International Journal of Scientific and Technological Research www.iiste.org
ISSN 2422-8702 (Online)
Vol 1, No.3, 2015

Morphological Analysis of the Antibacterial Action of Chitosan on Gram-Negative Bacteria Using Atomic Force Microscopy

Belgin Erdem (Corresponding author)
Department of Biology, Faculty of Arts and Sciences, Ahi Evran University,
Bagbası Campus, Kirsehir, Turkey.
E-mail: berdem@ahievran.edu.tr

Mustafa Kurt
Department of Physics, Faculty of Arts and Sciences, Ahi Evran University,
Bagbası Campus, Kirsehir, Turkey.

Salih Okur
Department of Material Science and Engineering, Faculty of Engineering and Architecture,
Izmir Katip Celebi University, Izmir, Turkey.

Abstract
Chitosan, as a cationic natural polymer, *has been* widely used as antibacterial, nontoxic, *biocompatible* and *biodegradable* properties. The main objective of this study was to elucidate the antibacterial effect of chitosan upon Serratia marcescens and Enterobacter aerogenes, which are food contaminated an important organism in food production. Atomic force microscopy (AFM) was used to study effect of chitosan on the bacterial morphology.
The use of AFM imaging studies helped us to understand how chitosan act differently upon *Serratia marcescens* and *Enterobacter aerogenes.*
Chitosan thus appeared to bind to the outer membrane, explaining the loss of the barrier function. This property makes chitosan a potentially useful indirect antimicrobial for food protection. Analysis of surface topography by atomic force microscopy (AFM) showed a significant increase in roughness of all blends relative to chitosan. Observed antibacterial properties of chitosan could be primarily attributed to surface topographical changes.
Finally, both length and diameter of the microorganisms decreased after contact with chitosan.

Keywords: Atomic force microscopy; Chitosan; Antibacterial

1. Introduction

In recent years, demand for foods without chemical preservatives has revealed in the discovery of new natural additivies. Chitosan is one of the new generation food additives and has been accepted as a potential food preservative of natural origin (Devlieghere et al. 2004).
The use of chitosan in food applications is growing, and food preservation is a primary interest. In that sense, This polysaccharid has received much attention for a wide range of unique applications in food, including bioconversion for the production of value-added food products, preservation of food from microbial deterioration, formation of biodegradable films, purification of water and clarification and deacidification of fruit juices (Rhoades & Roller 2000).
AFM has recently emerged as a valuable tool. AFM can be used to image the sample surface topography at high resolution. Therefore, AFM has been widely applied to studies of bacterial morphology (Wright & Armstrong 2006).
A few previous studies evaluated the effects of different AFM scanning modes on the cell morphology of several bacteria (Pelling et al. 2005; Arce et al. 2009). Moreover, the bacterial ultrastructures, including flagella, pili, capsules and the study of antibacterial effects were also observed and quantified, based on the exquisite sensitivity and high spatial resolution of AFM (Touhami et al. 2006, Stukalov et al. 2008). For example, Atomic force microscopy has been applied to visualizing the antimicrobial action of peptides, chitosan and the β-lactam antibiotics penicillin and amoxicillin (Da Silva & Teschk 2003; Li et al. 2007; Meincken & Holroyd 2005; Eaton et al. 2008).

In order to demonstrate the mechanism, the morphological changes of S. *marcescens* and *E. aerogenes* treated with chitosan were examined by AFM.

This last described technique takes advantage of the lateral resolution of this communication describes the application of AFM imaging to study the antimicrobial effect of chitosans a on *S. marcescens E. aerogenes* (as model Gram-negative organisms), respectively. The results are correlated with cell-viability studies, and help us to understand how the bacteria react to the treatment by chitosan. In addition, nanoindentation of the bacterial cells is used to assess the effect of the chitosan on cell rigidity.

The aim of the present study is to perform a high resolution investigation on the surface and morphological alterations induced in *S. marcescens* and E. *aerogenes* cells after exposure to chitosan. It is envisaged that by employing a battery of imaging along with AFM, interaction of chitosan with bacterial cell surfaces could be elucidated.

2. Materials and Methods

2.1. Chitosans and microorganisms

Chitosan (degree of N-deacetylation 80-85%, from crab shells) was obtained from Sigma-Aldrich (USA). Tests were conducted in two sets: a test set with chitosan and a control set without chitosan. For the preparation of the chitosan solution, as received was dissolved in 1.0% (v/v) acetic acid to make a 2.5% (w/v) solution. In both cases, the pH was adjusted to 5.8 with10 M NaOH. After stirring overnight, the solutions were autoclaved at 120 ^{0}C for 15 min.

2.2. Bacterial strains

To obtain inocula for the examination, two strains of food spoilage bacteria, Microorganisms were purchased from S. *marcescens* (ATCC 21074) and *E. aerogenes* (ATCC 5402) were obtained from the the American Type Culture Collection (ATCC) and cultured were maintained by the transfer in tryptic soy broth (TSB). After cultivation, the washed cells, suspended and diluted in steriled saline solution (0.85% NaCl) to approximately 10^8 CFU/mL, were used as the inoculum.

2.3. Assays for antibacterial activity

Antimicrobial activity of the two compounds was tested against the two strains in Muller–Hinton broth, using inocula of 10^8 cell/mL. The solution of chitosan was added to reach a final concentration of 0.50% (w/v). After fixed treatment times of 0, 2, 8 and 24 h of incubation at 37 ^{0}C, 1mL of each sample was diluted and plated by the spread technique on Muller–Hinton agar. The plates were incubated at 37 ^{0}C for 24 h and the viable cell numbers were determined. Triplicate analyses of each sample were performed and each experiment was carried out in duplicate.

Simultaneous AFM height, amplitude and phase images were obtained in AC mode on the air-dried substrates using an Asylum MFP-3D AFM (Santa Barbara, CA).

Membrane roughness values were averaged from at least three different cells per species, agent and dose. Artificial color and light were added to the three dimensional reconstructions of height data to aid visualization of image detail.

The antibacterial process of chitosan against S. *marcescens* and *E. aerogenes* was elucidated by AFM observation at intervals. Chitosan were added to bacterial cultures grown to the late exponential phase. Samples were removed onto the surface of a piece of mica plate after 2h, 8h and 24 for AFM observation. S. *marcescens* and *E. aerogenes* which was not treated by chitosan was also visualized by the AFM as a control. AFM worked under the same conditions as described above.

3. Results

3.1. Morphological changes of S. *marcescens* and *E. aerogenes* under an AFM

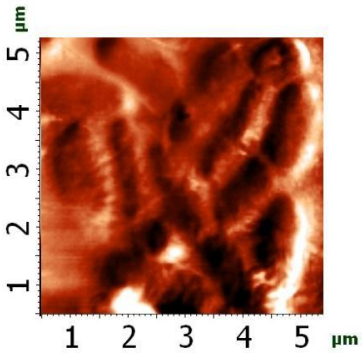
The morphology of chitosan S.*marcescens* and *E. aerogenes* was examined by AFM. The cells were degraded from a spherical shape to irregularly condensed masses when treated for 8h as shown in Figure 1.

The S. *marcescens* and *E. aerogenes* showed a regular rod shape about 1 μm wide by 2.0 μm long (Figure 1). 2 hour later, the bacteria changed from the original rod shape to a nearly spherical shape. A large amount of debris and a blurry cingulum with differently sized small particles around the bacterial cell appeared, as shown in Figure 1. The integrity of the bacterial cell disappeared and holes of various sizes appeared on the cell surface after treatment for 8 hour, as shown in Figure 1, which revealed signs of membrane disruption. After 24 hour, the debris filled the vision field, and no bacterial cell could be found, as shown in Figure 1. The morphological changes suggested that the action site of chitosan was

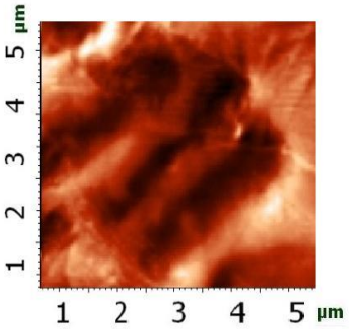
probably the bacterial envelope and that the killing of S. *marcescens* and *E. aerogenes* was the result of membrane disruption.

The images of S. *marcescens* and *E. aerogenes* strains show that treated cells are completely collapsed onto the slide surface. Furthermore, the surface of these cells appeared wrinkled, jagged and with numerous granules. These modifications are visible in Figure 1, showing a image of a single cell of *E. aerogenes* after treatment. Finally, the typical morphology of the normal cells of S.*marcescens* and *E. aerogenes* appeared severely affected after chitosan treatment.

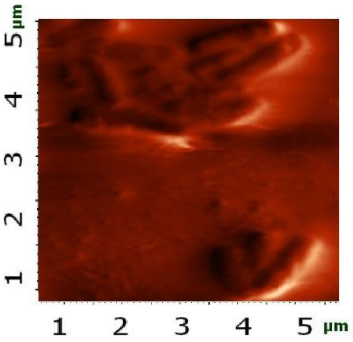
Our results showed that two microorganisms tested were sensitive to chitosan in liquid media. However, to the best of our knowledge, the detrimental effect of chitosan on growth of some bacterial species tested in this study S. *marcescens* and *E. aerogenes* is not reported in other works, we described the sensitivity of S.*marcescens* and *E. aerogenes* to chitosan.



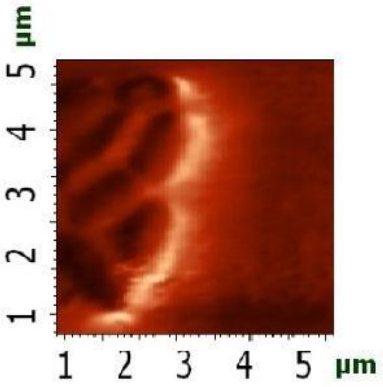
Nontreated S. *marcescens*



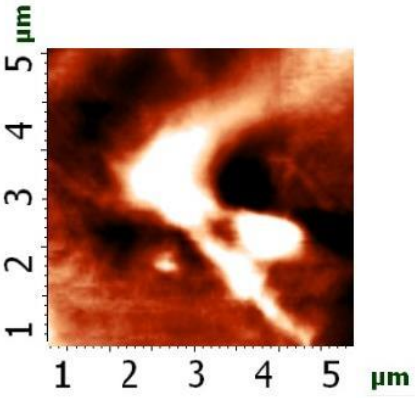
Nontreated *E. aerogenes*



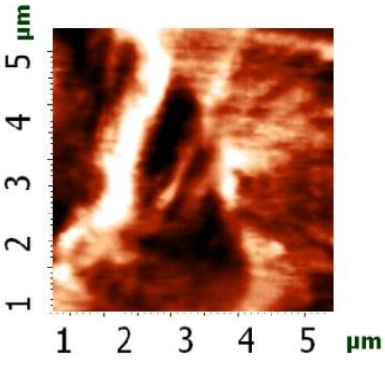
Treated for 2 hours S. *marcescens*



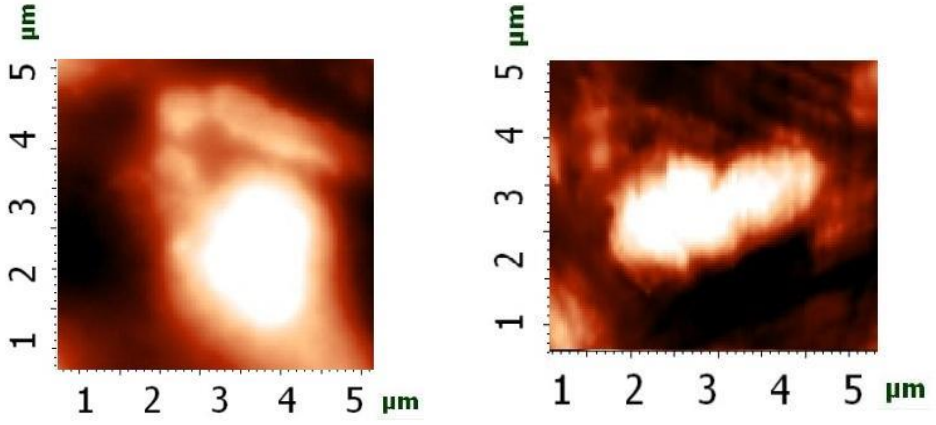
Treated for 2 hours *E. aerogenes*



Treated for 8 hours S. *marcescens*



Treated for 8 hours *E. aerogenes*



Treated for 24 hours S. *marcescens* Treated for 24 hours *E. aerogenes*

Figure.1. AFM images of S. *marcescens* and *E. aerogenes*, before and after treated by chitosan

4. Discussion

In this study, Representative AFM phase measurements before and after treatment with chitosan of Gram-negative strains are shown in Figure 1. In particular, chitosan treated cells of S.marcescens and E. aerogenes showed a loss of the original shape and had a marked bumpy surface, as also evidenced in image detail.

With a time delay, S. *marcescens* and *E. aerogenes* cells were disrupted to a considerable degree with the leakage of cytosolic components, membrane sloughing, breaching, and bledding. Chitosan exhibit higher antibacterial activity and the negatively charged surface of the bacterial cell is the target site of the polycation (Aranaz et al. 2009).

AFM of chitosan nanoparticles-treated cultures of S. choleraesuis revealed that the antibacterial action was probably via membrane disruption and leakage of cellular protein so as to kill the bacteria cells due to the change of membrane penetrability. Furthermore, AFM can be used to investigate the mechanical properties of cell surfaces in force spectroscopy mode (Qi et al. 2004).

Morphological changes of *E. coli* K88 treated by chitosan nanoparticle loaded copper ions has also been studied by AFM. It was found that chitosan nanoparticle loaded copper ions killed *E. coli* K88 through damage to the cell membrane (Du et al. 2008).

Our results showed the morphology of Gram-negative cells as being much more affected by chitosan. This finding was somewhat expected, given the presence of the outer membrane in the Gram-negatives and the fact that the biological membranes are among possible targets of chitosan.

5. Conclusion

Specifically, cell lysis, surface roughening and cell clustering were observed. S. *marcescens* and *E. aerogenes* exhibited less morphological change on treatment. Moreover, chitosan killed S. *marcescens* and *E. aerogenes* through damage to the cell membrane, as indicated by images of morphological changes.

These studies show that chitosan could inhibit the growth of various microorganisms markedly and exhibit higher antibacterial activity. AFM of chitosan treated cultures of S. *marcescens* and *E. aerogenes* revealed that the antibacterial action was probably via membrane disruption and leakage of cellular protein so as to kill the bacteria cells due to the change of membrane penetrability.

References

Aranaz, I., Mengibar, M., Harris, R., Panos, I., Miralles, B., Acosta, N., Galed, G. & Heras, A. (2009). Functional Characterization of Chitin and Chitosan. Current Chemical Biology, 3, 203-230.

Arce, F.T., Carlson, R., Monds, J., Veeh, R., Hu, F.Z., Stewart, P.S., Lal, R., Ehrlich, G.D. & Avci, R. (2009). Nanoscale structural and mechanical properties of nontypeable *Haemophilus influenzae* biofilms. Journal of Bacteriology, 191, 2512–2520.

Da Silva, A. & Teschke, O. (2003). Effects of the antimicrobial peptide PGLa on live Escherichia coli. Biochimica et Biophysica Acta,1643, 95–103.

Devlieghere, F., Vermeiren, L. & Debevere, J. (2004). New preservation technologies: possibilities and limitations. International Dairy Journal, 14, 273-285.

Du, W.L., Xu, Y.L., Xu, Z.R. & Fan, C.L. (2008). Preparation, characterization and antibacterial properties against E. coli K(88) of chitosan nanoparticle loaded copper ions. Nanotechnology, 19, 085707.

Eaton, P., Fernandes, J.C., Pereira, E., Pintado, M.E. & Malcata, F.X. (2008). Atomic force microscopy study of the antibacterial effects of chitosans on Escherichia coli and Staphylococcus aureus. Ultramicroscopy, 108, 1128–34.

Li, A. Lee, P.Y., Ho, B., Ding, J.L. & Lim, C.T. (2007). Atomic force microscopy study of the antimicrobial action of Sushi peptides on Gram-negative bacteria. Biochimica et Biophysica Acta, 1768, 411–8.

Meincken, M., Holroyd, D.L. & Rautenbach, M. (2005). Atomic force microscopy study of the effect of antimicrobial peptides on the cell envelope of Escherichia coli. Antimicrobial Agents and Chemotherapy, 49, 4085–92.

Pelling, A.E., Li, Y., Shi, W. & Gimzewski, J.K. (2005). Nanoscale visualization and characterization of *Myxococcus xanthus* cells with atomic force microscopy. Proceedings of the National Academy of Sciences of the United States of America, 102, 6484–6489

Qi, L., Xu, Z., Jiang, X., Hu, C. & Zou, X. (2004). Preparation and antibacterial activity of chitosan nanoparticles. Carbohydrate Research, 339, 2693–270.

Rhoades, J. & Roller, S. (2000). Antimicrobial Actions of Degraded and Native Chitosan against Spoilage Organisms in Laboratory Media and Foods. Applied and Environmental Microbiology, 66, 80-86.

Stukalov, O., Korenevsky, A., Beveridge, T.J. & Dutcher, J.R. (2008). Use of atomic force microscopy and transmission electron microscopy for correlative studies of bacterial capsules. Applied and Environmental Microbiology, 74, 5457–5465.

Touhami, A., Jericho, M.H., Boyd, J.M. & Beveridge, T.J. (2006). Nanoscale characterization and determination of adhesion forces of *Pseudomonas aeruginosa* pili by using atomic force microscopy. Journal of Bacteriology,188, 370–377.

Wright, C.J. & Armstrong, I. (2006). The application of atomic force microscopy force measurements to the characterisation of microbial surfaces. Surface and Interface Analysis, 38, 1419.