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Design, preparation and assessment of citrate-linked monosaccharide cellulose conjugates with elastase-lowering activity

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Abstract

Elastase-sequestering wound dressings were prepared as the fructose and glucose citrate–cellulose conjugates on the cotton fibers of gauze dressings. The modified dressings were designed for chronic wounds, which contain destructively high levels of neutrophil elastase. The design of the 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 cellulose–citrate conjugates of glucose and fructose were prepared on cotton gauze by rapid acid-catalyzed esterification of fibrous cellulose with citric acid as an ester cross-linking agent. The glucose and fructose citrate ester conjugates of cellulose were characterized as both intermolecular ester bonds of citrate-cross-linked cellulose and as citrate esters of glucose and fructose. Glucose and fructose were released from the fabric through hydrolysis of the citrate ester of the monosaccharide and were characterized using high performance anion exchange chromatography with pulsed amperometric detection and a modified Fehling's test. Chromatographic analysis of the hydrolyzed glucose and fructose ester products revealed the cellulose conjugates to be formed at levels of 4 mg of glucose per gram of cotton and 0.9 mg of fructose per gram of cotton. Fourier transform infrared spectroscopy was employed to detect the citrate-esterified conjugates on the cotton fiber. The modified cotton gauzes containing the monosaccharide ester conjugates and citrate-cross-linked cellulose were compared with untreated gauze for reduction of elastase activity in buffered saline, and in solutions of chronic wound fluid. The monosaccharide and citric acid conjugates of cellulose demonstrate a sequestration effect by extracting elastase from solution. The order of elastase-lowering potency of the monosaccharide–citrate cellulose conjugates in wound fluid was judged to be fructose–citrate > glucose–citrate = citrate. Elastase-lowering activity displayed a linear response over a range of 10–75 mg of gauze samples with the fructose–citrate cellulose conjugate (11.5–0.5 μmol of sugar/unit of elastase activity). © 2002 Published by Elsevier Science Ltd.

Keywords: Elastase; Monosaccharide; Aldehyde; Cellulose

1. Introduction

Elastase is a serine protease that has been associated with a variety of inflammatory diseases (Bonney & Smith, 1986; Powers, 1983), and recently has been implicated as a destructive protease that impedes wound healing. The presence of elevated levels of elastase in non-healing wounds has been associated with the degradation of important growth factors (Yager et al., 1997) and fibronectin (Grinell & Zhu, 1994) necessary for wound healing. Serine-195 of the elastase active site acylates hydrolyzable peptide and protein substrates at residues COOH-terminal to valine (Schechter & Berger, 1967), and may be inhibited by derivatized electrophilic functional groups, such as aldehydes and ketones (Edwards & Bernstein, 1994). The application of elastase recognition sequences to the fibers of wound dressings is a possible way of inhibiting high levels of elastase in the chronic wound. Previously, we have modified cellulose in cotton wound dressings through the incorporation of peptide and anionic or aldehydic elastase-binding functionalities with the goal of lowering elastase activity in the chronic wound environment Edwards, Batiste, Gibbins, & Goheen, 1999; Edwards et al., 1999; Edwards et al., 2001).

The esterification of cellulose with citrate-linked esters of carbohydrates provides a novel conjugate of cellulose to

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explore structure function interactions with enzymes. Monosaccharides conjugated to cellulose on cotton gauze were designed to assess potential affinity-enhancing properties of modified cellulose gauze to bind elastase. The open chain ketone and aldehyde isomers of fructose and glucose have electrophilic character that might enhance binding to the active site of elastase. To form cellulose conjugates of glucose and fructose on cotton gauze, we have utilized a cross-linking technique employing rapid esterification of fibrous cellulose with citric acid as an ester cross-linking agent (Welch & Andrews, 1989a). Citrate-cross-linked esterification of cellulose also forms esters between the anhydroglucose hydroxyls of cellulose and citrate, and gives rise to unesterified carboxyls of citrate. The preparation, characterization, and elastase-lowering activity of the conjugates are presented.

2. Materials and methods

USP Type VII cotton gauze sponges (12 ply—4 in. × 4 in.) were treated in solution pad baths consisting of 7% citric acid and 7% sodium hypophosphite and either 0.12 M glucose or fructose. The gauzes were padded by two repetitions of dipping in the treatment solutions followed by removal of excess solution on a laboratory mangle with about 90% wet add-on (Andrews, 1990). The padded fabrics were dried and cured at 155 °C in ovens with mechanically circulated air. The treated gauzes were then washed under deionized water for 1 h following the treatment.

2.1. Colorimetric assessment of glucose and fructose on cotton gauze

A modified colorimetric test for determination of ketone and aldehyde groups present in glucose and fructose, respectively, was employed to determine the amount of monosaccharide present on the cotton. The copper number of cellulose and the citric acid cellulose conjugates was determined by a test using a modified Fehling's solution (Fieser & Fieser, 1967). Concentrations of glucose and fructose were determined by measuring the copper number of monosaccharide-modified cellulose in both treated and untreated samples (Garner, 1967) and subtracting the concentration of monosaccharide in untreated from treated samples.

2.2. Chromatographic analysis of glucose and fructose on cotton gauze

The monosaccharide conjugates of the cotton gauze were analyzed for glucose and fructose content by injecting hydrolyzed fructose and glucose samples onto high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD). Cotton gauze samples weighing approximately 800–900 mg that contain the cellulose–citrate conjugates of glucose and fructose were soaked in 1 M NaOH for 30 min. The gauze samples were filtered and the eluant-containing glucose and fructose was neutralized with 3N HCl to pH 7. All samples were filtered through a 0.45 μm filter. The glucose-treated samples were first diluted 10-fold. Glucose and fructose concentrations in duplicate samples were determined on HPAE-PAD using a Dionex (Sunnyvale, CA) BioLC instrument. Glucose and fructose were separated on Dionex CarboPac PA-1 guard ($25 \times 4\ mm^2$) and analytical ($250 \times 4\ mm^2$) anion exchange columns, at a flow rate of 1.0 ml/min at ambient temperature (~25 °C). Column eluant conditions were: 16 mM NaOH isocratic (inject; 0.0–2.0 min), a gradient of 16–160 mM NaOH (2.0–26.0 min), followed by isocratic 200 mM NaOH (26.1–29.0 min), and return to 16 mM NaOH (29.1–32.0 min) to reequilibriate the column with the initial mobile phase prior to the next sample injection. The monosaccharides (25 μl injections) were detected using integrated pulsed amperometric detection (IPAD). The PED-2 detector was equipped with Au working and Ag/AgCl reference electrodes, operating with the following working electrode pulse potentials and durations: $E_1 = +0.05$ V ($t_0 = 0.00$ s), $E_2 = 0.05$ V ($t_2 = 0.42$ s), $E_3 = +0.75$ V ($t_3 = 0.43$ s), $E_4 = +0.75$ V ($t_4 = 0.60$ s), $E_5 = -0.60$ V ($t_5 = 0.61$ s), $E_6 = -0.60$ V ($t_6 = 0.96$ s). The duration of the IPAD integration interval was set at 0.2–0.4 s. Using a Spectra-Physics SP8880 autoinjector and Dionex Peaknet chromatography software, runs were accumulated of multiple samples and standards. Response factors were generated for each of the monosaccharides using check standards.

2.3. Fourier transform infrared spectroscopic measurements

A Nicolet Magna-IR 550 spectrometer was used for the FT-IR measurements. Resolution for all infrared spectra was 2 cm^{-1}, and 250 scans for each spectrum. The finished cotton gauzes analyzed were ground in a Wiley mill to pass a 80 mesh screen. FT-IR spectra were taken of cotton powder samples prepared 0.5% by weight in potassium bromide pellets.

2.4. Enzyme assays

Enzyme assays of the solutions containing unbound human neutrophil elastase were conducted in pH 7.6 buffer composed of 0.1 M sodium phosphate, 0.5 M NaCl, and 3.3% DMSO. Measurement of enzyme activity was demonstrated by spectrophotometric monitoring of the release of *p*-nitroaniline at 410 nm from the enzymatic hydrolysis of the substrate *N*-methoxy-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MeOSuc-Ala-Ala-Pro-Val-*p*NA) (Sigma) (Nakajima, Powers, Ashe, & Zimmerman, 1979). The spectrophotometric kinetic assays were performed in a Bio-Rad Microplate Reader (Hercules, CA) with a 96-well format. Two hundred microliter aliquots of an elastase solution (0.2 units) were assayed per well, and 20 μl of a 60 μM substrate solution was added to initiate the enzyme reaction.

2.5. Sequestration and inhibition of elastase activity by finished cotton gauze

The effect of linking glucose and fructose to citric acid conjugates of cellulose cotton gauze was tested for extraction of elastase from solution. Treated and untreated gauze samples were submerged in 1 ml of buffer containing 1 unit/ml of human neutrophil elastase. The samples were allowed to incubate for 1 h at room temperature, and each individual gauze sample was removed and placed in an Autovial press filter (Whatman) to extract unbound buffer and enzyme. The filtered fraction of each individual sample was recombined with solution not taken up by the gauze and, the combined solutions were assayed for elastase activity.

2.6. Patients and wound fluids

Informed consent was obtained for all procedures, and approval was received from the Virginia Commonwealth University Committee on the Conduct of Human Research, in accordance with the 1975 Declaration of Helsinki. Fluids were harvested from a grade III trochanteric pressure ulcer of a patient with a spinal cord injury using a sub-atmospheric device (V.A.C., KCI, San Antonio, TX). Fluids were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C. The protein concentration was determined with the Bio-Rad Protein assay (Richmond, CA) with bovine serum albumin as a quantitation standard.

2.7. Assay of wound fluid

The patient wound fluid was diluted (1:100 (wound fluid/buffer; v/v)) at a volume of 3 ml with pH 7.6 buffer (0.1 M sodium phosphate, 0.5 M NaCl, and 3.3% DMSO). The gauze samples were soaked in the wound fluid solutions for 1 h, then the solutions were filtered under pressure from the gauze using a Whatman Autovial (0.45 μm PFTE membrane). Recovery of the wound fluid solution from the gauze was 90%. The wound fluid solution was assayed for elastase activity in a manner similar to the elastase enzyme assay described earlier. Rates of substrate hydrolysis were measured on a reaction progress curve of absorbance versus time.

3. Results

3.1. Cellulose conjugates

The modifications made to the cotton cellulose in this study were citric acid conjugates of fructose and glucose. The monosaccharides were cross-linked to the cotton cellulose with an acid-catalyzed citric acid reaction (Andrews, 1990; Welch & Andrews, 1989a). The structures in Fig. 1 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 acid-catalyzed cross-linking 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.

Table 1
Description and abbreviation of cellulose conjugates

Structures (Fig. 1)	Description of modified gauze	Abbreviation
I	Citric acid–cellulose conjugate	CAC
II	Fructose–citric acid cellulose conjugate	FAC
III	Glucose–citric acid cellulose conjugate	GAC

Polycarboxylic acids, such as citric acid have previously been found to be effective cross-linking agents for cotton and other cellulosics (Andrews, 1990; Caulifield; Welch & Andrews, 1989a). The use of the tricarboxylic acid, citric acid, as a cross-linking agent for cellulose was first introduced for its inexpensive, and non-toxic use for durable press finishes of cotton. The cross-linking of cellulose with citric acid is optimized by use of an acid catalyst. Sodium hypophosphite has been shown to be the most effective catalyst for ester cross-linking of cellulose (Welch & Andrews, 1989b). The citric acid cross-linking of cellulose has been adopted in this study for the attachment of monosaccharides to cellulose. Yang showed that the cross-linking reaction of citric acid and other polycarboxylic acids containing three or more carboxyls proceeds through a cyclic anhydride (Yang and Wang, 1996). Thus, the reaction of a monosaccharide in the presence of citrate-esterified cellulose (CAC) provides two available carboxyls for cyclic anhydride-mediated esterification of the monosaccharide and yields a monosaccharide ester of CAC. However, the preferred sites of esterification in cellulose and the monosaccharides are not known. The functional group modifications made on the cotton cellulose and the abbreviations are listed in Table 1.

The citrate glucose and fructose conjugates of cellulose were characterized through base hydrolysis of the monosaccharide ester linked to cellulose followed by HPAE-PAD of the hydrolysis products. Since the monosaccharides are attached to the cellulose fiber through an ester linkage to citrate-cross-linked 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. The weakly acidic character of the monosaccharides allows partial ionization at the high pH of the chromatography eluant, which means they can be separated by anion-exchange mechanisms. The pulsed amperometric detection of glucose and fructose is possible through measurement of the electrical current generated by their

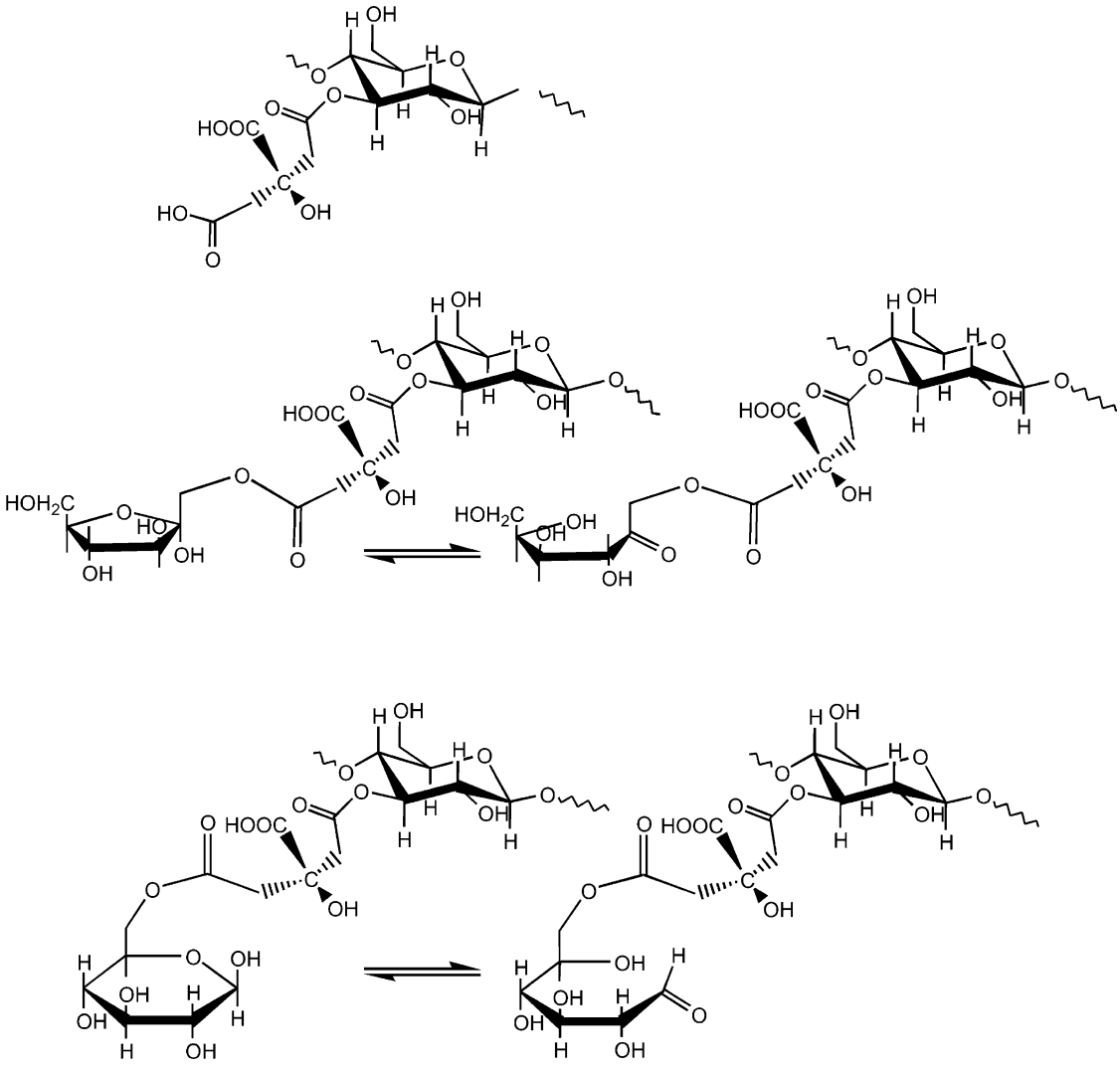

Fig. 1. Representative structures of fructose–citrate and GACs of cellulose shown as the interconverting cyclic and open chain forms of the sugars that occur in water. Structure I is a citric acid–cellulose conjugate. Structure II is of a fructose–citric acid cellulose conjugate. Structure III is of a glucose–citric acid cellulose conjugate.

Table 2
Glucose and fructose levels on citrate–cellulose conjugates (citrate, fructose, and glucose conjugates were treated as outlined in Section 2 to quantitate glucose and fructose. The glucose and fructose concentrations on cotton gauze were determined from HPAE-PAD chromatograms as described in Section 2. Quantities of the monosaccharides were calculated based on internal standards of known amounts of glucose and fructose eluted on the chromatography column)

Gauze	Glucose (μg/g cotton)	Fructose (μg/g cotton)
UT[a]	17.0 ± 0.6	3.6 ± 0.1
CAC	40.6 ± 4.0	2.5 ± 1.1
GAC	4076.4 ± 334.0	162.2 ± 61.0
FAC	156.8 ± 14.5	902.4 ± 132.0

[a] UT refers to untreated cotton gauze.

oxidation at the surface of a gold electrode. Fig. 2(A) shows the elution profile of glucose and fructose samples injected onto the HPAE-PAD system for chromatographic analysis. 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 and 0.9 mg per gram of cotton, respectively. The lower levels of glucose and fructose observed in both the fructose and glucose conjugate hydrolysates were due to the alkaline degradation of the monosaccharide. During the alkaline hydrolysis of the monosaccharide, ester glucose and fructose are in equilibrium through the same 1,2- and 2,3-enediol anion species, which goes through the Lobry de Brujn–Alberda van Ekenstein rearrangement (Lobry de Brujn & Alberda van Ekenstein, 1895).

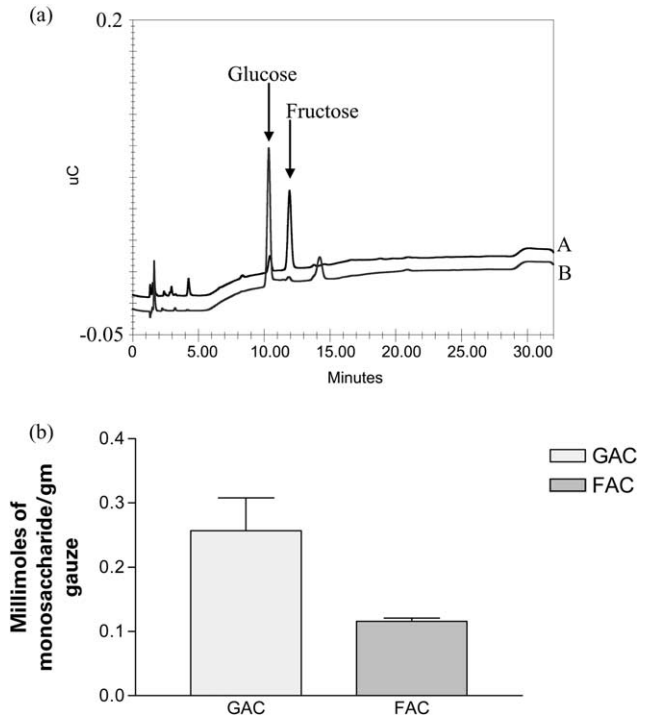

Fig. 2. (A) High performance anion exchange chromatogram with pulsed amperometric detection of glucose and fructose hydrolyzed from the citrate ester of cellulose. Following base cleavage of the fructose and glucose from the cotton fiber neutralized solutions containing the products were analyzed using chromatographic analysis as described in Section 2. (B) The quantitative determination of fructose and glucose conjugated to cellulose cotton gauze was determined as described in Section 2. Levels of glucose and fructose were measured by difference from citrate–cellulose used as a control employing a modified Fehling's solution.

In addition to the chromatographic measurement of fructose and glucose, a modified Fehling's test for ketone and aldehyde groups (Fieser & Fieser, 1967; Garner, 1967) was used as an additional assessment of the amount of fructose and glucose cross-linked on the cotton cellulose. As shown in Fig. 2(B), the concentration of monosaccharide per weight of cotton gauze is determined by this colorimetric method. Threefold more glucose–citric acid conjugate was found than the fructose conjugate. Base titration of free carboxyls due to cellulose bound citrate groups was employed to determine the degree of substitution (DS) levels of available citrate carboxyls esterified to cellulose. All citric acid conjugates had DS levels of 0.32, which indicates nearly 10% of the anhydroglucose hydroxyls of cellulose were esterified.

The esterified citrate conjugates of cellulose were also characterized by FT-IR spectral analysis. Attachment of the monosaccharides to cellulose requires that at least two ester bonds are formed between the anhydroglucose hydroxyls of cellulose and the citrate carboxyls, and between the citrate carboxyls and hydroxyls of the monosaccharide as shown in Fig. 1. Thus, the cross-linked cotton fiber has carbonyl groups that may be characterized as ester, carboxylic acid, and carboxylate anion functionalities and which may be detected by infrared bands. Previously, Yang (1991) showed that citrate ester cross-linkages in cotton cellulose can be distinguished from the corresponding citrate acid and carboxylate anions through IR analysis of acid and base treated fabric. FT-IR spectral analysis of the glucose and fructose conjugates was used to show that the spectral band of the ester carbonyl can be separated from the bands of the carboxylic acid and carboxylate anion found in the cotton fiber. Figs. 3 and 4 show the spectral results of the

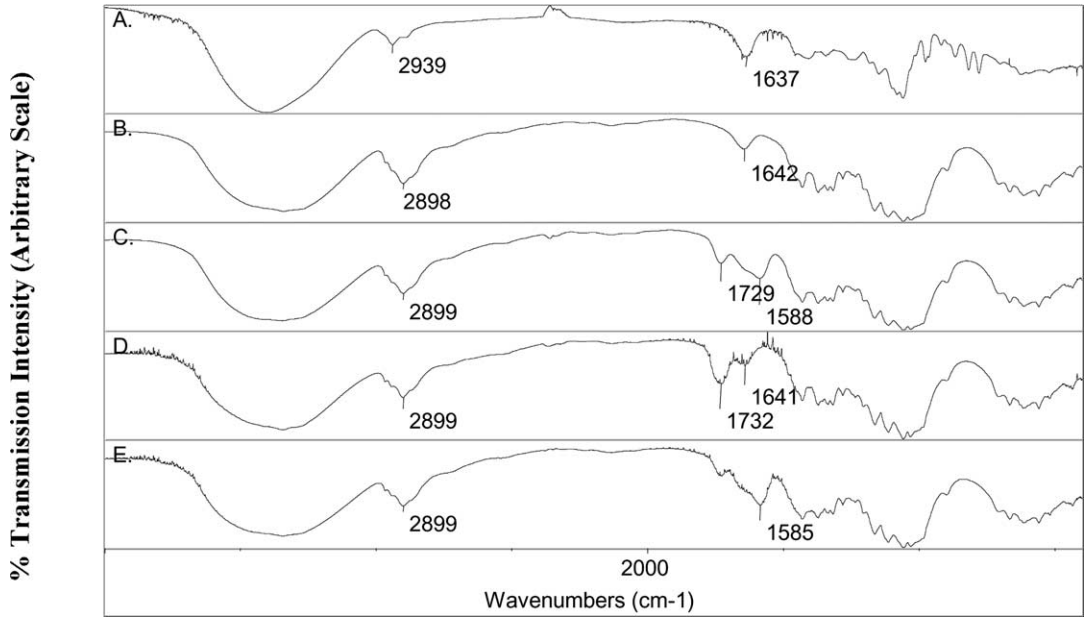

Fig. 3. Fourier transform infrared spectra of FAC of cellulose on gauze with acid and base treatments. (A) Spectrum of fructose (neat). (B) Spectrum of untreated gauze or cellulose. (C) Spectrum of FAC of cellulose. (D) Fibers treated with 0.1 M HCl solution. (E) Fibers treated with 0.1 M NaOH solution.

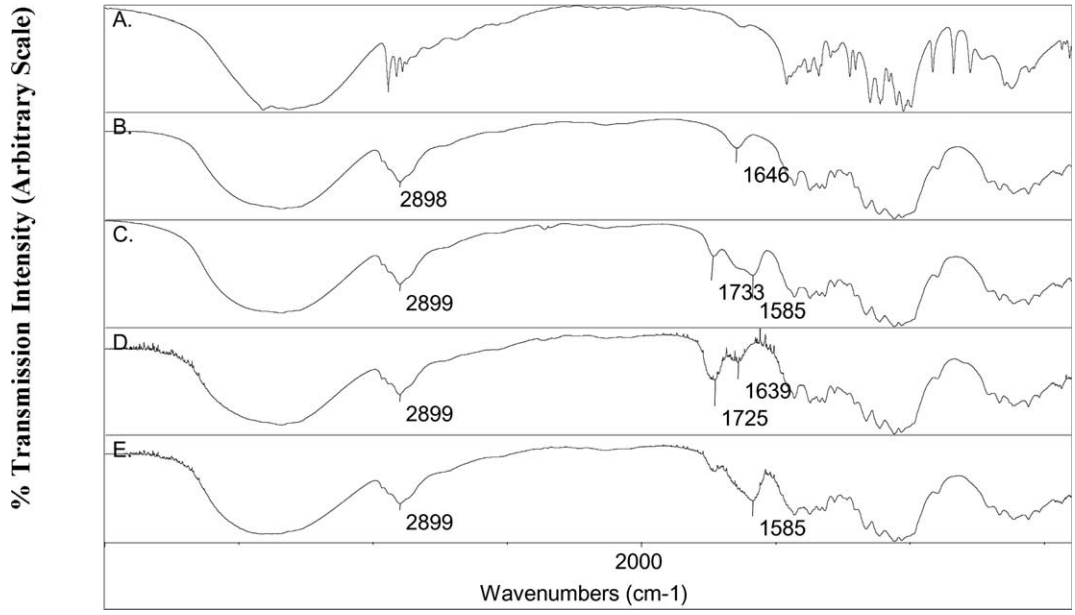

Fig. 4. Fourier transform infrared spectra of GAC of cellulose on gauze with acid and base treatment. (A) Spectrum of glucose (neat). (B) Spectrum of untreated gauze or cellulose. (C) Spectrum of GAC of cellulose. (D) Treated with 0.1 M HCl solution. (E) Treated with a 0.1 M NaOH solution.

hydrolysis products of the fructose–citrate conjugate (FAC; Fig. 3) and glucose–citrate conjugate (GAC; Fig. 4) gauze. Both types of cross-linked fabrics containing the glucose and fructose citrate conjugates were treated with 0.1 M NaOH for 2 min at room temperature. The resulting spectra of that modification are seen in Figs. 3(E) and 4(E). Base treatment of the conjugate gives an increase in the intensity of the band at 1585 cm^{-1} and a decrease in the 1732 and 1725 cm^{-1} band intensity (Figs. 3(D) and 4(D), respectively) occurred in the spectrum of the cotton gauze fibers. When the gauze is treated with 0.1 M HCl for 2 min at room temperature, an increase in the band at 1725 cm^{-1} occurs and the band at 1588 and 1585 cm^{-1} disappear (as shown in Figs. 3(D) and 4(D)). Thus, the band at 1585 and 1588 cm^{-1} (Figs. 3(C) and 4(C)) observed in the spectra of the conjugates is due to the carbonyl of the carboxylate anion. However, the carbonyls of the carboxylic acid and the ester overlap for the fructose–citrate and GACs, respectively, at 1732 and 1725 cm^{-1} as seen in Figs. 3(D) and 4(D).

3.2. Effect of modified gauze on elastase activity

The ability of the modified cotton gauze to absorb neutrophil elastase in solution was measured. Quantities of conjugated cotton gauze were soaked to saturation for an hour in elastase solutions, and the kinetics of elastase activity were measured from these solutions based on the relative initial velocity (v_0) values for enzyme solutions exposed to cotton gauze as described previously (Edwards et al., 2001).

The measurement of elastase activity remaining in solution upon treatment with the gauze was accomplished by monitoring the reaction rate within a 30-min time frame. The reaction progress curves for treated samples are shown in Fig. 5. Citric acid cross-linking 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 GAC, the FAC lowered elastase activity and demonstrated a more potent dose response per gram of gauze. The elastase-lowering activity of FAC gauze was linear at 75, 25, and 10 mg. Larger amounts of the GAC conjugate were required to achieve a similar elastase-lowering activity. CAC also lowered elastase activity in solution at a rate equivalent to GAC.

An assessment of the kinetics of elastase-lowering activity in wound fluid for all three gauzes is shown in Fig. 6(A)–(C). In Fig. 6(A), it can be seen that the fructose conjugate (FAC) lowered elastase activity in wound fluid in a dose dependent manner at 10, 25, and 50 mg quantities of gauze. With increasing FAC gauze concentration lower activity is observed, when compared with untreated gauze. Citric acid (CAC) and citric acid glucose (GAC) conjugates were compared at 50 and 100 mg quantities of gauze. The elastase activity remaining in solution following treatment with CAC and GAC gauze was comparable. However, FAC gauze appeared the most effective in removing elastase activity from the wound fluid, since more elastase activity was removed from solution per weight of FAC gauze than with GAC and CAC. Fig. 7 demonstrates the lowered elastase activity (recorded as enzyme activity based on initial velocity v_0) remaining in solution as a function of gauze weight. The relationship between elastase activity remaining in wound fluid and FAC, CAC, and GAC gauze concentrations is shown. Based on the amounts of elastase activity

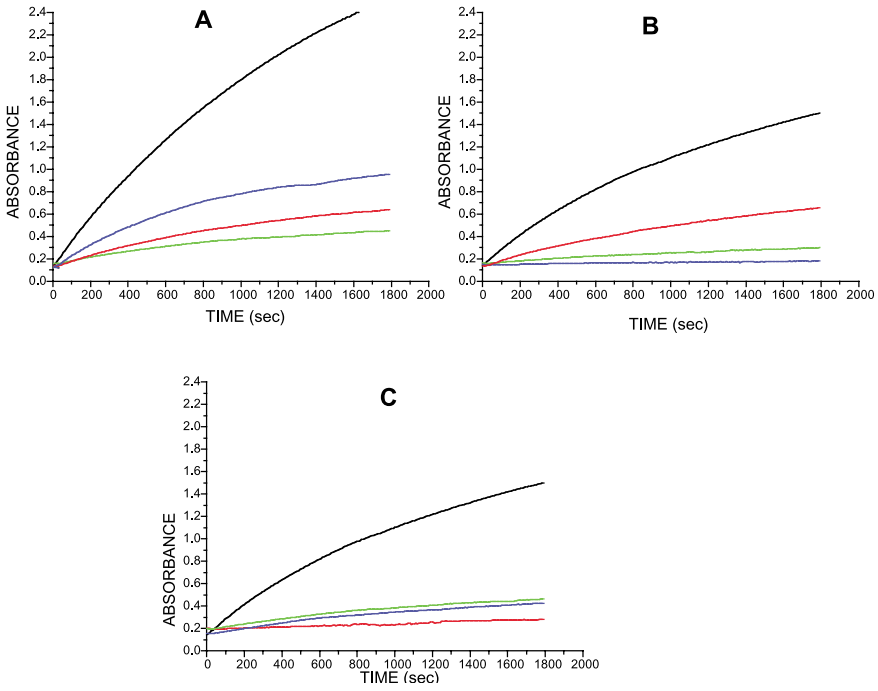

Fig. 5. Reaction progress curves for fructose- and glucose–citrate cellulose gauze-treated solutions of elastase. The reactions are described in Section 2. The substrate hydrolysis was performed with a 60 μM solution of MeOSuc-Ala-Ala-Pro-Val-*p*NA. Reaction rates were monitored by spectrophotometric measurement of the release of *p*-nitroaniline at 405 nm. (A) Control gauze at 75 mg (black); FAC gauze at 10 mg (blue); FAC gauze at 25 mg (red); FAC gauze at 75 mg (green). (B) Elastase control without gauze (black); CAC at 25 mg (red); CAC gauze at 50 mg (green); CAC gauze at 100 mg (blue). (C) Elastase control without gauze (black); GAC gauze at 25 mg (blue); GAC gauze at 50 mg (green); GAC gauze at 100 mg (red).

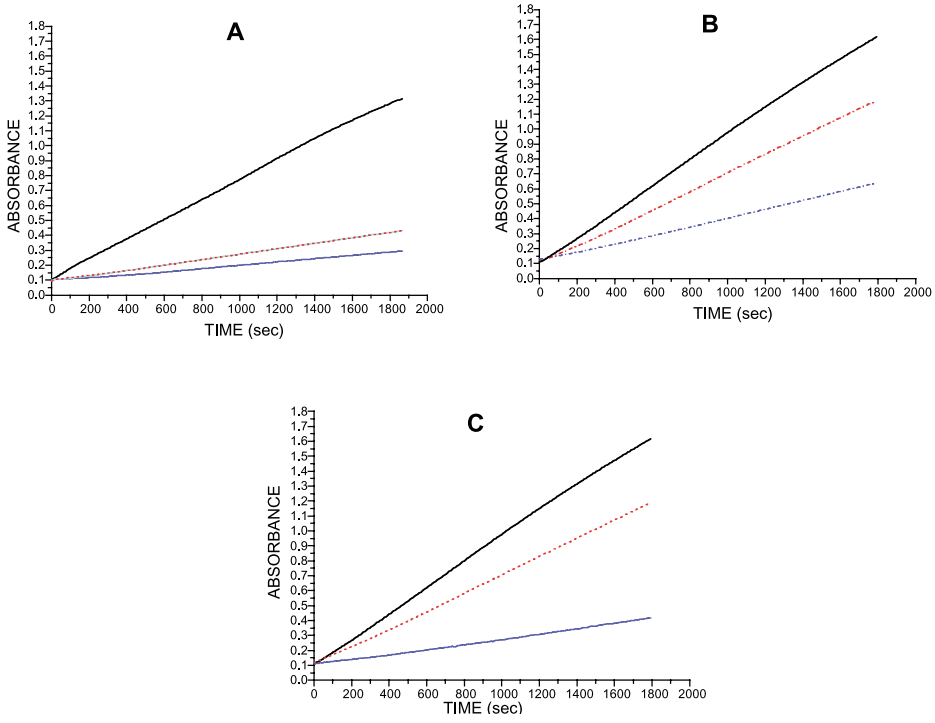

Fig. 6. Elastase activities from conjugate-soaked solutions containing human wound fluid. The assays are described in Section 2. The wound fluid solution was diluted in buffer 1:100 (wound fluid/buffer, v/v). The substrate hydrolysis was performed with a 60 μM solution of MeOSuc-Ala-Ala-Pro-Val-*p*NA in the wound fluid solution as described in Section 2. Reaction rates were monitored by spectrophotometric measurement of the release of *p*-nitroaniline at 405 nm. (A) Elastase-containing wound fluid (black); FAC gauze at 10 and 25 mg (yellow/pink); FAC gauze at 50 mg (blue). (B) Elastase-containing wound fluid with no gauze (black); CAC gauze at 50 mg (red); CAC at 100 mg (blue). (C) Elastase-containing wound fluid (black); GAC gauze at 50 mg (red); GAC gauze at 100 mg (blue).

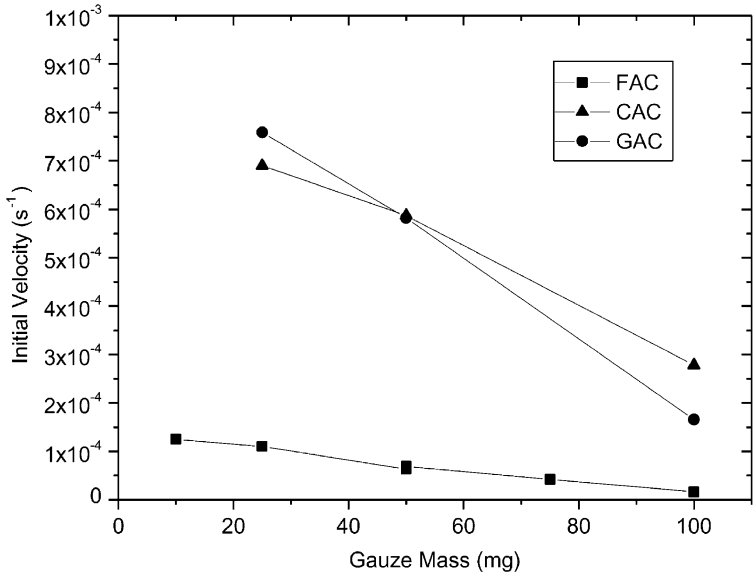

Fig. 7. A plot of the relationship between FAC, GAC, and CAC concentration and elastase activity remaining in the wound fluid. The plot is of elastase activity (measured as initial velocities, v_0) versus the mass of the cotton gauze conjugates.

remaining in wound fluid solution relative to GAC and CAC gauze, FAC gauze appears to lower elastase activity in wound fluid in a dose-dependent manner more effectively than GAC or CAC.

The fructose–citric acid conjugate (FAC gauze) was selected to compare levels of elastase extracted from solutions of pure human neutrophil elastase and from wound fluid. Measurement of protein levels remaining in solution and in wound fluid following incubation with elastase was performed to compare the relative amounts of protein taken up by the modified and untreated gauze. Fig. 8 shows a decrease in protein levels of both pure elastase (Fig. 8(A)) and with wound fluid-containing protein (Fig. 8(B)) with increasing sample weight. Untreated gauze did not show a similar decrease in protein levels.

4. Discussion

This study presents a method to prepare, characterize, and assay monosaccharide–citrate conjugates of cotton cellulose gauze for their ability to sequester elastase from wound fluid. Fructose and glucose citrate esters and citrate–cellulose conjugates were assessed in a saline solution simulated to represent the chronic wound environment. The conjugates were designed to assess the electrophilic and anionic character of the monosaccharide–citrate ester and citrate carboxyl of cellulose to enhance the affinity of cotton cellulose for elastase. Previously, it has been shown that both anionically charged cotton and addition of aldehyde functionality in cotton gauze results in sequestration of elastase from solution (Edwards et al., 2001). The heterogeneity of anionically charged citrate carboxyls and fructose and glucose-esterified citrate in the cellulose fiber creates two potential types of molecular sites on the modified cotton fiber for binding of elastase. Thus, the anionic sites of the conjugates may confer affinity for the positively charged residues of elastase and the aldehyde and ketone character of the monosaccharides may confer affinity for the active site of elastase.

Two monosaccharide analogs of CAC were compared in this study with CAC, and structures of the cellulose conjugates, as they may be bonded to the cellulose backbone, are depicted in Fig. 1. The conjugates were characterized by removing the esterified monosaccharides from the cotton by way of base hydrolysis and then monitoring the released glucose and fructose by HPAE-PAD and Fehling's solution. HPAE-PAD is considered to be a more sensitive and accurate measure of monosaccharide than the Fehling's test, which is partially attributable to the direct measurement of monosaccharides. In comparison, Fehling's measures all reducing sugars including the reducing sugars associated with cellulose and is not specific for reducing sugars, since ordinary aldehydes reduce the reagent. This difference in reducing sugars most likely accounts for the higher levels of glucose and fructose found on the cotton samples with the Fehling's solution.

An assessment of the elastase-lowering activity of cellulose conjugates (FAC, GAC, CAC) revealed that the citrate-linked cellulose conjugate of fructose and glucose, as well as the citrate–cellulose conjugate lowered elastase activity. When the citrate–cellulose conjugates were tested in solution, the most effective conjugate per weight of cellulose was the FAC conjugate gauze. However, the elastase-lowering activity observed with the citrate–cellulose conjugate (CAC) suggests that the cellulose-linked citrate carboxyls anionically bind positively charged elastase. The GAC and CAC conjugates possessed comparable activity in wound

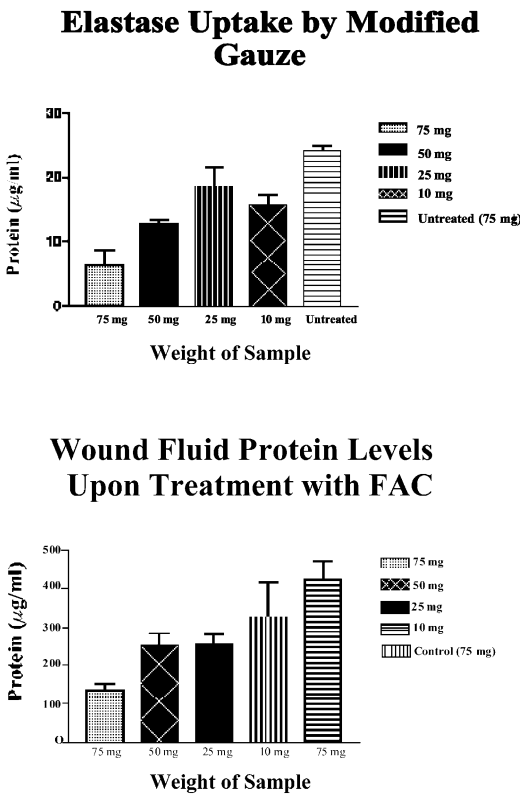

Fig. 8. (A) Protein uptake by elastase-soaked modified gauze, fructose–citric acid cellulose conjugate (FAC). Weighed FAC gauze was soaked in solutions containing elastase (1 unit/ml). Solutions were treated as described in Fig. 5(A). (B) Weighed FAC gauze samples were soaked in wound fluid solutions (diluted 1:100, wound fluid/buffer). Solutions were incubated with the specified weight of modified gauze for 1 h whereupon the solutions were filtered from the gauze under pressure applied to the gauze using a Whatman Autovial (0.45 μm PFTE membrane). The protein concentration was determined with the Bio-Rad Protein assay (Richmond, CA) with bovine serum albumin as a quantitation standard.

fluid. The effect of reducing elastase activity in solution with cotton containing aldehydic and negatively charged cellulose fibers has been observed with dialdehyde cotton and sulfonated, phosphorylated and carboxymethylated cotton (Edwards et al., 2001). Previously, the dialdehyde cotton gauze was designed for active site uptake of elastase and the negatively charged fibers were designed for counter-ion binding to the quanidinium-rich groups of elastase-containing arginines. A similar design has been applied to the cotton fibers discussed in this paper. The cotton gauzes of this study combine a negatively charged citrate cross-linked fiber with esterified glucose and fructose, which have some aldehyde and ketone functionality, respectively. Reducing sugars, such as fructose and glucose contain a free aldehyde or keto group, which reduce indicators, such as cupric ion (Cu^{2+}) complexes to the cuprous ion form (Cu^{+}) used in the assessment of the copper number employed for quantitative assessment of the monosaccharide titer on the cotton gauze. The elastase activity associated with the fructose conjugates (FAC) was significantly lower than the citrate and glucose conjugate (Fig. 8), which suggests the open chain hemiketal (keto group) of the fructose conjugate may enhance affinity of the elastase for the gauze.

The development of improved wound dressings usually arises from the synergy of a medical or surgical need for improved healing of the patient wound, coupled with the ability to design and enhance the wound dressing composition to meet that need. For example, the finding that moist wounds heal faster than those left exposed to the air gave rise to occlusive dressings (Winter, 1962). Cotton gauze is an important standard in the management of chronic wounds and continues to be utilized in hospitals and long-term care facilities for that purpose. However, little has been reported regarding the application of sugars and polysaccharides to cotton gauze. Here, we have presented a method for linking carbohydrates to cellulose through citrate esterification. This approach will provide a way of further assessing carbohydrates on cotton. On the other hand, the singular use of sugars and polysaccharides alone in promoting wound healing has been documented over the ages. For example, honey which contains fructose, glucose and other simple sugars and oligosaccharides is recorded in the Edwin Smith papyrus as being used by the ancient Egyptians, when combined with lard or resin into wounds, and 1000 years later Hippocrates recommended its use as an ointment or salve (Elliot, 1964). Granulated sucrose has also been evaluated with some mixed results over the years for its effect on improved healing of pressure ulcers (Chirife et al., 1982). Commercial wound dressings made from polysaccharides have principally been formulated as pastes, granules, and beads (Thomas, 1990).

The results of these studies indicate that elastase is removed from wound fluid by monosaccharide–citrate conjugates of cellulose. It is our premise that by reducing the activity of destructive proteases in the non-healing wound, while allowing important growth factors needed for healing to remain in the wound, accelerated healing should take place. We have taken a molecular-based mechanistic approach to explore this possibility for improved wound healing. The effect of this approach to promote selective uptake of elastase in the presence of growth factors, such as platelet derived growth factor, that promote wound healing remains to be studied. However, the significant increase in activity of the fructose conjugates of cellulose demonstrates that there may be an added benefit in applying monosaccharide functional groups to cotton gauze for removal of elastase from wound fluid. Future work will address optimizing the elastase-sequestering effect of citrate cross-linked cotton gauze by probing the types of polysaccharide conjugates that can be prepared and employed to increase selective elastase affinity for modified cotton gauze.

References

Andrews, B. A. (1990). Nonformaldehyde DP finishing of cotton with citric acid. *Textile Chemist and Colorist*, *22* (9), 63–67.

Bonney, R. J., & Smith, R. J. (1986). In I. Otterness, et al., *Advances in inflammation research*, Vol. II. New York: Raven Press.

Caulifield, D. F. (1994) Ester crosslinking to improve wet performance of paper using multifunctional carboxylic acids, butanetetracarboxylic and citric acid. *TAPPI Journal*, *77* (3), 205–212.

Chirife, J., et al. (1982). Scientific basis for use of granulated sugar in treatment of infected wounds. *Lancet*, *1*, 560–561.

Edwards, P. D., & Bernstein, P. R. (1994). Synthetic inhibitors of elastase. *Medical Research Review*, *14*, 127–194.

Edwards, J. V., Batiste, S. L., Gibbins, E. M., & Goheen, S. C. (1999). Synthesis and activity of NH_2- and COOH-terminal elastase recognition sequences on cotton. *Journal of Peptide Research*, *54*, 536–543.

Edwards, J. V., Bopp, A. F., Batiste, S., Ullah, A. J., Cohen, I. K., Diegelmann, R. F., Montante, S. J. (1999) Inhibition of elastase by a synthetic cotton-bound serine protease inhibitor; in vitro kinetics and inhibitor release. *Wound Repair and Regeneration*, *7*, 106–118.

Edwards, J. V., Yager, D. R., Cohen, I. K., Diegelmann, R. F., Montante, S., Bertoniere, N., & Bopp, A. F. (2001). Modified cotton gauze dressings that selectively absorb neutrophil elastase activity in solution. *Wound Repair and Regeneration*, *9*, 50–58.

Elliot, I. M. Z. (1964). *A short history of surgical dressings*, London: Pharmaceutical Press.

Fieser, L. F., & Fieser, M. (1967). *Reagents for organic synthesis*, Vol. 1 p. 360.

Garner, W. (1967). *Textile laboratory manual*, Vol. 5. Amsterdam: American Elsevier Publishing Company pp. 70–72.

Grinell, F., & Zhu, M. (1994). Identification of neutrophil elastase as the proteinase in burn wound fluid responsible for degradation of fibronectin. *Journal of Investigative Dermatology*, *103*, 155–161.

Lobry de Brujn, C. A., & Alberda van Ekenstein, W. (1895). Action des alcalis sur les sucres. *Recueil des Travaux Chimiques du Pays-Bas*, *14*, 203.

Nakajima, K., Powers, J. C., Ashe, B. M., & Zimmerman, M. (1979). Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. *Journal of Biological Chemistry*, *254*, 4027–4032.

Powers, J. C. (1983). Synthetic elastase inhibitors: Prospects for use in the treatment of emphysema. *American Review of Respiratory Disease*, *127*, 54–58.

Schechter, I., & Berger, A. (1967). *Biochemical and Biophysical Research Communications*, *27*, 157–162.

Thomas, S. (1990). *Polysaccharide pastes, granules and beads in wound management and dressings*, London: The Pharmaceutical Press pp. 62–68.

Welch, C. M., Andrews, B. K. (1989a). US Patent 4,820,307.

Welch, C. M., & Andrews, B. A. K. (1989b). Cross-links: A route to high performance nonformaldehyde finishing of cotton. *Textile Chemist and Colorist*, *21* (2), 13–17.

Winter, G. D. (1962). Formation of the scab and the rate of epithelization of superficial wound in the skin of the young domestic pig. *Nature*, *193*, 293–294.

Yager, D. R., Chen, S. M., Ward, S. I., Olutoye, O. O., Diegelmann, R. F., & Cohen, I. K. (1997). Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of proteinase inhibitors. *Wound Repair and Regeneration*, *5*, 23–32.

Yang, C. Q. (1991). Characterizing ester crosslinkages in cotton cellulose with FT-IR photoacoustic spectroscopy. *Textile Research Journal*, *61*, 298–305.

Yang, C. Q., & Wang, X. (1996). Formation of cyclic anhydride intermediates and esterification of cotton cellulose by multifunctional carboxylic acids: An infrared spectroscopy study. *Textile Research Journal*, *66* (9), 595–603.