Antimicrobial Properties of a Chitosan Dextran-Based Hydrogel for Surgical Use

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A chitosan dextran-based (CD) hydrogel, developed for use in endoscopic sinus surgery, was tested for antimicrobial activity *in vitro* against a range of pathogenic microorganisms. The microdilution technique was used to determine minimum inhibitory, minimum bactericidal, and minimum fungicidal concentrations. In addition, the time-kill efficacy of CD hydrogel was determined for two bacterial species. Scanning and transmission electron microscopy were carried out to elucidate the antimicrobial mechanism of this compound. CD hydrogel was found to be effective against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Clostridium perfringens* at its surgical concentration of 50,000 mg/liter. Minimum bactericidal concentrations ranged from 2,000 to 50,000 mg/liter. Dextran aldehyde (DA) was found to be the antimicrobial component of the CD hydrogel with MBC ranging from 2,000 to 32,000 mg/liter. *S. aureus* appeared to be killed at a slightly faster rate than *E. coli*. *Candida albicans* and *Pseudomonas aeruginosa* were more resistant to CD hydrogel and DA. Scanning and transmission electron microscopy of *E. coli* and *S. aureus* incubated with CD hydrogel and DA alone revealed morphological damage, disrupted cell walls, and loss of cytosolic contents, compatible with the proposed mode of action involving binding to cell wall proteins and disruption of peptide bonds. Motility and chemotaxis tests showed *E. coli* to be inhibited when incubated with DA. The antibacterial activity of CD hydrogel may make it a useful postsurgical aid at other body sites, especially where there is a risk of Gram-positive infections.

Due to their high water content and mechanical properties, hydrogels are very similar to human tissue and are implicated in a wide variety of biomedical applications. More specifically, hydrogels can be used to facilitate the postoperative healing process. They do so by controlling bleeding, allowing tissue to remain in the proper orientation, and by preventing the formation of adhesions (13). A chitosan/dextran-based (CD) hydrogel has been developed as a postsurgical aid in endoscopic sinus surgeries (ESS). The CD hydrogel significantly reduced the number of adhesions, as well as exhibiting excellent haemostatic, mucoadhesive, and antimicrobial properties (4, 24). Chitosan, a natural polymer derived from the alkaline deacetylation of chitin, was selected due to its nontoxicity, biocompatibility, and biodegradability (9).

In the health care arena, the development of a hydrogel that is biocidal for a broad range of pathogenic microorganisms is of great importance. The antimicrobial activity of chitosan against a wide range of Gram-positive and Gram-negative bacteria, filamentous fungi, and yeasts is well documented in the literature (10). The antibacterial activity of chitosan is attributed to its polycationic structure, which exerts a strong electrostatic interaction with the negatively charged bacterial cell surface, disturbing the cell membrane and inducing leakage (10). Chitosan is soluble in dilute acid and antimicrobial activity is pH dependent, with the molecule becoming polycationic at a pH below the pK_a (14). For biomedical purposes, a biocidal hydrogel should ideally be effective under neutral physiological rather than acidic conditions.

Toward this end, a modified chitosan was developed that is soluble in aqueous physiological conditions and neutral pH. The substitution of a hydrophilic group, usually with a negative charge, is needed in order to create a more water-soluble derivative. To achieve this, *N*-succinyl chitosan (SC) was prepared. The CD hydrogel was synthesized by the reaction of this amine functionalized SC (Fig. 1) and an oxidized dextran aldehyde (DA) (Fig.

2). Characterization of the CD hydrogel has previously been reported (14). Dextran, a bacterium-derived polysaccharide consisting of α -1,6-linked D-glucopyranose units, was selected due to its biocompatibility, biodegradability, and large number of hydroxyl groups that could be easily oxidized to aldehydes (18). These aldehyde groups allow the oxidized dextran to function as a macromolecular cross-linker for polymers containing large amounts of amino groups for hydrogel synthesis (6). The CD hydrogel used here was formed using a 1:1 mixture of 5% SC and 5% DA dissolved in water and once mixed, formed a gel in under a minute.

Chitosan derivatives that dissolve under neutral conditions have been found to lose some of their bactericidal activity (10). However, from preliminary work in animal models the CD hydrogel appeared to be antimicrobial. The aims of the present study were to determine the *in vitro* activity of CD hydrogel and its components against a range of microbial pathogens and to investigate the mode(s) of action. The antimicrobial characterization of DA has not been previously reported. MICs, minimum bactericidal concentrations (MBCs), and minimum fungicidal concentrations (MFCs) for various Gram-negative, Gram-positive, and one fungal species were determined by using the broth microdilution method. To evaluate the cellular effects on target bacteria, scanning electron microscopy (SEM) and transmission electron

Received 7 August 2011 Returned for modification 27 September 2011
Accepted 16 October 2011

Published ahead of print 24 October 2011

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doi:10.1128/AAC.05463-11

FIG 1 Molecular structure of N-succinyl chitosan.

microscopy (TEM) were used. The inhibition of motility was also investigated.

MATERIALS AND METHODS

Materials. Except for dimethylformamide (DMF; Univar, Ingleburn, New South Wales, Australia), all chemicals, antibiotics, and antifungals were supplied by Sigma-Aldrich (Castle Hill, New South Wales, Australia). Chitosan was supplied as practical grade and dextran (from *Leuconostoc mesenteroides*) had a stated molecular weight of 48,000 to 90,000. Stock solutions of penicillin G, fluconazole, and amphotericin B were filter sterilized; gentamicin was purchased as a sterile solution. Dehydrated bacteriological culture media were supplied by BD Company (Auckland, New Zealand), except for Columbia sheep blood agar plates which were purchased preprepared from Fort Richard Laboratories (Auckland, New Zealand). Roswell Park Memorial Institute (RPMI) 1640 medium was supplied by Invitrogen (Auckland, New Zealand).

Synthesis of succinyl chitosan and dextran aldehyde. SC and DA were prepared as previously described (14). Briefly, SC was obtained by heating chitosan with succinic anhydride in DMF followed by ethanol washing of the product. The polymer was then dissolved by high-speed stirring into sodium hydroxide solution and heating to the required temperature overnight. The solution was filtered, dialyzed, and lyophilized to give the product, a white fibrous material with a 75 to 80% yield. For DA, an aqueous solution of sodium periodate was gradually added to an aqueous solution of dextran with vigorous stirring. The mixture was then dialyzed and lyophilized to give a white, solid product. The product was very slow to dissolve, except at higher pH (7.0), possibly due to intramolecular hemiacetal formation (Fig. 2). The CD hydrogel was formed using a 1:1 mixture of 5% SC and 5% DA.

As previously described, analysis of the CD hydrogel was by proton nuclear magnetic resonance spectroscopy (^1H NMR) and gel permeation chromatography (GPC) (14). GPC was performed on a Polymer Laboratory PL1 using 0.02 M sodium phosphate buffer at pH 7.2, through a

mixed C column at 1.0 ml min^{-1} with dextran standards (Fluka, Biochemika, Buchs, Switzerland). Gel times were taken as the point where a 2-ml solution failed to flow in an inverted 5-ml glass sample vial. Aldehyde levels were measured by reaction with hydroxylamine hydrochloride and titration of the released acid (25).

Microorganisms and culture conditions. The microorganisms used in the present study included *Candida albicans* NZCC 1212, *Clostridium perfringens* ATCC 13124, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 9144, and *Streptococcus pyogenes* ATCC 12344 (New Zealand Culture Collection, Environmental Science and Research, Porirua, New Zealand).

For MIC, MBC, and MFC determinations, *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes* were cultured aerobically in Muller-Hinton broth (MHB) and subcultured on tryptic soy agar (TSA). *C. perfringens* was cultured anaerobically in brain heart infusion broth supplemented with 1% hemin and vitamin K₁ and subcultured on supplemented brain heart infusion agar. *C. albicans* was cultured aerobically in RPMI 1640 (with glutamine), buffered at pH 7.0 with morpholinepropanesulfonic acid, and subcultured on Sabouraud's agar.

MIC, MBC, and MFC determinations. The MICs, MBCs, and MFCs of DA, SC, and CD hydrogel were determined by the broth microdilution method (2). The range of concentrations tested are given in Table 1, along with those of the antimicrobial controls. Gentamicin was the antimicrobial control for Gram-negative species, penicillin was used for Gram-positive species, and amphotericin B was used for *C. albicans* (preliminary experiments showed the *C. albicans* strain to be fluconazole resistant [MIC > 64 mg/liter]). For CD hydrogel, a concentration of 50,000 mg/liter was included in the dilution series since this is the concentration of the product in clinical use. The SC solution was steam sterilized prior to use. DA was dissolved in distilled water then filter sterilized using $0.2\text{-}\mu\text{m}$

FIG 2 Molecular structure of dextran aldehyde showing likely formation of hemiacetal structure.

TABLE 1 Antimicrobial concentrations tested for MIC, MBC, and MFC determinations

Compound	Concn(s) (mg/liter) ^a
CD hydrogel	50,000, 32,000, 16,000, 8,000, 4,000, 1,000, 500, 250, 125, 62.5
SC	32,000, 16,000, 8,000, 4,000, 1,000, 500, 250, 125, 62.5
DA	(100,000, 64,000) 50,000, 32,000, 16,000, 8,000, 4,000, 1,000, 500, 250, 125, 62.5
Gentamicin	32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06
Penicillin G	32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06
Amphotericin B	32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06
Fluconazole	64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06

^a Additional DA concentrations for MFC determination in *C. albicans* are indicated in parentheses.

TABLE 2 MICs and MBCs of CD hydrogel, SC alone, DA alone, and antimicrobial positive controls

Microorganism (strain)	Concn (mg/liter)							
	CD hydrogel		SC		DA		Antimicrobial ^a	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Escherichia coli</i> (ATCC 25922)	32,000	50,000	>32,000	>32,000	32,000	32,000	1*	1*
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	>50,000	>50,000	>32,000	>32,000	32,000	32,000	0.5*	1*
<i>Staphylococcus aureus</i> (ATCC 9144)	8,000	8,000	>32,000	>32,000	4,000	4,000	1†	1†
<i>Streptococcus pyogenes</i> (ATCC 12344)	4,000	4,000	>32,000	>32,000	2,000	4,000	0.0019†	0.0019†
<i>Candida albicans</i> (NZCC 1212)	>50,000	>50,000	>32,000	>32,000	100,000	>100,000	0.125‡	0.25‡, >64§
<i>Clostridium perfringens</i> (ATCC 13124)	2,000	2,000	>32,000	>32,000	1,000	2,000	0.125†	0.125†

^a *, Gentamicin antibiotic; †, penicillin G antibiotic; ‡, amphotericin B antifungal; §, fluconazole antifungal.

pore-size cellulose acetate filters (Millipore, North Ryde, New South Wales, Australia). Inocula were prepared by adjusting overnight cultures to 10^6 CFU/ml in the appropriate culture medium with reference to standard curves of optical density versus viable count. Optical density readings were obtained out in a microtiter plate reader at 600-nm absorbance (Tecan Infinite m200; Tecan Group Ltd, Männedorf, Switzerland). Equal volumes (75 μ l) of inocula and dilutions of DA, SC, CD hydrogel, and antimicrobial controls, prepared in the same growth medium as the bacterial species being tested, were mixed in 96-well plate microtiter plates (Falcon; BD Company) to give a final concentration of 5×10^5 CFU/ml. After incubation at $35^\circ\text{C} \pm 2^\circ\text{C}$ for 24 h in the appropriate atmospheric condition, wells were examined for growth to determine the MIC. *C. albicans* microtiter plates were examined at 24 and 48 h. For MBC, wells showing no growth were subcultured onto the appropriate agar plates and examined for colonies after 48 h of incubation at $35^\circ\text{C} \pm 2^\circ\text{C}$. The MIC, MBC, and MFC determinations were carried out in triplicate in two independent experiments.

Stability of dextran aldehyde in aqueous solution. DA samples were dissolved in distilled water, stored at room temperature, and then filter sterilized prior to use. Individual MICs and MBCs were determined for *E. coli* and *S. aureus* at 1-, 5-, and 14-day intervals.

Time-dependent killing efficacy of dextran aldehyde. Time-dependent killing efficacy of DA was determined for *E. coli* and *S. aureus*. Overnight cultures were adjusted in MHB so that a final concentration of 5×10^5 CFU/ml was achieved when mixed with equal volumes of DA (final concentrations of 32,000 mg/liter for *E. coli* and 4,000 mg/liter for *S. aureus*). The cultures were incubated at $35^\circ\text{C} \pm 2^\circ\text{C}$ with shaking. Samples were taken every 30 min for 6 h, serially diluted 10-fold, and spread plated on TSA to obtain viable counts. Spread plates were incubated at $35^\circ\text{C} \pm 2^\circ\text{C}$ for 24 h.

SEM. Overnight *E. coli* and *S. aureus* cultures were washed once with 0.85% NaCl solution and then resuspended in 5 mM HEPES (pH 5.3 ± 0.1) to an optical density at 600 nm of ~ 0.4 . After incubation for 3 h at $35^\circ\text{C} \pm 2^\circ\text{C}$ with or without 50,000 mg or 8,000 mg of CD hydrogel/liter, respectively, the cells were washed once with phosphate-buffered saline (PBS) and fixed in 2.5% glutaraldehyde-PBS (pH 7.3 ± 0.1). Samples were postfixed in osmium tetroxide, dehydrated in a series of increasing ethanol concentrations (25 to 100%), and then air dried using a Bal-Tec CPD-030 (Bal-Tac AG, Balzers, Liechtenstein). Samples were sputter coated with 5-nm gold palladium in an Emitech K575X (Veranstaltungen, Germany) and viewed in a field emission scanning electron microscope (JSM-6700F; JEOL, Tokyo, Japan).

Preparation of cells for TEM. Bacteria for TEM samples were prepared as previously described (12). Overnight cultures of *E. coli* or *S. aureus* in MHB were adjusted to 10^8 CFU/ml, and aliquots were added to CD hydrogel, DA, and Triton X-100 in separate experiments. The final concentrations for the *E. coli* experiments were 50,000 mg of CD hydrogel/liter, 32,000 and 16,000 mg of DA/liter, and 0.02% (vol/vol) Triton X-100. For *S. aureus*, final concentrations were 16,000 mg of CD hydrogel/liter, 8,000 mg of DA/liter, and 0.01% (vol/vol) Triton X-100. After incu-

bation for 3 h at $35^\circ\text{C} \pm 2^\circ\text{C}$, the suspension was centrifuged at $8,100 \times g$ for 2 min at room temperature (Eppendorf centrifuge 5415). The cells were washed twice with 5 mM sodium phosphate buffer solution (PB; pH 7.2), centrifuged at $3,500 \times g$ at room temperature (Heraeus Sepatech Biofuge 15 centrifuge; Heraeus Sepatech GmbH, Osterode, Germany), and then fixed with 1% OsO₄ in 5 mM PBS for 2 h. The samples were washed three times with PB, dehydrated using an ethanol gradient, and then embedded in Quetol 651 epoxy resin (1). Thin sections of the specimens were cut with a diamond knife on an Ultracut Ultramicrotome (Reichert, Depew, NY). The grids were examined with a Philips CM100 BioTWIN transmission electron microscope (Philips/FEI Corp, Eindhoven, Holland), LaB6 emitter fitted with MegaView III digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Motility and chemotaxis. Motility was determined in motility test medium with or without 1% triphenyl tetrazolium chloride (TTC) according to the manufacturer's instructions. TTC is converted to a red color by succinate dehydrogenase and was used to assist in visualizing the bacteria. *E. coli* and *K. pneumoniae* were grown in successive inoculations of overnight cultures in TSB for 3 days at $35^\circ\text{C} \pm 2^\circ\text{C}$. A portion (100 μ l) of the last inoculated broth was added to 10 ml of TSB, followed by incubation for 3 h with shaking (200 rpm) at $35 \pm 2^\circ\text{C}$ (Innova 4000; New Brunswick Scientific). The motility of the bacteria was checked microscopically using the hanging drop method. Aliquots of the motile *E. coli* broth were mixed with equal volumes of DA in TSB (16,000 and 32,000 mg/liter), followed by incubation at $35 \pm 2^\circ\text{C}$ for 2 h. *E. coli* (motile) and *K. pneumoniae* (nonmotile) cultures prepared as described above but without DA served as controls. The motility medium was stab inoculated with treated and untreated cultures and incubated for 24 h at $35 \pm 2^\circ\text{C}$. An uninoculated tube served as a sterility control. Motility appeared as clouding (TTC-negative tubes) or a red color (TTC-positive tubes) in the growth medium that progressed laterally and downward from the stab inoculation. Motility was scored from 0 to 4 as previously described (17); no growth beyond the line of inoculation was scored as 0, and growth throughout the culture medium scored as 4.

For the chemotaxis assay, semisolid, non-nutritive plates were prepared (PBS with 0.3% agar and 1% TTC). Circular plugs (7-mm diameter) of MacConkey agar and firm PBS agar were inserted at a distance of 60 mm from each other, as measured between the center of each plug. *E. coli* and *K. pneumoniae* were prepared and treated with DA as for the motility test. Bacterial cells were centrifuged at 2,000 rpm for 10 min and then washed twice with PBS. The washed bacteria (50 μ l) were injected in the center of each plate between the two plugs and incubated at $35^\circ\text{C} \pm 2^\circ\text{C}$ for 24 h.

RESULTS

Evaluation of antibacterial activity. The MICs, MBCs, and MFCs for CD hydrogel and its components are shown in Table 2. *C. perfringens*, *S. aureus*, and *S. pyogenes* were inhibited by CD hydrogel at concentrations well below the 50,000 mg/liter used clin-

FIG 3 Time-kill efficacy of 4,000 mg of dextran aldehyde (DA)/liter for *S. aureus* (a) and 32,000 mg of dextran aldehyde/liter for *E. coli* (b). Each data point is the average of two independent experiments, and the vertical bar is the standard deviation.

ically. The MBCs were no different from the MICs for these organisms. *E. coli* was more resistant but still killed by 50,000 mg of CD hydrogel/liter. *P. aeruginosa* and *C. albicans* were resistant to CD hydrogel at the concentrations tested. None of the microorganisms were susceptible to SC alone at the maximum concentration tested (32,000 mg/liter). DA alone was generally more inhibitory for the bacteria tested than CD hydrogel, but *C. albicans* showed reduced susceptibility, being inhibited at 100,000 mg/liter, a concentration well above that used clinically. For all experiments, the antimicrobial controls were within the expected ranges (5).

Stability of dextran aldehyde in aqueous solution. MIC and MBCs for *E. coli* remained the same as stated in Table 2 for DA stored for 1- and 5-day intervals. After 14 days, the MICs remained the same but the MBCs for *E. coli* increased from 32,000 mg/liter to 50,000 mg/liter. MICs and MBCs for *S. aureus* remained the same as stated in Table 2 when tested on days 1, 5, and 14 of the storage period.

Time-kill efficacy. DA produced a gradual reduction in the viable counts of *S. aureus* and *E. coli* over the 6-h time period (Fig. 3). Killing of *S. aureus* appeared to proceed at a slightly faster rate. Overall, decreases of 3 log₁₀ and 2.5 log₁₀ in the viable counts for DA-treated *S. aureus* and *E. coli*, respectively, contrasted with the 3- to 4-log₁₀ increase seen in the untreated controls.

Electron microscopy. Scanning and transmission electron mi-

FIG 4 SEM of *S. aureus* either untreated (a) or incubated with 8,000 mg of CD hydrogel/liter (the arrow indicates leaking of cellular contents) (b).

croscopy (SEM and TEM) were used to investigate the cellular effects of CD hydrogel on *S. aureus* and *E. coli*. In the SEM images, increased cell aggregation was observed for *S. aureus* under low magnification compared to untreated control cells (not shown). After 3 h of incubation with 8,000 mg of CD hydrogel/liter, high-magnification images of *S. aureus* revealed leakage of cellular contents and cells with a shrunken appearance compared to the untreated control (Fig. 4). After the same incubation period, *E. coli* cells treated with 32,000 mg of CD hydrogel/liter showed cracks in the cell wall in addition to clumped flagella and a slightly shrunken appearance compared to the untreated control (Fig. 5).

TEM of untreated *E. coli* control cells revealed an even distribution of cytoplasm and good membrane integrity as seen by electron-dense lines (Fig. 6a). Cells incubated with Triton X-100 showed a rippling effect of the cell membrane, uneven distribution, and a loss of cytoplasm (Fig. 6b). At an MIC concentration of 32,000 mg/liter, CD hydrogel produced a similar rippling of the

FIG 5 SEM of *E. coli* either untreated (a) or incubated with 32,000 mg of CD hydrogel/liter (b). The arrow indicates a large crack and clumped flagella.

cell membrane and the formation of surface blebs, with part of the released membrane lipids forming vesicle-like structures (Fig. 6c and d). When incubated with DA below the MIC (16,000 mg/liter) leakage of cytoplasmic contents was seen in some cells (Fig. 6e). At the MBC (32,000 mg/liter), there appeared to be an increased number of bacteria with clear zones in the cytoplasm and cytoplasmic leakage (Fig. 6f).

TEM results for *S. aureus* are shown in Fig. 7. Untreated *S. aureus* (Fig. 5a) showed round, complete cell walls, in addition to well delineated membranes and uniform cytoplasmic regions. Cells incubated with Triton-X (Fig. 5b and c) showed a thickening and uneven rippling of the cell wall, the formation of mesosome-like structures, and in some cases, lysis. When incubated with DA at double the MIC/MBC (8,000 mg/liter) (Fig. 5d), mesosome-like structures, indentations at the interface between the cytoplasm and cell membrane, and rippling of the cell wall were seen. At double the MIC/MBC (16,000 mg/liter), CD hydrogel produced a similar effect, with rippling of the cell wall and the presence of mesosome-like structures, a thickening of the cell membrane, and an uneven distribution of cytoplasmic contents (Fig. 5e and f). Cell lysis was observed only in Triton X-100-treated samples (data not shown).

Motility and chemotaxis. In stab-inoculated, semisolid motility medium, with or without TTC dye, nonmotile *K. pneumoniae* grew along the line of inoculation only (score = 0), while the growth of motile *E. coli* radiated away from the inoculation line (score = 3). The motility of *E. coli* pretreated with 16,000 mg of DA/liter was reduced (score = 2). When pretreated with 32,000 mg of DA/liter, growth extended only slightly beyond the inoculation line (score = 1). The TTC had no apparent inhibitory effect since the same results were obtained in medium without the dye. In the chemotaxis test, *K. pneumoniae* remained at the original point of inoculation, while untreated *E. coli* showed movement toward the MacConkey agar plug, surrounding it after 24 h of incubation. Pretreatment with 16,000 mg of DA/liter slowed the motility of *E. coli* slightly, whereas at a concentration of 32,000 mg/liter the bacterial motility was minimal in that movement away from the inoculation point occurred but the bacteria failed to surround the nutritive plug after 24 h of incubation. In none of the experiments was movement toward the non-nutritive plug observed.

DISCUSSION

The antimicrobial activity of CD hydrogel, SC alone, and DA alone was determined for a variety of microorganisms. The CD hydrogel was found to have a bactericidal effect against *E. coli*, *S. aureus*, *S. pyogenes*, and *C. perfringens* at or below the 50,000-mg/liter concentration used in its surgical application. The active, antimicrobial component of the CD hydrogel was shown to be DA since SC alone exhibited no antibacterial effect. The MIC and MBCs for DA alone were typically around half those obtained for CD hydrogel. This is most likely a dilution effect reflecting the composition of CD hydrogel (50% SC and 50% DA) and indicating an absence of synergistic or antagonistic effects. The antibacterial activity of DA was probably primarily due to the reaction of the aldehyde groups with amino groups in proteins located in cell membranes (22) but may also be due to breakage of the peptide bonds in cell wall peptidoglycan. Aldehyde-containing biocides, such as *ortho*-phthalaldehyde and glutaraldehyde, are reported to react with primary amino groups located on the outer envelope or cell wall, producing a strong adhesive effect (3, 16, 26).

The lack of antimicrobial activity of SC is in stark contrast to its starting material, chitosan, which has been well characterized as an effective antimicrobial agent (19). This is most likely due to the loss of the chitosans' polycationic nature. The amine groups of SC carry no positive charge at neutral pH and therefore are not able to electrostatically interact with negatively charged bacterial membranes (18). Although not antimicrobial on its own, SC has the important function of contributing free amine groups that react with the aldehyde groups of DA through a Schiff base reaction to form imine bonds that cross-link the hydrogel. The oxidized dextran functions as a macromolecular cross-linker for polymers containing large amounts of amino groups for hydrogel synthesis (6, 8).

CD hydrogel was an ineffective antimicrobial agent against *P. aeruginosa*, and the yeast, *C. albicans*, although both were inhibited by high concentrations of DA. *P. aeruginosa* is well documented in the literature for its resistance to antibiotics and disinfectants. This resistance has been attributed to factors such as chromosomal mutation, low permeability of the cell wall, and genetic capability to express a cache of resistance mechanisms (11). However, these mechanisms may not account for reduced

FIG 6 Effect of membrane ultrastructure and intracellular distribution as shown by TEM of *E. coli*. (a) Untreated *E. coli*. (b to f) *E. coli* treated with 0.02% Triton X-100 (b), 32,000 mg of CD hydrogel/liter (c and d), 16,000 mg of DA/liter (e), or 32,000 mg of DA/liter (f). Arrows indicate blebbing.

susceptibility to DA, and it is more likely that strong lipopolysaccharide (LPS)-LPS links in the outer membrane are responsible (16). A possible mechanism for resistance in *C. albicans* is that the DA becomes bound to the abundant proteins in the thick, fungal cell wall and is unable to reach the more vulnerable cell membrane (23). The strain chosen was found to be fluconazole resistant, but it seems unlikely that the related mechanisms, such as the presence of resistance genes or upregulation of transporter genes, have any bearing on DA resistance (15, 21). The Gram-positive strains tested were more susceptible to DA and CD hydrogel than the Gram-negative strains, in that the MIC and MBC were lower. Similarly, when *S. aureus* was compared to *E. coli* in a time-kill experiment, the Gram-positive strain showed a slightly faster rate of killing, especially during the first 2 h of incubation. The reason for these differences is not apparent since Gram-positive bacteria have a thicker cell wall and might be expected to be less, rather than more, susceptible than Gram-negative bacteria. The anti-staphylococcal activity of CD hydrogel is of particular importance since intraoperative infection with *S. aureus* during ESS has been shown to frequently lead to postoperative infection and abnormal mucosal healing (7).

Due to the high chemical reactivity of DA, it was not possible to use fluorescent probes to assess membrane integrity. Membrane integrity experiments using propidium iodide (PI), 1-*N*-phenyl-naphthylamine (NPN), and Nile red (NR) in *E. coli* showed that DA alone reacted with all of these probes. This was due to the reaction between negatively charged aldehyde groups of DA with positively charged amine groups present in the chemical structures of these probes. The values of DA as a negative control were higher than the positive control and the utilized samples. The integrity of *E. coli* membranes were also examined by measuring the release of intracellular components (i.e., protein and DNA/RNA) by measuring absorption values at 260 and 280 nm via UV-vis spectrophotometry. The results obtained in these experiments were not conclusive because DA by itself absorbed at both 260 and 280 nm.

In order to help clarify the mechanism of action, information on the morphology of *S. aureus* and *E. coli* following treatment with CD hydrogel was sought using electron microscopy. SEM images revealed cell wall damage compatible with the mechanisms of action proposed above. The TEM images of the bacteria treated separately with CD hydrogel and DA were quite similar to those obtained with the surfactant Triton X-100 and also indicated that

FIG 7 Effect of membrane ultrastructure and intracellular distribution as shown by TEM of *S. aureus*. (a) Untreated *S. aureus*. (b to f) *S. aureus* treated with 0.01% Triton X-100 (b and c), 8,000 mg of DA/liter (d), or 16,000 mg of CD hydrogel/liter (e and f). Arrows indicate mesosome-like structures and external rippling; the cytoplasmic membrane (CM) and cell wall (CW) are also indicated.

the cell wall, particularly the cell membrane(s), is the primary site of action. The degree to which CD hydrogel and DA combine with intracellular proteins is not known. However, the visible effects of these compounds on the cell wall and the loss of cytoplasmic contents seem sufficient to explain their inhibitory activity.

Following the observation that CD hydrogel treatment resulted in clumping of *E. coli* flagella, the effect of DA on motility and chemotaxis was determined. A short treatment time was used so that even at the MIC some *E. coli* would survive and could be assessed using motility agar. Inhibition of motility and chemotaxis by DA was demonstrated, and this finding is compatible with the SEM pictures. However, it is not possible to be specific about the mode of action of DA, which could act through binding directly to the flagellum proteins and/or to the basal body. Motility and chemotaxis in *E. coli* are generally regarded as contributing to virulence in that they enable the bacteria to move away from hostile environments and access more favorable ones (20). DA may prevent motile *E. coli* from moving away from the site of CD hydrogel application and escaping into the surrounding tissues.

CD hydrogel, used in ESS for its hemostatic and antiadhesion properties, has demonstrable antibacterial activity *in vitro*, especially against Gram-positive bacteria. The antimicrobial component of the CD hydrogel is DA. It is proposed that the mechanism

of action primarily involves binding of the DA component with amino groups in proteins located in the bacterial cell wall. It is anticipated that CD hydrogel could be used after surgery at other body sites, particularly where postsurgical Gram-positive infections are likely to occur.

ACKNOWLEDGMENTS

We thank the following staff at Microscopy Otago: Liz Girvan for help with the SEM images and Richard Easingwood for assistance with the TEM images.

Some of the authors (S.C.M. and L.R.H.) have patented the gel for use in surgical applications, and it has been licensed to Medtronic Ltd for use in ear, nose, and throat surgery.

This study was supported by the New Economy Research Fund of the Foundation for Research, Science, and Technology (NERF grant UOO-X0808) and the Department of Chemistry, University of Otago.

REFERENCES

1. Abad A. 1992. Medium temperature epoxy resin for immunocytochemistry: Quetol 651 with water. *Microsc. Res. Tech.* 20:274–280.
2. Andrews JM. 2001. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* 48(Suppl 1):5–16.
3. Artzi N, Shazly T, Baker AB, Bon A, Edelman ER. 2009. Aldehyde-amine chemistry enables modulated biosealants with tissue-specific adhesion. *Adv. Mater.* 21:3399–3403.
4. Athanasiadis T, et al. 2008. Effects of a novel chitosan gel on mucosal wound healing following endoscopic sinus surgery in a sheep model of chronic rhinosinusitis. *Laryngoscope* 118:1088–1094.
5. Clinical and Laboratory Standards Institute. 2010. Performance standards for antimicrobial susceptibility testing: 20th informational susceptibility supplement, M100–S20. CLSI, Wayne, PA.
6. Draye J, et al. 1998. In vitro release characteristics of bioactive molecules from dextran dialdehyde cross-linked gelatin hydrogel films. *Biomaterials* 19:99–107.
7. Jervis-Bardy J, Foreman A, Field J, Wormald PJ. 2009. Impaired mucosal healing and infection associated with *Staphylococcus aureus* after endoscopic sinus surgery. *Am. J. Rhinol. Allergy* 23:549–552.
8. Jia X, et al. 2004. Synthesis and characterization of in situ cross-linkable hyaluronic acid-based hydrogels with potential application for vocal fold regeneration. *Macromolecules* 37:3239–3248.
9. Khor E. 2002. Chitin: a biomaterial in waiting. *Curr. Opin. Solid St. M.* 6:313–317.
10. Kong M, Chen XG, Xing K, Park HY. 2010. Antimicrobial properties of chitosan and mode of action: a state of the art review. *Int. J. Food Microbiol.* 144:51–63.
11. Lambert PA. 2002. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *J. R. Soc. Med.* 95(Suppl 41):22–26.
12. Li X, et al. 2010. Chitosan kills *Escherichia coli* through damage to be of cell membrane mechanism. *Carbohydr. Polym.* 79:493–499.
13. Lim SH, Hudson SM. 2004. Synthesis and antimicrobial activity of a water-soluble chitosan derivative with a fiber-reactive group. *Carbohydr. Res.* 339:313–319.
14. Liu G, et al. 2009. Synthesis and characterization of chitosan/dextran-based hydrogels for surgical use. *Macromol. Symp.* 279:151–157.
15. Masiá C, Gutiérrez F. 2002. Antifungal drug resistance to azole and polyenes. *Lancet Infect. Dis.* 2:550–563.
16. McDonnell G, Russell AD. 1999. Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev.* 12:147–179.
17. Murinda SE, Nguyen LT, Ivey SJ, Almeida RA, Olivier SP. 2002. Novel single-tube, agar-based test system for motility enhancement and immunocapture of *Escherichia coli* O157:H7 by H7 flagellar antigen-specific antibodies. *J. Clin. Microbiol.* 40:4685–4690.
18. Nowakowska M, Zapotoczny S, Sterzel M, Kot E. 2004. Novel water-soluble photosensitizers from dextrans. *Biomacromolecules* 5:1009–1014.
19. Park BK, Kim MM. 2010. Applications of chitin and its derivatives in biological medicine. *Int. J. Mol. Sci.* 11:5152–5164.
20. Penn CW, Luke CJ. 1992. Bacterial flagellar diversity and significance in pathogenesis. *FEMS Microbiol. Lett.* 79:331–336.

21. Sanglard D, Coste A, Ferrari S. 2009. Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation. *FEMS Yeast Res.* 9:1029–1050.
22. Simons C, Walsh SE, Maillard JY, Russell AD. 2000. A note: ortho-phthalaldehyde: proposed mechanism of action of a new antimicrobial agent. *Lett. Appl. Microbiol.* 31:299–302.
23. Sohn K, et al. 2006. Getting in touch with *Candida albicans*: the cell wall of a fungal pathogen. *Curr. Drug Targets* 7:505–512.
24. Valentine R, et al. 2010. The efficacy of a novel chitosan gel on hemostasis and wound healing after endoscopic sinus surgery. *Am. J. Rhinol. Allergy* 24:70–75.
25. Zhao H, Hendel ND. 1991. Determination of the degree of substitution of formyl groups in polyaldehyde dextran by the hydroxylamine hydrochloride method. *Pharm. Res.* 8:400–402.
26. Zhu PC, Roberts CG, Favero MS. 2005. Solvent or matrix-mediated “molecular switches”: the lipophilic dialdehyde (OPA) and the amphilic 1,3-pthalandiol and OPA disinfection mechanism. *Curr. Org. Chem.* 9:1155–1166.