J Mater Sci: Mater Med (2008) 19:1083–1090
DOI 10.1007/s10856-007-3072-z

Antibacterial activity of chitosan-based matrices on oral pathogens

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Received: 5 June 2006 / Accepted: 2 April 2007 / Published online: 15 August 2007

Abstract Chitosan is a well sought-after polysaccharide in biomedical applications due to its biocompatibility, biodegradability to non-toxic substances, and ease of fabrication into various configurations. However, alterations in the anti-bacterial properties of chitosan in various forms is not completely understood. The objective of this study was to evaluate the anti-bacterial properties of chitosan matrices in different configurations against two pathogens—Gram-positive *Streptococcus mutans* and Gram-negative *Actinobacillus actinomycetemcomitans*. Two-dimensional (2-D) membranes and three-dimensional (3-D) porous scaffolds were synthesized by air drying and controlled-rate freeze drying. Matrices were suspended in bacterial broths with or without lysozyme (enzyme that degrades chitosan). Influences of pore size, blending with Polycaprolactone (PCL, a synthetic polymer), and neutralization process on bacterial proliferation were studied. Transient changes in optical density of the broth, adhesion characteristics, viability, and contact-dependent bacterial activity were assessed. 3-D porous scaffolds were more effective in reducing the proliferation of *S. mutans* in suspension than 2-D membranes. However, no significant differences were observed on the proliferation of *A. actinomycetemcomitans*. Presence of lysozyme significantly increased the antibacterial activity of chitosan against *A. actinomycetemcomitans*. Pore size did not affect the proliferation kinetics of either species, with or without lysozyme. NaOH neutralization of chitosan increased bacterial adhesion whereas ethanol neutralization inhibited adhesion without lowering proliferation. Mat culture tests indicated that chitosan does not allow proliferation on its surface and it loses antibacterial activity upon blending with PCL. Results suggest that the chemical and structural characteristics of chitosan-based matrices can be manipulated to influence the interaction of different bacterial species.

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Introduction

With the recent developments in stem cell technologies, tissue engineering offers a promising avenue for regenerating lost oral structures, including the periodontium [1–3]. Conceptually, biodegradable scaffolds guide the in-growth of cells that constitute the required structure, while disappearing from the site [4]. The feasibility of tissue engineering [5], guided tissue regeneration, bone grafting and the use of enamel matrix derivatives have been demonstrated by restoring all three tooth-supporting tissues i.e. periodontal ligament fibers, alveolar bone, and cementum [2, 6]. Several studies have shown that optimum tissue regeneration cannot be expected in sites infected with oral microorganisms [7] and the clinical predictability is impaired by a lack of understanding of many fundamental questions including how scaffold microarchitecture influences the cell organization while abating oral pathogens. In addition, post-operative problems are also common, often due to adverse tissue reactions to implanted materials and microbial contamination [8, 9].

Chitosan, a polysaccharide, has shown promise for use as a biomaterial in oral tissue engineering [10], with the potential in regenerating a functional periodontium [11] and osteoconductive properties in animal models [12, 13]. Chitosan is biocompatible, available in large quantities, and inexpensive. Further, chitosan is metabolized into non-toxic D-glucosamines by lysozyme [14, 15], an antibacterial enzyme present in human saliva. Importantly, antimicrobial activity of chitosan solution against many pathogens including *Porphyromonas gingivalis* [16], *Escherichia coli* [17], *Actinobacillus actinomycetemcomitans* [18], *Pseudomonas aeroginosa*, *Staphylococcus aureus* [17] and *Streptococcus mutans* [18, 19] is documented. Although the exact mode in which chitosan exhibits anti-microbial action is still not clear [20], it is partially attributed to the protonation of chitosan in solution; the positive charge attracts the negatively charged bacterial cell walls. Interaction between the two charges breaks the cell wall of bacteria leading to leakage of their cytoplasm, eventually causing death [18, 21, 22]. It was also found that the anti-bacterial activities of chitosan are strain-dependent [23]. Further, increased molecular weight (MW) and degree of deacetylation (DD) of chitosan increase the potency of antibacterial activities [24]. However, increased DD decreases biodegradation by lysozyme [14, 15, 25]. Nevertheless, most of these studies investigating antimicrobial properties have utilized chitosan either in solution or as a thin sheet or membrane.

Blending of other molecules with chitosan is of interest because this could result in improved biomechanical properties [19, 26]. Chitosan can be blended with polymers such as polycaprolactone (PCL) to overcome mechanical limitations [27] or gelatin to overcome biological limitations [28]. Chitosan blended with synthetic polymers also show improved film quality and oral delivery [29]. Matrices can be formed in a variety of configurations [30], and porous matrices show better support for cell growth than films [28]. Despite these advances, it is not clear how the presence of other non-antibacterial components, three-dimensional (3-D) configurations, or biodegradation of chitosan affect antibacterial activity.

This study focused on assessing the anti-bacterial properties of chitosan-based matrices against Gram-positive *S. mutans* and Gram-negative *A. actinomycetemcomitans*; *S. mutans* is a spherical bacterium that is considered to be the most cariogenic (causing tooth decay) [31] and *A. actinomycetemcomitans* is a rod-shaped coccobacillus involved in various forms of periodontitis and other non-oral infections such as septicemia and meningitis [32]. The effects of 2-D membranes and 3-D scaffolds of chitosan and chitosan-PCL blends were investigated. These results show that the antibacterial properties of chitosan are surface-contact dependent and can vary with different species of oral bacteria.

Materials and methods

Chitosan (~85% deacetylated) of MW > 310 kD, PCL of 80 kD MW, and hen egg white lysozyme (46,400 U/mg) were purchased from Sigma Aldrich (St. Louis, MO). *S. mutans* (ATCC 25175, NCTC 10449) and *A. actinomycetemcomitans* (ATCC 43719, NRRL 2501) were purchased from ATCC and grown aerobically in brain heart infusion (BHI) broth at 37 °C for 48 h. All experiments used culture in exponential growth phase. LIVE/DEAD BacLight™ Bacterial Viability Kit (L7012) was purchased from Molecular Probes (Eugene, OR). All other chemicals used were of reagent grade.

Formation of 2-D membranes and 3-D chitosan scaffolds

Three-dimensional (3D) scaffolds and 2D membranes of chitosan were formed by methods reported previously [28]. In brief, chitosan (0.5% w/v for 3D scaffolds and 1%w/v for membranes), was dissolved in 0.5 M acetic acid. For making 3D scaffolds, approximately 300 µL of this solution was added to each well of a 24-well plate. Some solutions were frozen at –20 °C and others at –85 °C for 24 h. All frozen samples were lyophilized for 24 h at –85 °C to obtain porous scaffolds.

Membranes of approximate thickness 80–100 µm were formed by pouring 20 mL of solution into 10 cm diameter Teflon petri dishes and air drying overnight. From these membranes, 2 cm × 2 cm size samples were cut and used in antibacterial analyses. Membranes and scaffolds were neutralized in 1 N sodium hydroxide (NaOH), rinsed with sterile water and sterilized in 90% ethanol. To test the effect of neutralization protocol, some membranes were directly neutralized and sterilized in 90% alcohol. All samples were washed with sterile phosphate buffered saline (PBS) before testing with bacteria.

Hydrated porous matrices were assessed for pore size using the technique described previously [33]. In brief, digital micrographs were captured from different locations using an inverted microscope (Nikon TE2000, Melville, NY) outfitted with a CCD camera. Pore sizes were quantified using an image analysis software (Sigma Scan Pro, Chicago, IL).

Formation of chitosan-PCL blend membranes

Chitosan solutions (3 mL, 2% w/v) were slowly added to 10 mL of PCL (80 kD MW; Sigma Aldrich, St. Louis, MO) solutions of different concentrations in glacial acetic acid to obtain 25, 50 and 75% PCL (by mass) blends [27]. Formed solutions were poured into Teflon Petri dishes and dried for 24 h in an oven at 55 °C to obtain uniform membranes.

Analysis of bacterial proliferation in suspension

Sterile chitosan samples were suspended in 5 mL of bacterial broth of known optical density. Cultures were conducted in 20 mL glass vials with rubber caps. The vials were divided into four groups: control (no chitosan matrices), 2D membranes, 3D scaffolds pre-freezed at –20 °C, and 3D scaffolds pre-freezed at –85 °C. Cultures were incubated with and without lysozyme solution (1,000 mg/L) at 37 °C with constant gentle shaking. At 3, 6, 9, 12, and 24 h, 0.5 mL of these cultures were retrieved, diluted to 1 mL with deionized water and optical density was measured using a spectrophotometer at 600 nm. After 24 h, the matrices were removed and analyzed for bacterial adhesion by Scanning Electron Microscopy (SEM) and bacterial viability by *Bac*Light™ fluorescence staining as described in next section.

For SEM analysis, samples were fixed using 3.7% paraformaldehyde followed by rinsing with sterile water and ethanol. Subsequently, samples were air-dried in a vacuum desiccator, sputter-coated with gold and imaged using a JEOL 6360 SEM (Jeol USA Inc., Peabody, MA).

Viability analysis

Samples were washed twice with 0.85% NaCl solution, and stained on each side with 50 μL of the LIVE/DEAD *Bac*Light™ viability stain. The stain was prepared by mixing 1.5 μL each of SYTO 9 and propidium iodide with 1 mL of sterile deionized water. The stained chitosan structures were incubated in dark for 2 h at room temperature and observed under an inverted microscope (Nikon TE2000, Melville, NY). Further, samples were analyzed layer by layer at 1 μm steps up to a depth of 50 μm using a confocal microscope (Leica TCS, Heidelberg, Germany). Live bacteria fluoresced green and dead bacteria fluoresced red.

Contact-dependent anti-bacterial property of chitosan surface

Dense bacterial 'mats' were generated on BHI agar plates by spreading 25 μL of bacterial suspension (OD ≥ 1.5) over approximately 2 cm × 2 cm area and incubating at 37 °C for 12 h. Membranes were placed on these mats and gently pressed to ensure complete contact. Visible changes in the morphology of the mats underneath the membranes were monitored with respect to control (mats with no membranes on top) and digital images were obtained.

After 24 h, one set of membranes were removed and analyzed by SEM. Another set was rehydrated by adding few drops of media, then gently removed and placed on a clean agar plate, bottom side facing down. Bacterial growth accompanying the membranes was monitored as before for additional 24 h. In tandem with mat cultures, membranes suspended in bacterial broth for 24 h were also tested on an agar plate for adherent bacterial growth.

Statistical analysis

All experiments were repeated three or more times with triplicate samples in each experiment. Significant differences between groups were evaluated using a one-way Analysis of Variance (ANOVA) with 99% confidence interval.

Results

Anti-bacterial activity of chitosan in suspension

Streptococcus mutans showed an initial increase in OD of all cultures similar to control (Fig. 1A). However, OD of 3-D membrane suspensions decreased from 6 h onwards and was significantly lower than those of 2-D membranes by 12 h, suggesting chitosan was potent against *S. mutans* and was more effective in 3-D form than 2-D membranes. On the contrary, *A. actinomycetemcomitans* cultures containing chitosan structures had ODs higher than control (Fig. 1B). 3-D structures showed a marginal decrease in OD compared to 2-D after 6 h.

Influence of pore size

Pre-freezing matrices at –20 and –85 °C resulted in scaffolds with average pore-size of 140 ± 35–60 ± 20 μm respectively, similar to previous results [28]. When the effect on *A. actinomycetemcomitans* was tested, there was no significant difference (Fig. 1B) in the proliferation between the two scaffolds. SEM analysis showed that both the scaffolds were completely covered by *A. actinomycetemcomitans* (Fig. 1C). When viability was tested, a green color rim was observed on the pore openings of both the scaffolds (Fig. 1D and E), indicating a viable bacterial population. Interestingly, there was a red colony beneath the live colonies, suggesting that most of the bacteria in contact with chitosan matrix were dead. *S. mutans* also showed similar effect (Fig. 1F, G, and H), indicating that the effects of pore morphology on anti-bacterial activity of chitosan are independent of the species.

Effects of lysozyme and matrix degradation

Lysozyme did not significantly influence the growth of *S. mutans* in control and 3-D scaffolds (Fig. 1A). However, combination of 2-D chitosan and lysozyme decreased the growth of *S. mutans* compared to chitosan alone. With

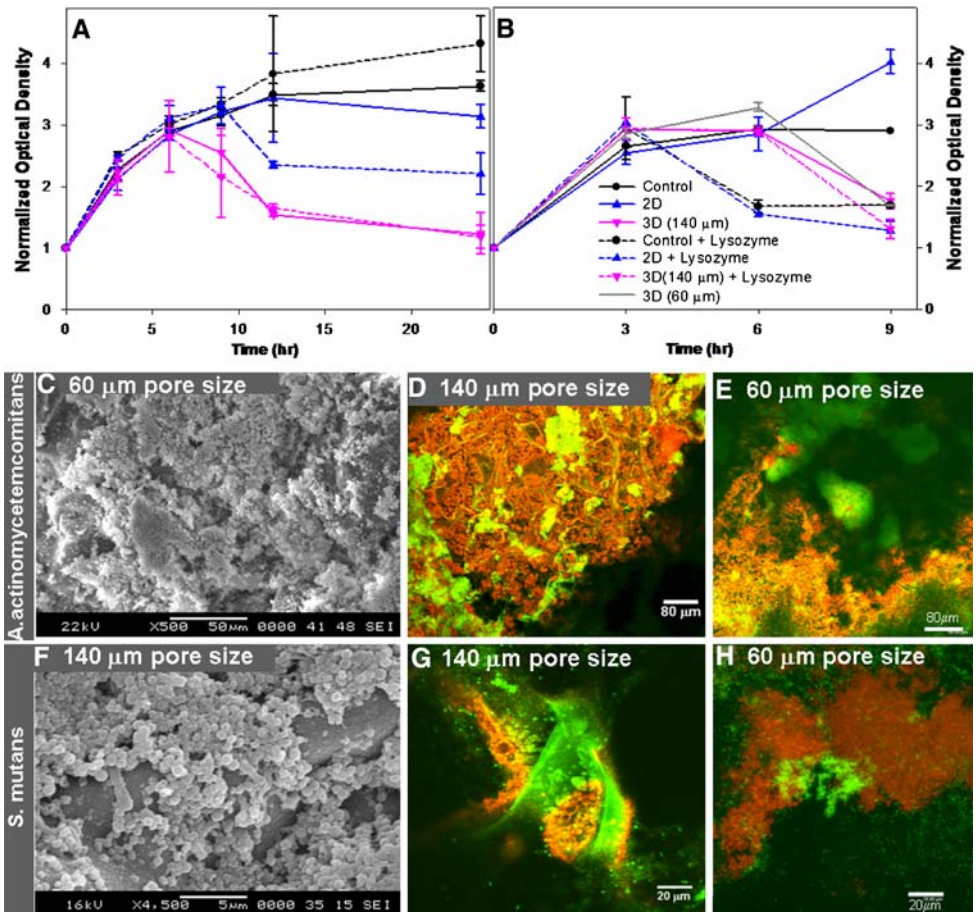

Fig. 1 Influence of matrix architecture on anti-bacterial activity of scaffolds. Matrices were suspended in cultures of *S. mutans* and *A. actinomycetemcomitans* with and without lysozyme. (**A**) *S. mutans*. (**B**) *A. actinomycetemcomitans*. (**C**) Scanning Electron Micrograph showing adherence of *A. actinomycetemcomitans* to scaffold. (**D** and **E**) Confocal micrographs showing viable (green) and non-viable (red) *A.actinomycetemcomitans* on scaffolds of different pore-sizes. (**F**, **G** and **H**) Micrographs showing adherence and viability of *S. mutans*

A. actinomycetemcomitans, there was a significant reduction in growth in the presence of lysozyme in control and 2-D membrane cultures (Fig. 1B). In 3-D scaffold cultures, lysozyme showed a delayed antibacterial activity. Thus, lysozymal effects on the anti-bacterial activity of chitosan were species-dependent. Nevertheless, all broths containing chitosan matrices and lysozyme showed a reduction in turbidity. When the matrices were disturbed, bacterial clumps flushed out of the matrices into the surrounding media suggesting a preferential aggregation of bacteria onto the chitosan matrices. When analyzed by SEM, however, no differences were observed in the bacterial colonization on control and lysozyme-treated matrices (Fig. 2C, D). Furthermore, viability of the adherent bacteria was not significantly different in 2-D (Fig. 2A, B) or 3-D forms (Fig. 2E, F) from those without lysozyme (Fig. 1D, E).

Effects of neutralization by EtOH and NaOH

Chitosan matrices are commonly prepared after dissolving in acidic water (pH < 6.3) and are neutralized in NaOH or ethanol to remove acidity prior to rehydration. Since neutralization could affect surface properties, influence of neutralization of matrices were also tested. The OD of *A. actinomycetemcomitans* cultures containing NaOH-neutralized chitosan membranes (Fig. 2G) showed no difference relative to control cultures at different time points. However, OD of cultures containing ethanol-neutralized chitosan membranes showed significant increase relative to control. Interestingly, ethanol-neutralized membranes showed negligible bacterial adhesion (Fig. 2I) whereas NaOH-neutralized membranes showed significant bacterial adhesion (Fig. 2H). This difference in antibacterial activity of chitosan could be attributed to the contact-dependent characteristics of chitosan.

Anti-bacterial properties of chitosan surfaces and effects of blending with PCL

To test the contact-dependent influence directly, chitosan and chitosan-PCL blend membranes were placed on bacterial mats (Fig. 3A and B). After 24h, the transparency of chitosan membrane did not change (Fig. 3C), suggesting minimal growth of *A. actinomycetemcomitans*. PCL and chitosan-PCL blends appeared cloudier, although growth underneath could not be observed due to the opaque nature of the membranes. SEM analysis of these membranes

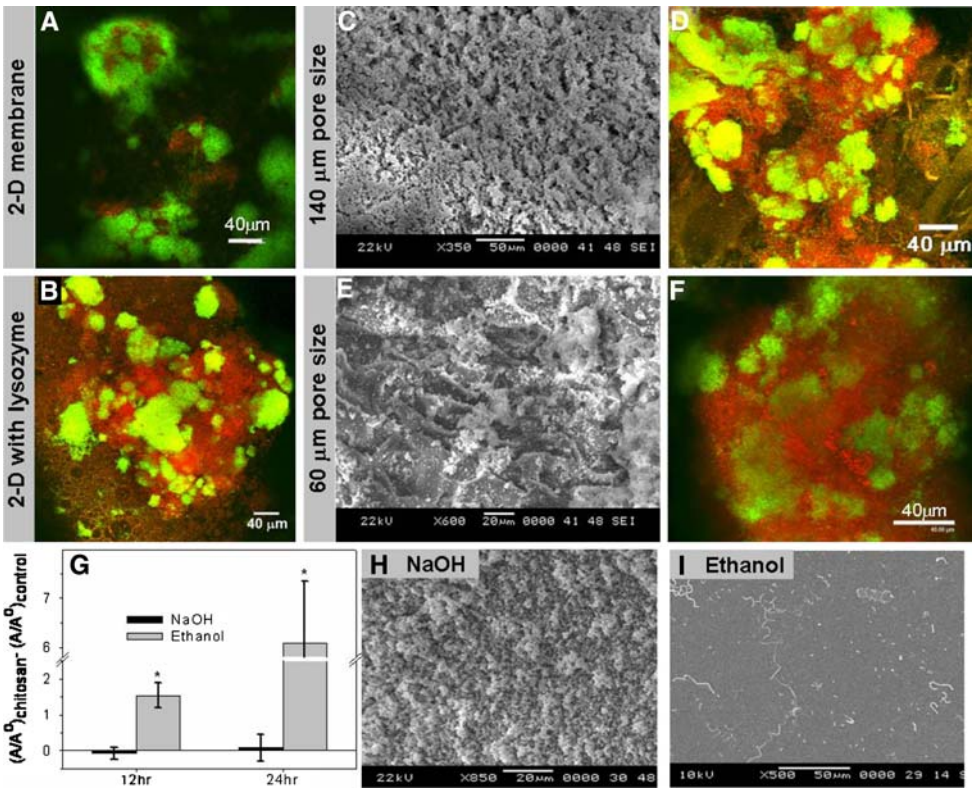

Fig. 2 Effect of lysozyme and neutralization on adherence and viability of bacteria. Chitosan matrices were suspended in *A. actinomycetemcomitans* broth containing lysozyme. Viability of bacteria on (**A**) 2-D membrane. (**B**) 2-D membrane with lysozyme. Adherence and viability of bacteria on 3-D scaffolds of pore size (**C** and **D**) 140 μm and (**E** and **F**) 60 μm. (**G**) Effects of neutralization by EtOH and NaOH. 2-D membranes were incubated in *A. actinomycetemcomitans* broth after neutralization and sterilization**.** Difference in normalized OD of chitosan and control at various time points (*$P < 0.01$ relative to NaOH neutralized samples). Panels (**H** and **I**) Micrographs showing the adherent bacteria on membranes

showed negligible adhesion to chitosan surfaces (Fig. 4A) but presence of PCL significantly increased bacterial adhesion, as shown in the blend membranes, suggesting loss of antibacterial property of chitosan in the blends. Nevertheless, these membranes were transferred to a fresh agar plate with no mats to understand the proliferative ability of adherent bacteria. There was no visible bacterial growth underneath the chitosan membranes (Fig. 3D), confirming contact-dependent anti-bacterial property of chitosan. On the other hand, significant bacterial growth

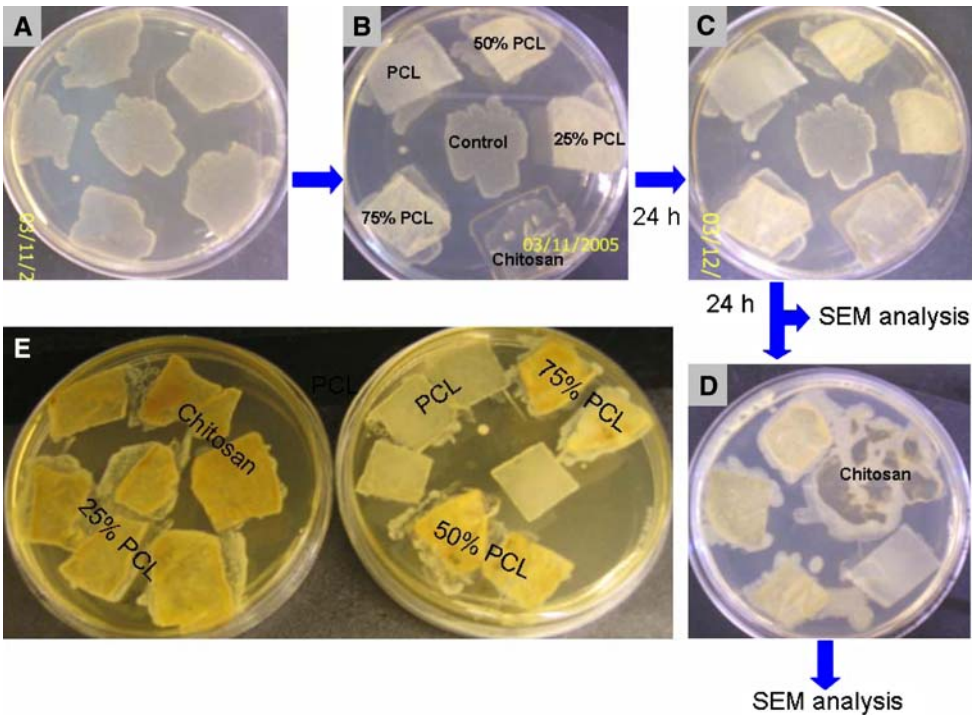

Fig. 3 Contact dependent growth of *A. actinomycetemcomitans* on chitosan- PCL blend membranes. (**A**) Dense mats without membranes. (**B**) Ethanol sterilized membranes seeded on mats. (**C**) Bacterial growth after 24 h. (**D**) Membranes from (**C**) seeded on fresh agar plate. (**E**) Membranes were incubated in *A. actinomycetemcomitans* broth and placed on fresh agar plates; growth surrounding membranes after 24 h

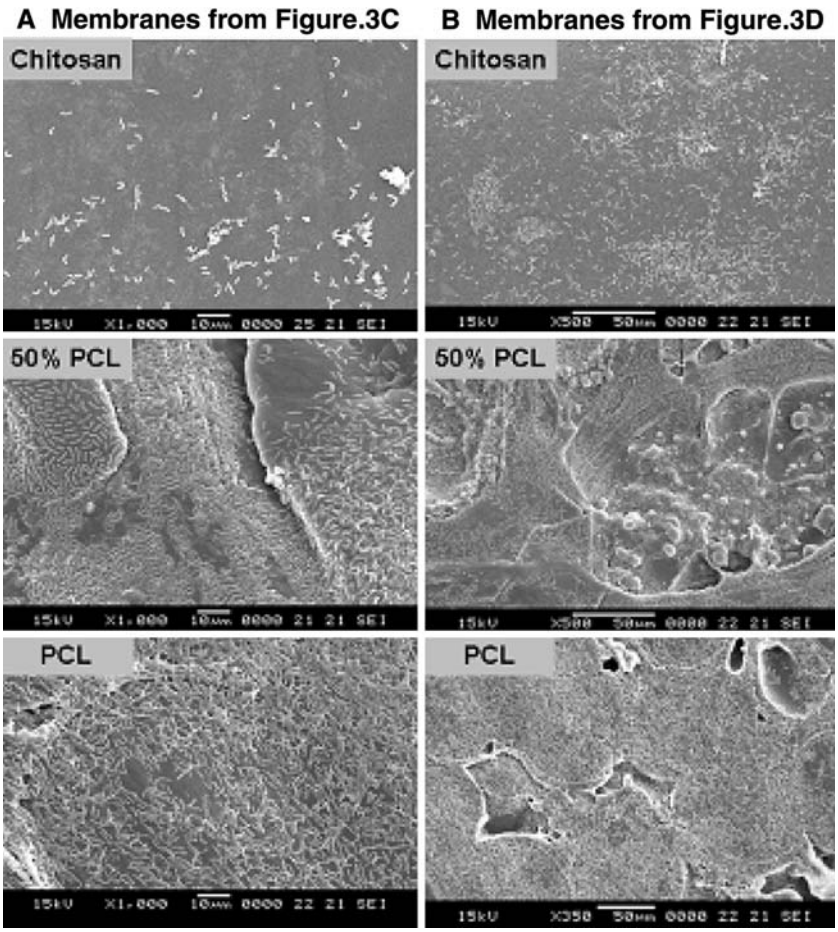

Fig. 4 Influence of blending with PCL on adherence of bacteria. Panel A. SEM images of membranes from Fig. 3C. (**B**) SEM images of membranes from Fig. 3D

was observed surrounding blend and PCL membranes, suggesting that attached bacteria were proliferative. SEM analysis verified the presence of bacteria on these membranes (Fig. 4B), similar to Fig. 4A. Identical results were obtained with *S. mutans* (data not shown). Incubation of membranes previously cultured in bacterial broths for 24 h, on agar cultures also showed similar results, confirming that the anti-bacterial property of chitosan is contact-dependent.

Discussion

This study focused on evaluating the anti-bacterial activity of chitosan in 2-D membranes, 3-D scaffolds pre-frozen at –20 and –85 °C, with and without lysozyme, and PCL-blended matrices. 3D matrices were formed using a well-established controlled rate freezing and lyophilization technique to produce scaffolds of 60 and 140 μm pore sizes [30]. In addition, it has been shown that scaffolds of 140 μm pore size are more conducive for cellular colonization of various cell types including fibroblasts and endothelial cells [28].

First, the influence of porous architecture in abating colonization of *S. mutans* and *A. actinomycetemcomitans* was explored. 3D matrices were more effective in reducing the optical density of bacterial broth, particularly of Gram-positive *S. mutans*. This suggests that chitosan 3D retains stronger antibacterial property towards Gram-positive bacteria than Gram-negative bacteria, similar to chitosan solutions that others have reported [23]. There are several reasons that may explain the greater antibacterial properties of the porous scaffolds compared to 2D membranes**.** One of the reasons could be decreased oxygen tension, since both the bacteria studied here are facultative anaerobes; small pore sizes could introduce a steep oxygen gradient across the thickness of the sample, which could be more conducive to the bacterial growth. In addition, there was a thin film noted on the 60 μm pore scaffolds due to heterogeneous cooling conditions which was not observed on the 140 μm pore scaffolds. This thin layer could interact more as a 2D membrane which were less antibacterial compared to 3D matrices. Further, 2D membranes have less surface area for bacterial adhesion, decreasing the contact-dependent antibacterial characteristics of chitosan. Therefore, freely floating bacteria were able to readily proliferate in

suspension. Irrespective of pore-sizes, both *A. actinomycetemcomitans* and *S. mutans* showed complete adhesion and no significant difference in their viability, suggesting that the effects of pore size of chitosan on bacterial adhesion are not species-dependent.

Next, the effects of lysozyme-mediated degradation on the antibacterial properties of chitosan were tested using nearly hundred fold the natural concentration of lysozyme in the body. Lysozyme alone showed species-dependent antibacterial activity, similar to other reports on the functionality and the redundancy of many salivary enzymes [34, 35]. Anti-bacterial activity of chitosan structures in presence of lysozyme was also species-dependent. However, lysozyme did not have any significant effect on adhesion and viability of either *S. mutans* or *A. actinomycetemcomitans* on the surface of chitosan scaffolds. In contrast, lysozyme has been shown to inhibit the adherence of *S. mutans* to saliva-treated hydroxyapatite [35]. On the other hand, the combination of chitosan and lysozyme was antibacterial for both bacterial species in suspension, probably due to the degradation products of chitosan or by independent mechanisms. Further investigation is necessary to understand these possibilities.

To better understand the influence of surface characteristics on contact-dependent antibacterial activity of chitosan, neutralization process and blending with other polymers were tested. Chitosan dissolves in acidic water (pH < 6.3) and the solutions are prepared using a mild acid such as acetic acid. Prior to hydrating, chitosan structures are neutralized to remove the acid. To understand the importance of the neutralization process, structures were either neutralized in NaOH followed by sterilization in alcohol or neutralized as well as sterilized in 90% ethanol. The NaOH neutralized membranes which allowed bacterial adhesion showed decreased bacterial growth relative to ethanol neutralized membranes that showed no bacterial adhesion. This confirms that antibacterial activity of chitosan is contact-dependent.

To directly assess the contact-dependent antibacterial activity of chitosan, a new assay was adapted using 2D membranes. To directly assess the contact-dependent antibacterial activity of chitosan, close contact with the membranes was created by spreading the bacteria (*A. actinomycetemcomitans* or *S. mutans*) in a small and specific size area on an agar plate and growing into dense mats. By placing the membranes on these mats, it was ensured that the bacteria can proliferate only on the surface of chitosan and chitosan/PCL blend membranes. Proliferation of adherent bacteria by serial plating indicated that there was minimal bacterial adhesion and viability on chitosan, whereas the blend and pure PCL membranes allowed bacterial proliferation and viability to a large extent. This suggests that blending chitosan with non-antibacterial membranes compromises the antibacterial activity of chitosan. This can be attributed to the altered surface characteristics of chitosan as well as the non-anti-bacterial nature of PCL [36]. However, one has to test the influence of blending chitosan with other macromolecules [29]. In a separate study, we have performed surface analysis by atomic force microscopy and other physico-chemical analysis such as changes in the crystal structure, chemical bonding between chitosan and PCL, and viscoelastic properties [37]. Based on those results, observed decrease in antibacterial activity of chitosan-PCL blends could be partially attributed to changed surface roughness characteristics. Nevertheless, decreased antibacterial activity could also be due to altered surface charge distribution, which needs further investigation to understand altered antibacterial activity. Although we have previously shown that 140 μm pore sized scaffolds are more conducive for colonization of fibroblasts and endothelial cells [28], testing cell colonization in presence of oral pathogens would help understand the regeneration of oral tissues.

In summary, this study showed that chitosan-mediated antibacterial activity is contact- dependent and that growth of bacteria occurs away from the membrane in suspension. Chitosan is more effective against bacterial colonization in 3D scaffolds of pore size 140 μm than in any other configuration. Blending chitosan with other components such as PCL compromised its antibacterial activity. Lastly, the choice of neutralization media of chitosan greatly affects its anti-bacterial activity. Chitosan can be neutralized with ethanol when using for tissue engineering applications where it is necessary to abate bacterial colonization on the matrix while promoting tissue cell growth. Testing the simultaneous colonization of oral pathogens and gingival fibroblasts for regenerating periodontal tissue forms the next step of this project.

Acknowledgements Financial support was provided by the Oklahoma Center for Advancement of Science and Technology (HR05–075), OUHSC-ORALS, INBRE Summer Research Program for Undergraduates, and the National Institutes of Health (NIH/NCRR P20RR018741). Initial guidance in bacterial cell culture work by Hector Cumba and Asma Ahmed at Oklahoma State University is deeply appreciated.

References

1. R. LANGER and D. A. TIRRELL, *Nature* **428** (2004) 487
2. M. A. REYNOLDS, M. E. AICHELMANN-REIDY, G. L. BRANCH-MAYS and J. C. GUNSOLLEY, *Ann. Periodontol.* **8** (2003) 227
3. L. E. FREED, G. VUNJAK-NOVAKOVIC, R. J. BIRON, D. B. EAGLES, D. C. LESNOY, S. K. BARLOW, et al., *Biotechnology (N Y)* **12** (1994) 689
4. M. NAKASHIMA and A. H. REDDI, *Nat. Biotechnol.* **21** (2003) 1025

5. A. J. PUTNAM and D. J. MOONEY, *Nat. Med.* **2** (1996) 824
6. G. R. YOUNG, *Dent. Today* **22** (2003) 73 quiz 77
7. S. J. LIN, L. T. HOU, C. M. LIU, C. S. LIAO, M. Y. WONG, J. Y. HO, et al., *J. Dent.* **28** (2000) 199
8. G. M. BOWERS, R. G. SCHALLHORN, P. K. MCCLAIN, G. M. MORRISON, R. MORGAN and M. A. REYNOLDS, *J. Periodontol.* **74** (2003) 1255
9. P. EICKHOLZ, T. S. KIM, R. HOLLE and E. HAUSMANN, *J. Periodontol.* **72** (2001) 35
10. C. H. HAMMERLE and R. E. JUNG, *Periodontology* **33** (2000) 36
11. R. MUZZARELLI, G. BIAGINI, A. PUGNALONI, O. FILIPPINI, V. BALDASSARRE, C. CASTALDINI, et al., *Biomaterials* **10** (1989) 598
12. J. S. PARK, S. H. CHOI, I. S. MOON, K. S. CHO, J. K. CHAI and C. K. KIM, *J. Clin. Periodontol.* **30** (2003) 443
13. Y. J. YEO, D. W. JEON, C. S. KIM, S. H. CHOI, K. S. CHO, Y. K. LEE, et al., *J. Biomed. Mater. Res. B Appl. Biomater.* **72** (2005) 86
14. K. TOMIHATA and Y. IKADA, *Biomaterials* **18** (1997) 567
15. F. L. MI, Y. C. TAN, H. F. LIANG and H. W. SUNG, *Biomaterials* **23** (2002) 181
16. G. IKINCI, S. SENEL, H. AKINCIBAY, S. KAS, S. ERCIS, C. G. WILSON, et al., *Int. J. Pharm.* **235** (2002) 121
17. Y. C. CHUNG, H. L. WANG, Y. M. CHEN and S. L. LI, *Bioresour. Technol.* **88** (2003) 179
18. B. K. CHOI, K. Y. KIM, Y. J. YOO, S. J. OH, J. H. CHOI and C. Y. KIM, *Int. J. Antimicrob. Agents* **18** (2001) 553
19. R. TARSI, R. A. MUZZARELLI, C. A. GUZMAN and C. PRUZZO, *J. Dent. Res.* **76** (1997) 665
20. E. I. RABEA, M. E. BADAWY, C. V. STEVENS, G. SMAGGHE and W. STEURBAUT, *Biomacromolecules* **4** (2003) 1457
21. I. M. HELANDER, E. L. NURMIAHO-LASSILA, R. AHVENAINEN, J. RHOADES and S. ROLLER, *Int. J. Food Microbiol.* **71** (2001) 235
22. L. QI, Z. XU, X. JIANG, C. HU and X. ZOU, *Carbohydr. Res.* **339** (2004) 2693
23. H. K. NO, S. H. LEE, N. Y. PARK and S. P. MEYERS, *J. Agric. Food. Chem.* **51** (2003) 7659
24. Y. M. CHEN, Y. C. CHUNG, L. W. WANG, K. T. CHEN and S. Y. LI, *J. Environ. Sci. Health. Part A Tox. Hazard. Subst. Environ. Eng.* **37** (2002) 1379
25. R. J. NORDTVEIT, K. M. VARUM, O. SMIDSROD, *Carbohydr. Polym.* 29 (1996) 163
26. A. MOSHFEGHIAN, J. TILLMAN and S. V. MADIHALLY, *J. Biomed. Mater. Res. A* **79** (2006) 418
27. A. SARASAM and S. V. MADIHALLY, *Biomaterials* **26** (2005) 5500
28. Y. HUANG, S. ONYERI, M. SIEWE, A. MOSHFEGHIAN and S. V. MADIHALLY, *Biomaterials* **26** (2005) 7616
29. C. G. L. KHOO, S. FRANTZICH, A. ROSINSKI, M. SJOSTROM and J. HOOGSTRAATE, *Eur. J. Pharm. Biopharm.* **55** (2003) 47
30. S. V. MADIHALLY and H. W. MATTHEW, *Biomaterials* **20** (1999) 1133
31. J. A. BANAS, *Front. Biosci.* **9** (2004) 1267
32. E. S. CHAVES, M. K. JEFFCOAT, C. C. RYERSON and B. SNYDER, *J. Clin. Periodontol.* **27** (2000) 897
33. Y. HUANG, M. SIEWE and S. V. MADIHALLY, *Biotechnol. Bioeng.* **93** (2006) 64
34. J. D. RUDNEY, K. L. HICKEY and Z. JI, *J. Dent. Res.* **78** (1999) 759
35. V. ROGER, J. TENOVUO, M. LENANDER-LUMIKARI, E. SODERLING and P. VILJA, *Caries Res.* **28** (1994) 421
36. D. S. JONES, J. D. JOKIC and S. P. GORMAN, *Biomaterials* **26** (2005) 2013
37. A. R. SARASAM, R. K. KRISHNASWAMY and S. V. MADIHALLY, *Biomacromolecules* **7** (2006) 1131