Available online at www.sciencerepository.org

Science Repository



Research Article

Titanate-Metal Complex as a Novel Antimicrobial in Dentistry

Lindsey Theda, Paul Kim, Daniel CN Chan and Whasun Oh Chung*

University of Washington School of Dentistry, USA

ARTICLE INFO

Article history:

Received 1 June, 2018

Accepted 11 June, 2018

Published 9 July 2018

Keywords:

*Streptococcus mutans**Lactobacillus casei*

adhesion of cariogenic bacteria

metal- titanate

ABSTRACT

Metal-titanates possess antimicrobial properties, and our previous studies showed that they could be beneficial in reducing bacterial load in dental materials and treatments. We investigated the mechanism of the antibiotic effect of Au(III)-monosodium titanate (MST) complex on known cariogenic bacteria, *Streptococcus mutans* and *Lactobacillus casei*. Bacteria were exposed sequentially to 10, 25, 50, 100, 200, and 400 mg/L of Au(III)-MST to determine their ability to develop resistance. Cells were grown for 4 and 18 hours following exposure to Au(III)-MST, and changes in adhesion gene expression were assessed by quantitative real-time PCR. Bacterial adhesion was assessed using Syto 13 dye and polystyrene plates. Bacterial counts were determined using OD reading. Expression of genes *srtA* and *fbpA* in *L. casei* was initially decreased after 4 hours of exposure. Expression of the *Pac* gene in the *S. mutans* culture was increased after 18 hours. The resistance study showed that the growth of *L. casei* in the presence of the metal complex was steadily decreased compared to the control, whereas growth of *S. mutans* was similar to the control up until 200 mg/L but significantly impaired in the presence of 400 mg/L Au(III)-MST. *L. casei* did not exhibit resistance to the Au(III)-MST complex, whereas *S. mutans* was able to grow in the presence of up to 200 mg/L Au(III)-MST. The mechanism underlying the antimicrobial effect of Au(III)-MST on *L. casei* may be via the regulation of *srtA* and *fbpA* genes and on *S. mutans* may be via the regulation of the *Pac* gene.

© 2018 Whasun Oh Chung. Hosting by Science Repository.

Introduction

The use of metal ions as medicinal drugs is made possible by their unique and wide variety of redox potentials, geometries, thermodynamic and kinetic properties, and other intrinsic properties as therapeutic agents [1]. Metal ions have been used in medicinal contexts in the treatment of cancers, inflammation, and arthritis, among others [2]. Metal ions have also long been known to contain chemical properties that cause them to successfully inhibit bacterial growth. Given the growing resistance of microbes to current antimicrobial agents, the need for a new class of

antimicrobials has become increasingly apparent, and metal ions may serve as a new treatment modality in this context. Concerns over the potential systemic toxicity of metals to the host have halted their widespread use as therapeutic agents, but if the use of metal ions is localized and controlled, their toxicity to the host could be greatly minimized [2]. This creates a unique niche for the novel approach of using metal-titanate complexes to fight pathogen-causing microbes due to the ability of such complexes to deliver metal ions in a controlled manner.

* Correspondence to: Whasun Oh Chung, Ph.D, University of Washington School of Dentistry; Tel: +206-543-4339; Fax number: +206-685-4258; E-mail: sochung@uw.edu

Titanates are insoluble particulate compounds of titanium and oxygen with crystalline surfaces that bind metal ions [2]. Titanates combined with metal ions are very effective as broad-spectrum antibiotics, antifungals, and anti-proliferative agents because of their redox potentials [3]. These compounds can also serve to remove metal ions from biologic environments, as well as successfully deliver them in a localized fashion without causing systemic toxicity. Additionally, metal-titanates have a higher affinity for more highly positively charged species (M3+) than for less positively charged species (M2+). Therefore, competition between protons, sodium ions, and metal ions with titanate binding sites makes the successful delivery of metals to tissues probable in biological settings [2].

Previous *in vitro* studies conducted by our research group assessed the ability of a number of metal-titanate complexes to inhibit planktonic bacterial growth [4,5]. These studies demonstrated that Au(III) loaded onto monosodium titanate (MST) was a potent inhibitor of bacterial growth. Au(III)-MST complex consistently inhibited the growth of cariogenic and periodontal bacteria *Aggregatibacter actinomycetemcomitans*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* by at least 50% when applied in concentrations above 10-50 µM, compared to other tested metals (Pd(II), Pt(II), and Pt(IV)) whose inhibition of the same bacterial strains ranged from 0-45% [4]. These results confirmed that metal-titanate compounds inhibit planktonic bacterial growth *in vitro*, but the mechanism whereby metal-titanates inhibit bacterial growth and whether or not bacteria can develop resistance to them remain unknown.

We hypothesize that Au(III)-titanates can act as broad-spectrum antimicrobials via their ability to alter the level of expression of specific genes that confer bacterial virulence factors. Given that adhesion is an important virulence factor for bacteria, and especially for cariogenic bacteria, we assessed the effects of Au(III)-MST on known adhesion genes. Understanding the mechanism of bacterial growth inhibition by metal ion species is important because we must verify that they will not cause significant adverse reactions within host cells. Understanding the mechanisms by which metal-titanate compounds inhibit the growth of bacteria, their metabolism, and locating their targets in bacterial cells are important steps to developing applications for metal-titanate complexes and assessing their long-term effects.

Experimental Design

Assay to test development of resistance: Bacteria were grown as previously described [4]. Once the initial exponential phase was reached, bacteria were passed to fresh media and exposed sequentially to 10, 25, 50, 100, 200, and 400 mg/L of Au(III)-MST after periods of 24 hours. Control bacteria were grown and passed to fresh media every 24 hours but were not exposed to Au(III)-MST. Bacterial counts were determined in liquid media via OD reading using the conversion factor for bacterial counts previously established in our lab [6].

Quantitative RT-PCR: Based on previous work that revealed inhibition of both species' growth and that gene changes were likely to be first evident at 25 mg/L metal titanate complex, bacteria species *S. mutans* and *L. casei* were exposed to 25 mg/L Au(III)-MST and grown for 4 and 18 hours [4, 5]. A control for both bacterial species was grown for the same amount of time, with no exposure to Au(III)-MST. RNA from each

experimental group was extracted using Trizol (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. RNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA). Changes in the expression of the genes involved in adhesion (Pac and GTF for *S. mutans*; fbpA and srtA for *L. casei*) were assessed by quantitative real-time PCR (QRT-PCR) with an optimization protocol previously established in our laboratory [6-8].

Adhesion Study: Examination of changes in adhesion properties of cariogenic bacteria was carried out as previously described [9]. *S. mutans* and *L. casei* cells grown (sub-cultured as previously described) to an exponential phase were washed 2x with Tris-buffered saline (TBS, pH 7.2) and resuspended to an OD600 of 0.5 (equivalent to $\sim 6 \times 10^7$ CFU/ml). The re-suspended bacterial cells were incubated with 10 mM Syto 13 dye (Life Technologies) in the dark for 20 min. Once the cells were stained, 150 µL of the cell suspension was added to a Microfluor 2 black plate (Thermo Scientific) and incubated aerobically for 3 h or 18 h with or without an overlay of mineral oil. After the wells were washed 3x with TBS, bacteria adhering to polystyrene surfaces were measured using Tecan spectrophotometer (excitation, 485 nm; emission, at 528 nm). Relative adhesion of each bacterial species and concentration was calculated based on the fold difference between the OD reading of the control and the OD reading of the Au(III)-MST -exposed bacteria.

Statistical methods: The Pffaf method was used to evaluate correlations of gene expression levels between treatment and control groups [10]. A two-tailed distribution, unequal variance student's t-test was used for all statistical analyses. Error bars were defined by the standard deviation of test values.

Results

Resistance Study

Figure 1 shows that the growth of *L. casei* continuously decreased as the Au(III)-MST concentration increased every 24 hours compared to the control. In every concentration at or above 50 mg/L, growth was significantly lower than the control (p value <0.05) for *L. casei*. Growth was inhibited completely when introduced to Au(III)-MST in the concentration of 400 mg/L.

Figure 2 shows that the growth of *S. mutans* was not impaired or slightly lowered while in the presence of increasing concentrations of Au(III)-MST every 24 hours up to a concentration of 200 mg/L. At a concentration of 400 mg/L Au(III)-MST, however, growth of *S. mutans* was almost completely arrested. *S. mutans* growth at the Au(III)-MST concentrations of 25, 100, and 400 mg/L were significantly lower than growth of the control (p values <0.05).

Gene Study

Figure 3 shows that after 4 hours of exposure to the Au(III)-MST complex, srtA gene expression of the *L. casei* bacteria was decreased by 60% when compared to the control (p<0.05). Furthermore, the figure shows that at 18 hours after exposure to 25 mg/L of the Au(III)-MST complex, the expression of srtA gene of *L. casei* was increased, although not significantly. Similarly, expression of the fbpA gene was decreased

significantly by 82% compared to the control after 4 hours of exposure ($p < 0.05$), but slightly, though not significantly, increased ($p > 0.05$) compared to the control after 18 hours (Figure 4).

Figure 5 demonstrates no statistically significant difference between the expression of Pac after 4 hours when *S. mutans* was grown in the presence of 25 mg/L of the Au(III)-MST complex compared to the control. However, Pac expression was significantly increased (by 91%) after 18 hours of exposure to 25 mg/L of the Au(III)-MST complex when compared to the control ($p < 0.05$). The expression of GTF gene in *S. mutans* was slightly increased after 4 & 18 hours of exposure to 25 mg/L of the Au(III)-MST complex, but this value was not significantly different from that for the control (Figure 6).

Adhesion Study

Figure 7 illustrates that the adhesion of *S. mutans* was not significantly different from the control (all p values > 0.05) when exposed to 10, 25, and 50 mg/L of Au(III)-MST after 3 hours. It appears, however, that adhesion at 10 mg/L was decreased by 11%, at 25 mg/L it was increased by 41%, and at 50 mg/L it was increased by 9%. Figure 8 shows that after 18 hours of exposure to either 10, 25, and 50 mg/L of Au(III)-MST, the adhesion of *S. mutans* was increased for all 3 conditions. It shows that at 10 mg/L the adhesion increased by 64%, at 25 mg/L by 149%, and at 50 mg/L by 128%. However, only the increase in adhesion in the 25 mg/L condition after 18 hours was statistically significant (p value < 0.05), whereas the 10 and 50 mg/L conditions were not.

Figures 9 and 10 shows that the adhesion of *L. casei* exposed to Au(III)-MST at the concentrations of 10, 25, and 50 mg/L changed little compared to the control after 3 & 18 hours of exposure (all p values > 0.05). At an earlier exposure time and at 10 mg/L, the adhesion was decreased by 9%, and at 50 mg/L it was decreased by 33% (Figure 9). For the exposure at 18 hours, exposure to 10 mg/L resulted in the adhesion decreasing by 9%, while at 25 mg/L it was increased by 16%, and at 50 mg/L it was increased by 32% compared to the control (Figure 10). However, all results on the adhesion ability of *L. casei* showed the changes were not statistically significant (all p values > 0.05).

Figures

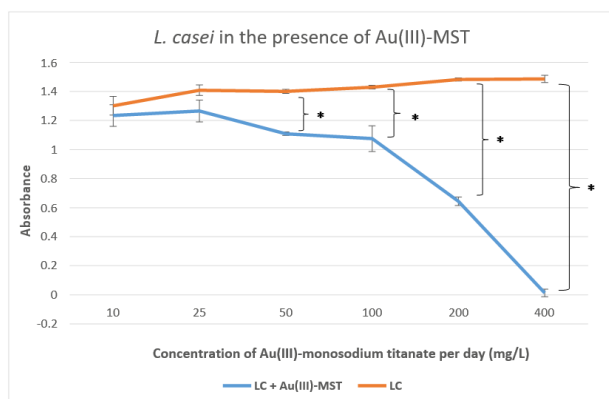


Figure 1

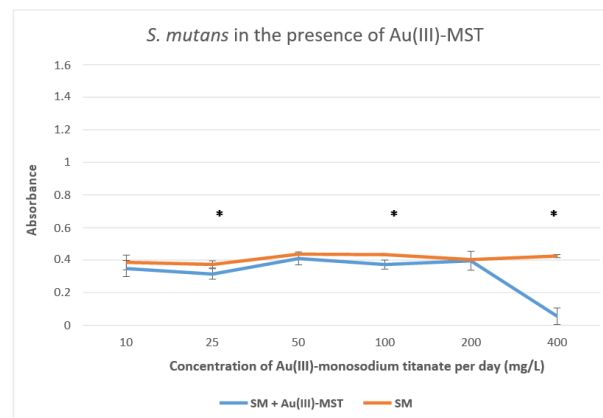


Figure 2

Figures 1 & 2: Sequential exposure every 24 hours to increased concentrations of Au(III)-MST shows that both *L. casei* and *S. mutans* are able to compensate for the presence of Au(III)-MST in low concentrations, but are not able to develop resistance to the compound at concentrations above 25 and 200 mg/L, respectively. * p -value < 0.05 .

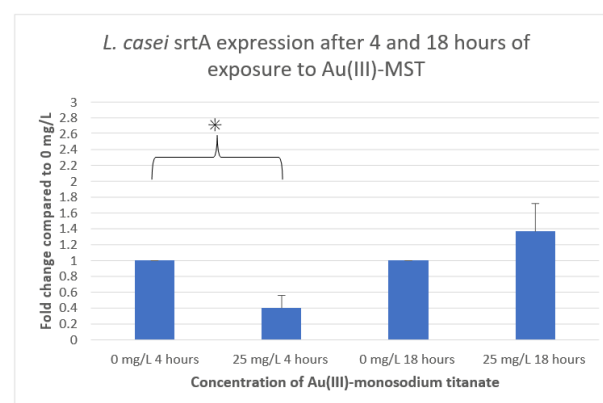


Figure 3: Changes in the expression of srtA gene in *L. casei* at 4 & 18 hours after exposure to Au(III)-MST are shown. * p -value < 0.05 .

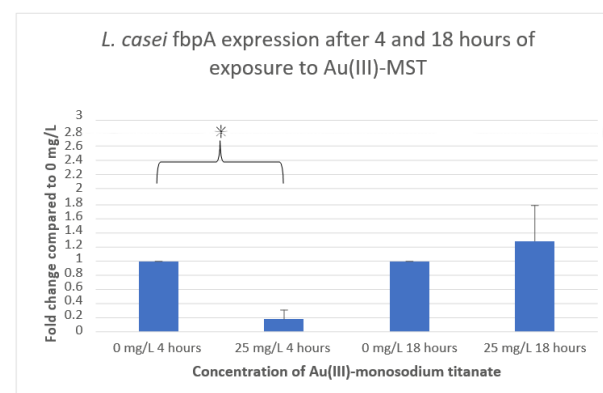


Figure 4: Changes in the expression of fbpA gene in *L. casei* at 4 & 18 hours after exposure to Au(III)-MST are shown. * p -value < 0.05 .

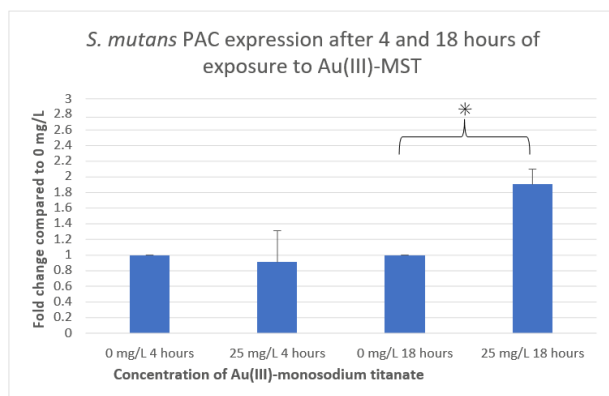


Figure 5: Changes in the expression of PAC gene in *S. mutans* at 4 & 18 hours after exposure to Au(III)-MST are shown. *p-value <0.05.

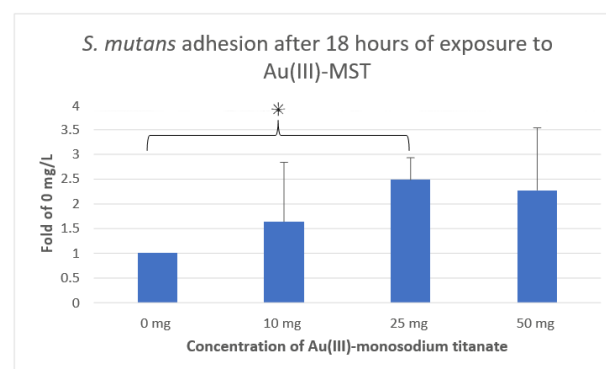


Figure 8: Differences in adhesion levels of *S. mutans* in different concentrations of Au(III)-MST after 18 hours is shown here. *p-value <0.05.

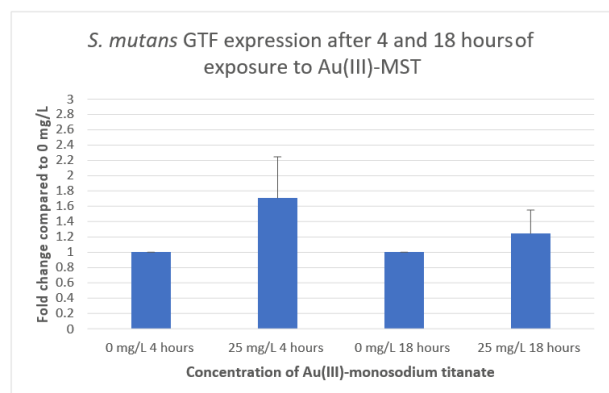


Figure 6: Changes in the expression of GTF gene in *S. mutans* at 4 & 18 hours after exposure to Au(III)-MST are shown. No statistically significant results were observed.

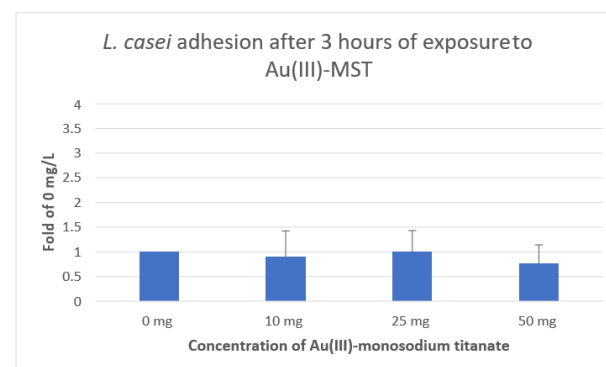


Figure 9: Differences in adhesion levels of *L. casei* in different concentrations of Au(III)-MST after 3 hours is shown here. No statistically significant results were observed.

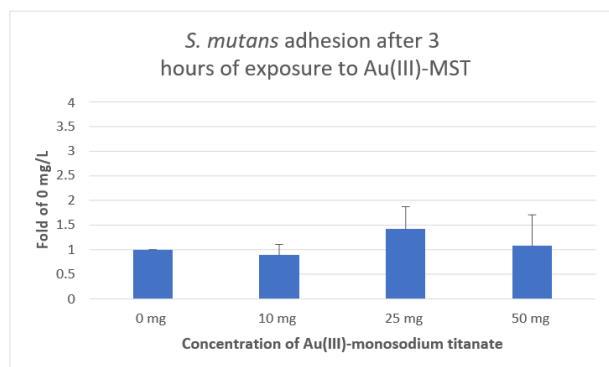


Figure 7: Differences in adhesion levels of *S. mutans* in different concentrations of Au(III)-MST after 3 hours is shown here. No statistically significant results were observed.

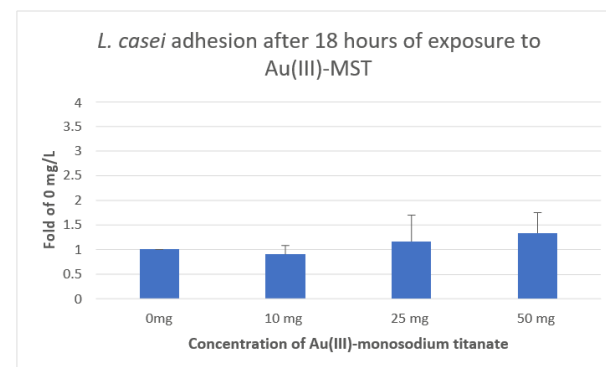


Figure 10: Differences in adhesion levels of *L. casei* in different concentrations of Au(III)-MST after 3 hours is shown here. No statistically significant results were observed.

Discussion

Titanates are inorganic ion exchangers that can readily exchange a variety of metal ions for sodium to produce the corresponding metal titanate. In the sodium form, titanates do not suppress cellular metabolism and exhibit minimal cytotoxicity in *in vitro* studies using fibroblasts and monocytes. Furthermore, sodium titanates do not trigger

cellular inflammatory responses and thus are well suited for delivering metal ions with little downstream alteration of cell function [11]. Many metal ions have inhibitory effects on bacteria, but systemic toxicity resulting from exposure to metal ions is an undesirable side effect that has limited the therapeutic use of metals. In this report, we have investigated the mechanism behind the antimicrobial properties of the Au(III)-MST complex on cariogenic bacteria *S. mutans* and *L. casei*. Our data suggest that bacteria may regulate their viability by counteracting the initial interference on adherence by the Au(III)-MST complex. We also found that with sequential exposure to higher concentrations (up to 400 mg/L) of the Au(III)-MST complex, cariogenic bacteria species, *S. mutans* and *L. casei*, show differential ability to adapt for growth inhibition.

For our gene study, the 25 mg/L concentration was chosen because previous studies showed that total protein levels of cariogenic bacteria were lowered significantly at this concentration [5]. We also wanted to make sure to assess the gene expression levels at small concentrations of our Au(III)-MST compound to make sure there were enough viable cells remaining to extract a sufficient amount of RNA from. In *L. casei*, expression of the genes *srtA* and *fbpA* was decreased significantly after 4 hours of exposure to 25 mg/L of Au(III)-MST complex, whereas after 18 hours their expression was comparable to the control condition. The *srtA* gene codes for an enzyme involved in the attachment of Gram-positive bacteria to surfaces through sortase-dependent proteins. Sortase A is responsible for the anchoring of the majority of conserved carboxylic sorting motif LPXTG of surface proteins [12]. The *fbpA* gene codes for the fibronectin binding protein that allows bacteria the ability to attach to fibronectin and fibrinogen which in turn allows it to adhere to host tissues. Our data showed that the effect of Au(III)-MST on *L. casei* involves initial stress within the first 4 hours of exposure resulting in a downregulation of general adhesion genes. After 18 hours of exposure, bacterial cells successfully compensated for the initial stress caused by the Au(III)-MST complex, as indicated by levels of attachment gene expression comparable to the control.

Our results for *S. mutans* suggest that the antimicrobial action of Au(III)-MST on this species also can be offset at low concentrations through adhesion gene upregulation. At 4 hours of exposure, no statistically significant changes in gene expression were observed between test and control groups. However, at 18 hours of exposure, the *Pac* gene, which is involved in the initial attachment of *S. mutans*, was upregulated. This may suggest that *S. mutans* undergoes some stress from the introduction of Au(III)-MST, but that its effects are not detectable until the 18-hour mark when changes in gene expression are evident. An explanation for toxicity may include positively charged ligands involved in the functional structure of surface molecules of *S. mutans*. The titanate complex may provide a molecular vehicle for Au(III), which is biologically non-toxic to humans, to affect the attachment of *S. mutans* at high concentrations through ion exchange. *S. mutans* in turn may compensate for this attack by upregulating the cell-surface protein antigen gene (*Pac*) which aids it in attachment. The GTF gene expression did not show significant differences between the test group and the control, suggesting that the adhesion mechanism through the extracellular matrix is not affected by the Au(III)-MST compound.

As suggested by our resistance study, *S. mutans* displays a greater ability to compensate growth in the presence of Au(III)-MST than does *L. casei*.

This may be caused in part by the response of *S. mutans* to upregulate attachment genes (such as the *Pac* gene) within 18 hours, whereas *L. casei* is stressed initially and therefore unable to adhere and grow as effectively.

Given a sufficiently elevated concentration of Au(III)-MST (400 mg/L), neither species of bacteria continued growing. We found that the adhesion of *S. mutans* after 18 hours of exposure was significantly higher in the 25 mg/L condition compared to the control. This correlates with our other studies suggesting that *S. mutans* is able to compensate for the initial stress after introduction to Au(III)-MST by increasing its adhesion properties through upregulation of adhesion genes including the *Pac* gene. Possible explanations for the variations in standard deviations may involve the wettability of the polystyrene wells that we used or inability of the cells to adhere to the polystyrene because of its surface properties. Future studies should consider introducing artificial saliva to simulate a more realistic oral environment and assessing the wettability of the polystyrene surface.

Future widespread use of metal-titanate complexes as antimicrobial agents in dentistry is highly plausible. Incorporation of these compounds could be key to disinfecting canals during endodontic treatment, reducing bacterial counts in periodontal pockets, and reducing the number of fillings needing to be replaced over time due to secondary caries infections. For this technology to move forward, the mechanism of microbial growth inhibition and arrest by metal-titanate compounds must be further characterized. This can also lead to the development of novel antimicrobials against complex biofilms for use in medical and hygienic applications.

Acknowledgements

The authors thank Dr. David Hobbs (Savannah River National Laboratory) for providing Au(III)-MST.

Funding

This work was funded by the University of Washington School of Dentistry Alumni Fund and Washington Dental Services. (These sources had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication)

REFERENCES

1. P. C. Bruijninx, P.J. Sadler (2008) New Trends for Metal Complexes with Anticancer Activity. *Curr Opin Chem Biol* 12: 197-206. [[Crossref](#)]
2. Yen-Wei Chen, Jeanie L. Drury, Whasun Oh Chung, David T. Hobbs, John C. Wataha (2015) Titanates and Titanate-Metal Compounds in Biological Contexts. *Int J Med Nano Res* 2: Epub, June 13. [[Crossref](#)]
3. Su YH, Yin ZF, Xin HL, Zhang HQ, Sheng JY et al. (2011) Optimized Antimicrobial and Antiproliferative Activities of Titanate Nanofibers Containing Silver. *Int J Nanomedicine* 6: 1579-1586. [[Crossref](#)]
4. Chung WO, Wataha JC, Hobbs DT, An J, Wong JJ et al. (2011) Peroxotitanate- and monosodium metal-titanate compounds as inhibitors of bacterial growth. *J Biomed Mater Res A* 97: 348-354. [[Crossref](#)]
5. Trinuch Eiampongpaiboon, Whasun O. Chung, James D. Bryers, Kwok-Hung Chung, Daniel C. N. Chan (2015) Antibacterial activity of gold-

- titanates on Gram-positive cariogenic bacteria. *Acta Biomater Odontol Scand* 1: 51-58. [[Crossref](#)]
6. W. O. Chung, B. A. Dale (2008) Differential utilization of NFκB signaling pathways for gingival epithelial cell responses to oral commensal and pathogenic bacteria. *Oral Microbiol Immunol* 23: 119-126. [[Crossref](#)]
 7. Yin L, Chung WO (2011) Epigenetic regulation of gingival innate immune responses to oral bacteria. *Mucosal Immunol* 4: 409-419. [[Crossref](#)]
 8. Gil S, Coldwell S, Drury JL, Arroyo F, Phi T et al. (2015) Genotype-specific regulation of oral innate immunity by T2R38 taste receptor. *Mol Immunol* 68: 663-670. [[Crossref](#)]
 9. Sug-Joon Ahn, Sang-Joon Ahn, Christopher M. Brownhardt, Robert A. Burne (2009) Changes in biochemical and phenotypic properties of *Streptococcus mutans* during growth with aeration. *Appl Environ Microbiol* 75: 2517-2527. [[Crossref](#)]
 10. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucl Acids Res* 29: 45. [[Crossref](#)]
 11. Davis RR, Hobbs DT, Khashaba R, Sehkar P, Seta FN et al. (2010) Titanate particles as agents to deliver gold compounds to fibroblasts and monocytes. *J Biomed Mater Res A* 93: 864-869. [[Crossref](#)]
 12. D Munoz-Provencio, J Rodriguez-Diaz, MC Collado, P Langella, LG Bermudez- Humaran, et al. (2012) Functional Analysis of the Lactobacillus Casei BL23 Sortases. *Applied and Environmental Microbiology* 78: 8684-8689.