

DEVELOPMENT AND EVALUATION OF SILVER ZEOLITE ANTIFOULING COATINGS ON STAINLESS STEEL FOR FOOD CONTACT SURFACES

ALEXANDER GRIFFITH¹, SURESH NEETHIRAJAN^{1,3} and KEITH WARRINER²

¹BioNano Laboratory, School of Engineering and ²Department of Food Science, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada

³Corresponding author.

TEL: 1-519-824-4120;

FAX: 1-519-836-0227;

EMAIL: sneethir@uoguelph.ca

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ABSTRACT

The majority of foodborne illness outbreaks can be linked to cross-contamination events through contact with contaminated surfaces. Consequently, there is a sustained interest in developing antimicrobial coatings such as silver zeolite to reduce contamination levels. In this study, we examined the efficacy of silver zeolite for preventing biofilm formation by common foodborne pathogens such as *Listeria innocua* Seeliger and *Escherichia coli* O157:H7. Biofilm formation was assessed by cresyl violet assay, quantification of colony-forming units and scanning electron microscopy, and atomic force microscopy. For silver zeolite suspended in growth media, concentrations of 0.3% w/v were effective at reducing bacterial growth of *L. innocua* after 8 h. For *E. coli* incubated with silver zeolite, there was a dose-dependent reduction in bacteria after 4 h. When coated stainless steel coupons were incubated with *Listeria* and *E. coli*, significant reductions in bacterial growth were achieved. Coating stainless steel food processing surfaces with silver zeolite may provide a means of reducing cross-contamination events of pathogens and spoilage microbes. The ability of the surface to resist the attachment of biofilms provides a complementary approach to chemical sanitation.

PRACTICAL APPLICATIONS

Antimicrobial coatings are gaining importance for the food manufacturing and food processing industries for food safety applications. The results of this study demonstrate that the silver zeolite could inhibit foodborne pathogenic biofilm formation and could potentially serve as an effective antimicrobial coating for food contact surfaces. The methodology for impregnating the silver zeolite in polymers followed by coating on stainless steel surface provides detailed procedure for preparing the antimicrobial surfaces.

INTRODUCTION

Current food contact surface materials completely lack defense against microbial growth. Thus, food pathogenic biofilms attached to any surface in a moist food manufacturing environment can survive and proliferate. Nano-engineered surfaces have the potential to prevent the growth of biofilms and will serve as the alternative way to inhibit the spread of microbial infections. These smart surfaces will not only repel microbes but can also eradicate them. The development of novel biofouling control strategies will

assure good production hygiene, effective and less usage of cleaning, and disinfectant products.

In the course of food processing and preparation, the product contacts numerous surfaces that can potentially result in cross-contamination events. Stainless steel surfaces, the most commonly used material in food industries, have rough and sponge-like surfaces to which bacteria can easily attach. Several high-profile foodborne illness outbreaks can be attributed to cross-contamination events leading to widespread dissemination of pathogens. For example, an outbreak of *Listeria monocytogenes* linked to deli meats

could be traced to biofilms formed on the surface of slicer units (Ferreira *et al.* 2014). The widespread contamination of beef and tenderized steaks is linked to outbreaks caused by biofilms of *Escherichia coli* O157:H7 on processing equipment (Giaouris *et al.* 2014). Once established within a biofilm, pathogens along with spoilage microbes cannot be readily inactivated using common sanitizing agents (Bridier *et al.* 2011).

Control of bacterial growth in the food processing environment is a key to food safety. Surfaces in contact with food during preparation typically do not incorporate an antimicrobial coating. Recent work has shown that reliance on disinfectants such as bleach to remove highly resistant foodborne pathogens may not be sufficient to eradicate the colonies (Bridier *et al.* 2011). Bacteria on such surfaces often form biofilms, which are collective colonies that attach to a surface and secrete a protective glycoprotein coating called an extracellular polymer matrix.

Silver zeolite is an anti-corrosive coating that has the potential of restricting microbial growth and thereby can inhibit biofilm formation. Zeolites are a porous crystalline aluminosilicate that are easy to apply at low temperatures, adhere strongly and exhibit resistance to corrosion. Silver has well-established antimicrobial properties (Marambio-Jones and Hoek 2010), and is easily applied to a zeolite base. The advantages of silver over other metallic zeolites include better thermal stability, ability to integrate or incorporate into matrices, and their health and environmental safety (Galeano *et al.* 2003; Marambio-Jones and Hoek 2010). Upon contact with water, silver zeolite releases silver ions, which then enters the cell and creates highly destructive reactive oxygen species (Matsumura *et al.* 2003). This further leads to cellular damage, and the silver ions bind with components to interfere with bacterial replication.

The application of a silver–zinc zeolite coating applied to stainless steel showed antimicrobial properties against resistant biofilms of *E. coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Cowan *et al.* 2003). Studies on the interaction of marine bacteria *Halomonas pacifica* with silver zeolite-coated surfaces indicated that the electrostatic interactions and the increased hydrophobicity of the coated surface were responsible for the reduction in bacterial attachment (Chen *et al.* 2009). As a coating on titanium, silver zeolite resisted attachment by methicillin-resistant *S. aureus* (Wang *et al.* 2011). In addition to metal surfaces, silver zeolites have also been applied to polyvinyl fluoride to enhance antibacterial activity in filtration membranes (Liao *et al.* 2011; Shi *et al.* 2013). Silver zeolite has also been applied to tissue containers where they kill *Candida albicans*, *S. aureus* and *P. aeruginosa* (Matsuura *et al.* 1997). Silver zeolite has the potential role in medical applications where catheters coated with silver zeolite have exhibited reduced bacterial colonization (Khare *et al.* 2007).

Two of the bacterial species responsible for a large portion of foodborne illness cases are *E. coli* (Stophforth *et al.* 2006) and *Listeria* (Blackman and Frank 1996; Simpson *et al.* 2008). These bacteria are capable of forming biofilms, providing a reservoir for cross-contamination (de Oliveira *et al.* 2010; Mafu *et al.* 2011). The acidic biofilm environment also causes biofouling of equipment such as surfaces, chutes, cutting tables, tube systems, pipes and conveyor belts. The resulting corrosion, equipment impairment and reduced heat transfer efficiency may cause equipment to require more frequent maintenance and replacement.

Many strains of foodborne pathogens are known to be resistant to traditional sanitation methods. This resistance may be due to the protective structure of the biofilm more than resistant mutations in the bacteria (Balaban *et al.* 2004). Chlorine resistance in *S. aureus* has been documented in the setting of poultry processing (Bolton *et al.* 1988). Furthermore, bacteria in biofilm form exhibit higher levels of tolerance to disinfectants and heat (Frank and Koffi 1990). *Listeria* in particular has been noted to be able to develop resistance to bleach (Norwood and Gilmour 2000). *E. coli* has also been reported to resist treatment with biocides upon attaching to a surface (Das *et al.* 1998).

Current food contact surface materials lack defense against microbial growth. Thus, food pathogenic biofilms attached to any surface in a moist food manufacturing environment can survive and proliferate (Bower *et al.* 1996). Understanding the ability of a surface material to resist possible bacterial colonization is essential for selecting and designing surfaces for food processing (Banerjee *et al.* 2011). Nano-engineered surfaces might prevent the growth of biofilms and may serve as an alternative way to inhibit the spread of microbial infections. Characterization and optimization of surface pretreatment with antimicrobial coatings provides a means to reduce biofilm formation and increase food safety.

The objective of this study was to understand the impact of silver zeolite on the initial biofilm formation of *E. coli* O157:H7 and *Listeria innocua* Seeliger on stainless steel surfaces. The formation was determined quantitatively using cresyl violet staining of biofilms exposed to silver zeolite in suspension according to previously established methods (Bhaduri *et al.* 1987; Djordjevic *et al.* 2002; Stepanovic *et al.* 2007). Second, bacteria recovered from silver zeolite-coated stainless steel plates were plated for quantification of colony-forming units. Last, the structure of the coating and the presence of bacteria were assessed using scanning electron microscopy (SEM) images, and the thickness of the coating was determined by atomic force microscopy (AFM). In this study, we assess the efficacy of silver zeolite as an antimicrobial and antifouling coating to prevent biofilm formation by *E. coli* and *L. innocua*.

MATERIALS AND METHODS

Bacterial Cultures

L. innocua Seeliger and *E. coli* O157:H7 were cultured in tryptic soy broth (TSB). *L. innocua* was aerobically grown over a 48-h period at 30C, and *E. coli* was grown over a 24-h period at 37C. The bacterial cells were harvested by centrifugation (10 min at 161 rcf) then suspended in 0.85% saline solution to a final cell density of 8 log cfu/mL ($OD_{600} = 0.2$).

Bacterial concentration was standardized by spectrophotometry at 600 nm, where an optical density of 0.2 represents approximately 10^8 bacteria per milliliter. The control growth study indicated that there was firm *Listeria* biofilm attachment as early as 8 h and firm *E. coli* O157:H7 attachment after 12 h.

Zeolite Preparation

Squares of stainless steel (grade 304), 1" and 1/4" square, were prepared with 0.05, 0.1 and 0.15% silver zeolite coating. Stainless steel plates were first cleaned with 1.3% Alconox detergent solution. A zeolite synthesis solution with molar composition of $10Na_2O:0.2Al_2O_3:SiO_2:200H_2O$ was prepared. Stainless steel sheets were floated on this solution on polypropylene balls. The deposition process was maintained at 65C by water bath immersion for 11 h. After coating, plates were washed with deionized (DI) water and dried with compressed air.

To exchange sodium in the zeolite for silver, zeolite-coated stainless sheets were floated on polypropylene balls in 0.01 M $AgNO_3$ solution for 6 h at room temperature in the dark. After incubation, the plates were rinsed with DI water followed by soaking in DI water for 1 h to remove impurities. Prior to use, plates were washed with 70% isopropyl alcohol and allowed to air dry for 1 day.

Cresyl Violet Assay

Standard round bottom 96-well plates (Corning Incorporated, Corning, NY) were inoculated with concentrations of *L. innocua* and *E. coli* O157:H7 (McFarland standard [$\sim 10^8$ cfu/mL] in TSB). Crushed and powdered silver zeolite particles were emulsified in TSB media over a 12-h period at 80C under constant stirring. The emulsion was diluted to four concentrations (0.3, 0.15, 0.075 and 0.0375% w/v) in TSB growth media. Bacteria were exposed to either control TSB alone or TSB with different concentrations of silver zeolite for a period of either 4 or 8 h. Experiments were performed with eight replicates per sample. Samples were incubated at 30C for *Listeria* and 37C for *E. coli* O157:H7.

After the incubation, growth media were gently aspirated from the wells and were rinsed three times with 0.02 M phosphate-buffered saline (PBS) to remove planktonic bacteria. Plates were heat fixed at 55C for 90 min before being exposed to 100 μ L per well of 2% cresyl violet dye for 15 min. Wells were then rinsed thoroughly with DI water and allowed to dry overnight. Once dried, 100 μ L of 95% ethanol was added to each of the wells for 20 min. The plates were read at 570 nm with 655 nm as a reference using a Bio-Rad 650 spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

Quantifying cfu from Silver Zeolite-Coated Plates

In order to quantify the cfus of bacteria adhering to stainless steel squares coated with silver zeolite upon incubation with bacteria, cells were scraped using a swab from the squares and plated onto agar for quantification. Various concentrations of silver zeolite were used (0, 0.05, 0.1 and 0.15%). The stainless steel coupons were placed in Petri dishes containing 20 mL of TSB and 200 μ L of freshly prepared bacterial suspension at a concentration of 10^8 cells per mL. Plates were incubated for 4 and 8 h at 30C for *Listeria* and 37C for *E. coli* O157:H7.

After incubation, plates were rinsed gently with 0.02 M PBS. Plates were scraped six times using 100 μ L inoculation swabs. The media collected in the loop were deposited into 900 μ L of 0.85% saline solution. Six (1:10) serial dilutions were prepared and inoculated onto tryptic soy agar with 0.01 M kanamycin (Sigma-Aldrich, Oakville, Canada) for *E. coli* and Oxford agar (Oxoid Ltd., Canada) for *Listeria*. Colonies were counted after 24 h of incubation at 30C for *Listeria* and 37C for *E. coli* O157:H7.

SEM Imaging

For SEM, 1/4" square stainless steel plates were coated with silver zeolite (0, 0.05, 0.1 and 0.15%) and incubated with *L. innocua* and *E. coli* O157:H7 as described in the previous section. After incubation with bacteria, the plates were immersed in PBS solution containing 2% glutaraldehyde for 48 h. Plates were then rinsed three times with PBS and incubated with osmium tetroxide (Sigma-Aldrich) for 1 h. Plates were again rinsed three times with PBS. Plates were then sequentially immersed in ethanol at concentrations of 50, 70, 80, 90 and 100%. Samples were dried of 100% ethanol using the critical point technique of 40 min of CO_2 at 1,200 kPa. Upon drying, the stainless steel plates were sputter coated with gold palladium in a vacuum chamber for 120 s, resulting in a deposition thickness of 20 nm. The stainless steel plates were then imaged using a Hitachi S-570 (Hitachi, Tokyo, Japan) SEM at varying angles and magnification. Images were analyzed using ImageJ software (NIH) for qualitative assessment and

comparison of bacterial attachment and the presence of the bacterial biofilm matrix.

AFM Imaging

The stainless steel coupons coated with 1.0 and 0.5% silver zeolite on the stainless coupons were imaged using an Agilent AFM (Agilent AFM/SPM 5500, Agilent Technologies, Chandler, AZ). Standard silicon cantilevers with a spring constant of 48 N/m were used. All AFM measurements were carried out in atmospheric air at room temperature of approximately 25°C using the intermittent contact mode with resonant frequency of around 190 kHz. The scan speeds were in the range of 0.2–0.3 Hz. Both topographic and error signal images were acquired simultaneously during AFM imaging. SPIP software (Image Metrology, Copenhagen, Denmark) was used for analyzing the AFM images of stainless steel coupons.

Statistical Analysis

Statistical tests were performed using the R programming language (The R Foundation for Statistical Computing, Vienna, Austria). The single-tailed Student's *t*-test was chosen due to the normal distribution of the data, the similar standard

deviations and the low number of comparisons. The similarity of the standard deviations was established using the *F* test. Because of the high variance of the sample, a *P* value of 0.05 was chosen to represent statistical significance.

RESULTS

Cresyl Violet Assay

For *L. innocua* incubated with silver zeolite in TSB at an initial inoculum concentration of 10^8 cfu/mL, there was a significant decrease in growth for the concentration of 0.075% after 4 h (Fig. 1a) but no difference after 8 h (Fig. 1b). For the lower inoculum concentration of 10^6 cfu/mL, there was a dose-dependent reduction in bacteria relative to silver zeolite dose after 4 h (Fig. 1c). After 8 h, only the 0.3% dose remained effective (Fig. 1d).

For *E. coli* O157:H7 incubated with silver zeolite at an initial inoculum concentration of 10^8 cfu/mL, there was a dose-dependent reduction in bacteria after 4 h (Fig. 2a) but no difference after 8 h (Fig. 2b). For the lower inoculum concentration of 10^6 cells per mL, there was no difference at 4 h (Fig. 2c) or 8 h (Fig. 2d). Using a single-tailed Student's *t*-test it was determined that the effect of silver zeolite was greater on *E. coli* O157:H7 samples with an initial inoculum of 10^8 .

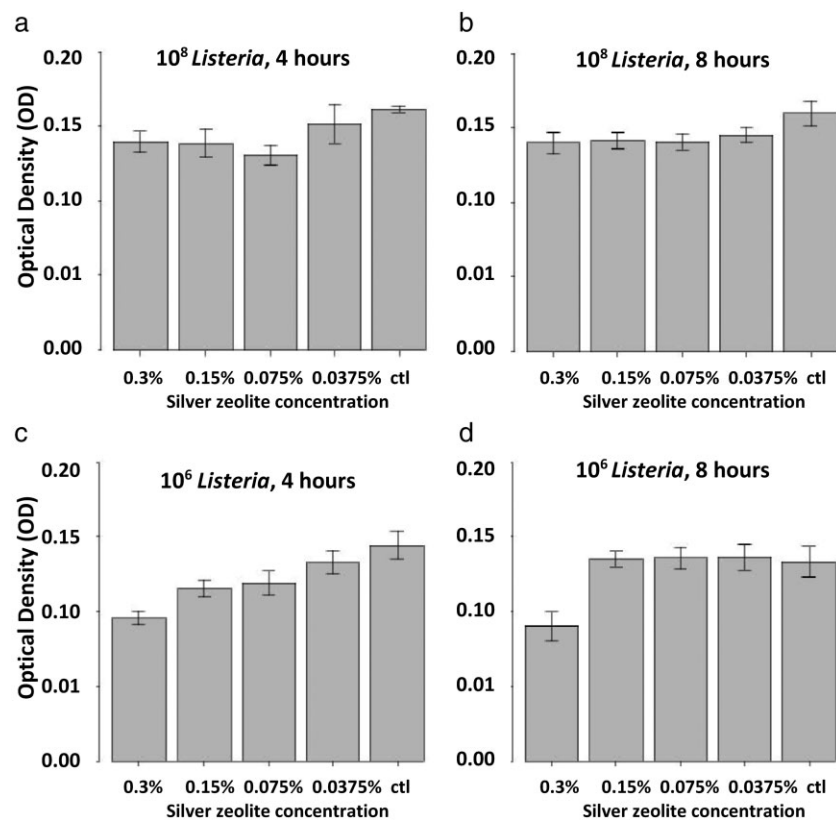


FIG. 1. BIOFILM FORMATION BY *LISTERIA INNOCUA* GROWN IN MEDIA AT AN INITIAL INOCULATION CONCENTRATION OF 10^6 OR 10^8 CELLS PER ML INCUBATED FOR 4 OR 8 H WITH TRYPTIC SOY BROTH MEDIA CONTAINING VARIOUS CONCENTRATIONS OF SILVER ZEOLITE. CTL, CONTROL SAMPLE.

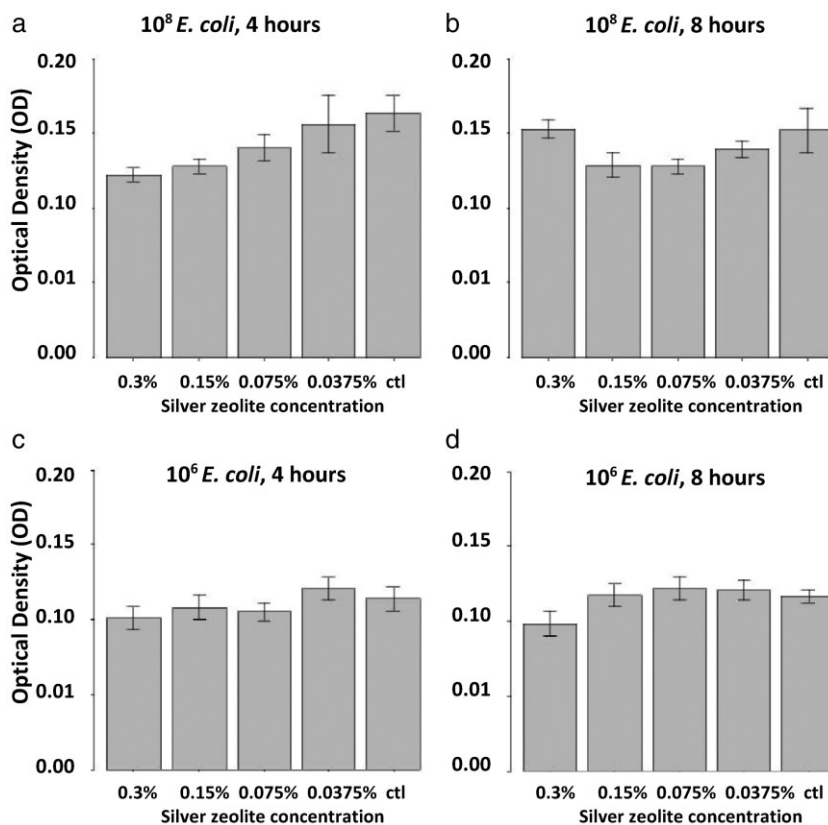


FIG. 2. BIOFILM FORMATION BY *ESCHERICHIA COLI* GROWN IN MEDIA AT AN INITIAL INOCULUM CONCENTRATION OF 10⁶ AND 10⁸ CELLS PER ML INCUBATED FOR 4 AND 8 H WITH TRYPTIC SOY BROTH MEDIA CONTAINING VARIOUS CONCENTRATIONS OF SILVER ZEOLITE. CTL, CONTROL SAMPLE.

Quantifying cfus from Silver Zeolite-Coated Plates

cfus from bacteria collected from silver zeolite-coated stainless steel plates were counted after 4 and 8 h for *L. innocua* and *E. coli* O157:H7. For *E. coli* at 4 h (Fig. 3a), cfus were

reduced by 2 logs at a concentration of 0.1% and 4 logs at a concentration of 0.15%. This reduction was only maintained at 8 h by the 0.15% concentration coating (Fig. 3b).

For *Listeria*, a 2 log reduction was maintained by the 0.15% concentration after 4 (Fig. 3c) and 8 h (Fig. 3d). At 8 h, concentrations of 0.1 and 0.05% silver zeolite reduced the cfu by 1 log.

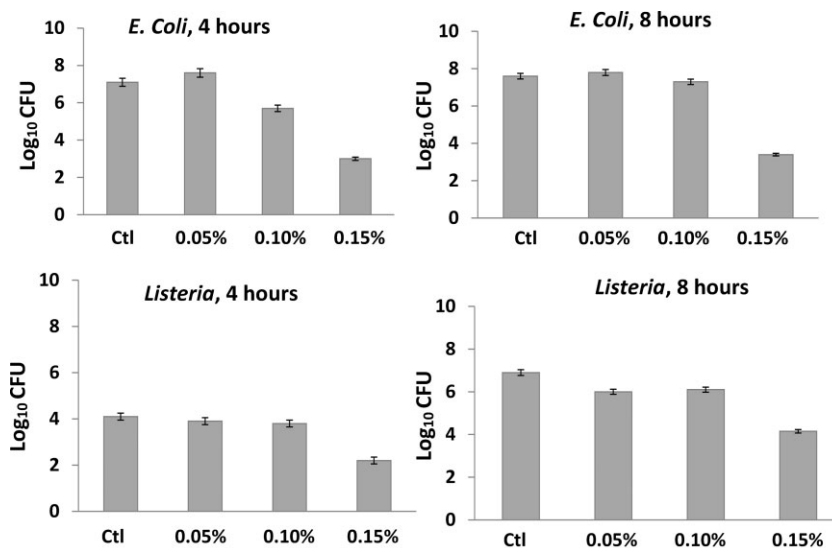


FIG. 3. BIOFILM FORMATION BY *ESCHERICHIA COLI* O157:H7 AND *LISTERIA INNOCUA* GROWN ON STAINLESS STEEL PLATES COATED WITH SILVER ZEOLITE IN VARIOUS CONCENTRATIONS INCUBATED FOR 4 OR 8 H

SEM Imaging

The SEM images revealed the presence of bacteria growing in clusters on the uncoated stainless steel squares (Figs. 4 and 5). Both *E. coli* O157:H7 and *L. innocua* were able to attach firmly to stainless steel coated with less than 0.1% silver zeolite. At silver zeolite concentrations greater than 0.1%, no bacterial attachment was observed (Figs. 4 and 5). Greater silver zeolite concentrations resulted in greater crystal size. At 0.05%, deposits ranging from 1 to 5 nm in width were observed. At 0.1% concentration the scattered deposits were overshadowed by large amorphous polyurethane regions and crystalline growths. Coating concentrations of 0.15% resulted in larger crystals.

AFM Imaging

Analysis of AFM images (Fig. 6) showed crystalline deposits of silver zeolites at 0.5% w/w emulsified in solution. AFM image analysis of the 0.05% silver zeolite coated on the stainless steel squares reveals that the deposition of the zeolite–polyurethane matrix is about 400–1,000 nm.

DISCUSSION

Cresyl Violet Assay

The results from this assay demonstrate a high degree of dependence on initial bacterial inoculation dose and time of exposure. *L. innocua* was reduced by the highest dose, 0.1% after 8 h of exposure when the initial inoculation was 10^6 , but not 10^8 . Similarly, doses as low as 0.05% were effective after 4 h of exposure to *L. innocua* but not 8 h. Similarly for *E. coli* O157:H7 at an inoculation dose of 10^8 , silver zeolite reduced bacteria after 4 h but not 8 h.

Quantifying cfus from Silver Zeolite-Coated Plates

In this assay, silver zeolite-coated stainless steel plates were exposed to *Listeria* and *E. coli* O157:H7 for 4 and 8 h. For both bacteria and both time points, increasing doses of silver zeolite cause a decrease in cfus recovered from the plates. Dramatic log scale reductions were observed, supporting the antimicrobial activity of the surface. The

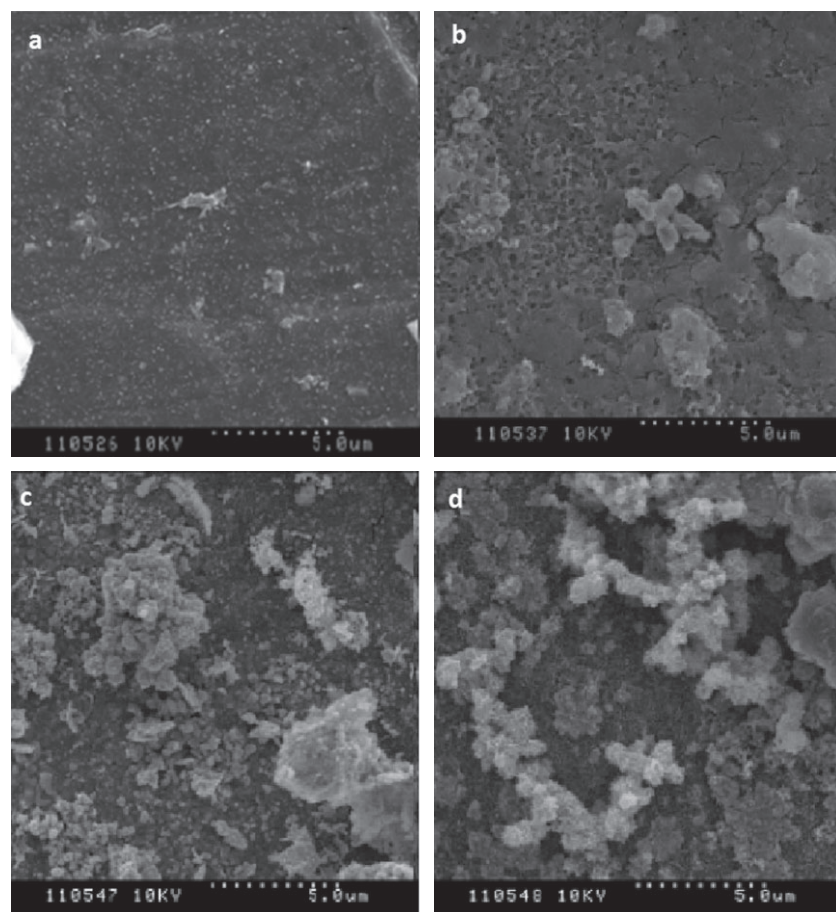


FIG. 4. STAINLESS STEEL PLATES COATED WITH (A) 0, (B) 0.05, (C) 0.1 AND (D) 0.15% SILVER ZEOLITE AFTER 4 H OF EXPOSURE TO *LISTERIA INNOCUA*

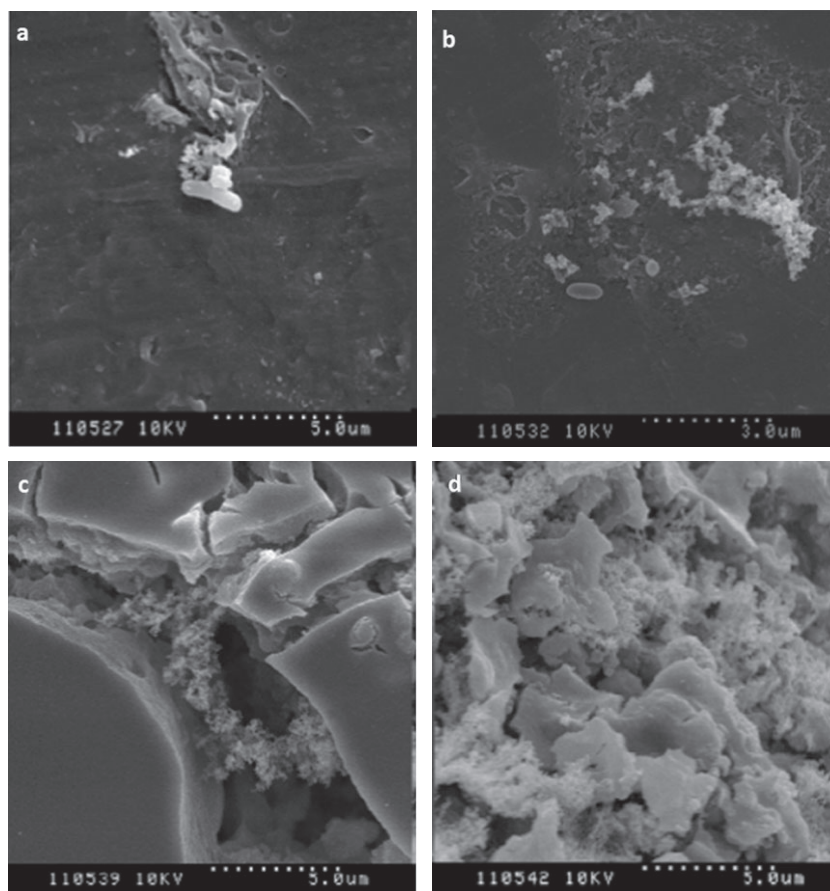


FIG. 5. STAINLESS STEEL PLATES COATED WITH (A) 0, (B) 0.05, (C) 0.1 AND (D) 0.15% SILVER ZEOLITE AFTER 4 H OF EXPOSURE TO *ESCHERICHIA COLI* O157:H7
 (a) Rod-shaped *E. coli* O157:H7 is clearly visible on the control nontreated stainless steel surface.

antimicrobial activity may be due to the action of the silver ions as well as changes in the hydrophilic nature of the coated surface.

The resistance to attachment exhibited second-order dampening characteristics. The rate at which the bacteria were attaching to the surface related directly to the dose of silver zeolite. Initially, the effectiveness of the silver zeolite was independent of the number of cfus. However, because the

peak concentration observed was consistently 2×10^7 the inhibition takes on the appearance of the derivative Michaelis–Menten curve.

SEM Imaging

Quantitative analysis of SEM images revealed the quality and degree of coating material deposition and the degree of

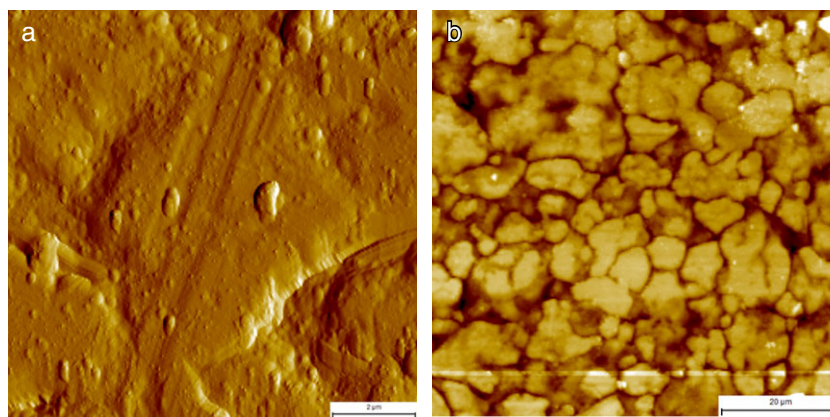


FIG. 6. ATOMIC FORCE MICROSCOPY IMAGE OF (A) UNCOATED STAINLESS STEEL AND (B) STAINLESS STEEL COATED WITH 0.1% SILVER ZEOLITE

bacterial population present on the samples. The number of polyurethane strands observed increased with the concentration of silver zeolite. Although there were few observable large silver zeolite crystals, the degree to which the metal surface became covered with amorphous deposits also increased with the silver zeolite concentration. The increase in polyurethane can be attributed to the increased oxidative conditions during the coating stage. The free radicals released from the silver zeolite interacted with the polyurethane strands, providing activation energy sufficient to drive polymerization. Apart from the control and the 0.05% silver zeolite plates, there were no observable bacteria on plates with silver concentrations above 0.05%. This may be due to the reduced bacterial adhesion as seen in the cfu assay, which showed 1×10^7 bacteria on the control and the 0.05% silver zeolite plates, while high concentration plates had 1×10^2 less bacteria.

AFM

AFM imaging reveals the thickness of the coating as 400–1,000 nm, making it favorable for use in industrial applications that would be unable to accommodate a thicker coating.

Krishnani *et al.* (2012) have demonstrated the antimicrobial activity of silver zeolite against *Vibrio harveyi* and *V. cholerae* using agar well diffusion assays for shrimp aquaculture applications. The researchers mixed 0.68 g silver nitrate with 1 g of silver zeolite in water for investigating the efficacies, which may not be a suitable concentration for food processing applications. Silver zeolites have been approved as a food contact material with restriction of 0.05 mg/kg of food by the European Food Safety Authority (Llorens *et al.* 2012; Fortunati *et al.* 2013). In this study, we developed a novel way of physical coating of silver zeolite of up to only 0.15% on stainless steel surface using polypropylene specific for food contact surfaces. We were able to successfully coat the steel surfaces with the minimal coating of 400–1,000 nm on stainless steel surfaces.

The cresyl violet assay approach was limited by the fact that silver zeolite was dissolved in the media as a suspension, possibly increasing the reactive surface area. In this form, it may also not be representative of coating that would protect the bottom of the plate from colonization. In order to compensate for the weaknesses of this assay we counted cfus from coated plates as a complementary assay. AFM and SEM image analysis data support the superior antimicrobial efficacy of silver zeolite coatings. Further studies are warranted to investigate the required duration of the surface coating for retaining the ability to release silver ions and the durability of the coating.

CONCLUSIONS

In this study, increasing concentrations of silver zeolite coating were found to reduce bacterial adhesion by *L. innocua* Seeliger and *E. coli* O157:H7 on stainless steel plates. This was particularly evident from the reduction in the number of cfus of bacteria recovered from the surface of silver zeolite-coated stainless steel plates. Cresyl violet quantification of bacterial colonies grown with silver zeolite suspension showed that the antimicrobial effect was dependent on the initial inoculation dose and the duration of exposure. Stainless steel plates imaged using SEM showed no bacterial adhesion with a silver zeolite concentration greater than 0.05%. AFM results suggest that the thickness of the coating preparations was determined to range between 400 and 1,000 nm. Silver zeolite may be a useful coating for food contact surfaces for reducing bacterial attachment and the spread of foodborne illness. For application in the food industry, not only will the anti-fouling surfaces need appropriate food contact approval status but also their mode of application will need to be economically practical and environmentally safe. Hence, additional studies are needed to further characterize the practical use and durability of silver zeolite antimicrobial coating. Prevention of biofilm formation on food contact surfaces can be accomplished by avoiding conditions that promote bacterial cell attachment and selecting conditions that make the environment unfavorable for microbial growth. The production and modification of novel surfaces with nano- micro-sized features and altered hydrophobicity will make food processing and food contact surfaces less attractive for pathogenic biofilms.

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