Selenium-Based Antimicrobial Titanium

A Summary of SelenBio, Inc. Research and Development on Titanium Coatings

Background

SelenBio, Inc.'s Seldox™ Technology

SelenBio, Inc. is a biopharmaceutical company specializing in the prevention of biofilm formation using molecular coatings of redox active selenium. Our Seldox technology addresses the needs of many biomedical and industrial companies for an effective, low cost, and safe antibacterial or anticellular coating technology. This technology is designed to eliminate undesirable biological inhabitation including bacterial biofilm, fungi or other biologic colonization on surfaces. It has been applied to numerous substrates - from medical devices such as contacts lenses, bandages and FDA approved orthodontic adhesives, to industrial applications such as reverse osmosis filters, fracturing fluid proppants, and boat hulls. Our customized Seldox™ compounds are efficacious against gram negative and gram-positive bacteria, fungi, viruses and neoplastic growth. The Seldox technology is based on inexpensive, widely available materials, and is a CATALYTIC antimicrobial, which means that once the technology is incorporated into a given substrate, it will remain active for years, decades, or longer. In theory, it will continue to work as long as the substrate remains intact.

As part of ongoing research, SelenBio, Inc. has recently developed several methods to apply its revolutionary antimicrobial, anti-biofilm Seldox compounds to coat titanium, opening a host of potential medical and industrial applications.

Selenium and the Catalytic Generation of Superoxide Radicals

SelenBio, Inc. technology is based on the use of organoselenium Seldox compounds as a catalytic generator of superoxide radicals ($O_2^{-\cdot}$) from the oxidation of thiols. This catalytic attribute of selenium has been known for over two decades, but the pro-oxidative characteristics of several selenium compounds has only recently been elucidated and expanded upon by SelenBio's research team. In general, an organoselenium molecule (RSeH) is catalytic and produces superoxide by its interaction with thiols, which are ubiquitous in the environment as they are found in all body fluids, as well as on the surface of the bacteria themselves. The superoxide that is generated by the reaction outlined below accounts for SelenBio's toxicity to targeted cells.

As can be seen in Figure 1, the selenium compound catalyzes the formation of two moles of superoxide radical for every two moles of thiol compound, yet the selenium compound is unchanged. Since the superoxide radicals are catalytically generated without depleting the base compound, the antimicrobial benefit is effectively permanent, although this may depend on the resilience of the substrate itself in some cases. This is perhaps the most unique characteristic of selenium: many metals can act as catalysts, but none have been shown to catalyze the production of antimicrobial compounds from naturally occurring compounds while remaining covalently bound to a substrate.

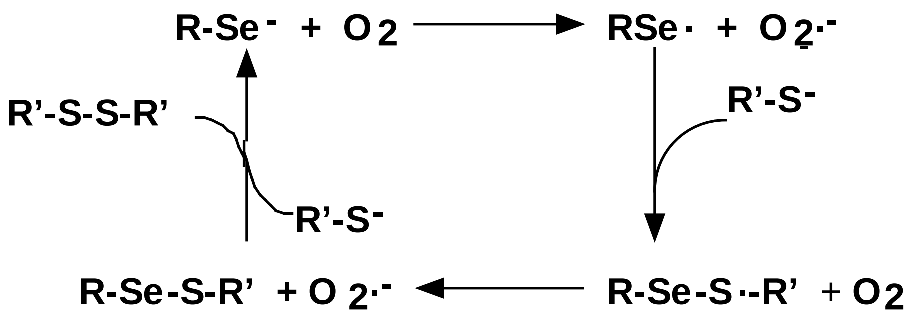

Figure 1: The Catalytic Cycle of Selenium. Since Selenium is catalytic, the lifetime of the coating is limited only by the lifetime of the material it is attached to.

An important characteristic of the superoxide radical is that it has a short half-life in the micro-seconds at high concentrations (>100 uM). Thus, it cannot damage remote organisms or create systemic toxicity since its path-length at effective concentrations is too short. Since the superoxide radical has only a very short diffusion lifetime, the selenium coatings will be only locally active and will not adversely affect non-attached cells or organisms, with an absolute maximum range measured in millimeters (some bacterial strains are highly sensitive to superoxide and its descendent species, even at nanomolar concentrations). Further, superoxide is quickly destroyed inside the body by the enzyme superoxide dismutase, which means that it can not last longer than a few microseconds, and will produce no toxicity at a distance, which is important for the protection of health tissue.

The above mechanism illustrates how selenium catalyzes a short-range antimicrobial effect against microorganisms. This translates neatly to a surface coating, where Seldox can be applied across the entire surface, or limited to certain areas. This is significant in medical devices as it allows one to coat areas of a titanium implant that will be exposed to blood flow (and thus infection), while leaving other areas without Seldox protection, allowing bone ingrowth. This property is also useful in industry, especially in bioreactors, where some portions of a waste or nutrient stream need to be able to flow (where Seldox compounds can ensure that there is no biofilm growth to foul pipes, filter membranes, etc), while other areas require bacteria growth, such as chambers that are used to break down waste or create useful products.

Titanium Coating Development

SelenBio, Inc. has made significant progress in the development of coating and copolymerization technologies to provide antimicrobial benefits to a wide variety of materials with our Seldox technology. This summary will discuss the coating process and results of work with titanium.

Experiments with commercially pure titanium have shown strong inhibition of bacteria *in vitro*. Selenium scientists developed a two-step coating process which has proven to be highly effective and stable. The process consists of an initial surface etch followed by a covalent linkage of an anchor group to the surface. The Seldox active group is then attached to the anchor.

Coating Properties:

1. Colony forming unit (CFU) microbiology assays have shown greater than four logs of inhibition (99.99+%) of representative gram-positive bacteria.
2. Accelerated stability studies have shown the coating to have a lifetime greater than fifteen years in brackish water at 27^{o}C.
3. This coating is resistant to solvents (including boiling toluene), is impervious to numerous sterilization methods, and does not leech into the environment.
4. Experiments on mammalian cell culture have shown that mammalian cells, which are among the most toxin-sensitive cells, are able to grow to within one cell width of the material.

Newer Coating Technology

More recently, SelenBio, Inc. scientists have developed a one step coating for titanium and other inorganic substrates. This coating procedure has the potential to be developed into a "spray and dry" formulation that can be rolled out on a large scale in numerous applications in both new and existing infrastructure. Initial testing against bacteria has revealed extremely powerful inhibition, with coated samples showing greater than seven logs of inhibition (99.99999+%). Coating optimization and stability studies are ongoing.

Methodology and Results

SelenBio, Inc. scientists use several assays to measure effectiveness of the Seldox-enabled antimicrobial coatings.

1. Chemiluminescence (CL) is used as a qualitative chemical screening technique, as it confirms the catalytic production of superoxide, which is the first requirement for a successful incorporation of the Seldox compounds.

To measure CL, Seldox-enabled materials are placed in a solution containing lucigenin, a compound that produces light on exposure to superoxide, and a reduced thiol compound. The experiment can be run in either aqueous or organic phase and will produce light in proportion to the amount of superoxide produced by the catalytic surface. The light is measured by a Turner Biosystems 20/20n luminometer.

CL assays have shown that our titanium coatings are highly active in terms of superoxide production, indicating they will be successful in the inhibition of biofilm formation against numerous types of microorganisms.

2. An internally-developed colony forming units (CFU) assay forms the basis for measuring antimicrobial efficacy, and is used by SelenBio, Inc. as an initial screening technique to that effect. SelenBio, Inc. scientists use this assay to quantify antimicrobial efficacy against a widely relevant strain, *Staphylococcus aureus*. Other strains may be used though, as discussed later, inhibition of some organisms cannot be adequately measured with this method. A brief description of the method follows:

Small, Seldox-enabled, commercially pure (CP) titanium washers (approximately 1 cm^2) and non-coated controls are incubated in bacterial culture media (LB broth) in the presence of *Staphylococcus aureus* bacteria overnight. After the incubation with the bacteria in the broth, each titanium washer is gently washed twice with sterile PBS to remove any planktonic bacteria. The washers are transferred to sterile 1.5-ml micro-centrifuge tubes containing PBS for enumeration of bacteria within the biofilms formed on the substrate. To separate the adherent cells from the biofilm matrix and substrate, the micro-centrifuge tubes are vigorously vortexed. Disaggregated bacterial CFU are then enumerated by 10-fold serial dilution in PBS and plating onto LB plates.

CFU assays have shown that Seldox-enabled titanium strongly inhibits bacterial growth, with greater than four logs of inhibition against *S. aureus* (Figure 2). This data shows that this coating inhibits bacterial biofilms. With this ability confirmed, stability studies are warranted to ensure that the coating is stable.

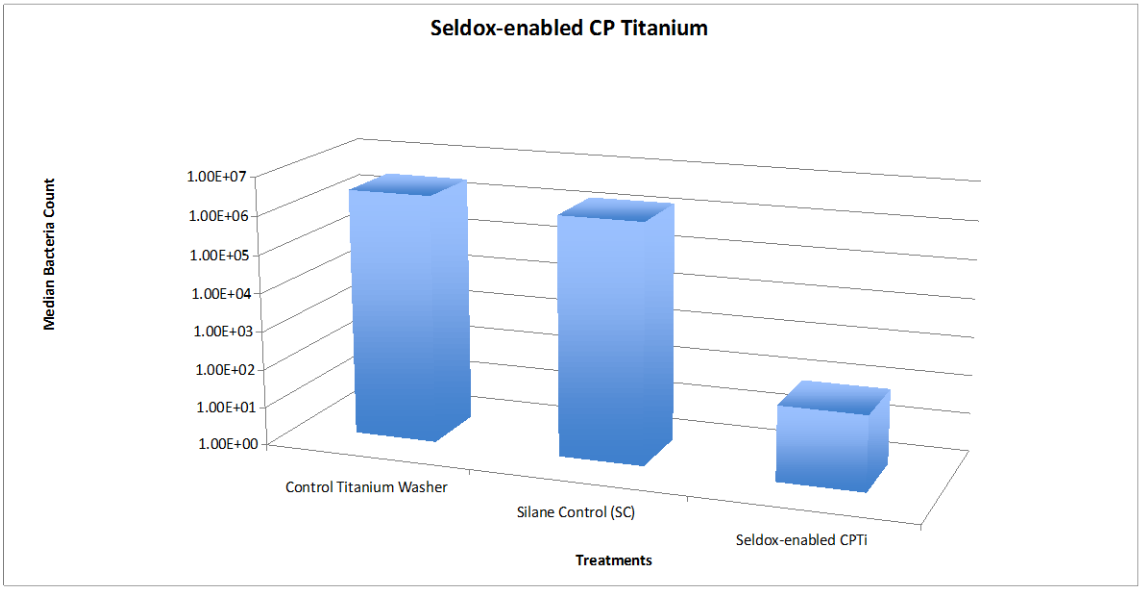

Figure 2: Inhibition of *S. aureus* on Seldox-Enabled Titanium

3. Stability studies are extremely important to demonstrate one of the primary features of Seldox technology: its longevity. In order to demonstrate that the Seldox coating is resilient under conditions harsher than it is likely to encounter, studies were performed as follows:

Seldox-enabled commercially pure titanium washers and uncoated controls are placed in 1X phosphate buffered saline (PBS) solution at pH 7.4 at 67-77°C for a given period of time, at which point samples are removed and subjected to the CFU assay described above. This is done to accelerate the possibility of any leeching that could occur as well as speeding decomposition.

Using this study, SelenBio, Inc. scientists have simulated more than fifteen years in brackish water at 27°C, without a loss of efficacy. Thus, this coating method is robust and long lasting.

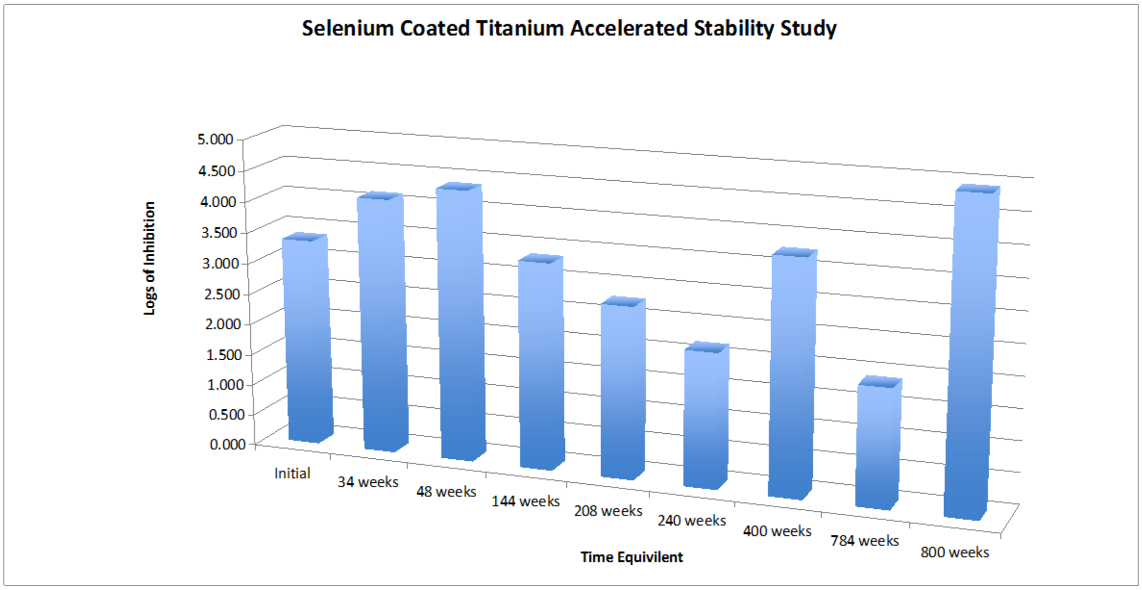

Figure 3: Stability of Seldox-enabled CP Titanium (x-axis denotes simulated time)

4. Scanning electron microscopy (SEM) is used to generate high quality images that contain qualitative data. SEM is also useful for understanding the impact of the Seldox coatings on strong biofilm formers, such as *Pseudomonas aeruginosa*, which can produce type two (false negative) errors in traditional CFU assays, as large masses of bacteria that stick together may be mistakenly read as a single colony count. SEM avoids this problem by allowing the researcher to plainly see what bacteria are present on the surface without the introduction of enumeration artifacts. Donlan and Costerton have performed extensive research on the subject of measuring biofilms, and have come to the following conclusion:

> *It should be obvious to the reader at this point, that any method that sets out to estimate the efficacy of a treatment against biofilms should use biofilms and not planktonic cells to do so. Standard NCCLS broth microdilution methods for susceptibility testing cannot accurately estimate antimicrobial efficacy against biofilms, because these techniques are based on the exposure of planktonic organisms to the antimicrobial agent.*[i]

They further tend to prefer the use either SEM or confocal laser scanning microscopy, as these methods allows direct examination of biofilms without the introduction of counting error. The general procedure for SEM used in SelenBio, Inc. test labs is as follows:

Seldox-enabled commercially pure titanium washers and non-coated controls are incubated in bacterial culture media in the presence of Seldoxed bacteria overnight. After incubation, the washers are quickly removed to a well containing a glutaraldehyde fixative solution and allowed to fix overnight. The samples are then chemically dehydrated, critically point dried, and mounted for SEM imaging. The samples can then be imaged on any commercial scanning electron microscope. SelenBio, Inc. primarily uses Hitachi equipment, including models S-570, S-3000, and S-4300.

Our SEM study produced the following imagery:

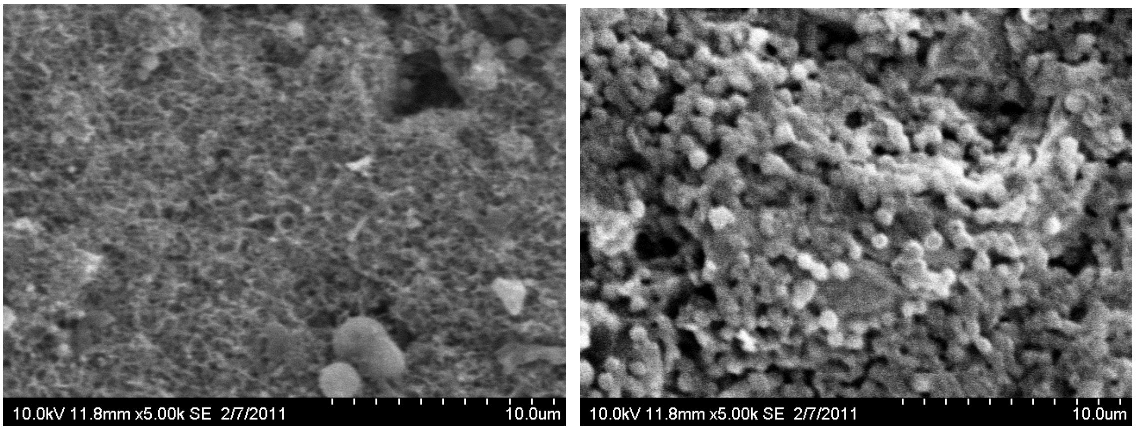

Figure 4: Seldox-enabled Titanium (left) compared to untreated titanium vs Staphylococcus aureus; 5000X magnification

Figure 4 shows a large amount of Staphylococcus aureus growing on the surface of the untreated titanium, while the Seldox-enabled titanium is totally bare of bacteria. The texture seen is the oxide layer of the titanium, while the shapes (approximately 2um spheres in the lower right) are titanium metal. Direct imaging confirms the CFU assay, showing total inhibition of biofilm on the surface.

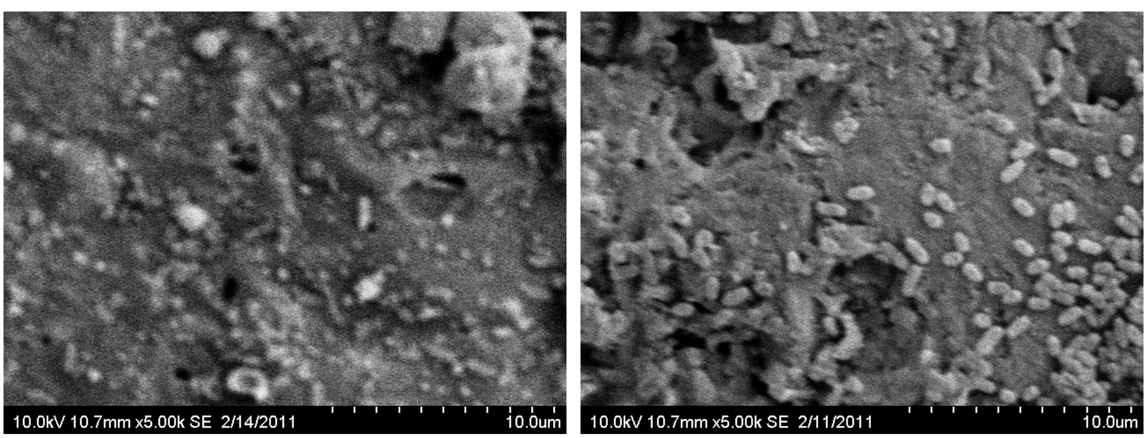

Figure 5: Seldox-enabled Titanium (left) compared to untreated titanium vs Pseudomonas aeruginosa; 5000X magnification

Figure 5 shows a monolayer of Pseudomonas growing on the surface of the untreated titanium, while the Seldox-enabled titanium is again bare of bacteria. The surface of the titanium is highly irregular due to the presence of an oxide layer combined with the natural texture of titanium metal.

5. Other assays are carried out at commercial laboratories or associated labs to bolster SelenBio, Inc.'s internal assays. These include assays that measure total selenium (TotSe) content of coated samples and of solutions from stability studies, performed by TraceAnalysis, Inc. of Lubbock, TX, and cellular toxicity assays performed by Professor Dan Webster at Texas Tech University Health Science Center.

TotSe quantifies the amount of selenium, if any, that leaves the sample during a stability study (generally due to erosion of the underlying material), which allows us to estimate a minimum lifetime of any given incarnation of the Seldox technology. This anticipates deployment in industry, where the effective lifetime of a given treatment, and costs associated with replacement or reapplication of that treatment can affect the bottom line. Cellular toxicity testing is done to ensure that a given Seldox treatment process will not produce an environmental hazard upon deployment. For Seldox-enabled titanium, the data from these assays has shown that the Seldox compound does not leech in detectable quantities, and is relatively non-toxic prior to attachment to a substrate, and completely non-toxic at remote distances after attachment.

Conclusions

SelenBio, Inc. has developed a powerful and long-lasting antimicrobial and anti-biofilm titanium coating using its Seldox compounds. This coating has shown stability over a long period of time and has allowed finicky mammalian cells to grow within a single cell length of the surface, all while preventing inhibition by bacteria strains. Thus, materials coated with Seldox compounds are both safe (for humans and animals), and effective (against microorganisms). Due to its two-step nature, the coating may be suited for application during the production process, though it may be applied to existing infrastructure given the correct conditions.

The one-step Seldox coating is still under development but shows great promise. SelenBio, Inc. scientists are currently working to optimize this coating, as they have optimized coatings for other applications (such as FDA grade silicone) so there is no leeching, yet the material retains a high degree of efficacy and maintains stability. Once developed, the one step Seldox coating will offer the opportunity to apply Seldox protection to a wide array of new and existing materials and infrastructure in a quick and easy manner.

Business Development Opportunities

SelenBio, Inc. is actively seeking commercialization partners for co-development and exclusive license agreements for antimicrobial titanium applications. The company continues to seek partnerships for other Seldox enabled applications, including coatings for a variety of substrates and incorporation into silicone, polyurethane and other plastics. Please visit our website at www.selenbio.com or contact Kenny Gallagher at 512-707-6878 or kenny@selenbio.com.

References

[i.] Donlan, RM, Costerton, WJ, "Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms," *Clinical Microbiology Reviews*, 2002: 167-193.

[ii.] Tran, PL, et al., "Organoselenium Coating on Cellulose Inhibits the Formation of Biofilms by Pseudomonas aeruginosa and Staphylococcus aureus," *Applied and Environmental Microbiology* 75 (11): 3586-3592.

[iii.] Reid, T., Tran, P., Cortez, J., Mosley, T., Shashtri, M., Spallholz, J., Pot, S. and Hamood., A. Medical Devices Coated with Organo-Selenium Inhibit Bacterial and Cellular Attachment. International Medical Devices 5: 23-31.