Chitosan-Silver Thin Film-Coated Titanium Coupons using Silane Linkers Inhibit Biofilm and Planktonic Growth

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ABSTRACT
Titanium is a component of many implants and orthopedic instruments, such as screws and rods; however, this and other materials may serve as a nidus for bacterial biofilm attachment. Chitosan is a biopolymer with advantages as a surface modifier, and silver ions have broad-spectrum antimicrobial properties. For this study, chitosan is bound to silver through a novel, patented process. The purpose of this research is to characterize silane-linked chitosan-silver coatings for titanium, including comparing antimicrobial efficacy. In this study, silane-linked chitosan-silver titanium coupons reduced *Staphylococcus aureus (S. aureus)* viability by 98% (planktonic) and 99.5% (biofilm) while supporting viability of Saos-2 osteoblast cells at levels of 75% compared with control uncoated titanium. Due to the observation of retaining osteoblast viability while reducing bacterial viability, silane-linked chitosan-silver coatings could be useful for titanium implants to reduce post-operative infection as well as support the healing process.

KEYWORDS
Titanium; *Staphylococcus aureus*; Silver; Chitosan; Silane; Osteoblast; Antimicrobial; Coating

INTRODUCTION

Titanium implants and instruments including bone plates, hip femoral stem components, shoulder arthroplasty stems, cranial plates, and intramedullary rods are widespread in the field of orthopedics due to the material's strength, resistance to corrosion, and bone-like mechanical properties.[1] They are also employed in a variety of spine surgery applications, including pedicle screws, rods, and interbody devices.[2] It is understood that irregularities on the surface of the implant promote the growth of bacteria by enhancing the attachment of bacterial species on rough and irregular surfaces.[3] Bacterial colonization can damage the surface TiO_2 layer. A study reported that the colonization of bacteria demonstrates prominent damage to the surface morphology and chemistry of implant surfaces.[4,5] Moreover, once implant-associated infections occur, they can be difficult to treat, as most implants have no inherent anti-bacterial activity. Contamination of the titanium implant leads to failure in function and causes a financial burden to the patient and the health care system due to requiring revision surgery or extended antimicrobial therapy. These conditions increase the possibility for surgical complications and severely worsen patients' regular activities.[6,7] Therefore, surface cleanliness seems to be essential for implant tissue integration. Producing coated medical implants that prevent antibiotic-resistant biofilm formation as well as inhibit planktonic viability offers health professionals and device manufacturers the potential to reduce the number and severity of postoperative infections.

Silver ions have broad-spectrum antimicrobial properties against bacteria and fungi and thus are advantageous as an implant coating.[8,9] Silver ions affect microorganisms through different modes of action: blocking substance transport in and out of the cell, inhibiting the production of energy, promoting the generation of reactive oxygen species in the presence of oxygen, interacting with DNA to prevent replication or combine protein sulfhydryl group on bacterial membrane, and disrupt bacterial membranes to kill the bacteria.[10-12] Recently, some works have demonstrated that implant surfaces modified by coatings embedded with nanoparticles benefit from an enhanced healing effect and exhibit long-lasting antibacterial capability with less cytotoxic silver concentration compared to other silver structures such as microparticles and ionic compounds.[13,14] Nano-structured surfaces usually provide multiple contact sites for the adhered bacteria, causing bacterial death via localized cell wall deformation.[12,15] Silver nanoparticles have many advantages such as good antibacterial activity, excellent biocompatibility, and satisfactory stability against antibiotics and antibiotic organic antimicrobials for medical applications.[16] An enhancement in the antimicrobial impact of silver ions is further observed in combination with biopolymer like polydopamine and calcium

phosphate.[15-17] Particularly, chitosan is a biopolymer that is gaining widespread attention as a surface modifying agent due to its versatility, and as a combination composite chitosan has revealed great antimicrobial behavior. Additionally, chitosan seems to have the ability to induce response of T helper cells.[18] Silver-loaded chitosan coating on poly (acrylic acid) coated titanium has been attempted previously via electrophoretic deposition.[19] Water-soluble catechol-containing chitosan coating on titanium surfaces have been used to induce the *in-situ* reduction of Ag^+ ions.[20] Recently, silver-based metal-organic framework incorporated into nanofibrous chitosan was coated on a titanium implant.[21] Silver-loaded chitosan-heparin polyelectrolyte multilayers (PEMs) were also constructed on alkali-heat treated titanium (Ti) substrates via layer-by-layer self-assembly technique.[22]

In this study, we investigated the effect of silane linking on the interaction of titanium (Ti-6Al-4V) with chitosan-silver as well as the interactions of titanium-silane-chitosan-silver composite on Saos-2 cells and *S. aureus*. The advantage of this coating is that chitosan and silver are linked through a patented process.[23,24] This novel approach involves creating a slurry of polysaccharide in a liquid containing silver ions, filtering, washing, reducing the silver ions to atomic silver, and drying the powder.[24] The linking method used in this study for triethoxysylilbutyraldehyde (TESBA) to titanium is similar to a previously reported process.[25] It is hypothesized that chitosan-silver thin film-coated titanium coupons using silane linkers inhibit biofilm as well as planktonic growth of bacteria and simultaneously promote osteoblast proliferation.

METHODS AND PROCEDURES

Treated Coupons

Ultra-corrosion-resistant grade two titanium rod with 1" diameter was obtained from McMaster Carr Supply Co. and cut into coupons. Titanium coupons were polished with 400, 600, 800, and 1200 grit sandpaper before being sonicated in soapy water, acetone, and ethanol to remove oil and residue for 10 minutes each. The coupons were then soaked in 5M NaOH for 24h at 60°C to allow accumulation of hydroxide reactive groups on the titanium surface and rinsed with deionized (DI) water twice. The coupons were treated with triethoxysylilbutyraldehyde (TESBA) to create a 2% (v/v) silane solution in ethanol, non-adhered silane was removed with ethanol, and coupons were dried for 10 minutes in a 110 °C oven. 1% chitosan-silver solution (Chitozan Health, LLC) was added and left to dry overnight. The coated coupons were immersed in phosphate buffer for 1h, rinsed with DI water, and dried fully. Fourier-Transform Infrared Spectroscopy (FTIR) (Frontier, Perkin-Elmer, Waltham, MA, USA) was used for surface functional group characterization of the different coated coupons.

Untreated Coupons

Titanium coupons were polished with 400, 600, 800, and 1200 grit sandpaper before being sonicated in soapy water, acetone, and ethanol to remove oil and residue for 10 minutes each. The uncoated coupons were rinsed with DI water and dried fully.

Morphology

Contact angles for Ti, Ti-chitosan-silver, and Ti-silane-chitosan-silver samples were collected to understand the hydrophobicity of the coupon surface. The morphology of the samples was visualized through Scanning electron microscope (SEM) (Nova NANOSEM 650 FEI™, Hillsboro, OR, USA) after 10 nm Au-Pt coating (EMS Quorum Q150T ES plus). Energy dispersive spectroscopy (EDS) (Oxford Plus) was used to evaluate the success of the coating process by determining the presence of silver even after 24-hour aqueous exposure for washing step. Dynamic Light Scattering (DLS) (Malvern Zetasizer) was used to determine the hydrodynamic diameter of the nanoparticles.

BacTiter-Glo™ Microbial Cell Viability Assay

Bacterial viability was also assessed over time when exposed to coated coupons. Coupons were treated with chitosan-silver, and then coupons were soaked in 1X phosphate buffered saline (PBS) for either seven or fourteen days. *Staphylococcus aureus (ATCC 29213)* was statically grown in tryptic soy broth (TSB) medium overnight at 37 °C with 5% CO_2. Thereafter, coupons were inoculated in sterile well plates with approximately 10^5 CFU of *S. aureus*. After 24 hours, coupons were removed from bacterial solution and washed with PBS to separate planktonic and biofilm samples (**Figure 1**), followed by analysis with BacTiter-Glo™ Microbial Cell Viability Assay (Promega) and luminescence readings on a Biotek plate reader with Gen5 software.

Cytocompatibility

Coupons were UV-sterilized for 20 minutes and washed in cell medium. Soas-2 cells, a cell line derived from primary osteosarcoma, were seeded at 90,000 cells/well in a sterile 12-well plate before incubation at 37 °C with 5% CO_2 and exposure to three test groups: treated coupons, untreated coupons, and tissue culture plastic (TCP) control. After 24 hours, cell viability was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) (n=3), and cell morphology was determined using LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific Inc).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0.0 software (GraphPad Software Incorporation, La Jolla, CA, USA). Data was assessed using ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

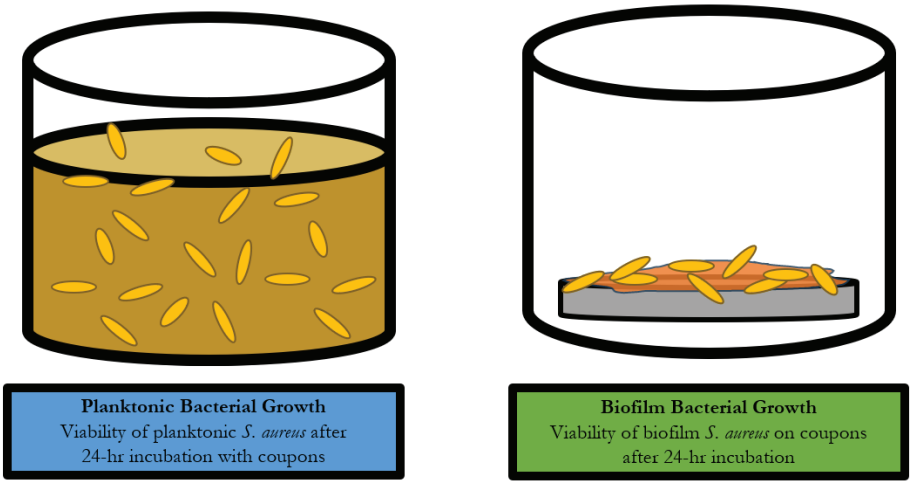

Figure 1. Division of Biofilm and Planktonic Bacteria during BacTiter-Glo Assay.

RESULTS AND DISCUSSION

Coating strategies could be implemented with many application methods including dip coating, solution casting, electrospraying, and electroplating. Of these, a simple, durable, and low-maintenance dip coating method was used in the present study. Direct titanium coating attempts with chitosan-silver solutions gave weak adherence to the substrate and delamination *i.e.* removal of the coating after contact with saline for non-silanated samples. Titanium coupons used for the study were Ti-6Al-4V, a biocompatible titanium alloy that has many medical applications and good corrosion resistance, but low antibacterial properties, which limits biological application.[26]

Morphology

SEM/EDX analysis showed the presence of relevant elements in the coupon (**Figure S1**). Silanization of the titanium with TESBA results in uniform silane coating (**Figure S2)** that provides adherence between titanium and chitosan-silver. Chitosan-silver was well-distributed over the silanated titanium coupon surface. EDX elemental mapping for the carbon and silver fraction reflected that it corresponds to the chitosan and silver content of the chitosan-silver coating solution (**Figure 2**). Chitosan silver solution gave a Z-average diameter of 166.6 nm with polydispersity index 0.248 using DLS (**Figure S3**). A similar study using polyacrylic acid to link titanium to chitosan-silver showed homogenous coating with a surface atomic percentage of 0.3% for silver.[19] Another study on the fabrication of a graphene oxide/chitosan/silver nanoparticle coating on titanium found uniform surface morphology during deposition.[27] Fourier transform infrared spectroscopy (FTIR) was used to examine the surface chemistry and bonding of silane and chitosan-silver to titanium coupon (**Figure 3A)**. No specific peaks are observed for cleaned titanium coupon, indicating no pre-existing chemical bonds. Peaks in titanium-silane curve that appear at wavenumbers 3380–3222 cm^{-1} and 1640–1555 cm^{-1} corresponding to OH stretching and OH bending confirm the formation of Ti-OH bond on the surface.[28] Further, the absorbance of Si-O-Si is shown by peaks appearing at wavenumbers 1100–1000 cm^{-1} and 846 cm^{-1} confirming the success of the silanization process of the Ti surface. The FTIR spectrum of pure chitosan indicates the presence of bands at 3359 cm^{-1} (N-H and O-H stretching), 2919 cm^{-1} (C-H) 1646 cm^{-1} (NH_2 bending) and 1024 cm^{-1} (C-O-C stretching). The FTIR spectra of chitosan silver coated on silanated titanium gave a broader peak at 3337 cm^{-1} with decrease in intensity of the peak at 1646 cm^{-1} (NH_2 bending) which indicates prevalence of more O-H group as the N-H group is involved in binding to the silver metal. Generally, both O-H and N-H groups have a strong affinity towards silver ions; however, difference in electronegativity between O and N atoms plays an important role as it dictates the deprotonation site which can favor the binding of free electrons to the metal.[29] The presence of peak at 1643 and 1552 cm^{-1} attributed to both the OH and NH_2 bending (overlapped region due to silane and chitosan) while a shift in the Si-O-Si peaks to 1071 and 852 cm^{-1} confirms the successful coating of the chitosan silver over silanated titanium. Contact angle analysis showed an increase in angle for treated samples, potentially indicating the presence of silver (**Figure 3B-D**).

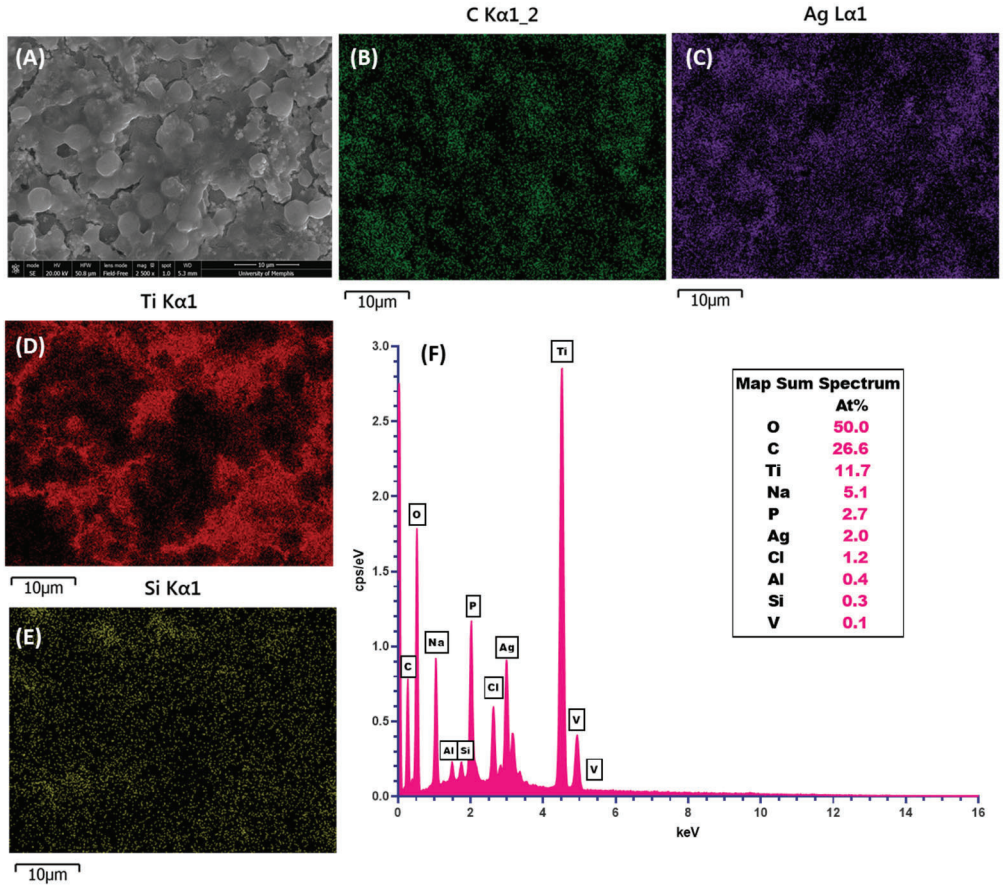

Figure 2. SEM/EDX for titanium silane chitosan silver. (A) SEM; EDX elemental mapping for (B) carbon, (c) silver, (D) titanium, (E) silica; (F) EDX spectra with atomic percentage distribution of different elements.

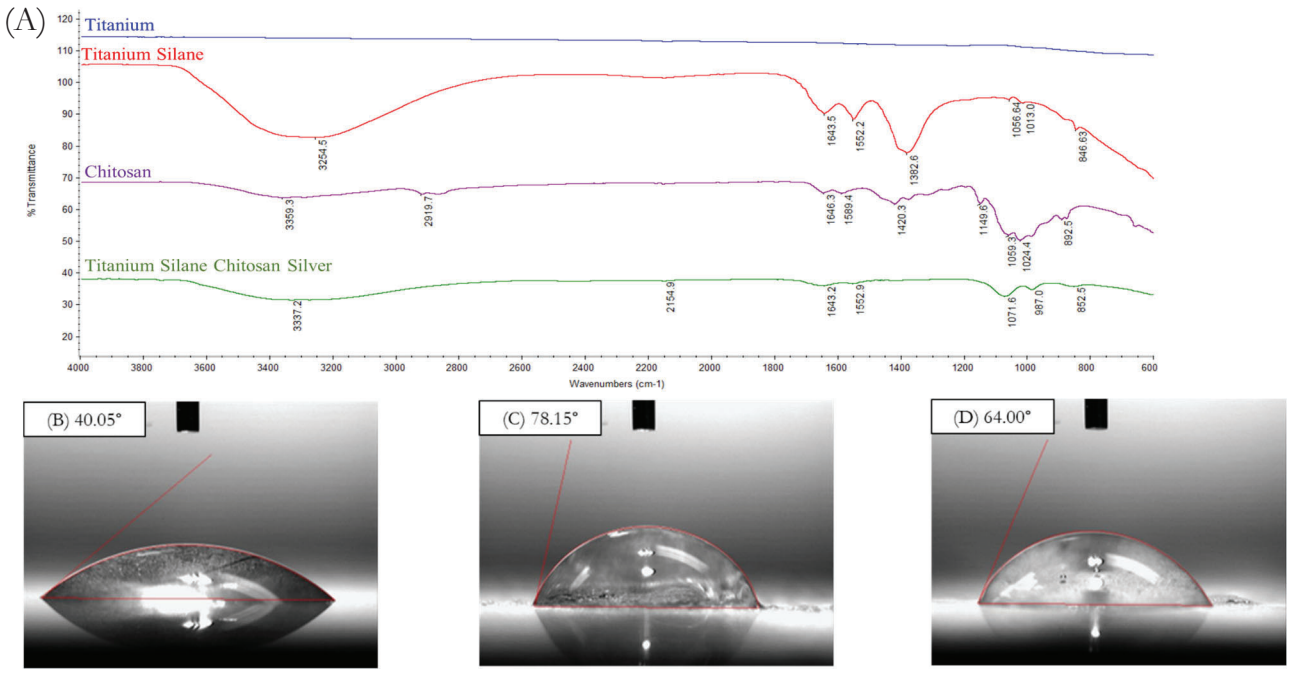

***Figure 3**. (A) FTIR spectra and contact angle images for (B) Ti coupon, (C) Ti-silane coupon, and (D) Ti-silane-chitosan-silver coupon.*

BacTiter-Glo™ Microbial Cell Viability Assay

S. aureus is the most common bacteria responsible for prosthesis-related infections, accounting for approximately half of the infections or more.[30] Therefore, the antimicrobial nature of the chitosan-silver coated silanated titanium coupons was evaluated against *S. aureus*. The results of the bacteria viability assay showed that bacterial attachment was inhibited for all loaded groups (**Figure 4**). The reduction in planktonic viability indicates that silver is being released from the surface of the coupons at a concentration suitable for antimicrobial applications up to 14 days in PBS after loading. Overall, results suggest that the linking procedure for coating titanium surfaces promotes significant retention of silver on the titanium surface which leads to decreased planktonic and biofilm viability of *S. aureus*. Even for 14-day immersion samples, planktonic and biofilm viabilities were significantly reduced with 98% and 99.5% reductions, respectively, suggesting retention and continued elution of silver at this timepoint.

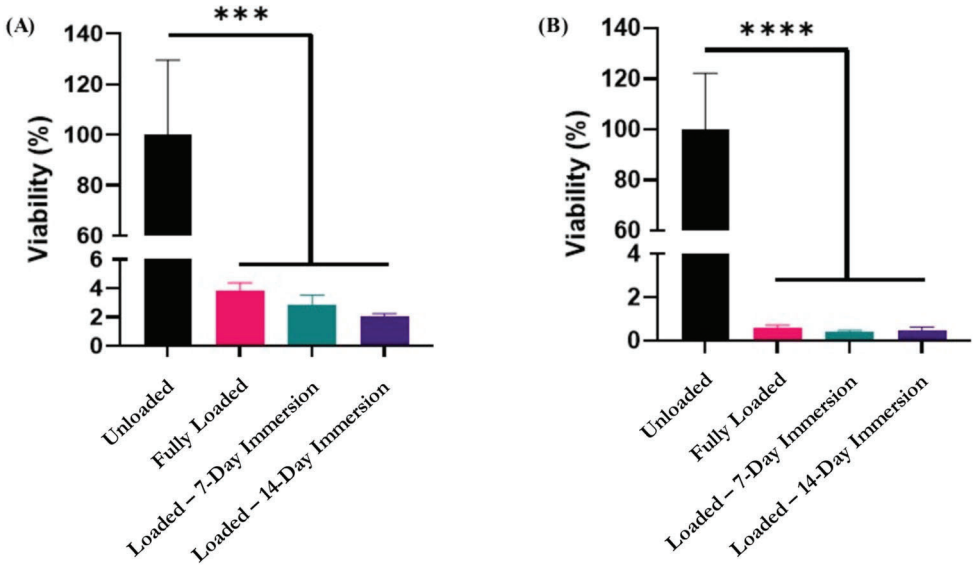

Figure 4. (A) Viability of planktonic *S. aureus* after 24-hour incubation with coupons (n=3); (B) Viability of biofilm *S. aureus* on coupons (n=3) after 24-hour incubation. Before incubating with S. *aureus*, loaded samples were immersed in PBS for 0, 7, and 14 days. **** indicates significant difference ($p<0.0001$) and *** indicates significant difference ($p<0.001$) as determined by ordinary one-way ANOVA with Tukey's multiple comparisons test.

Cytocompatibility

Silver is known to be toxic at even moderate levels, so the release of silver at non-cytotoxic levels is important in order to support cell viability.[31] The Saos-2 cell viability for treated coupons was not statistically different than untreated coupons and was about 70 percent of the TCP control (**Figure 5**). Due to the sustained viability of Saos-2 cells at 70% of the control, the conjugation of chitosan with silver may also be protective against toxicity, as defined by the ISO 10993-5 Biological Evaluations of Medical Devices standard when evaluating medical devices for in vitro cytotoxicity.[32] It can be observed that the addition of chitosan-silver coating does not decrease the cytocompatibility of titanium toward osteoblast cells. Silver content was greater on the surface of silanated titanium than non-silanated titanium, yet reduction in viability remained below toxic levels for both silver-containing groups.

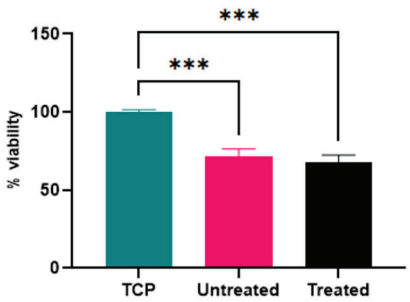

Figure 5. Percent viability of Saos-2 after 24-hour incubation with coupons (n=3). *** indicates significant difference ($p<0.001$) as determined by ordinary one-way ANOVA with Tukey's multiple comparisons test.

These results were also confirmed with Live/Dead staining, which produced similar results, with mostly living cells in all groups (**Figure 6**). Despite the presence of living cells in all groups, the treated coupons caused morphological changes in the cells and reduced the number of cells, potentially indicating apoptosis. In a similar study assessing cell response, silver nanoparticles induced significant changes in Saos-2 morphology and membrane damage.[33] Future studies could involve a lactate dehydrogenase-based or resazurin-based additional method of analysis to evaluate the long-term effects of coupons treated with chitosan-silver on Saos-2 morphology and growth as well as its *in vivo* response.

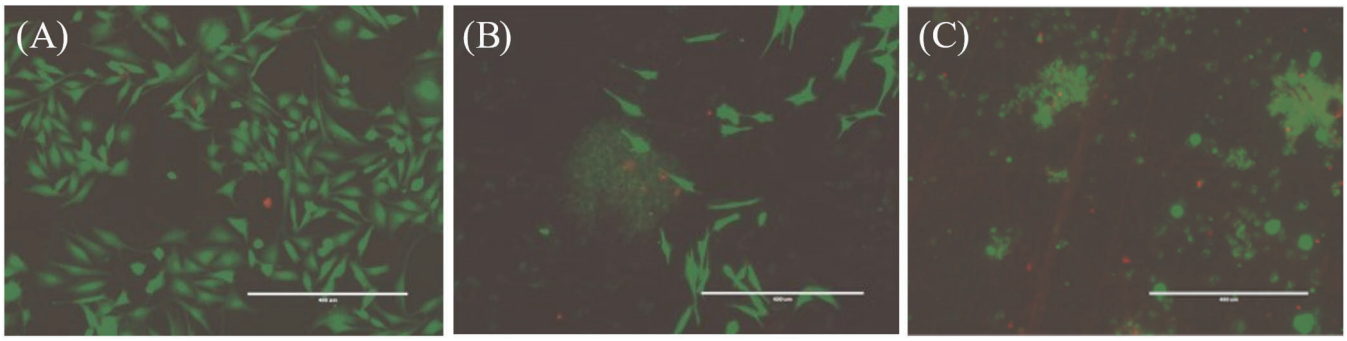

Figure 6. Live/Dead images of cells on (A) TCP control, (B) untreated coupons, and (C) treated coupons (n=1). Green and red coloration indicates live and dead cells, respectively. Scale bar is equal to 400 μm.

CONCLUSION

The dual behavior of chitosan-silver silane-linked titanium coupons to prevent biofilm and planktonic *S. aureus* growth while retaining viability of Saos-2 osteoblast cells was observed. Therefore, the approach of surface modification for titanium substrates presented here may provide an alternative strategy to simultaneously meet the desirable osteoblast growth while reducing bacterial infection for implants in clinical application.

ACKNOWLEDGEMENTS

The authors thank Ezzuddin Abuhussein for help with study completion as well as Chitozan Health, LLC for donation of chitosan-silver solution.

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ABOUT THE STUDENT AUTHORS

This work began when E.C. Montgomery was a senior undergraduate student. E.C. Montgomery graduated with a bachelor's degree in May 2022. Since that time, E.C. Montgomery has completed a master's degree in biomedical engineering and continued work under the supervision of the mentors mentioned.

PRESS SUMMARY

Titanium implants and instruments are important and commonly used in medicine because of the material's strength; however, titanium can encourage bacteria to attach to an implant surface, forming a biofilm, which complicates infection treatment. A common bacteria involved in these implant infections is *Staphylococcus aureus*. Chitosan is a natural material found in crustacean shells, and silver ions have broad-spectrum antimicrobial properties. The purpose of this research is to test if titanium coated with chitosan-silver reduces biofilm attachment compared to titanium alone. Specifically, in this study, chitosan-silver titanium coupons reduced *S. aureus* viability by 99%, compared to uncoated titanium, without significantly affecting the growth of bone cells. Due to this finding, silane-linked chitosan-silver coatings on titanium implants could reduce implant infection and support natural bone healing.