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Anti-biofilm properties of chitosan-coated surfaces

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Abstract—Surfaces coated with the naturally-occurring polysaccharide chitosan (partially deacetylated poly *N*-acetyl glucosamine) resisted biofilm formation by bacteria and yeast. Reductions in biofilm viable cell numbers ranging from 95% to 99.9997% were demonstrated for *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans* on chitosan-coated surfaces over a 54-h experiment in comparison to controls. For instance, chitosan-coated surfaces reduced *S. epidermidis* surface-associated growth more than 5.5 ^{10}log units (99.9997%) compared to a control surface. As a comparison, coatings containing a combination of the antibiotics minocycline and rifampin reduced *S. epidermidis* growth by 3.9 ^{10}log units (99.99%) and coatings containing the antiseptic chlorhexidine did not significantly reduce *S. epidermidis* surface associated growth as compared to controls. The chitosan effects were confirmed with microscopy. Using time-lapse fluorescence microscopy and fluorescent-dye-loaded *S. epidermidis*, the permeabilization of these cells was observed as they alighted on chitosan-coated surfaces. This suggests chitosan disrupts cell membranes as microbes settle on the surface. Chitosan offers a flexible, biocompatible platform for designing coatings to protect surfaces from infection.

Key words: Biofilm; chitosan; anti-biofilm.

INTRODUCTION

Microorganisms that attach to the surfaces of indwelling medical devices, such as catheters, heart valves and prosthetic joints, can cause acute and chronic infections [1]. These bacteria or yeast form multicellular biofilms in which the microorganisms evade clearance by the host defenses and are protected from killing by antibiotics. Often the only way to resolve such infections is by surgically removing the affected device. Because established biofilms are so persistent, an

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attractive strategy is to develop technologies that abort biofilm formation at its earliest stages.

One such technology is the impregnation of surfaces with conventional antiseptics or antibiotics (reviewed in Refs [2, 3]). The efficacy of this approach, which has undergone substantial clinical testing, remains uncertain. For example, after reviewing decades of studies, McConnell *et al*. [4] concluded that more rigorous investigations are required to either support or refute the hypothesis that central venous catheters coated with antimicrobial agents reduce the rate of blood stream infections. These data not only illustrate the need for additional research but also suggest the need for new strategies for retarding or preventing biofilm formation on medical devices.

Here, we report the potent anti-biofilm efficacy of chitosan (partially deacetylated poly (N-acetyl glucosamine)) coatings. Chitosan is an extensively studied biomacromolecule which is typically derived from chitin (2-acetamido-2-deoxy β-1,4-D-glucan), a major component of insect and crustacean exoskeletons. Chitin and its derivatives like chitosan are sold commercially as an FDA approved material for staunching blood loss, a nutritional supplement, a thickener in cosmetics, a flocculating agent in water treatment and in biomaterials (reviewed in Refs [5, 6]). As a biomaterial, chitosan has a track record for its non-toxicity, biocompatibility, ability to promote healing and its inherent antimicrobial properties [5, 6].

The antimicrobial properties of soluble chitosan against planktonic microbes are well known (reviewed in Refs [5, 6]). However, efficacy against planktonic cells does not always translate into efficacy against biofilm cultures. Many antimicrobial agents like antibiotics and bleach can be extremely effective against planktonic cultures but typically have limited efficacy against biofilm cultures [1, 7]. Biofilm cells are often 10–1000+ times less susceptible to various antimicrobial agents as compared to the same microbial cells grown in planktonic culture [7, 8]. As an example, a β-lactamase-negative *Klebsiella pneumoniae* strain had an ampicillin minimal inhibitory concentration of 2 μg/ml when grown in suspension culture. The same strain, when grown as a biofilm, was only slightly affected by a 4-h exposure to 5000 μg/ml ampicillin [9]. The reduced susceptibility of biofilm cultures has been linked to factors like limited agent penetration, varying microbial growth rates, altered microenvironments and resistant cellular phenotypes [1, 7, 8]. The authors were unable to find any published reports of chitosan surfaces being tested directly against biofilm cultures. The goal of the presented work is to fill a gap in the chitosan literature by reporting on the properties of chitosan against organisms grown specifically under biofilm forming conditions.

MATERIALS AND METHODS

Microbial strains

Experiments were performed with the following organisms. Gram-positive bacterial strains: *Staphylococcus epidermidis* ATCC 35984 and a medical isolate of *Staphy-*

lococcus aureus (kindly provided by Dr. C. Fux); Gram-negative bacterial strains: *Pseudomonas aeruginosa* PAO1 and *Klebsiella pneumoniae* KP1; eukaryotic yeast: *Candida albicans* SC5314.

Culturing techniques

Biofilm cultures were grown in the drip-flow biofilm reactor system described previously [11–13]. Figure 1 highlights the components of the drip-flow biofilm reactor system. The four chambered reactor is fabricated from polycarbonate plastic and has vented lids for each chamber which are affixed with screws. Gas exchange occurs through the lid-mounted 0.2-μm filters. Media enters each chamber through a 22 gauge needle inserted through the lid septum. The media runs the length of the coupon before leaving the reactor *via* the effluent port. The reactor is operated at 10° from horizontal. Media flow removes non-adherent cells favoring the growth of a biofilm culture. The drip-flow biofilm reactors were fed an iron-limited, minimal medium (SAPI) which had the following composition: 1 g/l glucose, 0.5 g/l

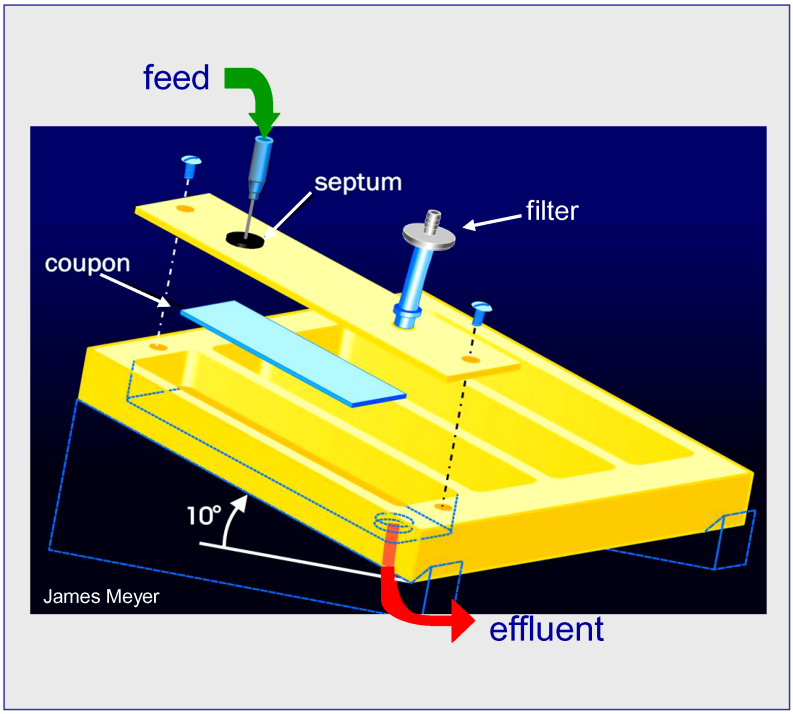


Figure 1. Drip-flow biofilm reactor system. Experimental surfaces were tested in the drip-flow biofilm reactor. All cultures were incubated for a 6-h batch phase (reactor in horizontal orientation) followed by a 48-h continuous feeding phase (reactor at 10° from horizontal orientation) before being analyzed. During the drip feed phase, non-adherent cells are washed off the coupon surface into the effluent port. Cultures were fed bovine serum supplemented medium at a rate of 20 ml/h at 37°C. Gas exchange occurs through the 0.2-μm filter attached to the reactor lid. This figure is published in colour at http://www.ingenta.com

NH_3NO_3, 0.25 g/l KCl, 0.25 g/l KH_2PO_4 and 0.25 g/l $MgSO_4 \cdot 7\ H_2O$ [10]. The autoclaved medium was supplemented with 5% (v/v) donor adult bovine serum (HyClone, Logan, UT, USA). The SAPI + serum medium had an average pH of 6.8.

Drip-flow biofilm reactor experiments began with the experimental surfaces being placed in a reactor well and each well being inoculated with 12 ml SAPI + serum medium containing 10^4 colony forming units/ml of the appropriate microorganism. To initiate microbe growth and attachment, the reactors were incubated for 6 h in a horizontal position at 37°C. After 6 h, the media was drained, removing most non-adherent cells. The reactors were set to an incline angle of 10° from horizontal and media was fed at a rate of 20 ml/h. Cultures were grown at 37°C. Coupons were harvested after 48 h of drip-feed cultivation (54 h in total) and surface-associated growth was analyzed using previously described biofilm scraping, homogenization and dilution methods [11–13]. Surface cell counts were obtained by plating aliquots from the homogenized, culture dilutions on tryptic soy agar (TSA) agar plates and counting colonies after 24 h of incubation at 37°C.

Tested surfaces consisted of a Plexiglas (poly(methylmethacrylate))coupon (approx. 9 cm^2), which was pre-coated with Rhoplex NW-1715K, a proprietary acrylic co-polymer emulsion (Rohm and Haas, Philadelphia, PA, USA). The pre-coat was employed to facilitate contiguous chitosan coatings. Without the pre-coat, the aqueous chitosan solution did not efficiently adhere to the hydrophobic Plexiglas surface. Experimental coatings were applied on top of the Rhoplex NW-1715K pre-coat. Control coupons consisted of only the Rhoplex NW-1715K pre-coat. The durability of the dried Rhoplex NW-1715K coat was assayed by soaking the coupons in SAPI medium. Briefly, the mass of Rhoplex NW-1715K on each coupon was determined by recording the mass of the coupon before and after Rhoplex NW-1715K coating (average mass 0.1313 g per coupon, standard deviation 0.005 g, $n = 4$). The Rhoplex NW-1715K coatings were permitted to dry for 2–3 days before the mass was recorded. The coupons were placed in 15 ml of SAPI medium without serum and incubated at 37°C for 2 days. Serum was not added because the absorbed protein changed the recorded mass of the coated coupon. The coupons were recovered, dried and the mass recorded. The mass of the Rhoplex NW-1715K coupons did not decrease during the soaking experiments, suggesting a stable coating in aqueous conditions. Rhoplex NW-1715K coating mass after 48 hours of soaking was 103% of presoaked Rhoplex NW-1715K mass (standard deviation 0.5%, $n = 4$).

Chitosan-coated test surfaces were produced as follows: Rhoplex NW-1715K pre-coated Plexiglas coupons were autoclaved for 20 min. Autoclaved coupons were then coated under sterile conditions with 0.5 ml of an autoclaved chitosan solution (1% (w/v) chitosan in 1% (v/v) glacial acetic acid). Coupons were permitted to dry 2–3 days under sterile conditions before being tested. The following commercial chitosan samples were tested: Medium molecular weight chitosan (Sigma-Aldrich Cat. No. 448877, 75–85% deacetylated) unless otherwise noted, this sample was utilized; Practical grade crab shell chitosan (Aldrich Cat. No. 417963, >85% deacetylated); Chitosan low viscous (Fluka BioChemika, Cat. No. 50494); and

Chitosan middle viscous (Fluka BioChemika, Cat. No. 28191). The durability of the chitosan coating was tested by recording the change in coated chitosan mass per coupon after soaking in SAPI medium. Briefly, the mass of each coupon was recorded before and after coating with chitosan (average chitosan mass 0.006 g per coupon, standard deviation 6×10^{-4} g, $n = 10$). Chitosan coatings were permitted to dry for 2–3 days before the mass was recorded. The coupon was then soaked in SAPI medium without serum at 37°C. Serum was not added because the absorbed protein changed the recorded mass of the chitosan-coated coupon. The coupon was removed from the SAPI medium, dried and the mass recorded. The chitosan mass was reduced by approximately 55% over the course of two days. The lost mass is likely from the dissolution of loosely associated surface polymers.

Antiseptic and antibiotic containing Rhoplex NW-1715K coatings were created by mixing the desired filter-sterilized antimicrobial agent(s) with the Rhoplex NW-1715K emulsion. The desired number of antimicrobial containing emulsion coats was applied either with an acetone disinfected brush or with a sterile pipette tip. Each coat was permitted to dry before additional coats of emulsion were applied. The mass of applied antibiotic was determined by either weighing the coupon before and after the coating process or by applying a known aliquot volume to the coupon surface. Chlorhexidine-containing coatings of Rhoplex NW1715K had an areal concentration of approx. 219 μg chlorhexidine/cm^2. Chlorhexidine- and deferoxamine-containing coatings of Rhoplex NW1715K had approximate areal concentrations of 173 μg chlorhexidine/cm^2 and 173 μg deferoxamine/cm^2. Rifampicin- and minocycline-containing coatings of Rhoplex NW1715K contained approx. 69 μg/cm^2 rifampicin and 69 μg/cm^2 minocycline.

Agent release from the Rhoplex NW-1715K coated surfaces was qualitatively analyzed using the antibiotic containing coupons. Briefly, 100 μl of an exponentially growing culture of *S. epidermidis* ($A_{600} = 0.1$) was spread onto a TSA plate. The antibiotic containing coupons were placed, coating side down, on the inoculated plate and incubated for 24 h at 37°C. Zones of inhibition for the antibiotic containing coatings averaged 0.54 cm (standard deviation 0.13 cm, $n = 8$) after 1 day. After 24 h, the coupons were transferred to freshly inoculated TSA plates (100 μl *S. epidermidis*, $A_{600} = 0.1$) and incubated again for 24 h at 37°C. Zones of inhibition for the antibiotic containing coatings averaged 0.29 cm (standard deviation 0.11 cm, $n = 8$) for day 2. In addition, the release of minocylcine and rifampicin was followed qualitatively by observing the color of the coating. Minocylcine and rifampicin are strongly yellow and orange, respectively. As they diffused from the coatings over the course the drip-flow experiments, the intensity of the coupon color diminished. These results were not quantified. The qualitative diffusion properties of chlorhexidine and chlorhexidine and deferoxamine were not determined.

Confocal scanning laser microscopy

Microbial biofilms were visualized using Live/Dead BacLight (Molecular Probes, Eugene, OR, USA) and a Leica AOBS confocal scanning laser microscope with excitation at 488 and 568 nm and with emission collected at 500 to 530 nm (green channel) and 585 to 615 nm (red channel). Images were taken using either a 10× or a 63× water objective. Microscope images were analyzed using the commercial software package MetaMorph (Universal Imaging, Downington, PA, USA).

Time-lapse analyses of *S. epidermidis* landing on chitosan-coated surfaces were acquired using confocal laser scanning microscopy movies taken on the Leica AOBS system. Briefly, exponentially growing *S. epidermidis* cultures (A_{600} = 0.05–0.1) were stained for approx. 2 h with Calcein AM (CAM, Molecular Probes; final CAM concentration in TSB 10 mM). The confocal microscope was focused at a single position on the surface of either a control coupon or a chitosan-coated coupon submerged in SAPI + serum medium. Approximately 200 µl of CAM-stained cell solution was injected into the medium directly above the coupon. Green fluorescence signal from the cells settling on the surface was recorded every 6 s for 0.5 h. Only one aliquot of cells were injected per sample. The green fluorescence signal for each image in the movie was quantified using MetaMorph (Universal Imaging, Downington, PA, USA). Experiments were run three times for the chitosan-coated and control surfaces. The presented data are representative of all experiments. Experiment-to-experiment variations included different numbers and rates of cells settling within the single, analyzed region and variations in the number and size of *S. epidermidis* cell clusters. The confocal, laser scanning microscopy movies represent a non-flow experimental set-up examining a non-motile microorganism.

RESULTS AND DISCUSSION

The efficacy of a chitosan-coated surface to inhibit biofilm formation was compared to some common antiseptic and antibiotic compounds impregnated in a polymer coating (Fig. 2A). Surfaces were challenged with *S. epidermidis,* a frequent bacterial culprit in device-associated infections [15]. Experiments were performed under slow, continuous flow conditions at 37°C for 48 h. Iron-limited, serum-supplemented medium was used to mimic *in vivo* conditions [10]. Chlorhexidine alone or a chlorhexidine/deferoxamine combination when incorporated into surface coatings reduced surface viable cell counts by less than one ^{10}log unit in comparison to the control surface (Fig. 2A). A combination of the antibiotics rifampicin and minocycline in a coating reduced cell densities by 3.9 ^{10}log units. Approximately 20% of the *S. epidermidis* colonies recovered from the rifampicin and minocycline surface had developed resistance to rifampicin. Surface-associated cell recovery techniques are referenced in the Materials and methods section. Resistance is defined here as cell growth on rifampicin (1.5 mg/ml) containing TSA plates. A chitosan coating demonstrated a 5.5 ^{10}log units reduction in areal viable cell density

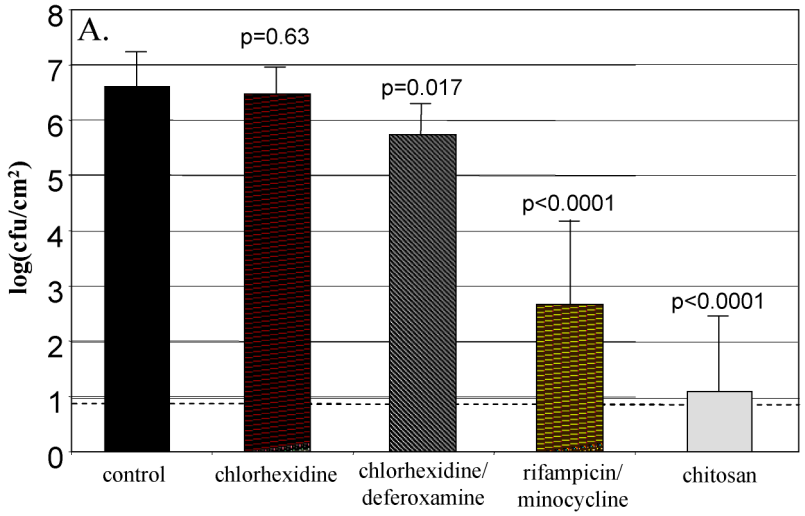


Figure 2. (A) Summary of antimicrobial coating experiments performed with Gram-positive *Staphylococcus epidermidis*. The data were obtained using a minimal iron-limited medium supplemented with 5% bovine serum. The control coating consisted of a layer of Rhoplex NW1715K on a Plexiglas coupon ($n = 9$), chlorhexidine containing coating of Rhoplex NW1715K (approx. 219 μg chlorhexidine/cm^2, $n = 4$), chlorhexidine and deferoxamine containing coating of Rhoplex NW1715K (approx. 173 μg chlorhexidine/cm^2, approx. 173 μg deferoxamine/cm^2, $n = 4$), rifampicin and minocycline containing coating of Rhoplex NW1715K (approx. 69 μg/cm^2 rifampicin, approx. 69 μg/cm^2 minocycline, $n = 4$). Chitosan coating over-lay on Rhoplex NW1715K precoat (approx. 667 μg/cm^2, $n = 9$; note: 5 of 9 experiments had cell counts below detection limit). All cultures were incubated for a 6-h batch phase followed by a 48-h feeding period at 37°C before being analyzed. The dotted line highlights the assay detection limit of 8.3 colony forming units (cfu)/cm^2.

compared to the control surface. Five out of the nine tested chitosan-coated surfaces had *S. epidermidis* cell counts below the detection limit of the assay.

Surfaces challenged with *S. epidermidis* were examined by confocal laser scanning microscopy (Fig. 2B–E). Control surfaces after 48 h of continuous feed had numerous, scattered, densely aggregated, biofilm cell clusters (Fig. 2B and 2C). The figures are presented for qualitative assessment of surface-associated growth and as supporting data for the surface-associated cell counts. Both the control surface, which was coated with an acrylic co-polymer, and the chitosan-coated surface absorbed some of the nucleic acid stain. These surfaces exhibited diffuse background fluorescence even in the absence of cells. No cells could be discerned on the chitosan-coated surface at the conclusion of the experiment (Fig. 2D and 2E). The textured appearance of the chitosan-coated surface is likely due to the sorption of serum proteins from the medium.

The anti-biofilm properties of chitosan-coated surfaces were evaluated against other medically-relevant microorganisms including the bacteria *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and the yeast *C. albicans* (Fig. 3). The following log reductions in viable cell numbers were observed on chitosan-coated surfaces in com-

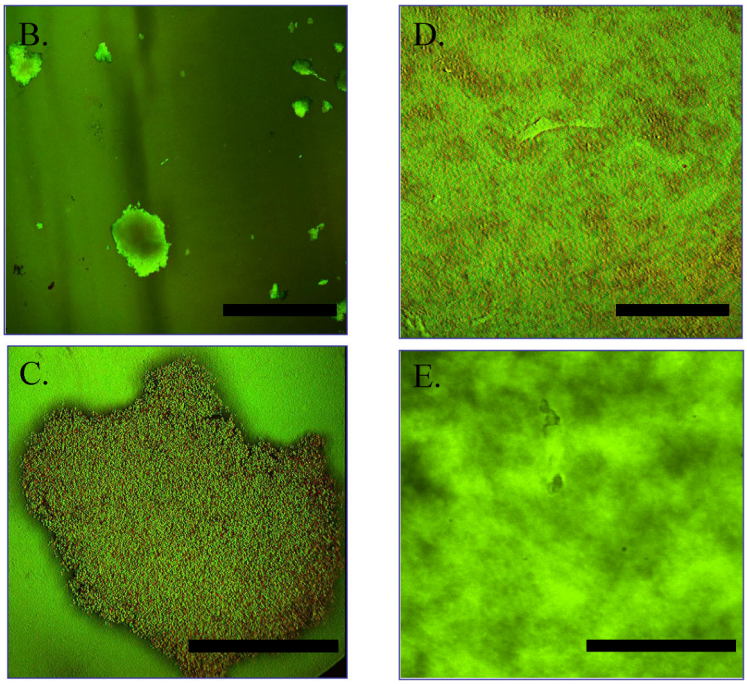


Figure 2. (Continued.) (B–E) Qualitative confocal scanning laser microscopy images of *S. epidermidis* on control surface and on chitosan-coated surface. (B) Control surface showing biofilm clusters viewed with 10× objective. Bar = 500 mm. (C) Control surface biofilm cluster viewed with 63× objective. Bar = 100 mm. (D) Chitosan surface viewed with 10× objective. Bar = 500 mm. (E) Chitosan surface viewed with 63× objective. Bar = 100 mm. No viable *S. epidermidis* cells were viewed on the chitosan-coated surface. The textured appearance of the chitosan-coated surface may be due to the sorption of serum proteins from the medium. This figure is published in colour at http://www.ingenta.com

parison to controls: *S. aureus* (2.0), *K. pneumoniae* (3.1), *P. aeruginosa* (1.4), and *C. albicans* (3.6). These reductions had probability values (P values) less than 0.05 for each case with the exception of *K. pneumoniae*. Even the smallest of these effects, recorded for *P. aeruginosa*, represents more than a 95% reduction on the chitosan-coated surface compared to the control. Reduced biofilm formation was confirmed by microscopic examination (data not shown). The microbe specific differences in chitosan efficacy is thought to be a result of varying microbe cell surface charge and differing cell wall/membrane structure (reviewed in Refs [5, 6]).

To rule out the possibility of an antimicrobial contaminant in the tested chitosan sample, chitosan was purchased from four different suppliers and representing different degrees of deacetylation and viscosities. The four samples were equally effective at inhibiting biofilm formation by *S. epidermidis* (data not shown). The data presented in Figs 2 and 3 were collected using chitosan coatings prepared from autoclaved chitosan solutions. Autoclaving the film after it had been cast abolished its anti-biofilm properties towards *S. epidermidis*. It is hypothesized that

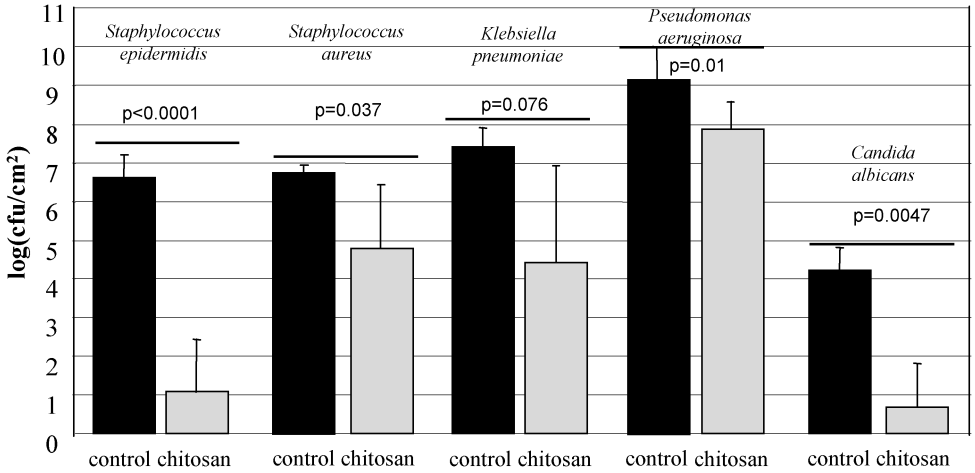


Figure 3. Effect of chitosan coating on biofilm formation/cell growth for microbial strains: *Staphylococcus epidermidis* (Gram positive), *Staphylococcus aureus* (Gram positive), *Klebsiella pneumoniae* (Gram negative), *Pseudomonas aeruginosa* (Gram negative) and eukaryotic fungus *Candida albicans*. All cultures were fed minimal medium supplemented with bovine serum. The control coupon, for each case, consisted of a Rhoplex NW1715K precoat on a Plexiglas coupon while the chitosan coated coupons had a precoat of Rhoplex NW1715K with a chitosan over-lay. All cultures were initially incubated for a 6-h batch phase followed by a 48-h feeding period at 37°C before being analyzed. *S. epidermidis* data $n = 9$, *S. aureus* data $n = 5$, *K. pneumoniae* data $n = 4$, *P. aeruginosa* data $n = 7$, *C. albicans* data $n = 4$.

autoclaving the cast film results in polymer cross-linking and chain breakage which may interfere with the antimicrobial properties of the positively charged amine groups [16, 17].

One proposed antimicrobial mechanism of soluble chitosan suggests the positively charged chitosan polymer compromises the integrity of the microbial cell membrane [5, 6]. We used time-lapse confocal laser scanning microscopy to investigate this mechanism with respect to the chitosan coatings. A planktonic culture of growing *S. epidermidis* cells were stained with calcein AM (CAM). CAM is a non-polar, non-fluorescent molecule that diffuses passively across cellular membranes. Inside a cell, native esterases cleave the CAM substrate generating a polar, green fluorescent molecule. The fluorescent product remains trapped inside the cell unless the cellular membrane is compromised. A permeable membrane would permit the green stain to diffuse out of the cell. A CAM-stained cell aliquot was injected into the medium directly above the coupon. A movie was recorded as the cells settled onto either the control surface or the chitosan-coated surface (Fig. 4A). Only one aliquot of cell solution was injected for each movie. Image recording began as the cells began depositing on the surface. On the control surface, individual cells and small aggregates of cells settled on the surface, retained their green fluorescence, and remained associated with the surface. On the chitosan-coated surface, the cells and small aggregates settle on the surface but began to lose their fluorescence. This

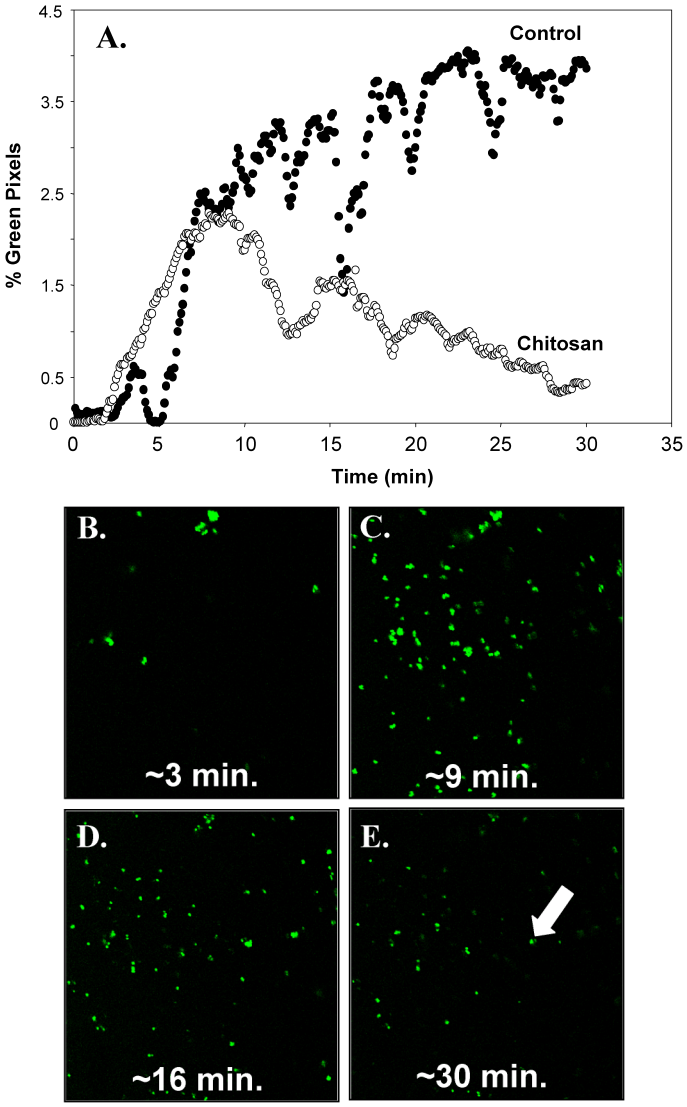


Figure 4. (A) Study of *Staphylococcus epidermidis* membrane integrity on chitosan and control surfaces. A calcein AM (CAM green) stained *Staphylococcus epidermidis* culture was applied to either a chitosan-coated (open circles) or a control surface (filled circles) and imaged using confocal laser scanning microscopy. Cells with intact membranes retain the CAM stain and fluoresce green. Cells with a compromised cell membrane lose the green fluorescent stain due to diffusion. The time-dependent green fluorescence signal is expressed as a percentage of total image pixels at a single depth corresponding with the coupon surface. The confocal laser scanning microscope is mounted on an air table. The oscillations in fluorescent data are thought due to perturbations in the air pressure of the air table. (B–E) Time sequence images from confocal laser scanning microscopy studies of chitosan surface. CAM-loaded *Staphylococcus epidermidis* cells are shown as a function of time on the chitosan-coated surface. The images from approx. 3 (B), 9 (C), 16 (D) and 30 min (E) were taken from the movie used to obtain the chitosan data in panel (A). The data were obtained from a single depth corresponding to the coupon surface.

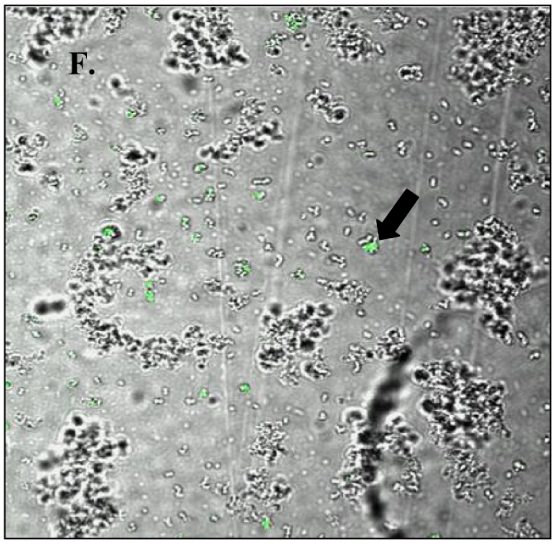


Figure 4. (Continued.) (F) Overlay of transmitted light and epifluorescent images. The image shows the chitosan surface after 30 min of cells landing on the surface. The image is from the same surface area shown in panels (B–E). The cell cluster highlighted by the arrow in panel (F) corresponds to the cell cluster highlighted by the arrow in panel (E). Cells with intact membranes are green while cells with compromised membranes have lost the fluorescent CAM stain. Numerous non-fluorescent cells are present on the surface. Large globular serum proteins are also seen associated with the surface. This figure is published in colour at http://www.ingenta.com

behavior is reflected in the quantification of the integrated green fluorescence on the surfaces as a function of time (Fig. 4B–E). An overlay of a transmitted light and epifluorescent image suggests the permeabilized cells remain associated with the chitosan surface (Fig 4F). The surface imaged in Fig. 4F is the same surface recorded in Fig 4B–E. These results suggest that chitosan coatings abort biofilm formation by permeabilizing microbial cells as they contact the surface.

CONCLUSIONS

Chitosan-coated surfaces resisted biofilm formation by bacteria and yeast, including *S. epidermidis*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *C. albicans*, during medium length, 54-h experiments. Chitosan was retained on the tested surfaces for the length of the experiments although some loss was observed likely due to polymer solubilization.

Using time lapse fluorescence microscopy and fluorescent-dye-loaded *S. epidermidis*, the likely permeabilization of these cells was observed as they alighted on chitosan-coated surfaces. This activity seems to echo reports of engineered antimicrobial surfaces in which cationic biocides have been covalently immobilized [18–21]. It has been suggested that cationic antimicrobial agents, like chitosan, are not likely to generate microbes with resistant phenotypes [22]. Chitosan also has

the unique advantages of proven non-toxicity, biocompatibility and the possibility of incorporating adjuvants into the coating. Chitosan offers a flexible, platform for designing coatings to protect surfaces from infection.

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