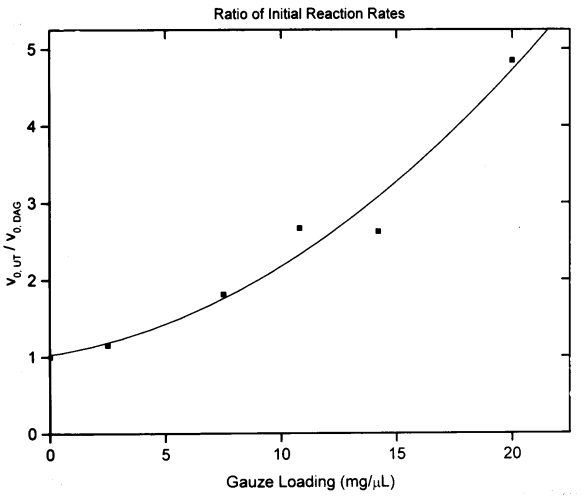

Figure 3. the untreated cotton gauze vs. dialdehyde cotton gauze ($v_{o,UT}/v_{o,DAG}$). $v_{o,UT}/V_{o,DAG}$ is the ratio of the slopes from the linear fit of absorbance-time data. The gauze loading from soaking in wound fluid is expressed as mg gauze/ul undiluted wound fluid.

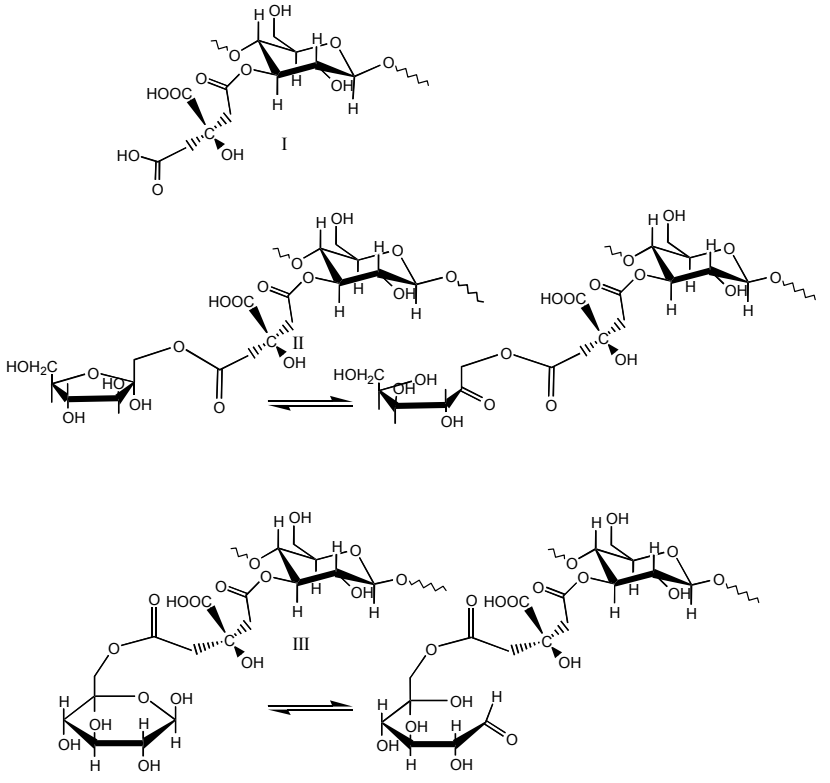

Figure 4. Representative structures of fructose-citrate and glucose-citrate conjugates of cellulose shown as the interconverting cyclic and open chain forms of the sugars that occur in water. Structure I is a citric acid cellulose conjugate (CAG). Structure II is of a fructose-citric acid cellulose conjugate (FAC). Structure III is of a glucose-citric acid cellulose conjugate (GAC).

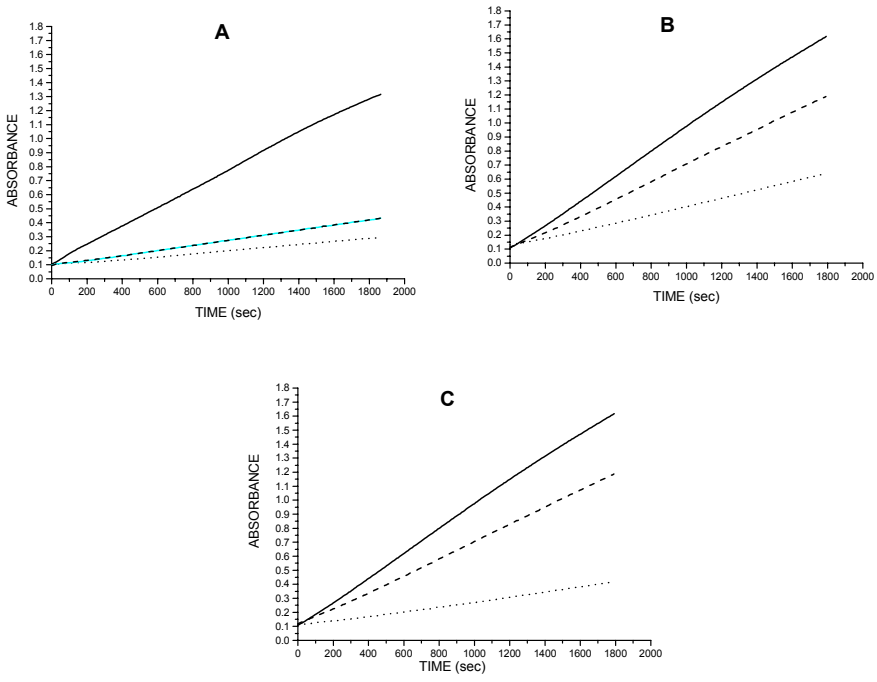

Figure 5. Elastase activities from conjugate-soaked solutions containing human wound fluid. The assays are described in above. The wound fluid solution was diluted in buffer 1:100, wound fluid:buffer, v:v. The substrate hydrolysis was performed with a 60 micromolar solution of MeOSuc-Ala-Ala-Pro-Val-pNA in the wound fluid solution. Reaction rates were monitored by spectrophotometric measurement of the release of p-nitroaniline at 405 nm. (A) Elastase-containing wound fluid (solid); FAC gauze at 25mg (dash); FAC gauze at 10 mg (dot). (B) Elastase-containing wound fluid (solid); CAG gauze at 50 mg (dot); CAG gauze at 100 mg (dash). (C) Elastase -containing wound fluid (solid); GAC gauze 50 mg (dot); GAC gauze at 100 mg (dash).

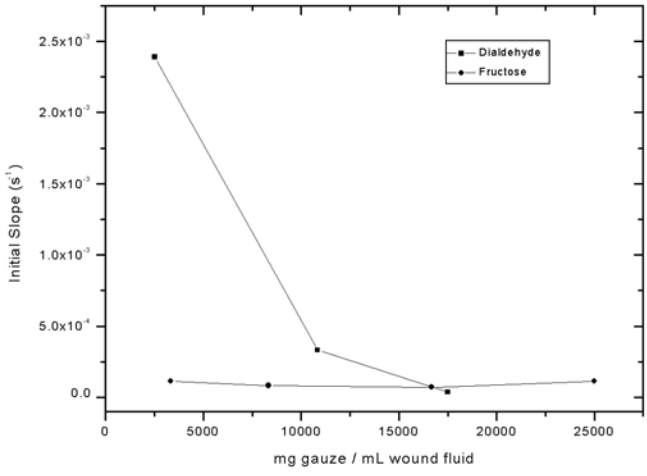

Figure 6. Initial velocity (v_o) measurements of reaction profiles showing protease inhibition by modified gauzes - dialdehyde and fructose conjugates. Diluted wound fluid was exposed to varying amounts of gauze then assayed for elastase activity. The initial velocity (slope) of the reaction profile is plotted against the mass of gauze normalized to the amount of wound fluid used in the trial.