

Chitosan films are NOT antimicrobial

L. John R. Foster · Julian Butt

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Abstract Chitosan is a promising biomaterial for biomedical applications and is currently applied as wound dressings. While chitosan solutions demonstrate strong bactericidal activity against a range of medically important bacteria, the study here reports a loss of this beneficial property in thin films cast from the same solutions. Chitosan films (20 μm) showed no inhibitory effects against *Escherichia coli*, *Staphylococcus aureus* or *S. epidermidis* species. In contrast, solutions used to prepare the films showed almost complete inhibition ($\sim 98 \pm 2\%$) when tested on bacterial lawns and in liquid cultures. Increased acidity of the chitosan solutions (pH 5) was shown to promote the bactericidal effects of this biopolymer. The concept that devices fabricated from chitosan have an inherent antimicrobial activity is suggested as an important misconception.

Keywords Chitosan · *Staphylococcus aureus* · *Staphylococcus epidermidis* · *Escherichia coli* · Biomaterial

Introduction

Chitosan is a natural biopolymer derived from crustacea and structurally similar to hyaluronic acid, a polymer component of the extracellular matrix (ECM), making it particularly attractive for tissue engineering (Muzzarelli et al. 1999). As an FDA (Food and Drug Administration, USA) approved biomaterial, chitosan is currently used in a number of commercial applications including wound dressings. Chitosan wound dressings are associated with greater macrophage infiltration to the wound site, greater numbers of mitotic cells in the wound bed, increased angiogenesis, faster re-epithelialisation and greater collagen deposition, leading to enhanced healing rates and tissue repair strengths. As a biomaterial chitosan shows increased cell attachment as well as their production of associated cytokines and growth factors (Biagini et al. 1991a, b; Cho et al. 1999; Mi et al. 2001).

This common biopolymer can be readily solvent-cast into a thin film with excellent mechanical properties and low toxicity. In addition, the positive charge of the amino groups along the biopolymer's chains are reported to confer unique mucoadhesive, haemostatic and antibacterial properties (Shigemasa et al. 1996; Rao and Shorma 1997; Lehr et al. 1992). The comparatively strong antimicrobial activity of chitosan in solution and gel-forms against a broad spectrum of both Gram-positive and -negative medically relevant microorganisms has been reported in detail [for a review see Raafat and Sahl 2009]. Despite

L. J. R. Foster (✉) · J. Butt
Bio/Polymer Research Group, School of Biotechnology & Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia
e-mail: J.Foster@unsw.edu.au

extensive studies on the antimicrobial efficacy of chitosan, its mode of action is still unclear; however there appears a consensus that its antimicrobial property is a product of surface-surface interactions between the biopolymer chains and microbial cell walls (Raafat and Sahl 2009; Rabea et al. 2003). Thus, chitosan based materials have the potential for antimicrobial action, the exploitation of which is of particular interest to the development of medical technologies [for a review see Khor and Lim 2003].

In the study here, we report on the apparent lack of antimicrobial activity of thin chitosan films cast from solution in dilute acetic acid.

Materials and methods

Microorganisms and materials

Chitosan (>85% DDA) was purchased from Sigma-Aldrich (Australia). All other chemicals such as acetic acid were of analytical grade and purchased from Univar, (Australia). Penicillin/Streptococcus antibiotic, nutrient agar and Luria broth were purchased from Gibco, (New Zealand). *Staphylococcus aureus* (ATCC12600), *S. epidermidis* (ATCC10145) and *Escherichia coli* (ATCC11775) were obtained from the UNSW Culture Collection.

Fabrication of chitosan solution and films

A solution of chitosan was prepared by dissolving the biopolymer at 2% (w/v) in deionised water containing analytical grade acetic acid (2% v/v) and stirring for 6 h. Chitosan films were subsequently prepared from the solution, the gelatinous chitosan solution (pH ~5.0) was poured into a clean, sterile, glass Petri dish (95 cm²) and dried for 6 days under clean conditions in the dark (atmospheric pressure, relative humidity: 30%, 25°C). The resulting chitosan films were carefully detached from the dishes without damage and their thickness measured using digital callipers. All films were thereafter placed between glass slides to preserve their shape and stored in the dark (rH: 30%, 25°C).

Assays for antimicrobial activity

Staphylococcus aureus, *S. epidermidis* and *E. coli* were grown as lawns on nutrient agar plates (37°C,

48 h). Chitosan solution (20 µl) at pH 5 and neutralised at pH 7 as well as square films (1 cm²) were centred on the lawns and incubated (37°C, 24 h), after which the appearance of clear zones were recorded. Antimicrobial activity of the chitosan solutions and films were also investigated through incubation (37°C, 160 rpm) in sterile Luria Broth (LB) growth media containing the same concentrations of the microbial species (c.f.u. = 10⁵). Samples of γ-ray sterilised films (1 and 3 cm²) were added to liquid cultures and incubated (6 ml, 37°C, 160 rpm). After 36 h, samples (100 µl) were removed and the cells counted as c.f.u. values after 24 h at 37°C. Triplicates of each film and five sample volumes from each were used ($n = 3 \times 5$). Films of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) were used as positive controls, other controls included, solution without the addition of chitosan.

Electron microscopy

Films cultivated in the presence of the bacteria were removed and washed with deionised water before sectioning with a scalpel (~5 × 5 mm) and fixed in Karnovsky's solution (2.5% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer) after being gently washed in 0.1 M phosphate buffer and dehydrated in alcohol at several dilutions (30, 50, and 70% v/v). The specimens were further dehydrated to the critical point and sputter coated with copper before being viewed at 10 kV by the microscope in high-vacuum mode (FEI Quanta 200; FEI, Hillsboro, Oregon, US).

Results and discussion

Solutions of chitosan, when incubated on lawns of *E. coli*, *S. aureus* and *S. epidermidis*, showed readily noticeable clear zones while a control of the solution without the solvated biopolymer apparently exhibited no such influence (Fig. 1). In contrast, thin films of chitosan showed no obvious signs of microbial inhibition (Fig. 1b).

Quantitative measurements of microbial inhibition were obtained by incubating the chitosan solutions and films in liquid cultures of the bacteria for 24 h at 37°C, prior to serial dilution and plating to determine viable cell numbers relative to controls (Table 1).

Fig. 1 Incubation of chitosan solution and thin films on microbial lawns of *Staphylococcus aureus* at 37°C for 24 h gram in Petri dishes. Antimicrobial activity, is observed as a clear zone for chitosan solution (a), but not for its film morphology (b)

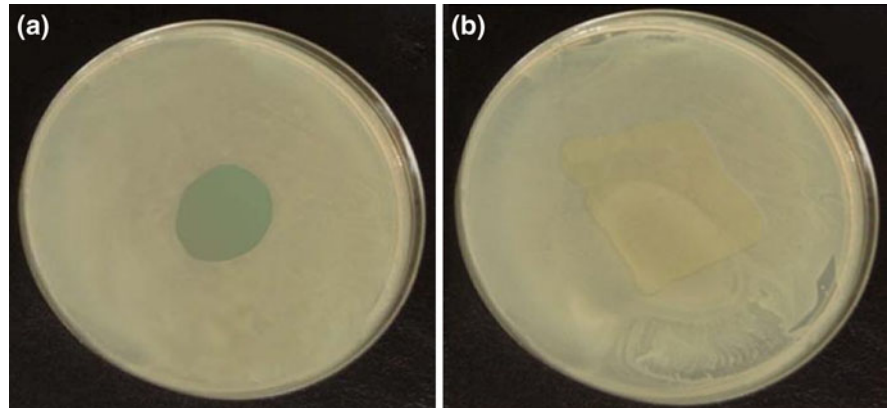


Table 1 Microbial inhibition exhibited by chitosan in solution compared to its film morphology

Microbial inhibition (%)				
Sample	Form	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>
Control	Solution (pH 7)	0 ± 4	0 ± 3	0 ± 4*
Chitosan	Solution (pH 7)	92 ± 4	90 ± 3	94 ± 3*
Chitosan	Solution (pH 5)	96 ± 4	93 ± 5	98 ± 4
Chitosan	Film (1:6) ^a	0 ± 3	2 ± 5	0 ± 5
Chitosan	Film (3:6)	3 ± 2	1 ± 5	1 ± 3*
PHBV ^a	Film (1:6)	−93 ± 5	−60 ± 2	−58 ± 3*
PHBV	Film (3:6)	−90 ± 4	−57 ± 4	−59 ± 3

^a Ratio of film surface area (cm²) to media volume (ml)

* Significant difference ($P > 0.05$)

Solutions of chitosan almost completely inhibited all microbial species with less than 8% viable cells remaining. The increased acidity of the control preparation solution also supported some antimicrobial activity with approx. 5% inhibition and this is consistent with that of Gil et al. who demonstrated increased antimicrobial activity of chitosan solutions with increasing acidity. The pK_a of chitosan is approx. 6 (Wicken and Knox 1983); thus, below this pH the amino groups at the C2 of the chitosan monomer units are positively charged. This charge would support the interaction of the chitosan chains with the anionic groups of the microbial cell surface. There was no significant difference between the antimicrobial effects on the chitosan solutions on the different microbial species.

In contrast to the strong antimicrobial action of the chitosan solution, thin films prepared from the

same solution showed no significant microbial inhibition. While increasing the film area to volume ratio from 1:6 to 3:6 appeared to show small levels of inhibition (<5%) these were not significant. Under the conditions used, the population of viable *E. coli* cells nearly doubled compared to the untested controls (93 ± 5%) while *S. aureus* and *S. epidermidis* increased by significantly less (~60 ± 5%, $P < 0.001$), clearly demonstrating the cells to be in their growth phases during the experiment. Poly(hydroxybutyrate-*co*-hydroxyvalerate), (PHBV), is another common biopolymer, that can serve as a common carbon source for microbial growth (Woolnough et al. 2008; Foster et al. 2001, 2008). We used films of P(HB-*co*-HV) as positive controls and those supported the proliferation of all the species tested (Table 1). Scanning electron microscopy was used to visualise the film surfaces and Fig. 2 clearly shows the

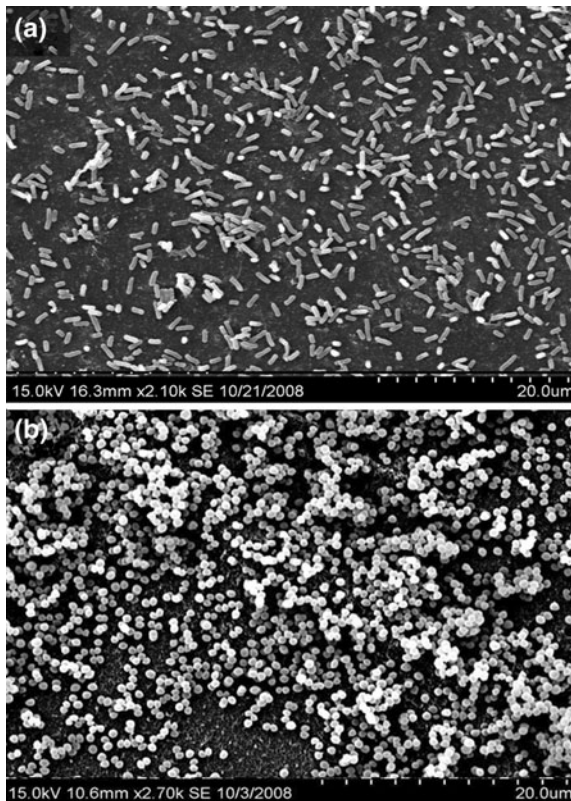


Fig. 2 Scanning electron micrographs showing proliferation of *Escherichia coli* (a) and *Staphylococcus aureus* (b) on thin films of chitosan after incubation (37°C, 24 h) and subsequent washing

rod-shaped cells of *E. coli* and coccoid-like cells of *S. aureus* attached to chitosan films even after washing (Fig. 2).

There are a number of studies reporting the comparatively strong bactericidal activity of chitosan in solution and as gels and our results are consistent with these, where solutions of chitosan significantly inhibited growth of all the species tested (Raafat and Sahl 2009). In the study here, *E. coli* and the *Staphylococci* species were selected as representatives of common Gram-negative and -positive pathogens respectively. *Escherichia coli* is a common enteric species while virulent strains can cause peritonitis and gastroenteritis. Similarly, *S. aureus* is one of the most common causes of postoperative infections and is implicated in a range of infections from impetigo to meningitis. *Staphylococcus epidermidis* is also a major concern for patients with surgical implants as it is primarily responsible for biofilm formation on these medical devices (Wilson and Henderson 2002).

In contrast to the comparative wealth of reports on the bactericidal effects of solvated chitosan, studies on antimicrobial activity of chitosan in film form are rare and limited to microorganisms commonly found in food (Beverly et al. 2008; Ouattar et al. 2000). Beverly et al. and Ouattar et al. have reported inhibitory effects of chitosan films against *Listeria* sp. when evaluated as food packaging materials (Beverly et al. 2008; Ouattar et al. 2000). However, this antimicrobial effect diminished with time and may have been due to growth limiting factors such as oxygen permeability. Furthermore, based on the film fabrication methodology used by these researchers we suggest that their films retained a degree of chitosan chain flexibility being more gel-like than the solid films used in this study. It has been postulated that the antimicrobial nature of chitosan is due to surface-surface interactions between the biopolymer chains and microbial cell walls (Raafat and Sahl 2009; Rabea et al. 2003). Thus, the presence of bacteria attached to the chitosan films, as per Fig. 2, suggests that the chitosan chains in the films used here did not interact with the microbial cell walls and consequently failed to exhibit any bactericidal properties.

Conclusion

Chitosan is a biomaterial already used in various medical devices and holds considerable potential in the fields of regenerative medicine and tissue engineering. The work reported here clearly shows that the final biomaterial morphology can significantly influence the antimicrobial activity of this promising biopolymer. Researchers operating under the impression that chitosan is antimicrobial should be aware that this may not be the case in their chitosan and chitosan-based devices.

References

- Beverly RL, Janes ME, Prinyawiwatkul W, No HK (2008) Edible chitosan films on ready-to-eat roast beef for the control of *Listeria monocytogenes*. *Food Microbiol* 25: 534–537
- Biagini G, Bertani A, Muzzarelli R, Damadei A, DiBenedetto G, Belligolli A, Riccotti G, Zucchini C, Rizzoli C (1991a) Wound management with *N*-carboxybutyl chitosan. *Bio-materials* 12(3):281–286

- Biagini G, Pugnali A, Damadei A, Bertani A, Belligolli A, Bicchiega V, Muzzarelli R (1991b) Morphological study of the capsular organization around tissue expanders coated with N-carboxybutyl chitosan. *Biomaterials* 12(3): 287–291
- Cho Y-W, Cho Y-N, Chung S-H, Yoo G, Ko S-W (1999) Water-soluble chitin as a wound healing accelerator. *Biomaterials* 20(22):2139–2145
- Foster LJR, Saufi A, Holden PJ (2001) Environmental concentrations of polyhydroxyalkanoates and their potential as bioindicators of pollution. *Biotechnol Lett* 23:893–898
- Foster LJR, Schwahn D, Pipich V, Holden PJ, Richter D (2008) SANS characterisation of polyhydroxyalkanoates and their bioPEGylated hybrids in solution. *Biomacromolecules* 9:314–320
- Gil G, del Mónaco S, Cerrutti P, Galvagno M (2004) Selective antimicrobial activity of chitosan on beer spoilage bacteria and brewing yeasts. *Biotechnol Lett* 26:569–574
- Khor L, Lim LY (2003) Implantable applications of chitin and chitosan. *Biomaterials* 24(13):2339–2349
- Lehr C-M, Bouwstra JA, Schacht EH, Junginger HE (1992) In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. *Int J Pharm* 78(1):43–48
- Mi F-L, Shyu S-S, Wu Y-B, Lee S-T, Shyong J-Y, Huang R-N (2001) Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. *Biomaterials* 22(2):165–173
- Muzzarelli RAA, Mattioli-Belmonte M, Pugnali A, Biagini G (1999) Biochemistry, histology and clinical uses of chitins and chitosans in wound healing. In: Jolles P, Muzzarelli RAA (eds) *Chitin and chitinases*. Birkhäuser Verlag, Switzerland, pp 251–264
- Ouattar B, Simard RE, Pielt G, Begin A, Holley RA (2000) Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. *Int J Food Microbiol* 62:139–148
- Raafat D, Sahl H-G (2009) Chitosan and its antimicrobial potential—a critical literature review. *Microbial Biotech* 2(2):186–201
- Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steurbaut W (2003) Chitosan as antimicrobial agent: applications and mode of action. *Biomacromolecules* 4:1457–1465
- Rao SB, Shorma CP (1997) Use of chitosan as a biomaterial: studies on its safety and homeostatic potential. *J Biomed Mater Res* 34:21–28
- Shigemasa Y, Shibasaki K, Minami S, Matsuhashi A, Tanioka S, Shigemasa Y (1996) Evaluation of chitin and chitosan for biomaterials. *Biotechnol Genet Eng Rev* 13:383–420
- Wicken AJ, Knox KW (1983) Cell surface amphiphiles of gram-positive bacteria. *Toxicon Suppl* 3:501–512
- Wilson M, Henderson B (2002) *Bacterial disease mechanisms, an introduction to cellular microbiology*. Cambridge University Press, UK. (ISBN-10: 052179689X)
- Woolnough CA, Charlton T, Yee L, Sarris M, Foster LJR (2008) Surface changes in polyhydroxyalkanoate films during biodegradation and biofouling. *Polym Int* 57(9): 1042–1051