

## Article

# Organo-Selenium Coatings Inhibit Gram-Negative and Gram-Positive Bacterial Attachment to Ophthalmic Scleral Buckle Material

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**Purpose:** Biofilm formation is a problem for solid and sponge-type scleral buckles. This can lead to complications that require removal of the buckle, and result in vision loss due to related ocular morbidity, primarily infection, or recurrent retinal detachment. We investigate the ability of a covalent organo-selenium coating to inhibit biofilm formation on a scleral buckle.

**Methods:** Sponge and solid Labtican brand scleral buckles were coated with organo-selenium coupled to a silylation reagent. *Staphylococcus aureus* biofilm formation was monitored by a standard colony-forming unit assay and the confocal laser scanning microscopy, while *Pseudomonas aeruginosa* biofilm formation was examined by scanning electron microscopy. Stability studies were done, by soaking in phosphate buffer saline (PBS) at room temperature for 2 months. Toxicity against human corneal epithelial cell was examined by growing the cells in the presence of organo-selenium-coated scleral buckles.

**Results:** The organo-selenium coating inhibited biofilm formation by gram-negative and gram-positive bacteria. The buckle coatings also were shown to be fully active after soaking in PBS for 2 months. The organo-selenium coatings had no effect on the viability of human corneal epithelial cells.

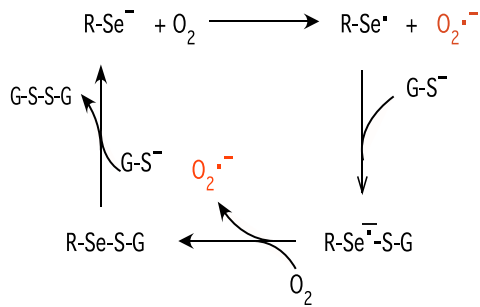
**Conclusions:** Organo-selenium can be used to covalently coat a scleral buckle, which is stable and inhibits biofilm formation for gram-negative and gram-positive bacteria. The organo-selenium buckle coating was stable and nontoxic to cell culture.

**Translational Relevance:** This technology provides a means to inhibit bacterial attachment to devices attached to the eye, without damage to ocular cells.

## Introduction

Biofilm formation can be a problem for solid and sponge-type scleral buckles.<sup>1–5</sup> This can lead to complications that can require removal of the buckle, which may result in vision loss due to related ocular morbidity, primarily infection, or recurrent retinal detachment. Thus, it would be desirable to have a coating on these buckles that would resist bacterial biofilm formation.

Organo-selenium is unlike other biocidal agents that inhibit biofilm formation, such as silver ions, in that organo-selenium can be attached covalently to various materials with no loss of its catalytic activity and unlike conventional eluting coatings it does not produce a deleterious systemic effect.<sup>6–8</sup> Equation 1 shows the catalytic mechanism of organo-selenium. Organo-selenium can produce superoxide radicals continuously by giving an electron to oxygen and taking one from sulfur compounds that are present in body fluids, such as glutathione.<sup>9</sup>



**Equation 1.** The catalytic mechanism of organo-selenium compounds.

This study is a determination of the ability of a covalent organo-selenium coating to inhibit biofilm formation on scleral buckles.

## Materials and Methods

### Bacterial Strains, Media, and Growth Conditions

The laboratory strains of bacteria tested were *Staphylococcus aureus* green fluorescent protein (GFP) strain AH133 and *Pseudomonas aeruginosa* PAO1. *S. aureus* GFP strain AH133 constitutively expresses green fluorescent protein from plasmids pCM11.<sup>10</sup> The strains were grown routinely in Luria-Bertani (LB) broth at 37°C with shaking (250 rpm). To maintain pCM11 in AH133, LB was supplemented with 1 µg/mL erythromycin. The prototrophic *P. aeruginosa* strain PAO1, originally isolated from an infected wound, was obtained from S. E. H. West (University of Wisconsin, Madison).<sup>11</sup> The efficacy of organo-selenium-coated samples was examined using LB medium (#113002022; MP Biomedicals, Solon, OH) and LB-Agar medium (#113002222; MP Biomedicals, Solon, OH) as the growth medium.

### Coating Polyurethane Foam Buckle Material with Polymerized Se-MAP

Sponge and solid Labtican (Oakville, Ontario, Canada) brand 4050 silicone band style buckles were used for all experiments. The Se-MAP monomer was purchased from Eburon Organics International (catalog no. 700.010; Lubbock, TX). Se-MAP (22% [wt/wt] selenium) stock solution was dissolved and diluted in 99.99% acetoacetoxyethyl methacrylate (AAEMA) to yield a concentrations of 0.25% selenium (wt/wt). The polymerized Se-MAP was coated on the surface of foam buckle material by coupling organo-selenium

in the form of a methacrylate (Se-3321) to a silylation reagent attached to the silicone. Unreacted material was removed by washing and the buckle then was sterilized with ethylene oxide.

### Biofilm Assay

The biofilm assay was done as described previously using the microtiter plate assay.<sup>6,7</sup> Biofilms were quantified by determining the colony-forming units (CFU) per scleral buckle sample. Organo-selenium-free control or organo-selenium-treated scleral buckles were incubated in 1 mL nutrient tryptic soy broth (TSB) media (#091010717; MP Biomedicals, Solon, OH) in the presence of approximately 10<sup>2</sup> to 10<sup>3</sup> initial CFU of *S. aureus* or *P. aeruginosa* in each well of the 24-well microtiter plate. The plates were incubated under aerobic conditions with gentle agitation at 37°C for 24 hours. To determine the CFU per segment, each segment was removed carefully from the well, rinsed gently with sterile distilled H<sub>2</sub>O three times and placed into a 1.5 mL microcentrifuge tube containing 1 mL phosphate-buffered saline (PBS; pH 7.4).

### Qualitative Analysis of the Biofilm by Scanning Electron Microscopy (SEM)

Biofilms formed on organo-selenium-free control and organo-selenium-treated scleral buckles were prepared for SEM by standard techniques.<sup>8,12,13</sup> *P. aeruginosa* biofilms were established on organo-selenium-free control and organo-selenium-treated scleral buckles as described above. After 24 hours of incubation, each sample and any adherent bacteria were fixed with 2% (wt/vol) glutaraldehyde in filter-sterilized 0.05 M PBS (pH 7.4) at room temperature for 16 hour and then rinsed three times for 15 minutes each in 0.05 M PBS. The fixed samples then were dehydrated in successive ethanol-water mixtures with increasing ethanol concentrations (20%, 40%, 60%, 80%, and 95% [vol/vol]) for 15 minutes each and then twice in absolute ethanol for 15 minutes. The ethanol-dehydrated samples then were placed in an absolute ethanol bath, which was placed in an EMS 850 critical point drier (Electron Microscopy Sciences, Hatfield, PA). The ethanol was replaced by successive additions of liquid carbon dioxide. Once the liquid CO<sub>2</sub> had replaced the ethanol, the chamber was heated under pressure to reach the critical evaporation point of carbon dioxide. The chamber then was slowly vented of gaseous CO<sub>2</sub> and the dry samples removed. The dried samples were affixed to aluminum mounts with double-sided carbon adhesive tape and sputter-coated

with platinum and palladium to a thickness of 18 nm. Observations were performed at 6 to 7 kV with a scanning electron microscope (Hitachi S-570; Tokyo, Japan). Three fields of view at  $\times 1500$  magnification were taken at randomly chosen areas from the optic surface of each sample. A biofilm-positive field will be defined as being occupied by biofilm over at least half of the visible area.

### Qualitative Analysis of the Biofilm by Confocal Laser Scanning Microscopy (CLSM)

The samples were prepared as described above in Materials and Methods using a 24-well plate assay as described previously.<sup>6</sup> Three organo-selenium-free control and three organo-selenium-treated scleral buckles were examined for the presence of *S. aureus* GFP AH133/pCM11 on the samples by CLSM. *S. aureus* GFP strain which constitutively express GFP from plasmids pCM11 when grown in the presence of 1  $\mu\text{g}/\text{mL}$  erythromycin. Visualization of the *S. aureus* GFP AH133 bacteria was accomplished with a Nikon Eclipse Ni-E upright confocal laser scanning microscope (Nikon, Melville, NY) as described previously.<sup>7</sup> The images were processed and analyzed using NIS-Elements Imaging Software. Three-dimensional biofilm image reconstructions were performed with NIS-Elements 2.2 software (Nikon) as described previously.<sup>7</sup> The biofilm structural features were analyzed with the COMSTAT program.<sup>14</sup> We obtained several image stacks of each biofilm by CLSM, and the images were analyzed as described previously (Tran et al.<sup>7</sup>).

### Quantitative Analysis of the Biofilm by the CFU Assay

The remaining microorganisms on the organo-selenium-free control and organo-selenium-treated scleral buckles were quantified by the CFU assay as described previously.<sup>6</sup> Following incubation, each sample was washed three times in 1X PBS and transferred to a sterile 1.5-mL microcentrifuge tube containing 1 mL of PBS (pH = 7.4) for enumeration of bacteria. The tubes were placed in a water bath sonicator for 10 minutes to loosen the cells within the sample and then vigorously vortexed 3 times for 1 minute to detach the cells. Suspended cells were serially diluted (10-fold) in PBS, and 10- $\mu\text{L}$  aliquots of each dilution were spotted onto LB agar plates. Thus, the equation for back calculating the bacterial concentration was  $\text{CFU} \times \text{dilution factor} \times 100$ . All experiments were done in triplicate, and all measurements were repeated three times.

### Assessing the Stability of Organo-Selenium Coating

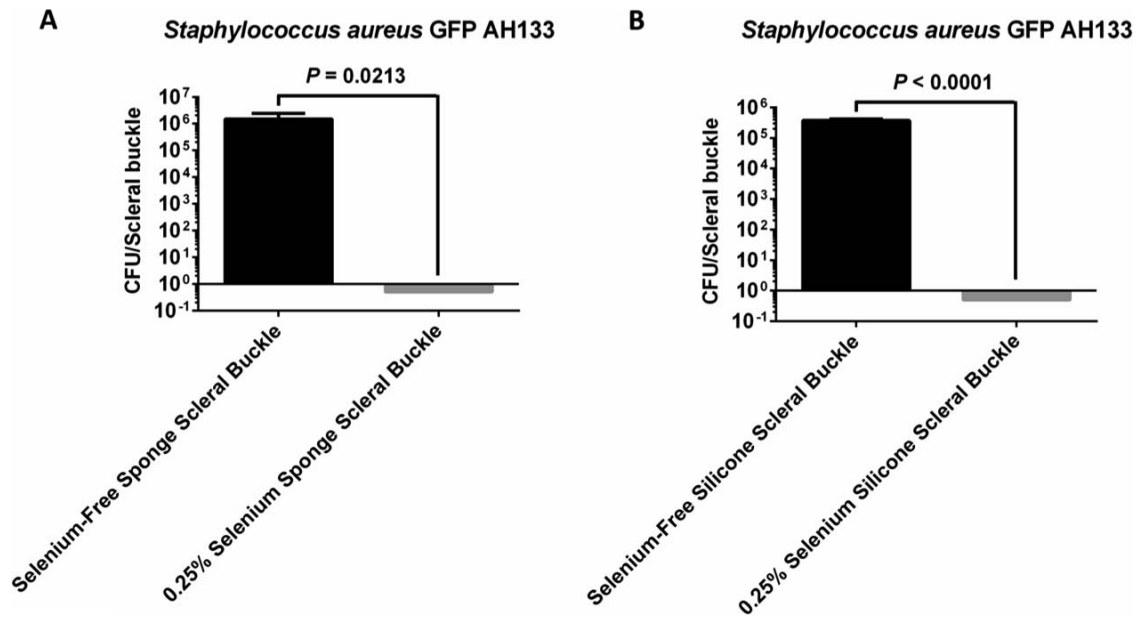
The stability of organo-selenium coating on the scleral buckle segments was tested in the following manner. Treated samples were polymerized with organo-selenium compound as described above. Organo-selenium-free control or organo-selenium-treated scleral buckles were placed in glass tubes containing 1X PBS in the ratio of one scleral buckle per milliliter of 1X PBS. After incubation at room temperature for 8 weeks, the control and treated scleral buckles were washed several times in 1X PBS, dried, and sterilized by ethylene oxide gas method. These samples then were used in the biofilm assays as described above to test for the loss of organo-selenium killing activity by the CFU assay as described above. All experiments were done in triplicate, and all measurements were repeated three times.

### In Vitro Toxicity

Immortalized human CECs (hTCEpi) were maintained at 37°C, 5% CO<sub>2</sub>, in Epilife medium (#MEPI500CA; Invitrogen, Carlsbad, CA) supplemented with Epilife defined growth supplement (#S0125; EDGS, Invitrogen) and 1% penicillin/streptomycin (#151410-122; Gibco, Carlsbad, CA) and were used between passages 40 and 60.<sup>15</sup> The medium was changed every 2 days. Organo-selenium-free control or organo-selenium-coated scleral buckles were attached on the bottom of each well of a 6-well plate (#14-832-11; Nunc; Nalge Nunc International, Rochester, NY) using Dow Corning Hi Vacuum Grease (#150G; Powell industries, Tukwilla, WA). Triplicate wells for each condition were analyzed. Supplemented Epilife medium then was added to each well. The hTCEpi telomerase-immortalized human corneal epithelial cells were plated into each well, and the plate was incubated for 48 hours. Images then were obtained using a Zeiss microscope equipped with a  $\times 10$  objective and a Canon EOS 1/Ds camera and optimized using Adobe Photoshop software. Following imaging, cells were removed from the surfaces of the wells and duplicate wells were counted using a hemocytometer. Cell counts are indicated for each condition.

### Statistical Analyses

Results of the CFU assays were analyzed statistically using GraphPad InStat 3.06 (GraphPad Software, San Diego, CA). Significance between pairs of values (Control versus one treatment group) was



**Figure 1.** Selenium in organo-selenium-treated scleral buckles inhibit *S. aureus* biofilm formation. Selenium-free, organo-selenium-coated silicone or organo-selenium-coated sponge scleral buckles were prepared as described in the Materials and Methods and inoculated with *S. aureus* GFP AH133. Biofilms were allowed to form for 24 hours. The samples were washed gently three times in 1X PBS to remove planktonic bacteria. Adherent bacteria (biofilm) were removed from the discs by vortexing in PBS, and CFU were determined by plating 10-fold serial dilutions on LB agar plates. Values represent the means of triplicate experiments  $\pm$  standard errors.

calculated using an unpaired 2-tailed *t*-test when the standard deviation (SD) was not significantly different and when a Gaussian distribution was observed. If the SD was significantly different, the Welch correction was applied to the unpaired 2-tailed *t*-test. When non-Gaussian distribution was observed (Kolmogorov-Smirnov test), significance was calculated by a nonparametric Mann-Whitney *U* test. Comparisons of the in vitro hTCepi telomerase-immortalized human corneal epithelial cell viability in the presence of selenium-free and organo-selenium scleral buckles were analyzed by 1-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons post-test and a 2-tailed unpaired *t*-test, to determine significant differences. Each experiment had 3 samples and each experiment was repeated 3 times. Differences were considered significant when the *P* value was  $<0.5$ .

## Results

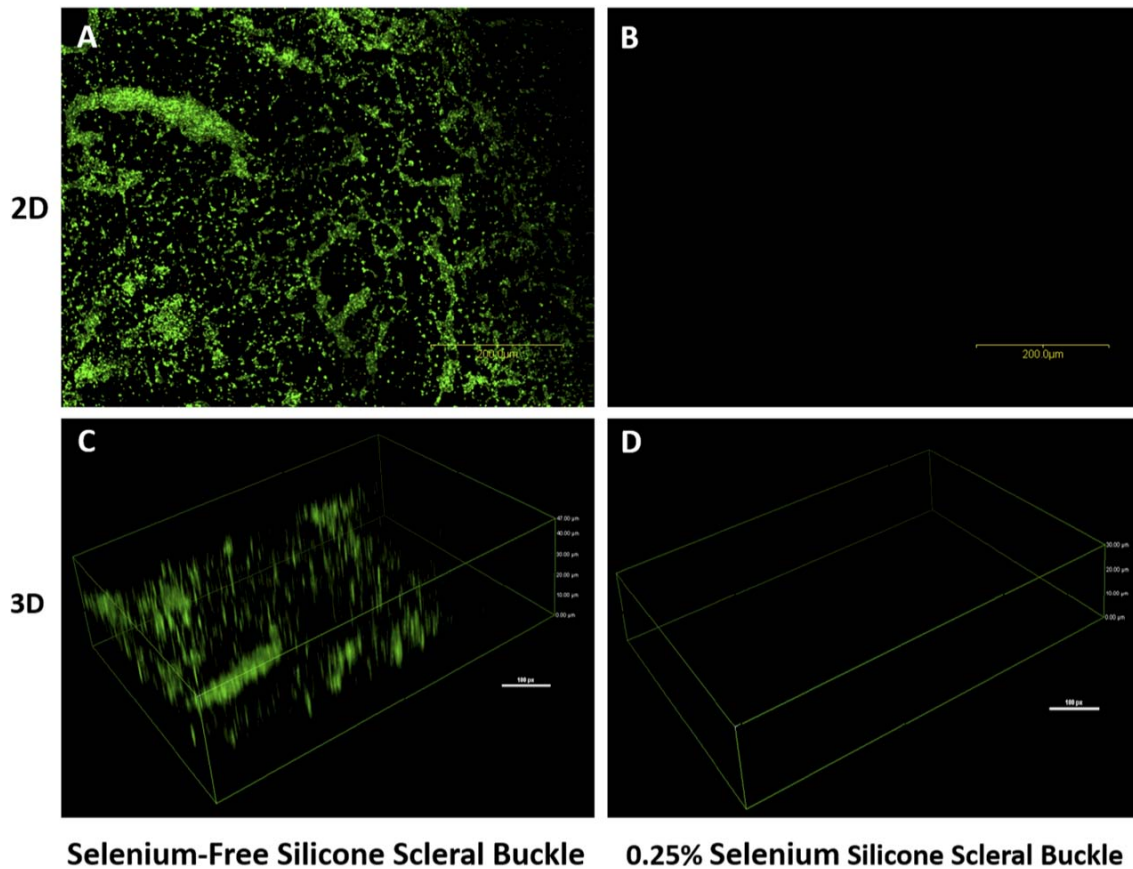
### CFU Study of the Bacterial Killing Ability of an Organo-Selenium-Coated Silicone Scleral Buckle and a Sponge Scleral Buckle

To test the bacterial killing ability of a 0.25% organo-selenium coating on the different buckles,

they were placed in a bacterial culture of *S. aureus* for 24 hours, along with untreated buckles. As can be seen in Figure 1, an average of over  $10^6$  and over  $10^5$  CFUs was found on the untreated sponge and the untreated silicone scleral buckles, respectively. However, no cells were found on the organo-selenium-treated buckles in each case. The organo-selenium-treated buckles were sonicated and an aliquot of the solution was plated to make sure that no bacteria remained attached to the buckle at the end of the experiment.

### CLSM of Organo-Selenium-Coated Silicone Scleral Buckles in the Presence of *S. aureus*

As a test on the CFU assays for the attachment of *S. aureus*, containing a gene for GFP, to the untreated and 0.25% organo-selenium-coated silicone scleral buckles, some of the buckles were examined by CLSM after overnight growth with the bacteria. Figure 2 shows the two-dimensional and three-dimensional growth of the bacteria on the buckles. The pictures demonstrate that, while a nice biofilm of *S. aureus* is attached to the untreated buckle, no bacteria are visible on the organo-selenium-treated silicone scleral buckle.

***Staphylococcus aureus* GFP AH133**

**Figure 2.** Organo-selenium-coated silicone scleral buckles visibly reduce *S. aureus* GFP AH133 biofilm formation on scleral buckles. Selenium-free and organo-selenium-coated silicone scleral buckles were examined by CLSM at  $\times 20$  magnification after 24 hours of incubation.

### Quantitative Analysis of the CLSM Results

By use of the COMSTAT program we were able to quantitate the amount of biofilm that formed in the CLSM study. This program analyzes the amount of fluorescence found in the different image stacks obtained from the CLSM study. This allowed us to

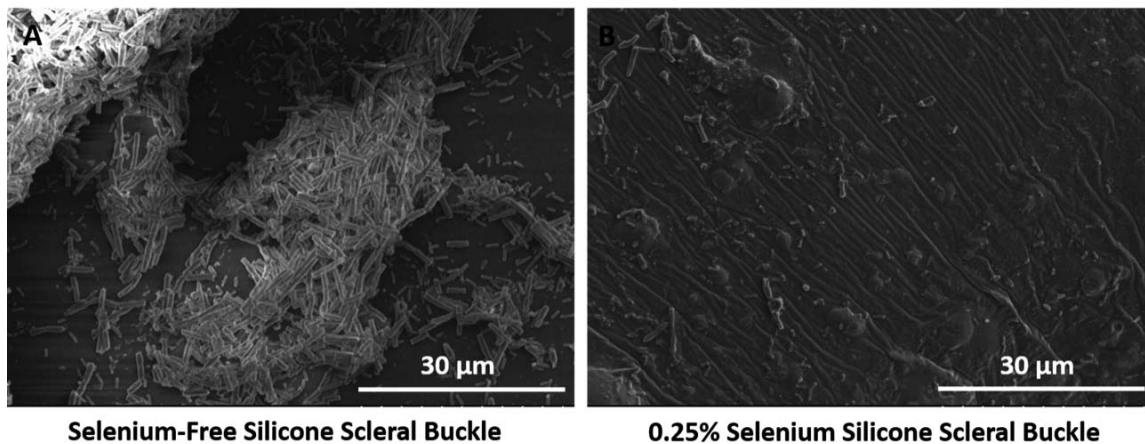
determine the total biomass, average thickness of the biomass, and surface area of the biomass. These results can be seen in Table 1. The untreated silicone scleral buckle material showed extensive biomass, while the organo-selenium-coated material showed no biomass.

**Table 1.** Quantitative Analysis of *S. aureus* GFP AH133 Biofilms Formed on Selenium-Free Silicone Scleral Buckle and Organo-Selenium-Coated Silicone Scleral Buckle<sup>a</sup>

Parameters measured	Uncoated Eye Buckle			OS-Eye Buckle		
	#1	#2	#3	#1	#2	#3
Total biomass ( $\mu\text{m}^3/\mu\text{m}^2$ )	7.52583	1.8295	2.69149	0	0	0
Average thickness ( $\mu\text{m}$ )	9.78667	2.1964	3.87766	0	0	0
Surface area of biomass ( $\mu\text{m}^2$ ) <sup>b</sup>	1.41E+06	359491	698265	0	0	0

<sup>a</sup> Several image stacks were acquired from each contact lens and analyzed by COMSTAT.

<sup>b</sup> Reflects the efficiency with which AH133 colonized the surface.

***Pseudomonas aeruginosa* PAO1**

**Figure 3.** SEM analysis of *P. aeruginosa* PAO1 biofilm formation on selenium-free silicone scleral buckle or 0.25% organo-selenium silicone scleral buckle. Biofilms were allowed to form as described in the Materials and Methods. After 24 hours of incubation at 37°C, the discs were fixed, dried, affixed to aluminum mounts, and sputter coated with platinum and palladium. Observations were performed at 6 to 7 kV with a scanning electron microscope. Five fields of view were examined from randomly chosen areas from the optical surface of each sample at magnification of  $\times 1500$ . Experiment was conducted in triplicate. Representative fields of view are shown. Scale bars: 30  $\mu\text{m}$ .

### SEM of Organo-Selenium-Coated Silicone Scleral Buckles in the Presence of *P. aeruginosa*

Since no GFP-containing construct for *P. aeruginosa* was available, samples were viewed by SEM. Figure 3 shows that extensive biofilms of *P. aeruginosa* were found on the untreated silicone buckle while only scattered bacterial attachment was observed for the 0.25% organo-selenium-coated buckle, after placement in cultures of *P. aeruginosa* for 24 hours. It would appear that while some attachment was possible, the bacteria were not able to establish good biofilms on the organo-selenium-coated buckles.

### Stability Studies of the Organo-Selenium Attachment to the Scleral Buckles

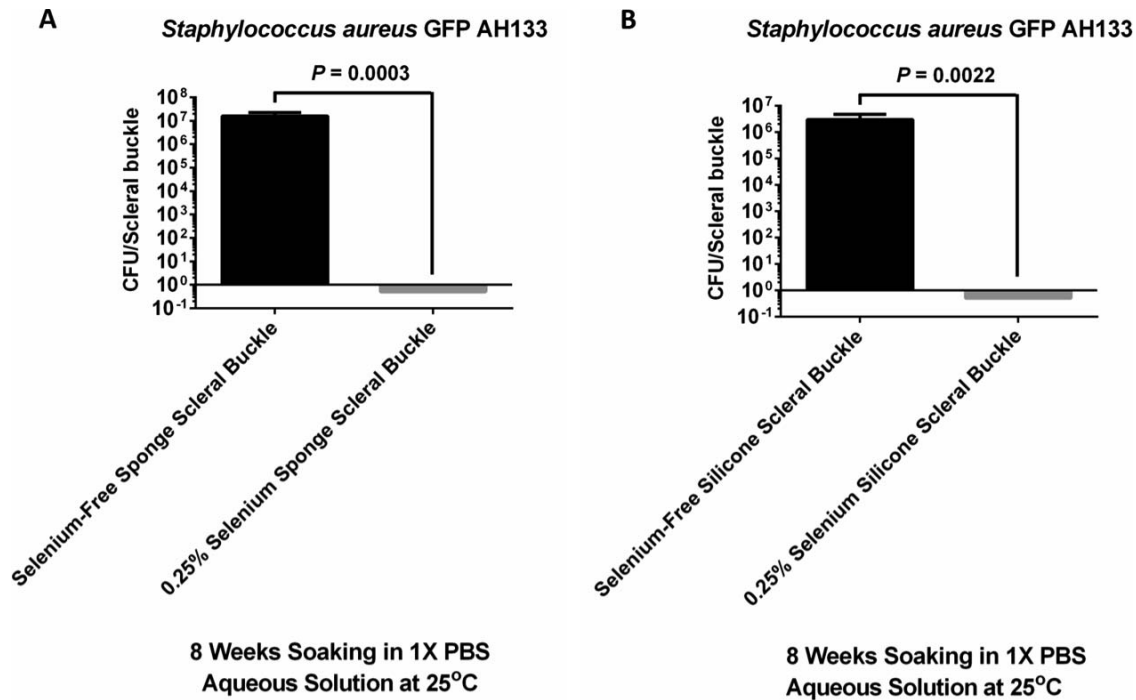
To determine the long-term stability of the organo-selenium coating to the different buckle materials, they were soaked in PBS solution for 8 weeks. After this time, they were placed into cultures of *S. aureus* for 24 hours. Figure 4 showed that the sponge and silicone scleral buckles, that were coated with 0.25% organo-selenium, still were completely resistant to the attachment of *S. aureus* after soaking for 8 weeks.

### Assessment of Toxicity of the Organo-Selenium-Coated Silicone Buckle Material to Human Corneal Epithelial Cells

Immortalized human corneal epithelial cells were grown for 48 hours in the presence of the organo-selenium-coated silicone buckle material. Figure 5 shows effect on the growth of these cells. The organo-selenium coating appears to have no effect on the growth of the cells either next to or under the buckle material. These results of the triplicate assays were confirmed by counting the cells in each well and are seen in Figure 6. The results varied from an average of 243,000 cells with no buckle present, 250,000 cells with an untreated buckle, and 225,000 in the presence of a 0.25% selenium-coated buckle. These results were not significantly different.

## Discussion

Implantation of a scleral buckle is the most established technique for the treatment of primary rhegmatogenous retinal detachment.<sup>16</sup> However, infections after insertion of a scleral buckle are a significant complication and the most frequently isolated organisms have been *S. epidermidis* and *S. aureus*.<sup>2-4,17</sup> Infection rates have varied from 0.1% to 0.2% to an average of 3.3%.<sup>2,18,19</sup> Cultures of 638



**Figure 4.** Organo-selenium coating on silicone scleral buckle remains stable for 2 months in aqueous solution. Silicone scleral buckles were prepared, coated with organo-selenium, and soaked for 2 months as described in Materials and Methods. The samples then were dried, sterilized, and tested for the ability to inhibit biofilm formation by *S. aureus* GFP AH133. Values represent the means of triplicate experiments  $\pm$  standard errors.

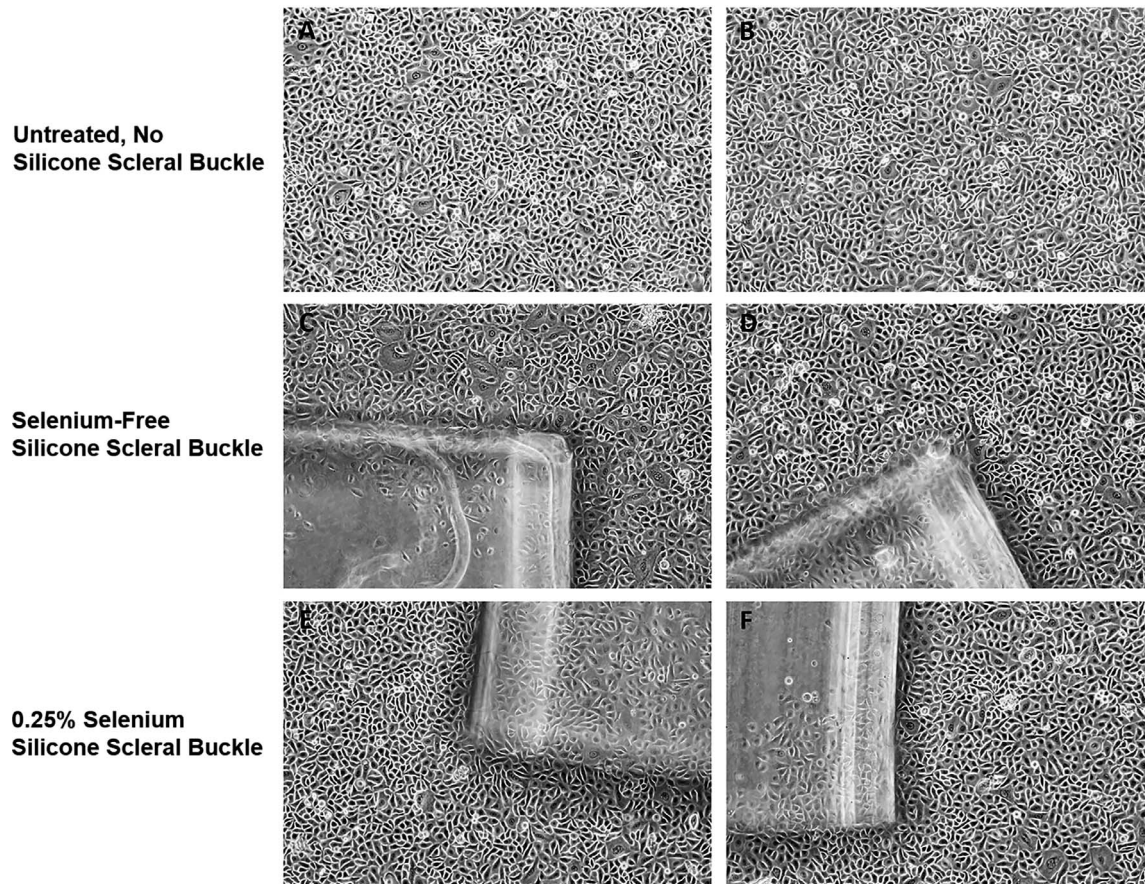
routine preoperative conjunctival specimens before retinal detachment surgery demonstrated bacterial contamination with *S. epidermidis* in 37%, *S. aureus* in 3%, *Proteus* in 1%, *Klebsiella* in 1%, and *Pseudomonas* in 0.2%. A more recent study found the most common etiological agent isolated was *S. epidermidis* (27/124, 21.77%) followed by *Mycobacterium* sp. (20/124, 16.13%) and *Corynebacterium* sp. (13/124, 10.48%), while the most common gram-negative bacilli identified was *P. aeruginosa* (9/124, 7.26%).<sup>2</sup> Postoperative infection with rejection of the scleral implants occurred in 4% (37) of 878 operations (Ulrich and Burton<sup>20</sup>), while in a more recent study the rate was 0.2% (31 of 15,022).<sup>2,20</sup> The management of these infections includes the use of systemic and local antibiotics, although removal of the scleral buckle usually is necessary to eliminate infection.<sup>4,17</sup> The risk of recurrent detachment following surgical removal of the infected materials was 33%.<sup>20</sup>

Scleral buckle infections appear to be caused by biofilm formation. To determine if bacteria are able to persist on scleral buckles by elaborating a glycocalyx matrix or biofilm that offers protection against host defenses and antimicrobial treatment, 28 scleral buckle elements removed for infection and

extrusion were cultured.<sup>5</sup> Bacteria were isolated from 18 elements (64%).<sup>5</sup> Of 17 buckles evaluated with SEM, 11 (65%) demonstrated the presence of bacteria encased in biofilm and biofilm was demonstrated on the surfaces and ends of solid silicon elements. In the silicon sponges, biofilm also extended into the matrix of the sponges. It was proposed that bacterial production of biofilm offers an explanation for the persistence of scleral buckle infections and their ability to withstand antimicrobial treatment.<sup>5</sup> In a later study, biofilms also were found on scleral buckles that did not demonstrate infection but were only removed for technical reasons at repeat surgery.<sup>1</sup>

Since chronic infections remain an important complication of biomaterial-mediated infections, the current studies were done to see if the covalent coating of organo-selenium onto scleral buckles would block biofilm formation on these materials.

When two different kinds of scleral buckle material (silicone and sponge) were placed in bacterial media containing *S. aureus*, for different time periods, biofilms were observed on the uncoated material but not on the organo-selenium-coated material. This was determined by counting the bacteria on the surface (CFU assays), as well as by CLSM, which



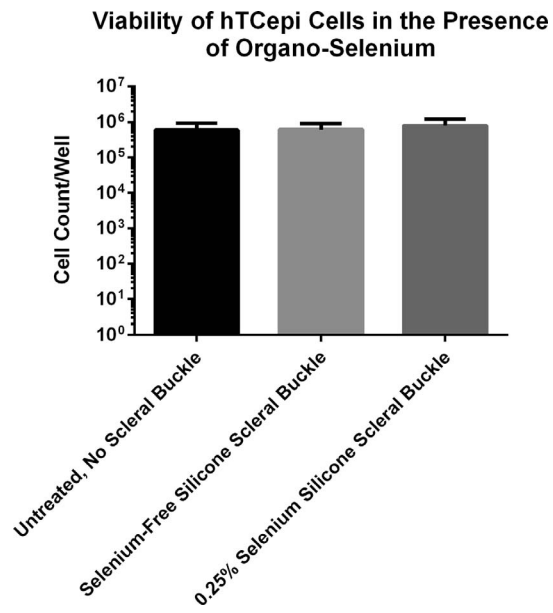
**Figure 5.** Organo-selenium–treated silicone scleral buckle is not cytotoxic. Immortalized hTCEpi cells were plated and incubated for 48 hours with Epilife medium supplemented with Epilife defined growth supplement and 1% penicillin/streptomycin containing no scleral buckle (A, B), selenium-free silicone scleral buckle (C, D), or (d) organo-selenium silicone scleral buckle (E, F). Magnification is  $\times 53$ . Following imaging, cells were removed from the surfaces of the wells and duplicate wells were counted using a hemocytometer.

showed a robust biofilm on the surface of the buckle (Figs. 1, 2). The CLSM was possible because the bacteria that were used expressed a GFP. This also made it possible to quantitate the amount of attachment of the bacteria to the buckle. As seen in Table 1, a considerable biomass was present on the different buckle samples that did not contain organo-selenium, but none on the samples with the 0.25% organo-selenium coating.

Scanning electron microscopy was used to evaluate the ability of *P. aeruginosa* to form biofilms on the buckle. While a small amount of bacteria was observed on the 0.25% selenium-coated silicone scleral buckle, a robust biofilm was observed on the untreated samples (Fig. 3). These results would imply that a higher organo-selenium than 0.25% would totally eliminate the attachment of a *P. aeruginosa* biofilm, as we have seen from dose response studies on other materials.<sup>8</sup>

Because of the possibility of toxicity of the organo-selenium coating to cells in the vicinity of the buckle, the coated buckle material was placed in tissue culture with human corneal epithelial cells. It was found that the organo-selenium–coated buckle material had no effect on the cells after 48 hours. As seen in Figure 5, cells grew under and around the buckle material with no apparent effect. In addition, when the cells were counted there was no statistical difference in the wells with the organo-selenium buckle material and the uncoated buckle material (Fig. 6). We used cells in culture growing next to the buckle as a sensitive test since cells in culture are more sensitive to toxic compounds than is tissue in an animal. This is because cells in culture are bathed in the same media and have no way to replenish the surrounding fluid, while cells in a tissue have a fresh supply of bathing fluid. This also is consistent with the finding that after 8 weeks of soaking in PBS, the





**Figure 6.** Viability results of hTCepi, telomerase-immortalized human corneal epithelial cells, grown in the presence of organo-selenium-coated silicone scleral buckles for 48 hours.

organo-selenium-coated material still showed complete inhibition of attachment of *S. aureus*. The 8-week fluid was sent to a trace analysis lab and no selenium could be detected in the fluid. This is consistent with our published results with media from soaking of other devices that have selenium on them, which were tested for toxicity with no effects found.<sup>7</sup> We also have published the results of placing contact lenses coated with selenium in rabbit eyes with no toxic effect after 2 months of continuous wear.<sup>21</sup> Thus, little or no organo-selenium is leaching from the material.

This paper represents a preliminary proof of concept for the use of organo-selenium polymers for blocking bacterial attachment to a scleral buckle. We plan to carry out future animal experiments, which will be more definitive in testing the ability of this material to function in an eye with no harmful effects over an extended period of time.

## Conclusions

The results demonstrated that it is possible to covalently attach an organo-selenium coating to foam and silicone buckle materials and this coating is stable and inhibits biofilm formation on the buckle. In addition, no toxicity was demonstrated by this coated material toward human corneal epithelial cells in tissue culture.

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