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Incorporating zosteric acid into silicone coatings to achieve its slow release while reducing fresh water bacterial attachment

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Abstract

Biofouling has posed serious problems in maritime industry including increased fuel consumptions, economic loss from ship-hull maintenances, contamination of drinking water, and serious corrosion for mechanical instruments. Minimizing the attachment of bacteria and formation of biofilm could be advantageous in reducing the early stages of biofouling. Zosteric acid, a natural product present in eelgrass, was found to have ability for preventing the attachment of some bacteria and barnacles. In this study, the antifouling ability of zosteric acid during the early stages of fouling was evaluated using attachment studies of fresh water bacteria. Simultaneously, various methods were sought for incorporating zosteric acid into silicone to prolong the release of the compound. The main results from this study were that zosteric acid exhibited anti-bacterial attachment regardless of whether it dispersed in water or incorporated into a coating. In addition, the release rate of zosteric acid from the incorporated coatings, particularly those where zosteric acid was uniformly dispersed with aggregates size of 4 μm or less, was orders of magnitude slower than those of previous reports. The release results indicate that the service life of our coatings could be far extended even with a small amount of zosteric acid incorporated.

Keywords: Zosteric acid; Natural product antifoulants; Bulk entrapment; *Pseudomonas putida*; Anti-bacterial attachment

1. Introduction

Fouling, the settlement and growth of a variety of organisms on structure surfaces exposed to an aqueous environment, has always posed serious problems. Primary fouling normally begins with the adsorption of organic material on the surface, followed by the attachment of a complex community of bacteria, diatoms, protozoa and algae spores to form the biofilms. The final step encompasses the attachment of higher ordered organisms, such as barnacles, algae, tubeworms, mollusks and sponges. Fouling not only leads to increased fuel and maintenance costs, damage of ship-hull and platform, but also results in contamination of drinking water and corrosion of mechanical equipment [1]. Substances that have the capability to prevent the attachment and subsequent growth of organisms on solid surfaces have been widely utilized to minimize fouling.

The common solution for keeping unwanted organisms away is to apply anti-fouling (AF) surface coatings. Many kinds of biocides, including organo-mercury, lead, and dichloro-diphenyl-trichloroethane (DDT), were used as antifoulants. However, such compounds pose severe environmental and human health risks, and were withdrawn voluntarily by the paint industry [2]. Health organizations and environmental legislation restrict the use of other antifouling paints containing tin (e.g. tributyltin TBT), copper, zinc, cadmium, chromium; all which pose serious environmental problems at even sub-parts per billion concentrations [3–6]. Therefore, there is an urgent need to ascertain suitable non-toxic or less-toxic alternatives. One of the alternatives is to generate antifouling coatings containing non-toxic or

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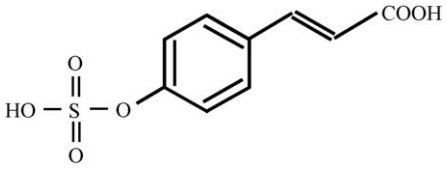

Fig. 1. The structure formula of zosteric acid is presented.

less toxic compounds, such as natural product antifoulants (NPAs).

Zosteric acid, the natural extract from zostera marina or eelgrass, has shown to prevent biofouling from some bacteria, algae, barnacles and tubeworms at non-toxic concentrations. The chemical name for zosteric acid is *p*-(sulphooxy) cinnamic acid. It contains a sulfate phenolic ester group at one end and a carboxylic acid group at the other (see Fig. 1). The AF effectiveness of zosteric acid has been demonstrated both in static laboratory assays with zosteric acid directly dispersed in marine water [7,8]. In laboratory assays using glass slides coated with zosteric acid, the attachment of *Acinetobacter* sp. on the slides dropped from 90 to 30% compared to the control surface as the concentration of zosteric acid increased from 1 to 200 $\mu g/cm^2$ [9]. In one field study, ceramic tiles were coated with crude zosteric acid and then placed into a marine environment for 1 week. No attachment of barnacles was found. In the other field study, zosteric acid was simply blended into a silicone foul release coating, and then applied to panels. The panels were immersed in marine water for 60 days, with no hard fouling and much less slime fouling being observed as compared to the panels without zosteric acid. Zosteric acid can dissolve in water easily. As such, its antifouling capability could be the result of the free-floating molecules interacting with bacteria. It was hypothesized that zosteric acid molecules present in an aqueous environment can block the surface interaction sites of the organisms, thus preventing their attachment to a surface [7]. Although the hypothesis has not been carefully evaluated, the latter field test [8] attributed the antifouling ability to the continuous release of zosteric acid. But the reported release rate, 1–100 mg/cm^2/day, was too fast, limiting the coatings effectiveness to only a short period of time. In order to prolong the anti-fouling life of the coating, better incorporation methods or controlled-release designs would be desired, which could be challenging due to the fact that most coating matrices and the NPA compounds have in-compatible physical characteristics [1]. For an example, while most of the coating matrices are hydrophobic polymers, some NPA compounds are hydrophilic, making the miscibility of NPA with the coating matrices difficult.

The compatibility of zosteric acid and various coating matrix has rarely been evaluated, and to our best knowledge, no literature associated with the leaching of zosteric acid from its entrapped coatings based on various incorporation methods has been documented. The current study focuses on developing methods to incorporate zosteric acid into silicone coatings and determines if the resulting coatings have sufficient release rates to deter bacterial attachment while slow enough to ensure the long service life of the coatings. The most suitable method for incorporating zosteric acid into silicone coatings to achieve at least a 70% reduction in bacterial coverage for two fresh water bacteria: enriched *Lake Erie* bacteria and *Pseudomonas putida*, were reported.

2. Experimental section

2.1. Materials and equipment

All experiments were performed with Polydimethylsiloxane (Sylgard® 184), an elastomeric silicone kit manufactured by Dow Corning. The components for preparing the elastomer were supplied in two parts, Sylgard® 184A (base elastomer) and Sylgard® 184B (curing agent). The mixture was primarily comprised of vinyl endcapped oligomeric dimethylsiloxanes and methyl hydrosiloxanes, and a platinum complex as the catalyst for the hydrosilation reaction. A 10:1 Sylgard® 184A/Sylgard® 184 B mixture by mass was prepared. Microscope glass slides of 2.5 × 7.5 cm purchased from VWR Scientific were used as substrates. Zosteric Acid (∼95% zosteric acid, ∼5% its salt and residual sodium chloride), synthesized in our own laboratory from *p*-coumaric acid (98% pure) and chlorosulphonic acid (99% pure), was used. *p*-Coumaric and chlorosulphonic acid along with certified ACS graded organic solvents, such as ethanol, methanol, pyridine and acetone, were purchased from Sigma-Aldrich and used as received. Two bacteria cultures were employed. The first was an indigenous enriched microbial consortium isolated from *Lake Erie*, the specification of the bacteria was not critically defined. Only the size, shape and color of different populations presented were semi-quantified using staining procedures. The second, *P. putida* (from American Type Culture Collection, # 12633), was used as the model fresh water bacteria. Both cultures were maintained as described elsewhere [10,11]. The characterization equipment included a contact angle goniometer equipped with a CCD video camera (Model 100-00 from Rame-Hart, Inc.), optical microscopes (IX 70, Olympus and Infini Tube, Edmund Scientific) equipped with CCD video cameras, and an atomic force microscope (Metrology 2000, Molecular Imaging). The concentration of zosteric acid in deionized (DI) water (purified with deionization polymer filters purchased from Easton Service Inc, Orwell, OH, and the purified DI water has a conductivity value less than 0.1 μΩ) for the leaching experiment was determined using a digital conductivity meter (Traceable®) and a UV-Visible spectrophotometer (UV-1601, Shimadzu).

2.2. Coating preparation

Microscope glass slides of 7.5 cm × 2.5 cm were cut into 7.5 cm × 1.25 cm pieces. Pure silicone, containing a 10:1 Sylgard® 184A/Sylgard® 184B mixture by mass, was pre-

pared. One drop (∼0.05 g) of the mixture was spread on the glass slides surfaces. A stream of industrial grade nitrogen was used to remove the dust particles before using a doctor blade to form coatings with a thickness of approximately 200 μm over an area of 2 cm × 1.25 cm. The mixture was allowed to flow and rearrange within this coated area while it was cured inside closed drawers (15 cm × 15 cm × 5 cm) under ambient conditions (20 °C and 1 atm) for 48 h. After curing, they were sterilized in an autoclave at 121 °C for 60 min prior to initiating the attachment study. The autoclaved coatings were also subjected to surface wettability characterizations and bulk modulus measurements.

The zosteric acid bulk entrapped silicones were prepared by blending a solution of zosteric acid with the silicone mixture. The solvents used for making the zosteric acid solution included DI water, ethanol, methanol, pyridine, acetone, and various organic-solvent/water mixtures. The organic-solvent/water ratios tested were 75/25, 50/50, 25/75 and pure solvents. The purpose of the organic solvent was to increase the miscibility of the zosteric acid solution with silicone, since silicone is immiscible with water while zosteric acid is generally insoluble in organic solvents. The zosteric acid was first dissolved in water and then the organic solvent was added to obtain a final solution containing ∼10 wt.% zosteric acid. The zosteric acid solution and the base silicone elastomer were first thoroughly mixed to form a homogeneous dispersion, and then heated at 150 °C with a vacuum of ∼50 mm Hg for 4 hours to totally remove the solvent. After evaporating the solvent (organic solvent and water), the curing agent (Sylgard® 184B) was added. The proper ratio of zosteric acid solution and the silicone mixture was adjusted to obtain silicone coatings with a desired amount of zosteric acid. Each of the coatings on the glass slides was prepared by spreading a small amount (∼0.05 g) of silicone mixture onto a glass slide using a doctor blade over an area of 2 cm × 1.25 cm. As with the non-bulk entrapped system, the coatings were allowed to flow and cured under ambient conditions for at least 48 h prior to auto-clave and subsequent bacterial attachment and leaching studies.

2.3. Bacterial attachment study

Each of the coatings was placed inside an amber bottle (60 mL) containing particular bacteria in 30 mL aqueous solution with or without zosteric acid. Care was taken to place the coatings at a 40° angle and face down to ensure the attachment was not simply the result of settlement of species and organic matter. Coatings were removed and observed at intervals of 1, 3, 7, 14, 21 and 28 days. The observations were made using a transmitted light optical microscope after rinsing the samples with fresh deionized water to remove the loosely attached matter. The video system attached to the microscope was used to capture the images of interest. The variations on the biofilm morphology on the surface of the silicone coatings due to the bacterial biofilm growth were examined. Various degrees of magnification were used to identify the differences in shapes and quantity of bacteria attached to the silicone surface.

The bacterial attachment was conducted using a 2 × 2 × 7 factorial design with three replicates. In this design, two different times of immersion (7 and 14 days), seven different concentrations (0, 5, 10, 20, 50, 100 and 500 ppm), and two types of bacteria (enriched *Lake Erie* and *P. putida*) were used. Three replicates for each combination were utilized. Using the enlarged morphology image, the percentage of bacterial coverage for each coating was estimated using the pixels occupied by the biofilm divided by the total pixels of the image.

A two-way analysis of variance (ANOVA) via MiniTab software was applied to evaluate the effect of concentration and time on attachment. The Tukey's honestly significant different test (HSD) was used to complete a pair-wise comparison to determine the significance of the data. With this approach, statistically significant results were depicted by P values <0.05, non-significant results were those with $P > 0.05$.

2.4. Coating properties evaluation

Coating properties, mainly surface wettability and bulk elastic modulus, and surface topography were evaluated before and after water immersion. The surface wettability of the coating was evaluated via water contact angle measurements using the contact angle goniometer video system. For coatings subjected to bacterial attachment, a portion of the biofilm was removed using a scotch tape immediately prior to measuring contact angle. Due to the low adhesion between the biofilm and the silicone coatings, the biofilm could be removed completely using a scotch tape, leaving behind a clean coating surface. Images of the sessile drops from on the surface were captured using a Dazzle DVC hardware and its software. During contact angle measurement, several liquid drops were randomly placed at different locations on the surface for each of the three replicates. Contact angle values were estimated using the Scion Image Software. The average of the angles measured on a particular surface was reported. The two-way ANOVA was conducted to determine the statistical significance of the data.

The elastic modulus of the coatings was measured using the JKR method, where a convex elastic lens of pure Sylgard® 184 was brought down into contact with the coating of interest. The force, determined using an electronic balance with an accuracy of 0.1 mg, acting between the two surfaces and the diameter of the circular contact, enlarged with a portable optical microscope, were measured to obtain the elastic modulus (E^*) of the system. The modulus of the coating (E_C) was deduced from the known value modulus of the elastic lens (E_L). An ANOVA similar to that of the contact angle analysis was conducted to determine any significant difference in the data.

The surface morphology was evaluated with an optical microscope (OM) and an atomic force microscope (AFM). With OM, various degrees of magnification were used under

the bright field transmission mode. AFM scans of the coating surface were obtained using the non-contact mode with a silicon cantilever having a spring constant of ∼42 nN/nm. The scan size was 80 μm × 80 μm and the scan rate was 0.2 Hz. The surface roughness of the coatings on an 80 μm × 80 μm area was attained from the AFM scans using the NanoScope III software version 4.42r4.

2.5. Leaching of zosteric acid

Leaching of zosteric acid from the bulk of the silicone coating was evaluated using a static cell where the coating was immersed in DI water. The quantity of zosteric acid leached into the solution was then determined via conductivity measurements. Cured and sterilized circular silicone sheets (8.4 cm in diameter and 0.05 cm in thickness) containing zosteric acid were prepared using the same conditions as those zosteric acid entrapped coatings for the bacterial attachment study. The conductivity of 200 mL of deionized water was determined before each sample was immersed. Then, the conductivity of the water containing a zosteric acid entrapped silicone sheet in it was measured periodically for up to 6 months. Any increase in conductivity was assumed to be the consequence of zosteric acid leaching into the water, and the zosteric acid leaching rate was estimated from the conductivity measurements. To ensure the increase in conductivity was directly associated with an increase in the zosteric acid concentration in DI water, samples of water from the static cell were also subjected to Ultra-violet spectroscopemetry (at an UV absorbance of λ = 275 nm) to determine the amount of zosteric acid in the solution. A linear relationship was found between the concentration determined using the conductivity meter (ppm) and the UV-absorbance detected using the UV-spectrometer (ABS). This corroborates that any changes in conductivity measurement was directly associated with the change in the zosteric acid concentration in the DI water.

3. Results and discussion

Before developing methods for incorporating zosteric acid into silicone coatings and studying the properties of such coatings and the leaching behaviors of zosteric acid from such coatings, we evaluated the antibacterial attachment capability of zosteric acid when it was simply present in water. The concentration of zosteric acid in water will provide us some insights on the amount of zosteric acid needed to be incorporated into silicone, and the optimum leaching rate.

3.1. Bacterial attachment on plain silicones using zosteric acid present in solution

It was hypothesized that NPAs can inhibit the attachment of bacteria and higher-ordered marine organisms by blocking the surface interaction sites of the organism, thus causing the organisms to lose their ability to attach to the surface [7]. If this hypothesis is correct, it is unnecessary for these NPAs to be as toxic as the currently used antifoulants, which prevents fouling by killing off the organisms, either targeted or non-targeted. Therefore, in our first study [10] we evaluated the toxicity of zosteric acid using both standard Microtox test and the quantitative toxicity assessment. EC_{50} (the concentration that causes 50% of the original microbial population to die) of zosteric acid with the Microtox test was found to be 440 mg/L (or ppm). Using the quantitative toxicity assessment, the values were 400 mg/L and 166 mg/L for the enriched *Lake Erie* consortium and *P. putida*, respectively. These values indicated that zosteric acid is approximately five to six orders of magnitude less toxic as compared to currently used antifoulant compounds, such as TBT and SeaNine 211. For TBT and SeaNine 211, the respective values of EC_{50} found from literatures were 0.022 and 0.036 mg/L for *V. Fischeri* (bacteria); and for *S. capricornotum* (an algae), they were 0.007 and 0.003 mg/L, respectively [12].

Bacterial attachment studies were first conducted on plain silicone coatings with zosteric acid simply dissolved in the water containing either enriched *Lake Erie* bacteria or *P. putida*. Several concentrations (5, 10, 20, 50, 100 and 500 ppm) of zosteric acid in water were evaluated. It is important to note that almost all of these concentrations were less than its EC_{50} value. The initial population of bacteria and environmental conditions in each bottle containing a plain silicone coating, either with or without zosteric acid, was controlled to be as identical as possible. Bacterial growth and subsequent attachment was allowed to occur for 2 weeks, and the biofilm morphology was taken and evaluated.

Fig. 2 contains representative biofilm morphologies of enriched *Lake Erie* bacteria (a, b, c and d) and *P. putida* (e, f, g and h) on silicone coatings after 14 days. Bacterial attachment on the plain silicone coatings without zosteric acid was used as the control for comparison. The controls presented approximately 45% of surface coverage with a branch-like biofilm for the enriched *Lake Erie* bacteria and 36% surface coverage with an elongated shaped biofilm for *P. putida*, respectively (Table 1). The attachment behaviors for *Lake Erie* bacteria showed the following trend. With 5 ppm of zosteric acid in water, the attachment was found to be slightly less than that of the control, showing a bacterial coverage of 33% (or a reduction of 25% of that depicted of control). As the concentration increased to 10 ppm, the bacterial surface coverage reduced to 13%, and it was 11% for coatings immersed in water with 20 ppm zosteric acid. A clear reduction (i.e. 92%) in the bacterial coverage occurred when the 50 ppm zosteric acid was used, depicted by a 3% coverage. When the zosteric acid concentration increased to 100 ppm, the coverage was even less. With 500 ppm of zosteric acid, even after 14 days of immersion, almost total inhibition of bacterial attachment (0.8% surface coverage) was observed. A similar trend was found for *P. putida*, the surface coverage by attached *P. putida* was 13, 1 and 0.4%, respectively, for coatings immersed in 20, 50 and 500 ppm zosteric acid solutions.

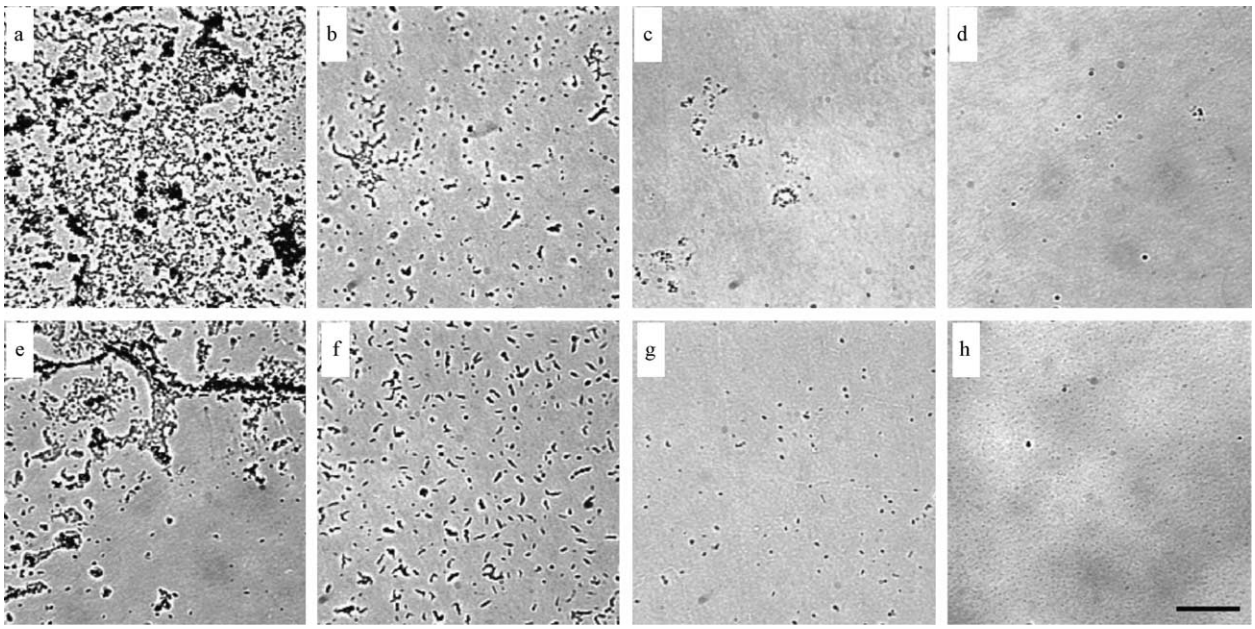

Fig. 2. Silicone coatings immersed in various water solutions containing bacteria and zosteric acid after 14 days: (a) *Lake Erie* bacteria without zosteric acid, (b), (c) and (d) *Lake Erie* bacteria with 20, 50, and 500 ppm of zosteric acid, respectively; (e) *P. putida* without zosteric acid, (f), (g), and (h) *P. putida* with 20, 50, and 500 ppm of zosteric acid. The coating surface was kept facing the bottom of the amber bottle and all samples were dipped serveral times in fresh deionized water before being analysized. The scale bar indicated in the image is 30 μm.

The attachment study showed that the concentration necessary to reduce the bacterial attachment to more than 90% was about 50 ppm. This concentration is substantially lower than the EC_{50} values (∼400 ppm for *Lake Erie* bacteria and ∼170 ppm for *P. Putida*) of zosteric acid for the two bacteria used in this study. Even though 50 ppm was higher than the effective concentration of the non-selective TBT in preventing attachment [13], when the EC_{50} values were compared, zosteric acid is actually 100–1000 times less toxic than TBT.

To further confirm that the zosteric acid concentration used in the attachment study posed limited toxicity, the aqueous bacterial population, as determined by the most probable number (MPN) was monitored. MPN utilizes agar plates to monitor heterotrophic microbial growth. At each sampling time, the aqueous bacterial population of a 2 mL sample was subjected to serial dilutions (10^{-2}–10^{-8}), and each bacterial solution was plated in triplicates. The colony forming unit (CFU) on each plate was counted after a 5-day, room temperature incubation in the dark. The average of the CFUs from the triplicate plates was used to evaluate the bacterial population in the aqueous solution. With zosteric acid concentrations up to 100 ppm, the number of bacteria present in the aqueous environment was actually higher ($P < 0.05$) than that of the solution without zosteric acid. The larger amount of bacteria could be attributed to fewer bacteria being attached to the coating surface. The extent of surface coverage and aqueous MPN clearly demonstrated that zosteric acid at concentrations ≤100 ppm exhibited minimum toxicity toward the bacteria employed in this study.

Table 1
The bacterial biofilm coverage on the surface of pure Sylgard® 184 silicone coatings after the coatings were immersed in the solution containing zosteric acid and either enriched *Lake Erie* bacteria or *P. putida* for 2 weeks

Zosteric acid concentration (ppm) in water containing either enriched *Lake Erie* bacteria or *P. putida*	%Surface covered by enriched *Lake Erie* bacteria	%Surface covered by *P. putida*
Control (0)	44.9	36.3
5	33.4 (25.8)	Not evaluated
10	13.2 (70.7)	Not evaluated
20	11.6 (74.1)	12.6 (65.3)
500	3.4 (92.5)	1.3 (96.5)
500	0.8 (98.2)	0.4 (99.0)
10	33.4 (25.8)	Not evaluated

A wide range of zosteric acid concentrations (0 to 500 ppm) were tested. The number inside the parenthesis represents the relative reduction (in percentage) in bacterial coverage as compared to that of the control.

The difference in bacterial attachment could also have resulted from the difference in coating properties, such as surface wettability and bulk modulus. In order to confirm that the difference in bacterial attachment is solely due to the presence of zosteric acid in the water, the variations of silicone coating properties after immersion in water were evaluated. The water contact angles (i.e. wettability) and bulk modulus of silicone coatings immersed in water containing *Lake Erie* bacteria and different concentrations of zosteric acid are presented in Fig. 3. Static contact angles, for the coatings, reduced significantly ($P < 0.05$) after the coating was immersed for 1 day and leveled off as immersion time was extended. Very similar trend was observed when the coating was immersed in DI water without bacteria. The increase in wettability after 1 day resulted from the slight reorganization of the side chain and backbone components of sili-

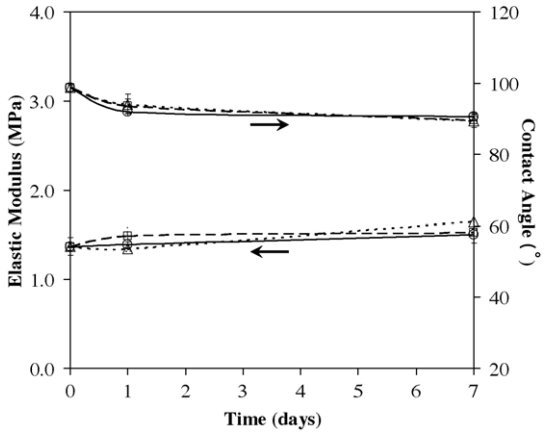

Fig. 3. The water immersion time dependent of elastic modulus (left axis) of the silicone coatings and static water contact angles (right axis) on the coatings immersed in various aqueous solutions containing *Lake Erie* bacteria are summarized. (◯), (△) and (□) represent the aqueous solution contains no zosteric acid, 50 ppm of zosteric acid, and 500 ppm of zosteric acid, respectively. The biofilm was totally removed before measuring the modulus and contact angle, and the measurement was performed within 15 min after the coating was taken out from the aqueous solution. The value of each data point reported was the average of five measurements, and the vertical lines represented the standard deviation of the five measurements.

cone as the system attempts to minimize the energy in the highly polar aqueous environment [14]. In all cases, bulk elastic modulus only fluctuated slightly ($P > 0.05$), and remained well within the standard deviation of the measurements. The elastic modulus, depending on the cross-linking density of Sylgard® 184 (an elastomer), was expected to be unchanged by immersion in water. In addition, because the aqueous zosteric acid concentration was less than or equaled to 500 ppm in all cases investigated, the effects of zosteric acid on the bulk properties were anticipated to be negligible. Therefore, the difference in bacterial attachments was not resulted from the surface wettability and bulk modulus of the coatings.

3.2. Entrapment of zosteric acid into silicone coatings

For practical applications, zosteric acid must be continuously released to the immediate vicinities of a surface in order to prevent fouling on the surface. One way of continuous discharge is to incorporate zosteric acid to a coating that will be applied to the surface, such as a ship hull. In earlier studies, zosteric acid was directly blended into silicone coatings in the form of a powder, and the coatings were applied to panels to perform attachment studies [8]. Neither the distribution of zosteric acid in the coating nor the dependency of zosteric acid leaching on the coating morphology has been investigated. In those earlier studies, zosteric acid leached with a rate of several mg/day-cm^2, which appeared inadequate for long-term service of the coatings. For example, a coating with a thickness of ∼100 μm and a zosteric acid concentration of 1 wt.% would leach all of the zosteric acid in less than one day. Even with the thickness increased to ∼1 mm and the zosteric acid concentration increased to ∼10 wt.%, all the zosteric acid would deplete from the coating in less than 1 week.

We investigated various techniques of incorporating zosteric acid into a model silicone coating to determine how the leaching can be controlled when zosteric acid was entrapped inside a carrier coating. Although Sylgard® 184 may not be the most suitable carrier for coating purposes, its properties are well understood [15]. Also, it is transparent, making it easier to examine the distribution of zosteric acid inside the coating. Initially, the direct blending of grounded zosteric acid power with silicone under vigorous mixing was adopted. This approach formed large zosteric acid aggregates (average ∼80 μm) (see Fig. 4(a)) with a majority of them having the size between 54 and 100 μm (Table 2). These aggregates likely span the entire thickness of the coating and create large pathways for water to enter and dissolve them, and the solution can also leave the coating quickly through these pathways. The details of leaching will be described in the next section.

As the distribution of zosteric acid became more uniform, the period of fast leaching extended while the leach rates reduced. To slow the leaching of zosteric acid, more homogenous distribution of smaller zosteric acid aggregates or even individual zosteric acid molecules within the silicone coating would be more desired. Methods for such distributions were sought by evaluating the properties of zosteric acid and the miscibility of various solvents with silicone. Zosteric acid is most soluble in water due to its inorganic sulfate characteristics. The highest soluble concentration of zosteric acid, synthesized in our lab, in water was experimentally determined to be 18 wt.% at ambient conditions (20 °C and 1 atm). Water, on the other hand, has a very low compatibility with nonpolar silicone polymers. In the initial attempt, the 18 wt.% zosteric acid/water solution was thoroughly mixed with the silicone base first, the water was then removed with heating and vacuum to obtain silicone base containing zosteric acid with a desired concentration (∼1 wt.% in this study). Then the silicone curing agent was added to generate cross-linked coatings with zosteric acid entrapped. The resulting coatings had more homogeneous distribution of zosteric acid, but still formed aggregates mostly spanned between 7 and 14 μm (see Fig. 4(b) and Table 2) after water was removed. Aggregates in this case were probably resulted from the zosteric acid/water domains created from the phase separation of this aqueous solution from the silicone base polymer. Therefore, it was necessary to find another solvent with intermediate polarity characteristics compatible with both zosteric acid and the silicone base to achieve a more desired bulk distribution of zosteric acid.

Some of the solvents that have the suitable properties are short chain alcohols, such as methanol and ethanol. These solvents have lower boiling points, so they can be easily removed from the mixture even if they have low miscibility with silicone. The solubility of zosteric acid in both methanol and ethanol were found to be 1.3 and 0.5 wt.%, respectively,

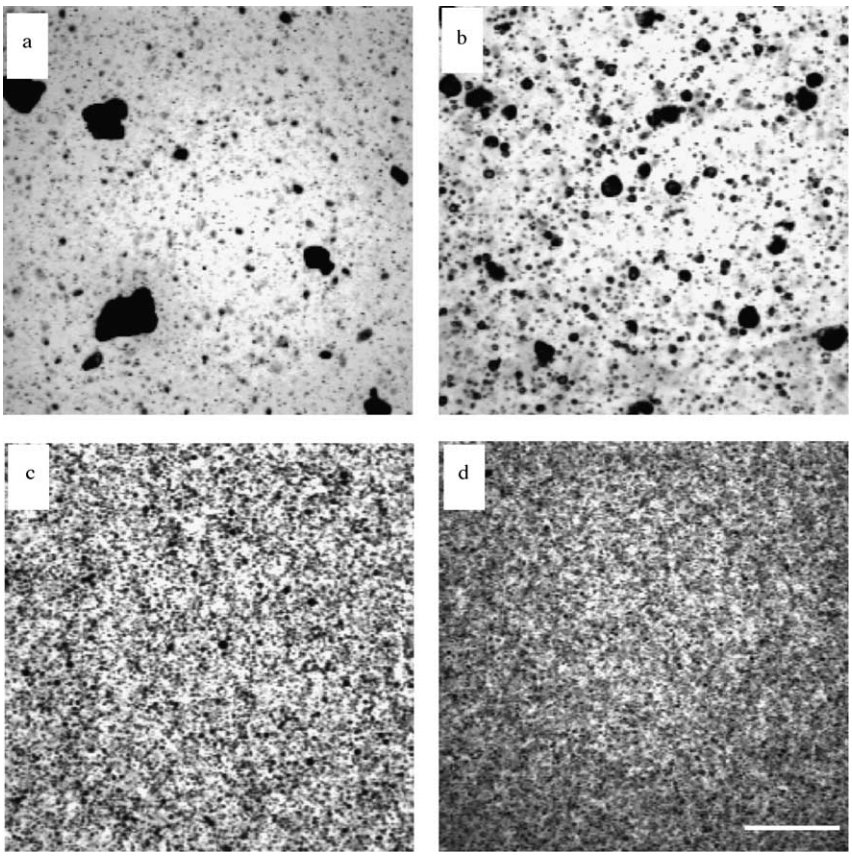

Fig. 4. Representing morphology of silicone coatings containing 1 wt.% of zosteric acid entrapped with various methods are shown. (a) zosteric acid in the form of fine power was simply blended into silicone mixture before curing; (b) zosteric acid was dissolved in water and the solution was mixed into the silicone base, and then water was allowed to evaporate before the curing agent was added for the coating to cure; (c) 50/50 mixed water/pyridine was used to dissolve zosteric acid and the solution was mixed in with the silicone base, the solvent was allowed to dry as completely as possible before adding the curing agent to cure the coating; and (d) 50/50 mixed water/acetone was used as the solvent for zosteric acid. The average size of the aggregates for coatings shown in (a), (b), (c) and (d) is 80, 11, 4 and 1 μm, respectively. The scale bar shown in the image is 200 μm.

under ambient conditions. Although the solubility of zosteric acid in these solvents was low, they were still much higher than other organic solvents, such as methyl-isobutyl ketone or toluene. In addition, these solvents were miscible with water; thus it is possible to obtain mixed water/alcohol solvent as the common solvent to generate higher concentration of zosteric acid solution before blending the solution with silicone base. When either methanol or ethanol or water/alcohol mixture was used as the solvent, zosteric acid formed slightly smaller aggregates as compared to that of pure water used as the incorporating solvent, and the aggregates distributed uniformly in the cured silicone coatings. The leaching rates of zosteric acid from these coating were much smaller than those of the coatings generated by simply blending in the zosteric acid power; however, these leaching rates were still higher than desired. A solvent that is more miscible with silicone could be better for entrapping zosteric acid with even smaller aggregates and a more uniform distribution; leading to a slower leaching.

Pyridine, a tertiary amine, is highly soluble in water and completely miscible with the silicone base. Although it is not totally compatible with zosteric acid, it appeared to be a good common solvent to bring the aqueous zosteric acid solution and silicone together. In addition, pyridine was used

Table 2
The aggregate size distribution on each zosteric acid entrapped coating was roughly evaluated using the particle analyzing features of ScionImage

Entrapping solvent	Aggregate size range (μm)	Aggregate size range (μm)	Aggregate size range (μm)	Average aggregate size (μm)
No solvent	<54 (15.4)	54–100 (76.9)	>100 (7.7)	80
Water	<7 (20.5)	7–14 (69.2)	>14 (10.3)	11
50/50 Pyridine/water	<2 (12.9)	2–6 (77.2)	>6 (9.9)	4
50/50 Acetone/water	<1 (61.7)	1–3 (29.4)	>3 (8.9)	1

The number inside the parenthesis is the percentage of number of aggregate at a particular aggregate size to the total number of aggregates evaluated in the three size-ranges chosen for each coating.

as the solvent for synthesizing zosteric acid. Therefore, it was anticipated that a high concentration of zosteric acid can be achieved when a mixed pyridine/water is used as the incorporating solvent, thus reducing the amount of solvent and subsequent solvent evaporation time for incorporation. A mixture 50/50 pyridine/water was used to dissolve zosteric acid. First, a solution containing 18 wt.% of zosteric acid in water was prepared, and then pyridine was added to this solution to finally obtain a mixture of zosteric acid:pyridine:water 10:45:45. This solution mixed very well with the silicone base but has the potential problem of poisoning the Pt-based catalyst used for curing Sylgard® 184. Therefore, it was necessary to completely remove all the pyridine present in the silicone base before adding the catalyst. The solvent was removed by heating above the boiling point of the pyridine followed with vacuum for four consecutive cycles of heating at 150 °C for 40 min, and vacuum approximately 50 mm Hg for 10 min. Four cycles of drying was found to be optimum for obtaining a lower and more controlled leaching rate. The coatings obtained after 4 cycles had very homogeneous distribution (Fig. 4(c)) of zosteric acid, with individual aggregates mostly having a size of 2–6 μm (Table 2).

Finally, acetone, a more environmentally friendly solvent that is highly miscible with water as well as silicone base and poses no poison effect to the catalyst, was tested. The detailed procedures were similar to those of using pyridine, but without the last rigorously solvent removal step. The coating generated with acetone/water mixed solvent resulted in the most uniform distributed zosteric acid. No individual aggregates of zosteric acid were observed without using a high magnification optical microscope (Fig. 4(d)). More than 60% of the aggregates were less than one micron, and another ∼30% had a size between 1 and 3 μm (Table 2).

The leaching of zosteric acid from coatings generated using various incorporated methods is summarized in Fig. 5. As can be seen, leaching from all the coatings showed a similar trend: a substantial high rate occurred during a first stage, and then the rate gradually leveled off, and approached an almost constant value in the second stage. The first fast leaching could come from dissolving and removal of zosteric acid aggregates having sizes greater than the coating thickness as well as zosteric acid on and near the surface of the coating. Zosteric acid leached out at the highest rate when the powder was simply blended into the coating. For this coating (Fig. 4(a)), during the first fast stage (0–6 h), the leaching rate, M_{ZA}, followed the trend of $M_{ZA} = 52.4/t$, where the units of M_{ZA} and t (time) are μg/cm^2-day and day, respectively. The average leaching rate for this interval was 437 μg/cm^2-day. Then the rate reduced to an average of 1.5 μg/cm^2-day and maintained for up to 6 months. For the coating (Fig. 4(b)) with zosteric acid incorporated by blending in a concentrated (18 wt.%) zosteric acid/water solution, the first fast stage (0–2 days) of leaching followed $M_{ZA} = 15.6/t$ and resulted in an average rate of 15.7 μg/cm^2-day. Then the rate reduced to almost a constant value of 0.2 μg/day-cm^2. Similar leaching behaviors and rates were observed for the coatings generated when ethanol, methanol, water/ethanol or water/methanol mixture was used as the incorporating solvent.

The leaching was slower for coatings with more homogeneous distribution and smaller aggregates. The slowest leaching was obtained when the 50/50 acetone/water mixed solvent was used to incorporate zosteric acid into silicone. In comparison, the leaching values were almost tripled when the pyridine/water mixed solvent was used. For zosteric acid incorporated silicone (Fig. 4(c)) with the pyridine/water mixed solvent, the leaching rate followed $M_{ZA} = 0.9/t$ from zero to three days, leading to an average rate of 0.64 μg/day-cm^2. The leaching rate leveled off to ∼0.07 μg/day-cm^2 after the fast stage of leaching. In the most homogeneous case (Fig. 4(d)), i.e. acetone/water mixed solvent, the leaching rates varied with time to a relationship of $M_{ZA} = 0.3/t$ for the first five days with an average value of 0.12 μg/day-cm^2, and then stayed at a constant of 0.03 μg/day-cm^2. For each coating, during the 6 months that were monitored, the continued leaching was at a rate very close to its slowest rate, suggesting the later stage of leaching could probably controlled by the diffusion of zosteric acid or water within the silicone network. In addition, the average leaching rate of the slow stage was found to correlate directly with the average zosteric acid aggregate size in the coating.

The more homogeneous the coatings, the less abrupt the change between the fast and slow stages. The transition between the first fast and the second slow leaching occurred at 6 h, 2 days, 3 days, and 5 days for the zosteric acid blended into the coating using dry powder, solution in water, solution in pyridine/water mixed solvent, and solution in acetone/water mixed solvent, respectively. The difference in the transition time and the leaching rate likely resulted from the aggregate size and distribution of zosteric acid in the coating.

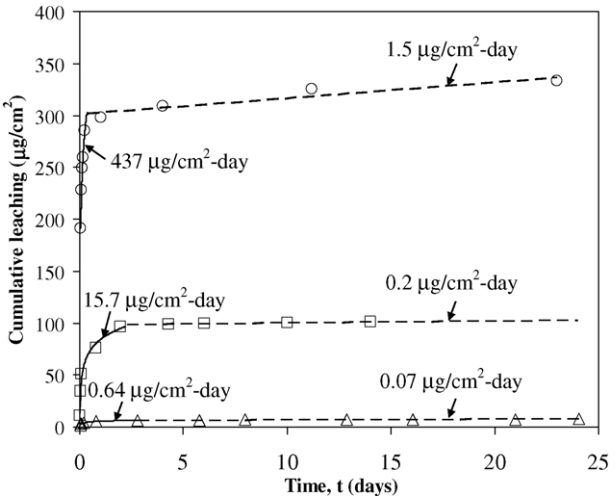

Fig. 5. Using a large static cell containing DI water as the leaching sink, cumulative leaching of zosteric acid from various silicone coatings containing 1 wt. % of zosteric acid was monitored. The coatings were prepared by (○) simply blending of the zosteric acid power with the coating mixture, (□) mixing of zosteric acid/water solution into coating mixture, and (△) mixing of zosteric acid/(50/50 water/pyridine) solution into coating mixture.

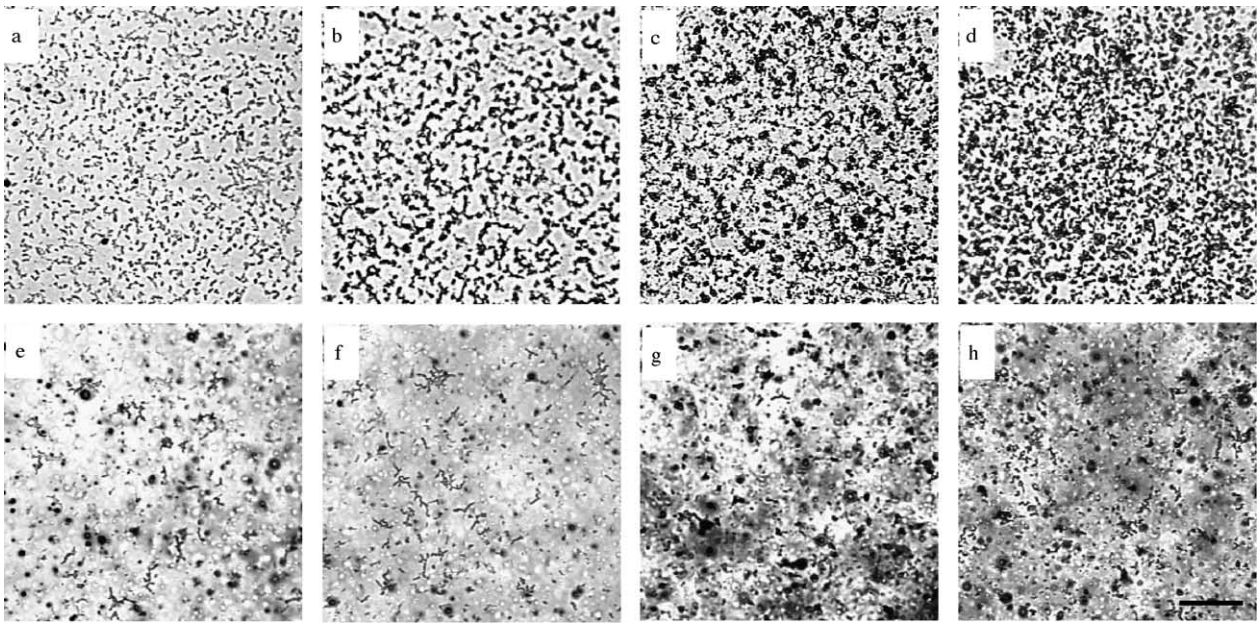

Fig. 6. Silicone coatings alone(first row) and coatings containing 1 wt.% of zosteric acid (second row) were immersed in aqueous solutions containing *Lake Erie* bacteria for (a) and (e) 7 days; (b) and (f) 14 days, (c) and (g) 21 days, and (d) and (h) 28 days. The dark spots seen in images (e) to (h) are the zosteric acid aggregates in the coating. The surface coverage by bacteria for the zosteric acid incorporated coating was about ∼30% of that of the silicone coating alone for each time interval. The scale bar shown in the image is 30 μm.

With bigger aggregates, the paths for water to travel and reach zosteric acid could be shorter and have lower resistance, thus zosteric acid dissolves in water and is carried out by water through the more straightforward paths and leading to a faster leaching. Conversely, for smaller aggregates distributed more uniformly inside silicone, the paths for water to travel into the coating could be more treacherous with possibility of thin silicone membranes encapsulating some of these aggregates to substantially increase the transporting resistance, resulting in a much slower leaching. The possible service life of each coating, by assuming that zosteric acid continued leaching at the slowest rate until zosteric acid completely depleted from the coating, was less than 2 months for the coating prepared by blending zosteric acid powder directly in the silicone. It takes about 5, 12 and 21 years for the coatings prepared using water, pyridine/water and acetone/water as the incorporation solvent, respectively, to exhaust its zosteric acid content. The details on how water diffuses into the silicone network (i.e. Sylgard® 184 elastomer) and how zosteric acid/water mixture transports out off the network are currently under study.

After obtaining homogenous coatings using the mixed solvent of pyridine/water or acetone/water to incorporate the zosteric acid within the silicone, attachment studies with enriched *Lake Erie* bacteria were performed using these coatings. The other two types of coatings with zosteric acid entrapped were not subjected to bacterial attachment due to the fact that zosteric acid depletes too fast from the coating.

The images of bacterial attachment on the pure silicone coatings and on the zosteric acid incorporated coatings prepared using pyridine/water as the mixed solvent are presented in Fig. 6. After 7 days of immersion, the difference in biofilm formation on the silicone coatings with and without zosteric acid entrapped was clearly visible. Substantially fewer bacteria (i.e. ∼70%) were attached to the coating with zosteric acid entrapped. The enriched *Lake Erie* bacteria attached to and covered ∼25% of the surface for those samples without zosteric acid. Once attached, the biofilm continued to grow, and the coverage increased to 33, 49 and 52%, respectively, for 14, 21, and 28 days of immersion. The bacterial coverage on pure

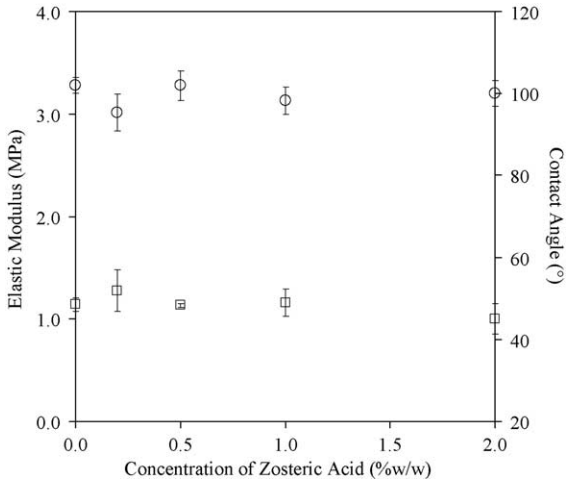

Fig. 7. Elastic modulus (□) and static water contact angle (◯) of silicone coatings containing zosteric acid ranging from 0 to 2 wt.% are summarized. Each value of contact angle and elastic modulus reported is the average of eight and four measurements, respectively. The standard deviation for each data point was represented by the vertical lines. No significant variation ($P > 0.05$) in either property was observed.

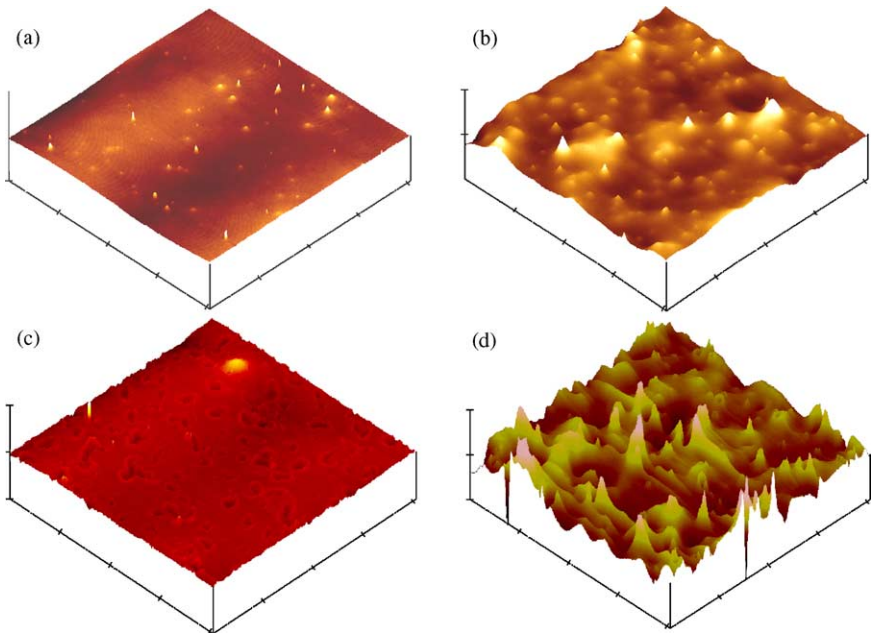

Fig. 8. Surface topographies (size: 80 μm × 80 μm, *z*-scale: 200 nm), generated with the non-contact mode AFM at a scan rate of 0.2 Hz, of silicone coatings containing 0 wt.% ((a) and (c)) and 1 wt.% ((b) and (d)) of zosteric acid before ((a) and (b)) and after ((c) and (d)) immersing in the aqueous solution containing *Lake Erie* bacteria for 14 days. The root mean square (RMS) surface roughness for the coatings shown in (a), (b), (c), and (d) were 4, 5, 13, and 66 nm, respectively. The shallow indentations seen in (c) could be resulted from the removal of the attached bacteria and/or the surface erosion.

silicone coating after 14 days of immersion differed from that obtained in the first part of this study. Possible reasons could be the sensitivity of bacteria to the ambient conditions and the variation in initial concentrations of bacteria. The first part of the study was carried out during the summer with an inoculum bacteria concentration of 1.0×10^{11} CFU/mL, while the entrapped study was conducted in winter using an inoculum concentration of 6.7×10^{10} CFU/mL. For coatings containing zosteric acid, the coverage was approximately about 31% of those that contained no zosteric acid for each particular immersion time (Fig. 6).

For coatings prepared using the acetone/water mixed solvent to incorporate zosteric acid, similar bacterial attachment, in term of surface coverage at a particular immersion time, was observed as that of the pure silicone coatings. This behavior could be the result of the low leaching of zosteric acid from the coating not providing sufficient amount of zosteric acid to deter the attachment of bacteria.

The coating properties are important factors that could affect bacterial attachment. Both bulk modulus and surface wettability on silicone coatings with and without zosteric acid were measured. No significant difference on both properties ($P < 0.05$) were found for Sylgard® 184 without and with zosteric acid bulk entrapped up to 2 wt.% (Fig. 7), suggesting differences in bacterial attachment would be the results of other factors. A rougher surface was believed to increase the attachment of bacteria by providing more area of contact. The surface roughness of the silicone coatings without zosteric acid and with 1 wt.% of entrapped zosteric acid before and after 14 days of immersion in bacterial solution were measured using the atomic force microscopy (Fig. 8). The respective average root mean square (RMS) surface roughness of the coatings without and with entrapped zosteric acid was 4 and 13 nm before immersion, and 5 and 66 nm after immersion, for a scan area of 80 μm × 80 μm. Even when the surface roughness for zosteric acid entrapped coatings increased significantly because of the erosion of the surface and the leaching of zosteric acid, no significant increment of bacteria attached to the surface was noticed. Therefore, the lower extent of bacteria attachment onto the rougher zosteric acid coating was due to the presence of zosteric acid and its ability to leach out at a sufficient rate, not the surface roughness or other coating properties.

4. Conclusions

The effectiveness of zosteric acid as a less-toxic antifoulant was evaluated by conducting bacterial attachment studies for plain silicone coatings with zosteric acid in the solution containing bacteria, as well as for coatings with zosteric acid entrapped. The surface wettability and bulk modulus of the coating immersed in aqueous solution remained almost constant, indicating the coating properties had little or no effect on the bacterial attachment behaviors. The bacteria population attached onto plain silicone coating surfaces was found to decrease as the concentration of zosteric acid in the solution increased. With 50 ppm zosteric acid in solution, the bacterial coverage was reduced by 93 and 96%, respectively, for *Lake Erie* bacteria and *Pseudomonas putida*. This concentration is significantly lower than the EC_{50} of the compound for each of the two types bacteria tested.

The homogeneity of the zosteric acid distributed inside the silicone is an important factor for slow release of the compound. The use of mixtures of pyridine/water and acetone/water as entrapment solvents produced homogeneous coatings; however the use of the acetone/water mixed solvent produced a coating with a leaching rate too low to reduce the bacterial attachment. When zosteric acid was entrapped into the bulk of silicone coatings with the pyridine/water mixed solvent, the reduction in bacterial attachment was clearly observed as compared to the pure silicone coatings. Again, the variation in coating properties, such as surface wettability, bulk elastic modulus, and surface roughness, was insignificant to affect bacterial attachment. Therefore, this study suggests that zosteric acid could be an effective antifoulant compound, which exhibited much less toxicity as compared to currently used antifoulants and still reduced bacterial attachment and biofilm formation. Bacterial attachment may not be a prerequisite for some macro fouling; the reduction in bacterial attachment is still advantageous in minimizing the occurrence of most fouling.

Acknowledgements

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