Final Progress Report

PI: Yuyu Sun
Biomedical Engineering Program
University of South Dakota
4800 N Career Ave, Suite 221
Sioux Falls, SD 57107
E-mail: yuyu.sun@usd.edu
Project Title: Antibiofilm tubing to reduce occupational exposure to biohazards in dentistry
Project Director: Yuyu Sun
Sponsor: NIOSH/CDC
Grant Number: R03 OH009325
Start and end date: 06/01/2008 to 05/31/2011

Table of Contents

Abstract 3
Section 1..........4
Section 2..........5
  Specific Aims 5
  Studies and Results 5
  Significance..........19
  Recommendations for Future Studies..........20
  New Publications 20

Abstract

Every dental unit is equipped with small-bore plastic tubing to bring water to the air/water syringe, the ultrasonic scaler, and the high-speed hand piece. Because of the formation and subsequent sloughing off of microbial biofilms from the inner surfaces of the plastic tubing, dental water is heavily contaminated with microorganisms and endotoxin. During dental procedures, the contaminated water will be aerosolized by dental equipment to spread the microbes and endotoxin into air. Dental health care personnel (DHCP) are exposed to these occupational biohazards repeatedly on a daily and long-term basis, which could cause respiratory infection, occupational asthma, and even fatality in DHCP.

The long-range goal of this project is to use iodine-based rechargeable antibiofilm tubing to control the formation of dental unit waterline biofilms so as to reduce occupational exposure of DHCP to the biohazards. Iodines are one of the most widely used disinfectants in dental and hospital settings. When tested in a model dental unit waterline delivery system, we found that iodine could form complexes with polyurethane (PU) dental tubing, which are more stable and less corrosive. Thus, after complexing iodine onto plastic tubes, the new tubes were able to inactivate any approaching planktonic cells to prevent microbial adhesion/colonization, the first step in the formation of biofilms. As a result, the tubes' inner surfaces were free of biohazards during dental operations, improving the microbial quality of dental unit water and aerosols. The antibiofilm activity was stable for a reasonably long period of time (e.g., weeks), and if it was lost due to extensive use, the tubing can be easily recharged by an iodine solution flushing treatment when the dental units are not in use to regenerate the antibiofilm function.

These findings shed new lights on the biofilm-control strategy in dental unit waterlines to improve the occupational safety and health of DCPs. The new materials can also be used in a wide range of related applications to fight infections.

Section 1

Significant (Key) Findings

(1) Iodine can form complexes with dental tubing polymers. Our studies showed that polyurethane (PU), one of the most versatile biomedical materials (the tubing material for dental unit waterlines), strongly binds iodine, one of the most effective antiseptics, through the formation of a charge-transfer complex. The PU-iodine complexes were characterized with UV/VIS study and X-ray photoelectron spectroscopy (XPS) analysis. The new materials evoked potent antimicrobial activity against Gram-negative and Gram-positive bacteria (including methicillin-resistant *Staphylococcus aureus,* vancomycin-resistant *Enterococcus faecium* and bacterial spores), fungi and viruses, as well as inhibited surface bacterial colonization and biofilm-formation. Base on the *Kirby-Bauer* test, the antimicrobial effects occurred through the slow release of iodine. The iodine release rate can be controlled by the preparation conditions of the PU-iodine complex. Trypan blue exclusion analysis indicated that PU-iodine has excellent mammalian cell viability.

(2) The new PU-iodine tubes can prevent biofilm formation. Thus, after complexing iodine onto plastic tubes, the new tubes were able to inactivate any approaching planktonic cells to prevent microbial adhesion/colonization, the first step in the formation of biofilms. As a result, the tubes' inner surfaces were free of biohazards during dental operations, improving the microbial quality of dental unit water and aerosols. The antibiofilm activity was stable for a reasonably long period of time (e.g., weeks), and if it was lost due to extensive use, the tubing can be easily recharged by an iodine solution flushing treatment when the dental units are not in use to regenerate the antibiofilm function.

Translation of Findings

We demonstrated that PU-iodine charge-transfer complexes can be formed by directly treating PU polymers with iodine in aqueous solutions for a short period of time under very mild conditions, regardless of the type and shape of the PU-based device. Iodine is one of the most widely used antiseptics in biomedical and dental settings with low cost and high efficacy, which has been safely used for more than 150 years. PU is one of the most widely used polymers for a wide range of medical/dental applications. The combination of these two can be easily performed in a broad range of settings. Further study of this technology will lead to a new strategy to achieve disinfection technique with long-lasting effects.

Outcomes/ Impact

The long-range goal of this project is to use antibiofilm tubing to control the formation of dental unit waterline biofilms so as to reduce occupational exposure of dental healthcare personnel to the biohazards. Our studies to date strongly indicate that by improving the qaulity of the new PU-iodine complexes, this goal is achieveable. The discovery of PU-iodine complexes as novel antimicrobial and biofilm-controlling device materials is particularlry encouraging. The popularity of PU as a versatile dental/biomedical material, the importance of iodine as an antiseptic, the ease in PU-iodine complex preparation, and the unique properties of the resultant materials pointed to great potentials of PU-iodine complexes as attractive candidates for a broad range of dental and biomedical applications, which will make significant contributions to a better and safer dental/healthcare environment. The significance of the implication and the broadness of the potential impacts will be way beyond the scope we originally proposed.

Section 2

A. Specific Aims:

The long-range goal of the project is to use the rechargeable antimicrobial tubing to improve the microbial quality of dental water.

The current specific aims of this project are to:

Aim 1. Develop iodine-polyurethane based charge-transfer complex tubing to control biofilm formation.

Aim 2. Evaluate the efficacy and safety of the new tubes in model dental water delivery systems.

B. Studies and Results:

B-1. Develop iodine-polyurethane based charge-transfer complex tubing to control biofilm formation. Given the fact that PU is one of the most widely used tubing materials, iodine is one of the most widely used antiseptics, and PU-iodine charge-transfer complexes are formed in a very simple treatment, it is anticipated that these complexes have great potentials for a broad range of medical, dental, and other related applications. In our study, polyether-based medical grade PU was a gift from The Lubrizol Corporation ((Estane 5714, Wickliffe, OH). Iodine (ACS reagent, ≥ 99.8%) was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were analytical grade and used as received.

Preparation of PU-iodine complex membranes

PU membranes were prepared by solvent casting from a 10% solution of PU in tetrahydrofuran (THF). After air-drying in a fume hood for 24 h, the resultant membranes were further dried under vacuum at 40 ^{o}C for 24 h, conditioned in a desiccator for 24 h at room temperature to reach equilibrium, and finally weighed before iodine treatment. Iodine solutions with different iodine contents were prepared by dissolving a predetermined amount of iodine in 4% potassium iodide (KI) aqueous solution. At each iodine concentration, a series of PU membrane samples were immersed in the iodine aqueous solution at room temperature for a certain period of time. The membranes were then taken out of the solution and rinsed thoroughly with Millipore water to remove un-bound free iodine (the rinsing solution was tested with 1% of starch solution to ensure that no further free iodine could be washed out from the membranes). The PU-iodine complex membranes were air dried, conditioned in a desiccator for 24 h at room temperature and weighed. Percentage iodine content was calculated according to the following equation:

$$I_2\% = (W_2-W_1)/W_1 \times 100 \qquad (1)$$

Where W_1 and W_2 were the weights of the PU membrane before and after iodine binding, respectively.

Characterization of the PU-iodine complex membranes

UV/Vis spectra of the samples were recorded on a Beckman Coulter DU 520 UV/Vis spectrophotometer. X-ray photoelectron spectra (XPS) of the samples were obtained by using a PHI 5700 X-ray photoelectron spectroscopy system equipped with dual Mg X-ray source and monochromated Al X-ray source.

Antimicrobial tests

The bacterial, fungal, and viral species were purchased from American Type Culture Collection (ATCC, Manassas, VA), and the spores were provided by North American Science Associates (Northwood, Ohio). All microbial tests were performed in a Biosafety Level 2 hood to ensure lab safety. The guidelines provided by the U. S. Department of Health and Human Services were followed, and appropriate personal protective equipment including gowns and gloves were used in all the microbial studies. The microbial tests were conducted following AATCC (American Association of Textile Chemists and Colorists) Test Method 100-1999 with modifications as specified below.

Antibacterial functions

Pseudomonas aeruginosa (*P. aeruginosa*, ATCC 10145, Gram-negative), *Legionella pneumophila* (*L. pneumophila,* ATCC 33155, Gram-negative), and *Staphylococcus aureus* (*S. aureus*, ATCC 6538, Gram-positive) were used as typical examples of non-resistant Gram-negative and Gram-positive bacteria, respectively. Methicillin-resistant *S. aureus* (MRSA, ATCC BAA-811) and vancomycin-resistant *Enterococcus faecium* (VRE, ATCC 700221) were selected to represent drug-resistant strains because these species have caused serious problems in both healthcare and various community settings. To prepare the bacteria suspensions, *P. aeruginosa* 10145 was grown in nutrient broth at 37 ^{o}C for 24 h, *S. aureus* 6538, MRSA BAA-81 and VRE 700221 were grown in tryptic soy broth at 37 ^{o}C for 24 h, and *L. pneumophila* 33155 was grown in 1099 Charcoal Yeast Extract (CYE) buffered broth at 37 ^{o}C for 48 h, respectively, according to the ATCC's recommendation. The bacteria were harvested by centrifuge, washed with phosphate buffered saline (PBS), and then resuspended in PBS to densities of 10^7-10^8 colony forming units per milliliter (CFU/mL). 10 μL of the freshly prepared bacterial suspensions were placed between two identical PU-iodine complex membranes (2.0 ± 0.1 cm^2). After a certain period of contact time, the membranes were transferred into 10 mL of sterilized sodium thiosulfate ($Na_2S_2O_3$) aqueous solution (0.03 wt%), vortexed for 1 min, and sonicated for 5 min to separate the films, quench the active iodine, and detach adherent cells from the membrane surfaces into the solution (our previous studies [22] found that this treatment did not affect the viability of the microorganism). The solution was then serially diluted, and 100 μL of each diluent were placed onto agar plates (nutrient agar for *P. aeruginosa* 10145, tryptic soy agar for *S. aureus* 6538*,* MRSA BAA-81 and VRE 700221, and 1099 CYE buffered agar for *L. pneumophila* 33155). Colony forming units on the agar plates were counted after incubation at 37 ^{o}C for 24 h for *P. aeruginosa* 10145, *S. aureus* 6538*,* MRSA BAA-81 and VRE 700221, and 48 h for *L. pneumophila* 33155. Pure PU membranes were tested under the same conditions to serve as controls. Each test was repeated three times, and the longest minimum contact time of the three tests for a total kill of the bacteria (the weakest antibacterial efficacy observed) was reported.

Antifungal functions

Candida albicans (*C. albicans*, ATCC 10231) and *Aspergillus niger* (*A. niger*, ATCC 1004) were used as representative examples of fungi. In the antifungal tests, cells were grown in broth solutions (YM broth for *C. albicans* 10231, and potato dextrose broth for *A. niger* 1004) at 26 ^{o}C for 48 h, harvested, washed, and resuspended in PBS to densities of 10^7-10^8 CFU/mL, as described above. 10 μL of the freshly prepared fungal suspensions were placed between two identical PU-iodine complex membranes (2.0 ± 0.1 cm^2). After a certain period of contact time, the membranes were transferred into 10 mL sterilized $Na_2S_2O_3$ aqueous solution (0.03wt%), vortexed, and sonicated. The solution was then serially diluted, and 100 μl of each diluent were placed onto agar plates (YM agar for *C. albicans* 10231, and potato dextrose agar for *A. niger* 1004). Colony forming units on the agar plates were counted after incubation at 26 ^{o}C for 48 h. Pure PU membranes were tested under the same conditions as controls. Each antifungal test was repeated three times, and the longest minimum contact time of the three tests for a total kill of the fungi (the weakest antifungal efficacy observed) was reported.

Antiviral functions

Stock solutions of MS2 virus (ATCC 15597-B1) were prepared using the agar overlay method suggested by ATCC; *E. coli* (ATCC 15597) was employed as the host for the MS2 virus. The stock solutions were diluted with PBS to 10^6-10^7 plaque forming units per milliliter (PFU/mL) of the virus. 10 μL of the freshly prepared viral suspensions were placed between two identical PU-iodine complex membranes (2.0 ± 0.1 cm^2). After a certain period of contact, the membranes were transferred into 10 mL of sterilized $Na_2S_2O_3$ aqueous solution (0.03 wt%), vortexed, and sonicated . The solution was then serially diluted, and 100 μL of each diluent were placed onto LB agar plates containing a "lawn" of 4 h-old *E. coli* 15597 as the host. Plaque forming units on the agar plates were counted after incubation at 37 ^{o}C for 24 h. Pure PU membranes were tested under the same conditions as controls. Each antiviral test was repeated three times, and the longest minimum contact time of the three tests for a total kill of the virus (the weakest antiviral efficacy observed) was reported.

Anti-spore functions

Bacillus subtilis (*B. subtilis*) spores (lot no. N24609) were used to challenge the anti-spore functions of the PU-iodine complex membranes. 10 μL of the spore solutions (10^4-10^5 spores/mL) were placed between two identical PU-iodine complex membranes (2.0 ± 0.1 cm^2). After a certain period of contact time, the membranes were transferred into 10 mL of sterilized $Na_2S_2O_3$ aqueous solution (0.03 wt%), vortexed, and sonicated. The solution was then serially diluted, and 100 μL of each diluent were placed onto tryptic soy agar plates. Colony forming units on the agar plates were counted after incubation at 37 °C for 24 h. Pure PU membranes were tested under the same conditions as controls. Each anti-spore test was repeated three times, and the longest minimum contact time of the three tests for a total inactivation of the spores (the weakest anti-spore efficacy observed) was reported.

Kirby-Bauer test

The antimicrobial function of the samples was also assessed by a *Kirby-Bauer* (KB) technique. In this study, the surface of a tryptic soy agar plate was overlaid with 1 mL of 10^7– 10^8 CFU/mL *S. aureus* 6538. The plates were then allowed to stand at 37 °C for 2 h. A series of PU-iodine discs (ca. 8 mm) with different iodine contents were placed onto the surface of the bacteria-containing agar plate. The membranes were gently pressed with a sterile forceps to ensure full contact between the membranes and the agar. The same procedure was also applied to the pure PU membranes to serve as controls. After incubation at 37 °C for 24 h, the inhibition zone around the membranes (if any) was measured.

Biofilm-controlling functions

The ability of the PU-iodine complex membranes to prevent microbial biofilm formation was evaluated against *S. aureus* 6538 (Gram-positive) and *P. aeruginosa* 10145 (Gram-negative); both species were known to readily form biofilms. In this test, *S. aureus* 6538 was grown in tryptic soy broth and *P. aeruginosa* 10145 was grown in nutrient broth at 37 °C for 24 h, respectively. The bacteria were harvested and resuspended into sterile PBS, as described above. Each PU-iodine complex membrane to be tested (1.0 ± 0.1 cm^2) was immersed individually in a vial containing 10 mL 10^7-10^8 CFU/mL of *S. aureus* 6538 or *P. aeruginosa* 10145 suspension in PBS. The vials were shaken gently at 37 °C for 1 h to allow initial bacterial adhesion. Each membrane was taken out of the bacterial suspension with sterile forceps, and gently washed 3 times with PBS to remove any non-adherent bacteria. The membranes were then immersed into 10 mL tryptic soy broth (for *S. aureus* 6538) or nutrient broth (for *P. aeruginosa* 10145) at 37 °C for 24 h. Afterwards, some of the immersed membranes were removed sterilely from the broth solutions, washed gently with non-flowing PBS (3×10 mL) to remove loosely attached cells, and sonicated in 10 mL PBS for 5 min to transfer the adherent cells into the solution. The solution was serially diluted, and 100 μL of each diluent were plated onto the correspondent agar plates. Recoverable microbial colonies were counted after incubation at 37 °C for 24 h.

The remaining membranes were washed gently with PBS, fixed with 2.5% of glutaraldehyde in 0.1M sodium cacodylate buffer (SCB), and stored at 4 °C overnight. At the end of fixation, the membranes were taken out, gently rinsed 3 times with PBS, and dehydrated through an alcohol gradient. Thereafter, the samples were mounted onto sample holders, sputter coated with gold, and observed under a Hitachi S-3200N scanning electron microscope. The same procedure was also applied to pure PU membranes to serve as controls.

Mammalian cell viability analysis

The effect of the PU-iodine complexes on mammalian cell viability was assayed by the trypan blue dye exclusion method. Briefly, test membranes were cut under sterile conditions and placed into a 96-well plate. The rat skin cell line CRL-1213™ (from ATCC) was cultured in DMEM / High Glucose medium supplemented with 10% fetal bovine serum at 37ºC in humidified air atmosphere of 5% CO_2, trypsinized, counted and then plated into the study wells of the 96-well plate. Cells seeded in wells without the membranes were designated as untreated controls. Each well was plated with 1.25×10^4 cells in 200 μL of the medium. Six and 24 hours

after the treatments commenced, the cells were exposed for 5 min to trypan blue 0.2% (diluted from 0.4% solution; Sigma-Aldrich). The numbers of stain-positive (dead and dying cells) and stain-negative cells in each culture were counted in a hemocytometer chamber. Four cultures were exposed to each membrane for each period. The data were analyzed with Student's t-test for statistical significance.

Stability and rechargeability of the PU-iodine complexes

The stability of the PU-iodine complexes in aqueous medium was monitored under static conditions. Membranes with different iodine contents weighing 0.20g each were suspended individually in 10 mL PBS at 37 ^{o}C for a certain period of time. The iodine contents at different time intervals in the immersing solution were determined by UV-VIS. The calibration curves of standard iodine solutions were prepared by measuring the absorbance of iodine solutions with concentrations varying between 0.05 ppm and 100 ppm.

The PU-iodine complexes were tested for retention of iodine and antimicrobial functions under storage. Membranes with known iodine contents were stored under normal lab conditions (25 ^{o}C, 30-90 % RH). The iodine contents and the anti-infective functions were tested periodically.

To test rechargeability, the PU-iodine membranes were first treated with 10% sodium thiosulfate aqueous solution at room temperature for 24 h to partially quench the bound iodine. The membranes were washed with Millipore water, air dried, and stored in a desiccator for 24 h to reach constant weight. The membranes were then treated with iodine aqueous solution to regenerate the PU-iodine complex structure, using the same conditions as in the preparation of the first generation of the complexes. After different cycles of this "quenching-recharging" treatment, the iodine contents and anti-infective functions of the resultant membranes were re-evaluated.

Figure 1 showed the effect of original iodine concentration in the binding solution. In the test range (0-12.7 g/L of iodine in the solution), the extent of complex formation was clearly iodine-concentration dependent, and after the initial rapidly increasing stage, a near linear relationship was observed.

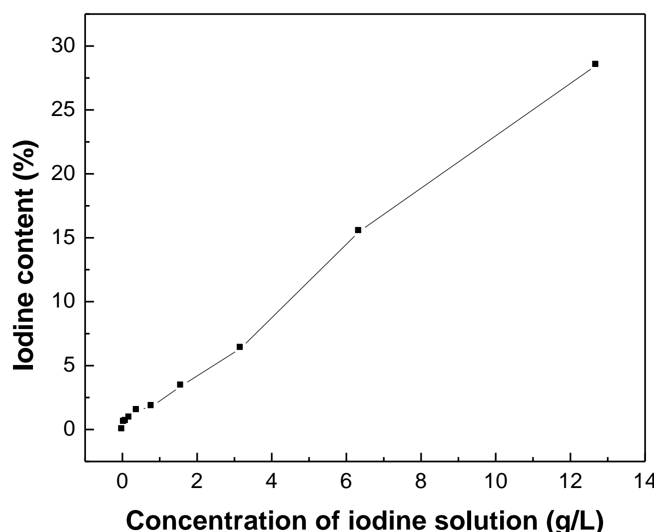

Figure 1. The effects of initial iodine concentration in the solution on iodine content in the resultant PU-iodine complexes (T = 21°C; t = 2 h)

Shown in Figure 2 were the effects of iodine binding time. With the increase of binding time, iodine contents in the resulting membranes increased rapidly until relatively constant values were reached within 1 h. Initial iodine concentration in the solution did not seem to significantly affect the time needed to reach equilibrium. In such a system, iodine binding was affected by three stages: (1) diffusion of iodine through bulk solution to the surface of PU membranes, (2) absorption of iodine molecules on this surface, and (3) diffusion of iodine from the surface to the interior of the PU sample. The second stage and third stage were accompanied by charge-

transfer complex formation between iodine and PU. It was reasonable to assume that on immersion of a PU membrane in iodine solution, with sufficient agitation, the first stage could be accomplished rapidly. The second stage then proceeded, followed by the third stage. In the third stage, the interior of PU membrane might be assumed to contain iodine solutions in its free volume. It was through these free volumes that iodine diffused, being bound to the benzene rings of PU molecules. Because of the high hydrophobicity and low swellability of PU polymers in aqueous solution, however, interior free volumes accessible to iodine solutions might be limited. Thus, the third stage could be of only secondary importance to the whole process, and iodine might be primarily bound to the surface layer of the membranes. This explained the relatively short period of time needed to reach absorption equilibrium, and the relatively low iodine content in the complexes under our experimental conditions (see below).

Factors promoting the third stage, such as using porous PU foams instead of dense PU membranes, using organic solvents (e.g., toluene) instead of water, using iodine sublimation instead of solution absorption, etc., have been shown to significantly increase iodine content in the resultant PU-iodine complexes (e.g., > 100% of iodine content). Nevertheless, the main purpose of the current study was to prepare PU-iodine complexes using simple and practical methods under mild conditions either before or after the target medical devices were fabricated to achieve durable and rechargeable antimicrobial and biofilm-controlling functions with little negative cytotoxicity effects. Thus, those harsh conditions to increase iodine contents were not of interest of this study. Furthermore, we found that higher than a certain value, further increasing iodine content in PU-iodine complexes could only slightly increase antimicrobial potency, but could dramatically affect cell viability, as discussed in the sections below.

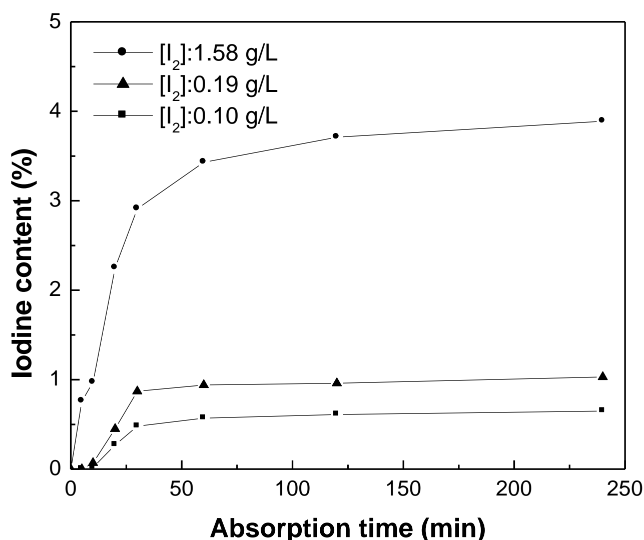

Figure 2. The effects of binding time on iodine content in the resultant PU-iodine complexes at different iodine concentration in the solution (T = 21°C)

Figure 3 presented the XPS survey scans of the samples. The original PU membrane (Figure 3a) showed three peaks corresponding to C-1s (285 eV), N-1s (403 eV) and O-1s (533 eV), respectively, in good agreement with the literature data. Upon iodine binding, two new peaks at 618.8 eV (I3d3) and 630.0 eV (I3d5) could be observed in the XPS spectrum of the PU-iodine membrane (Figure 3b), confirming the formation of PU-iodine charge-transfer complexes.

The PU-iodine complexes were further characterized with UV/Vis studies, as shown in Figure 4. Before iodine binding, the original PU membrane (Figure 4a) showed little UV absorption at higher than 300 nm. After iodine binding, however, a broad band centered at 350 nm could be observed in the UV spectrum of PU-iodine complexes (Figure 4b). The new band was assigned to the UV absorption of I_3^-, a widely reported polyiodide form in iodine-based charge-transfer complexes.

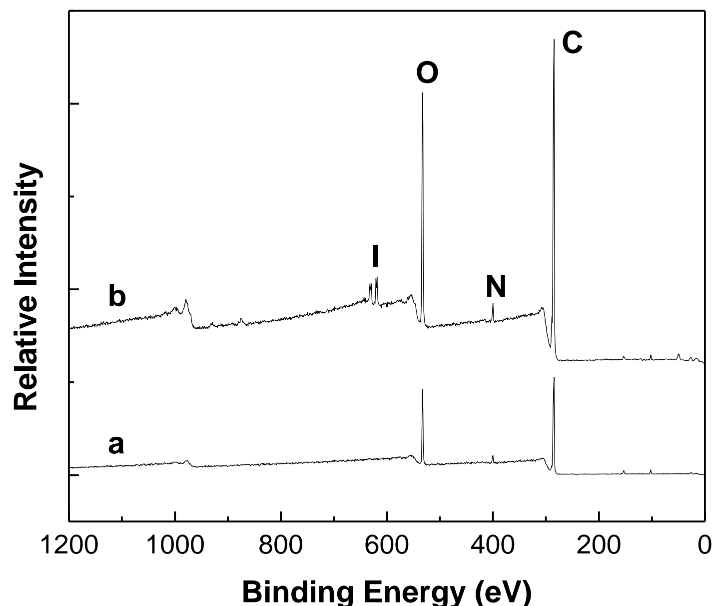

Figure 3. XPS spectra of (a) pure PU membrane, and (b) PU-iodine complex membrane containing 0.96% of iodine

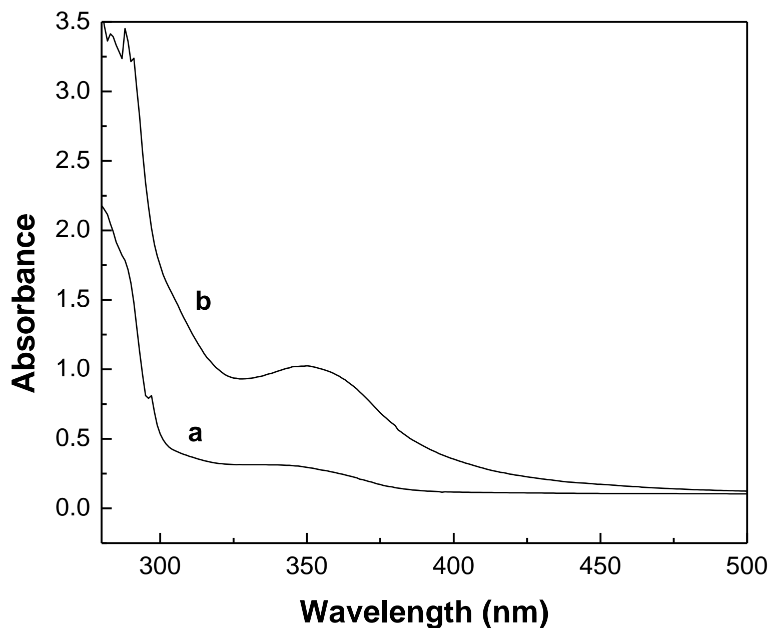

Figure 4. UV/VIS spectra of (a) pure PU membrane, and (b) PU-iodine complex membrane containing 0.96% of iodine

The antimicrobial efficacies of the PU-iodine complexes were evaluated against Gram-positive and Gram-negative bacteria (including drug-resistant species and bacterial spores), fungi, and viruses. Pure PU membranes were used as controls throughout this study, which did not show any antimicrobial effects. The PU-iodine complexes, however, demonstrated encouraging antimicrobial efficacy. The minimum contact times of the PU-iodine complexes with different iodine contents for a total kill of each microbial species were listed in Table 1. As expected, higher iodine content generally led to more rapid antimicrobial effects. For example, with 0.59%

of iodine, it took the PU-iodine complexes 10 min to provide a total kill of 10^7-10^8 CFU/mL of *P. aeruginosa* 10145 (Gram-negative bacteria) and *S. aureus* 6538 (Gram-positive bacteria). When the iodine content was increased to 3.46%, the contact time for a total kill of the same species dramatically decreased to 2 min. On the other hand, *L. pneumophila* (Gram-negative bacteria), the leading cause of Legionnaire's disease [44], showed relatively higher resistance toward the complexes, and it took the complexes 40 min and 20 min to provide a total kill of 10^7-10^8 CFU/mL of the organisms at 0.59% and 3.46% of iodine contents, respectively.

Table 1. Antimicrobial efficacies of the PU-iodine complex membranes with different iodine contents

Microorganisms	Minimum contact time for a total kill of the microorganisms at different iodine contents		
	Percentage iodine in the membrane		
	3.46%	0.96%	0.59%
P. aeruginosa 10145*	2 min	5 min	10 min
*S. aureus 6538**	2 min	5 min	10 min
L. pneumophila 33155*	20 min	40 min	40 min
MRSA BAA-81 *	2 min	5 min	10 min
VRE 700221*	2 min	5 min	10 min
C. albicans 10231*	2 min	5 min	10 min
*A. niger 1004**	20 min	40 min	40 min
MS2 virus 15597-B1**	15 min	20 min	40 min
*B. subtilis****	16 h	24 h	24 h****

***** Bacterial or fungal concentration: 10^7-10^8 CFU/mL;
****** Viral concentration: 10^6-10^7 PFU/mL;
*** Spore concentration: 10^5-10^6 spores/mL;
****After 24 h, this sample inactivated 99% of the spores.

It was a striking finding that the PU-iodine complexes provided potent antibacterial activity against drug-resistant species including MRSA BAA-811 and VRE 700221 (see Table 1), which have become major concerns in healthcare settings and a wide range of related community facilities, causing serious healthcare-related infections and community-acquired infections. These results pointed to great potentials of the PU-iodine complexes for use in a wide range of high-touch, high-risk applications to achieve antimicrobial effects.

The antifungal function of the PU-iodine complexes was evaluated with *C. albicans*, a mucosal organism and opportunistic pathogen of the immunocompromised , and *A. niger*, a common microbial contaminant of food and an important cause of otomycosis and aspergillosis. At 0.59% of iodine content, the PU-iodine complexes provided a total kill of 10^7-10^8 CFU/mL of *C. albicans* 10231 and *A. niger* 1004 in 10 min and 40 min, respectively. Higher iodine contents led to faster antifungal action. The virus (*E. coli* bacteriophage MS2), which has been widely used as surrogate of enteric viral pathogens, was also susceptible to the PU-iodine complexes. At 0.59% of iodine content, a total kill of 10^6-10^7 PFU/mL of the virus was achieved in 40 min. When the iodine content was increased to 3.46%, the minimum contact time for a total kill of the same species was decreased to 15 min.

The anti-spore activities of the PU-iodine complexes were of significant research interests. It has been well established that spores are highly resistant to disinfection. A number of chemical disinfectants (phenolics, quaternary ammonium compounds, alcohols, etc.) are effective antibacterial agents, but they have little or no sporicidal activity. Other disinfectants, such as glutaraldehyde, chlorine, and iodine, can inactivate both bacteria and spores, but the sporicidal effect often requires much higher disinfectant concentrations and much longer contact time. The resistance of spores to chemical agents has been attributed to the spore's coat and cortex, which act as effective barriers that prevent the access of disinfectants to the underlying spore protoplast. Although the sporicidal efficacies and inactivation mechanisms of *monomeric* disinfectants including iodine have been reported, studies concerning the sporicidal activities of *polymeric* biocides are still lacking.

Since sporicidal efficacies are regarded as a direct indication of the antimicrobial power of anti-infective polymers, in the present study, the PU-iodine complexes were challenged with *B. subtilis* spores, which have been used as biological indicators in sterilizations as well as surrogates of anthrax spores because of their high resistance. The PU-iodine complexes demonstrated encouraging anti-spore activities, which were concentration-dependant. At 0.59% of iodine content, the samples inactivated 99% of the spore (original spore concentration: 10^5-10^6 spores/mL) after 24 h of contact. When the iodine content was increased to 0.96% and 3.46%, a total inactivation of the same spores was achieved within 24 h and 16 h, respectively.

Although the biocidal mechanism of iodine is still not clear, it is generally accepted that iodine can rapidly penetrate into microorganisms and attack key groups of proteins (in particular the free-sulfur amino acids cysteine and methionine), nucleotides, and fatty acids. In addition, iodine may also destabilize membrane fatty acids by reacting with unsaturated carbon bond. All these factors can lead to cell death.

Because the PU-iodine complexes were able to effectively kill microbes, it was highly possible that they could prevent the formation and development of biofilms. To evaluate this effect, the original PU membrane and the PU-iodine membranes containing different amounts of iodine were contacted with *S. aureus* 6538 or *P. aeruginosa* 10145 (both of which are known to readily form biofilms) for 1 h to allow initial adhesion, and then immersed in the corresponding broth solutions for 24 h to facilitate formation and development of bacterial biofilms. The level of recoverable bacteria from pure PU membrane and PU-iodine complex membranes was presented in Table 2. The pure PU membrane surfaces had the highest level of recoverable bacteria: after sonication, 10^8 CFU/cm^2 of adherent *S. aureus* 6538 and 10^6 CFU/cm^2 of adherent *P. aeruginosa* 10145 could be recovered after 24 h of contact, suggesting that both of the bacteria had strong abilities to adhere onto pure PU surfaces, which would lead to biofilm formation. However, after iodine binding, the recoverable levels from membranes with 0.59% of iodine decreased to 10^4 CFU/cm^2, and with 0.96% and 3.46% of bound iodine, no bacteria could be recovered from the membrane surfaces after 24 h of incubation with the bacteria, pointing to potent biofilm-controlling effects.

Table 2. Recoverable S. aureus 6538 and P. aeruginosa 10145 from the pure PU membrane and PU-iodine complex membranes with different iodine contents after incubation in broth solutions for 24 h

Microorganisms	Recoverable adherent microorganisms (CFU/cm^2)*			
	Pure PU	PU-I_2complex (0.59%)	PU-I_2complex (0.96%)	PU-I_2complex (3.46%)
*S. aureus***	$(1.7 \pm 0.37) \times 10^8$	$(9.2 \pm 0.18) \times 10^4$	0	0
P. aeruginosa	$(4.2 \pm 0.87) \times 10^6$	$(7.1 \pm 0.15) \times 10^4$	0	0

*Every recoverable test was repeated three times.
** The *S. aureus* and *P. aeruginosa* concentrations were 10^7-10^8 CFU/mL.

The recovery study results agreed well with SEM observation. As shown in Figure 5, after 24 h of incubation, both species demonstrated strong tendency to adhere onto the surface of the original PU, forming layered (Figure 5a) or aggregated (Figure 5b) micro-colonies that develop into continuous bacterial biofilms. On the other hand, the PU-iodine membrane with 0.96% of iodine showed much clearer surfaces (Figure 5a' and 5b'): no adherent bacteria could be observed, and no biofilms were formed, suggesting powerful biofilm-controlling activity against the test organisms.

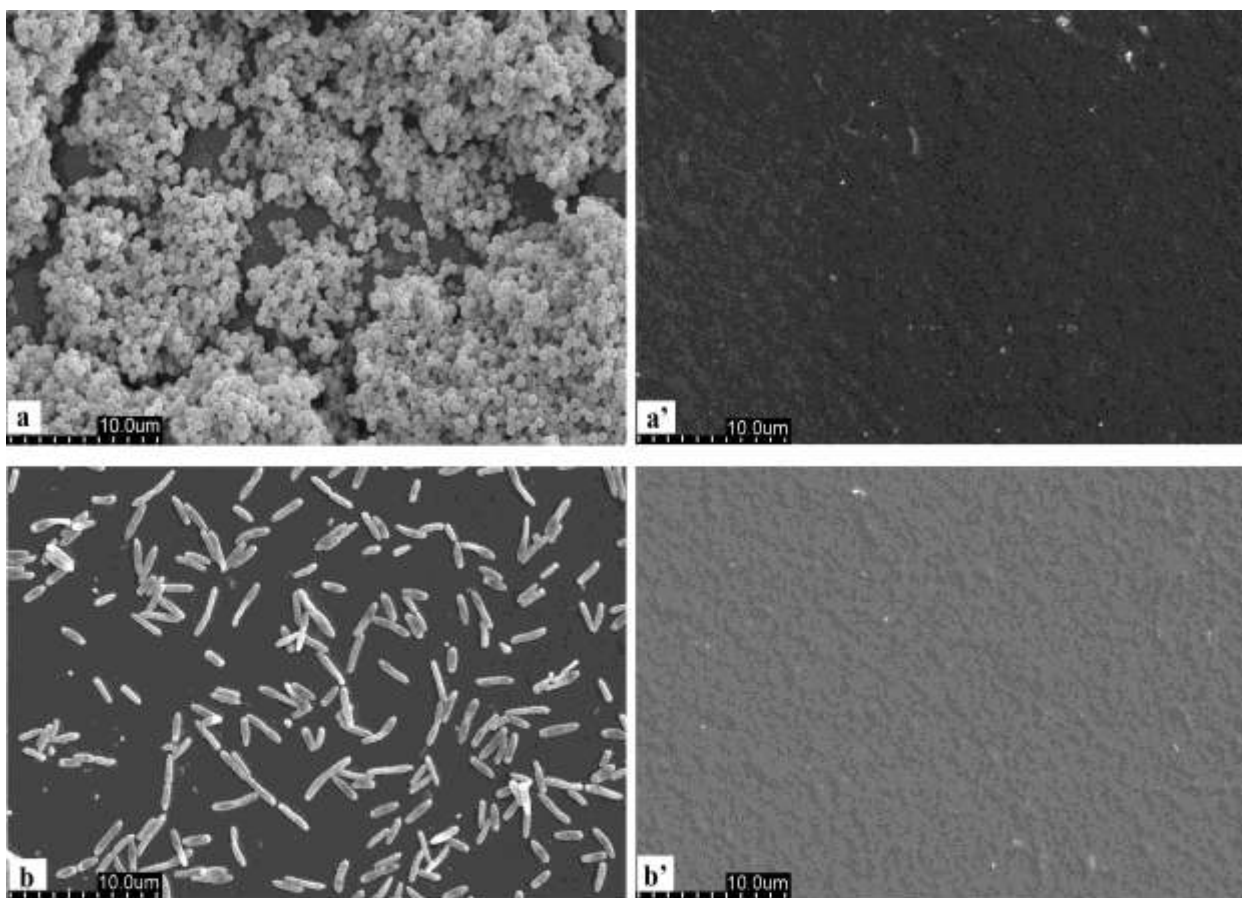

Figure 5. SEM results of biofilm-controlling activities of: (a) pure PU membrane challenged with *S. aureus* 6538*,* (a') PU-iodine complex membrane containing 0.96% of iodine challenged with *S. aureus* 6538*,* (b) pure PU membrane challenged with *P. aeruginosa* 10145, and (b') PU-iodine complex membrane containing 0.96% of iodine challenged with *P. aeruginosa* 10145; the contact time was 24 h.

To provide further information about the antimicrobial action of the PU-iodine complex systems, *Kirby-Bauer* tests of samples with different iodine contents were performed against *S. aureus* 6538 after incubation at 37 ^{o}C for 24 h, using pure PU membrane as controls. As presented in Figure 6, no inhibition zone could be observed around pure PU membrane (Figure 6a). With 0.59% of iodine, the inhibition zone of the PU-iodine complex membrane could be barely detected (Figure 6b). However, PU-iodine complex membrane containing 0.96% of iodine (Figure 6c) generated a zone of 0.8 ± 0.1 mm (n=3), and when the iodine content was increased to 3.46% (Figure 6d), the zone size dramatically increased to 7.0 ± 0.6 mm.

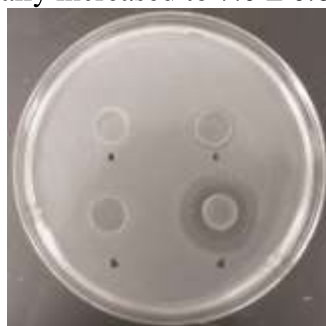

Figure 6. Zone of inhibition test results of: (a) pure PU membrane, (b) PU-iodine complex membrane with 0.59% of iodine content, (c) PU-iodine complex membrane with 0.96% of iodine content, and (d) PU-iodine complex membrane with 3.46% of iodine content

These findings suggested that during the test, some of the iodine liberated from the PU-iodine complex systems and killed the microbes. As expected, the higher the initial iodine content, the more iodine was released, and the larger the inhibition zone was. In such a system, the release rate of iodine from the polymer could have two opposite effects: for antimicrobial action, a fast release was preferred because it would lead to powerful and instant efficacies. On the other hand, however, the environmental impacts posed by releasing iodine and the short-term effectiveness due to the exhaustion of these agents were also very important factors that should be taken into account in real applications.

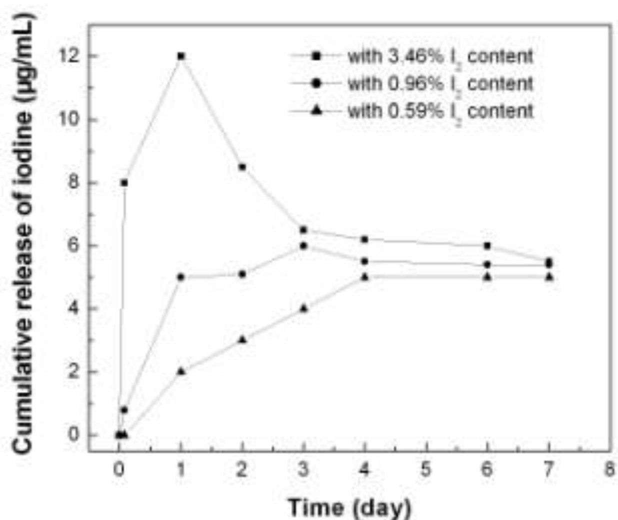

Figure 7. Iodine release into PBS at 37 ^oC from PU-iodine complex membranes with different iodine contents

To test to what extent iodine could leach from the PU-iodine complex, a quantitative evaluation of the release of iodine from samples with different initial iodine contents was conducted *in vitro* at 37 ^oC in PBS. Figure 7 showed the iodine content in the solution as a function of releasing time. With 0.59% of initial iodine content, it was found that in the first three days, the iodine content in the solution continuously increased; after that, when the equilibrium of the dissociation of the PU-iodine charge-transfer complex was achieved, the iodine content in the solution was kept constant at about 4.5 μg/mL (4.5 ppm). In other words, although the membrane contained 0.59% of bound iodine, only 4.5μg/mL of iodine was released from the membrane to the solution. These results suggested that the disassociation rate constant of the charge-transfer complex was very low, and this also explained the fact that at this iodine content, the PU-iodine complex did not provide any noticeable inhibition zones in the *Kirby-Bauer* test (see Figure 6b).

In the iodine release tests of PU-iodine complexes with higher initial iodine contents, however, an interesting "increasing → decreasing → constant" trend was observed, as shown in Figure 7. For example, with 3.46% of initial iodine content in the membrane, iodine content in the solution reached 8.0 μg/mL after 2 h, and increased to as high as 12.0 μg/mL after 24 h of release. After that, however, the iodine content in the solution begun to decrease, and finally a relatively constant value around 6.5μg/mL was detected after three days of release. Similarly, with 0.96% of initial iodine content in the membrane, the highest solution iodine content of 6.0 μg/mL was achieved at day 3. Afterwards, a slightly lower value of 5.5 μg/mL was observed, which remained constant during the test period of seven days. The decreasing trend could be caused by the sublimation of the released free iodine in the solution, particularly at high iodine contents. That is to say, disassociation of the charge-transfer complex released iodine into the solution, which could sublimate, particularly at high iodine content. The observed iodine content in the solution was thus the equilibrium of these two effects.

Apparently, the release of iodine will gradually reduce antimicrobial activity, but many real applications need long-term antimicrobial effects. One example of such applications is dental unit waterline tubing. As mentioned earlier, biofilms on the inner wall of dental tubing could lead to heavy microbial contamination of

dental treatment water and aerosols. Our results indicate that if PU-based dental tubing is treated with iodine aqueous solution, PU-iodine complexes will be formed to provide antimicrobial effects to prevent microbial adhesion and biofilm formation. Nevertheless, iodine release will gradually reduce this desirable function. One potential solution to this problem is to repeatedly recharge the released iodine. To confirm rechargeability, freshly prepared PU-iodine with different iodine contents (0.59%, 0.96%, and 3.46%) were first treated with 10% sodium thiosulfate aqueous solution at room temperature for 24 h to quench the bound iodine, and were then treated with iodine aqueous solution to regenerate the complex structure, using the same conditions in the preparation of the first generation of the PU-iodine complex membranes. As shown in Figure 8, after 5 cycles of this "quenching-recharging" treatment, iodine contents in the complexes were essentially unchanged. While many applications may not need this "rechargeable" feature, some applications, particularly dental tubing, may find this function very attractive for long-term antimicrobial effects. Besides, in dental tubing and other related applications, recharging can be easily performed using a pump on weekends, at nights, or when the systems are not in use.

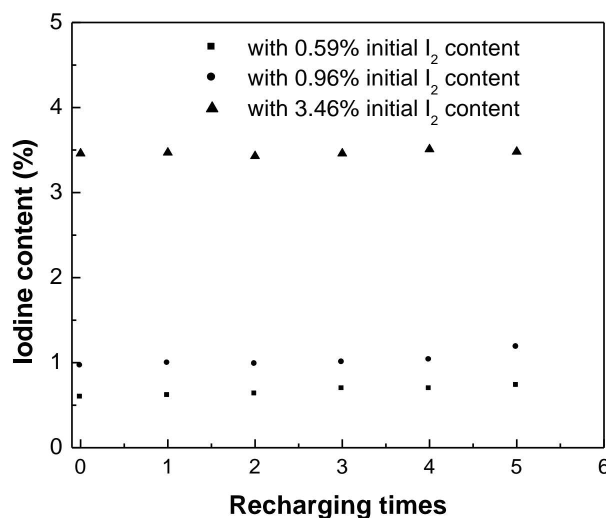

Figure 8. The effects of recharging on iodine contents in the PU-iodine complexes

On the other hand, many biomedical applications require good cell viability. To evaluate this, the effects of PU-iodine complex membranes on rat skin cell line CRL-1213™ viability were summarized in Table 3. As demonstrated by trypan blue assay, the viability of the CRL-1213™ cells was not affected by iodine contents in the range of 0.59%-3.46% within 6 h of contact. Of all the CRL-1213™ cells exposed to PU-iodine complex membranes, only a few had trypan blue–stained nuclei (indicating cell death), and when viewed by phase-contrast microscopy, the stained cells were of the same size and shape as the unstained cells (images not shown). Cultures of the cells exposed to pure PU membrane, and PU-iodine complex membranes with different iodine contents (0.59%, 0.96%, or 3.46%) showed similar percentages of stained cells and intact cellular morphology, and there were no significant differences in the proportions of trypan blue–stainable cells in cultures exposed for 6 h to the control or any test groups. On the other hand, after 24 h of exposure, the proportion of trypan blue–stained cells in most solutions decreased and there were no significant differences among control and PU-iodine complex membranes with 0.59% and 0.96% of initial iodine contents. When the iodine content was increased to 3.46%, however, the proportion of trypan blue–stained cells increased. Thus, this sample showed statistically significant ($P<0.05$) cell cytotoxicity effect on CRL-1213™ cells after 24 h of contact. This could be caused by higher releasing and subsequent accumulation of iodine from PU-iodine complex membranes in culture medium (see Figure 7), which could affect cell viability.

Table 3. The effects of PU- iodine complex membranes on CRL-1213™ cell viability evaluated with the trypan blue assay

Samples	% of undamaged cells after 6 h	% of undamaged cells after 24 h
Cell-only Control	93.7% ± 4.7%	97.8% ± 2.7%
Pure PU membrane	90.9% ± 7.5%	95.4% ± 1.4%
PU-iodine with 0.59% I_2	85.2% ± 8.2%	95.3% ± 4.2%
PU-iodine with 0.96% I_2	86.4% ± 4.6%	94.7% ± 1.6%
PU-iodine with 3.46% I_2	89.3% ± 10.3%	67.9% ± 9.9% *

* Significant difference ($P<0.05$).

Free iodine has a high sublimation tendency and thus low storage stability. However, after forming complex with PU, the PU-iodine membranes showed good stability. Under normal lab conditions (25 ^{o}C, 30-90 % RH), the PU-iodine samples with different initial iodine contents (0.59%, 0.96%, and 3.46%) have been stored for more than 6 months in a closed container. More than 95.5% of the initial iodine was retained, and no significant changes of the antimicrobial efficacies against the microbial species were observed, pointing to long-term storage stability.

B-2 Evaluate the efficacy and safety of the new tubes in model dental water delivery systems:

The dental unit water delivery system

The efficacy of the PU-iodine tubing was tested in a dental unit waterline (DUWL) delivery system that was a modified model previously described by the American Dental Association/American National Standards Institute (ADA/ANSI) Working Group 9.48. The model was designed to replicate biofilm growth during simulated clinical use with source tap water.

Materials and Methods

An initial experiment was designed for a four-week period to calibrate and test the model. We used a 10-liter carboy, retrofitted to allow connection of peroxide cured silicon tubing (Cole Parmer L/S 14), to hold the source tap water. A 5-ft section of the silicon tubing was connected to the carboy and threaded through the pump head. Fourteen sections of polyurethane tubing (A-dec) one-sixteenth of an inch in diameter, each measuring 5-cm in length, were first connected together with 2 cm-length silicon tubing (Figure 1) and then to the 5-ft section of silicon tubing. The end of the tubing was left to drain into a collection flask contained in a metal basket as seen in Figure 2.

Figure 1. Test line consisting of 5-cm sections of test tubing connected together with 2-cm sections of silicon tubing.

Figure 2. The model dental water delivery system

At the beginning of each day, the carboy was filled with 1,500 ml of tap water. A computerized system (Cole Parmer Masterflex System) was used to set the flow rate equivalent to 1.4 mL/min. to simulate a typical workday at the University of Texas health Science Center at San Antonio (UTHSCSA) Outpatient Clinic. The

system was turned on at the beginning of each workday Monday-Friday and operated for 8 hours. The pump was run for 5 min. on and 25 min. off to produce 7mL over the 5 min-on-period or 112 mL/day of output water.

Tap Water and Effluent Samples

Source tap water was cultured at the beginning of each day on Days 1-5 and periodically during the next three weeks. Effluent water samples were taken at the end of each day for the first week and thereafter at the end of each working week, as follows: the end of the tubing was wiped with an alcohol pad and the pump was run for a 5-minute collection cycle. The first mL of water was discarded and the remaining 6 mL was collected in a sterile collection tube and neutralized with 0.03mg/L of sodium thiosulfate. One-tenth mL was spread on R2A agar using a spread plate technique and left to incubate at room temperature for 7-10 days.

Tubing

Three 5-cm sections of polyurethane tubing, one from each end of the line and one from the middle, were removed at the end of each week. A sterile gastight syringe plunger (Hamilton Co. NV) was pushed through each section of tubing to dislodge any adherent biofilm into three collection tubes containing sterile PBS. In order to detach any remaining biofilm, one half of a mL of existing PBS was drawn into a syringe and flushed through the tubing. The collected liquid samples were vortexed for 15 seconds. Ten-fold serial dilutions in PBS were made for each section of tubing and one-tenth of a mL was plated on R2A agar in triplicate. Bacterial colonies were counted after 7-10 days and reported as colony forming units per milliliter (CFU/mL).

Evaluation of PU-iodine tubing

After calibration and testing of the delivery system was completed, the efficacy of the rechargeable antimicrobial PU-iodine tubing (T) to prevent biofilm formation was tested in the DUWL model. As Figure 3A shows, two carboys were used for this 8-week experiment. One of the carboys had a T line connected and the other had an untreated control (C) line connected, as previously described. At the end of Week 4, the T and C lines were attached to the same carboy, using a three-way barb connector as seen in Figure 3B. The C line then became a treated control line, or recharge control (RC) line as T and RC were recharged by running a 1:10 dilution of an iodinated solution for 2 min, left overnight, and flushed with tap water the next morning. An additional untreated control line (C) was connected to the other carboy. Weekly, samples of effluent and adherent bacteria from inside T, C, and RC tubing, were taken and cultured on R2A agar at room temperature for seven days, as previously described.

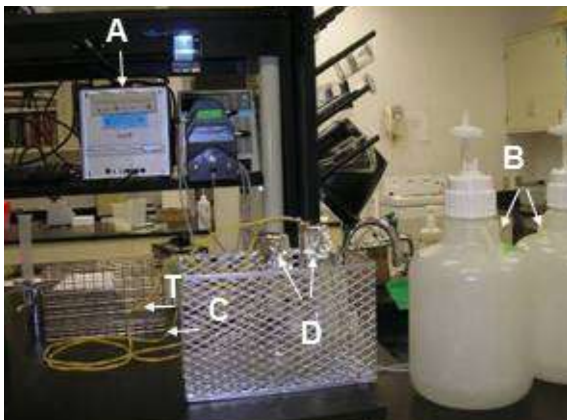

FIG 3A. The laboratory model comprised of a computerized system (A) used to pump water from carboys containing source water (B), through test (T) and control (C) lines, into collection flasks (D)

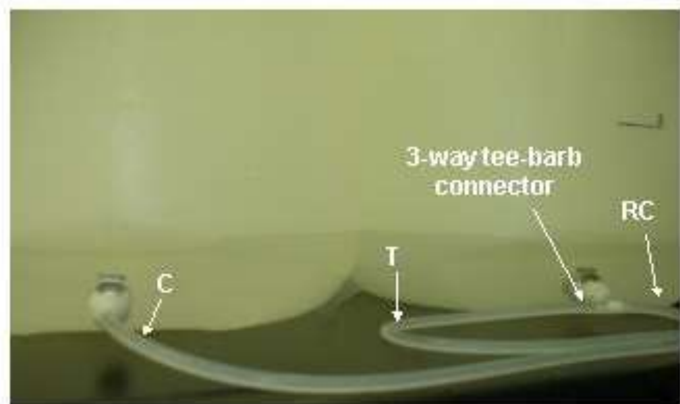

FIG 3B. The adapted model with a tee barb connector used to connect T and RC lines to the same reservoir for recharging and the untreated control (C) line to the second

The internal surfaces of T, RC, and C were examined for the presence of biofilm using scanning electron microscopy (SEM), as follows: Tubing sections were placed in a container with fixative of 4% formaldehyde and 1% glutaraldehyde in a phosphate buffer, rinsed (2 x 3 min) in 0.1 M PO_4 buffer. They were then placed in

Zetterquist's Osmium 1% for 30 minutes followed by a rinse in Zetterquist's buffer for 2 min. This was followed by a wash in 70% ethyl alcohol (ETOH) 3 x 15min, then 95% ETOH (3 x 15min), followed by 100% ETOH (2 x 20 min). Tubing sections were then immersed in hexamethyldisilazane (HMDS) for 5 minutes, decanted and let to air dry over night in a dessicator. They were finally coated with gold palladium before SEM imaging.

Statistical Analysis:

Two sample Student's t-tests were performed to determine if the means of CFU/ml and/or log CFU/ml were significantly different for test vs. control at each week and for all four weeks before recharge. For comparisons of test, recharged control (RC), and control tubing samples during the four weeks after recharge with iodine, one-way ANOVAs were performed to determine if the means of CFU/ml and/or log CFU/ml were significantly different for test, RC, and control at each week and for all four weeks. If the F-test for an ANOVA was significant, then post-hoc Games-Howell multiple comparison tests were performed to identify significant differences between test vs. control, RC vs. control, and test vs. RC. For all comparisons, $p<0.05$ was considered significant.

Results

As seen in Figure 4A, source tap water bacterial levels were consistently within the EPA Drinking Water Standard/CDC recommended level of 500 CFU/mL during the eight-week testing period. Effluent from T was the same as source tap water level for the first three weeks. At Week 4, an increase in counts was noted, which was followed by a further decline in Weeks 5 and 6 after recharge. By Weeks 7 and 8, the effluent bacterial levels emitted from T had increased again. On the other hand, bacterial counts in C effluent were consistently higher than 500 CFU/mL after Week 2.

With respect to the pre-recharge weeks, mean log CFU/ml for tubing samples (Figure 4B) was significantly greater for control compared to test at 2 ($t=11.60$, $p=0.007$), 3 ($t=48.38$, $p<0.001$), and 4 weeks ($t=6.24$, $p=0.016$), and for all four weeks combined ($t=3.98$, $p=0.001$). After iodine recharge, mean log CFU/ml was significantly different at 6 ($F=105.57$, $p=0.001$) and 7 weeks ($F=21.23$, $p=0.006$). In both cases, post hoc testing determined that mean log CFU/ml was significantly greater for control compared to test and recharged control, and test and recharged control means were not significantly different.

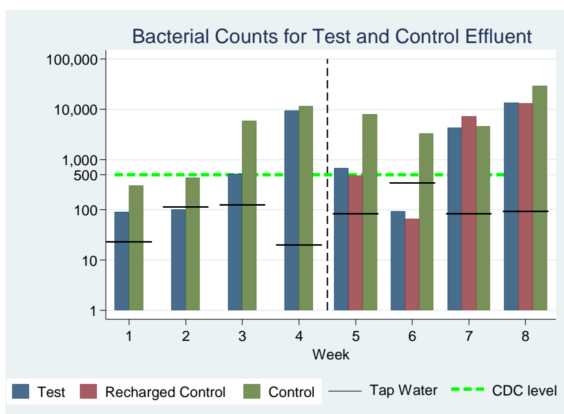

Figure 4A shows that T effluent CFU/mL was consistent with source tap water level for the first three weeks, with an increase in counts in Week 4. After recharge, a decline was seen in T in Weeks 5 and 6, and an increase again in Weeks 7 and 8. Bacterial counts in C effluent were consistently higher than 500 CFU/mL after Week 2.

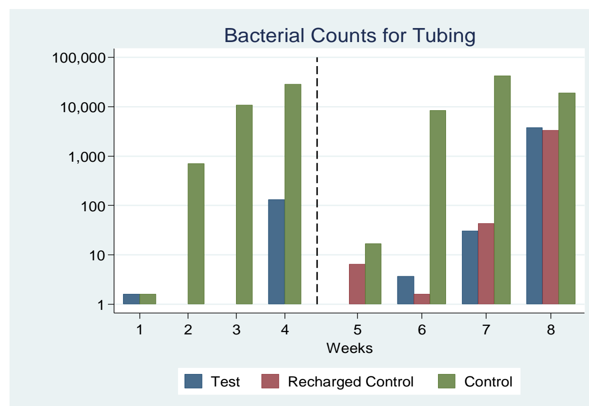

Figure 4B illustrates that adherent bacterial counts inside C tubing showed a 4 log CFU/mL increase over the first four weeks and again in Week 6, just two weeks after iodine recharge. Adherent bacterial counts inside T tubing were consistently low through week 7, with a 1-log increase noted in week 4, and a 2-log increase in week 8.

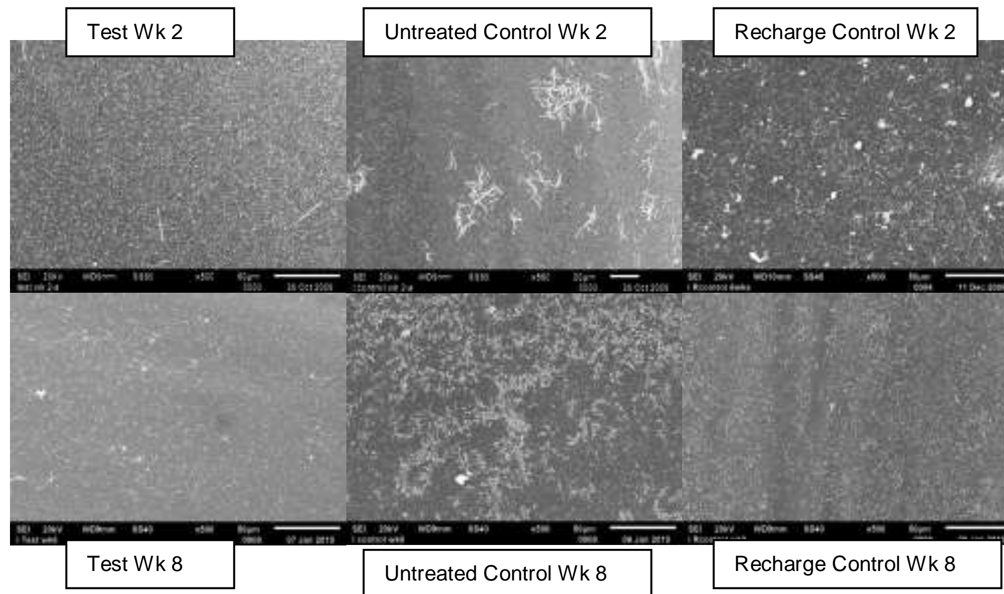

Figure 5. SEM images (x500) confirmed the development and formation of biofilm on the untreated control line (C) Weeks 2-8, to a lesser extent on the recharge control line (RC), but no apparent biofilm formation on the test line (T) even at Week 8.

Conclusions:

The antimicrobial agent initially prevented, and then reduced biofilm growth for limited periods of time in PU-iodine tubing. There was a significant difference in bacterial counts between T and RC versus C (p=0.002). Adherent bacteria inside T line, which are indicators of biofilm formation remained low for three weeks before and after recharge with iodine. This was confirmed with SEM images. Longer-term studies to test the efficacy of the PU-iodine tubing to prevent biofilm formation are necessary with an optimal recharge interval of three weeks.

C. Significance:

The long-range goal of this project is to use antibiofilm tubing to control the formation of dental unit waterline biofilms so as to reduce occupational exposure of dental healthcare personnel to the biohazards. Our studies to date strongly indicate that by improving the qaulity of the new PU-iodine complexes, this goal is achieveable. The discovery of PU-iodine complexes as novel antimicrobial and biofilm-controlling device materials is particulalry encouraging. The popularity of PU as a versatile dental/biomedical material, the importance of iodine as an antiseptic, the ease in PU-iodine complex preparation, and the unique properties of the resultant materials pointed to great potentials of PU-iodine complexes as attractive candidates for a broad range of dental and biomedical applications, which will make significant contributions to a better and safer dental/healthcare environment. The significance of the implication and the broadness of the potential applications will be way beyond the scope we originally proposed.

D. Recommendation for future studies

We have successfully followed the schedules outlined in our applications and completed all the original specific aims. It has been found that the new approach has great potential to controlling biofilms. Further studies should

use combination of different halogens for the treatment of the tubes, and test the efficacy and safety of the new materials in purified water to further improve activity and reduce recharge frequency.

E. New Publications:

1. Luo, J, Deng, Y, and **Sun, Y** (2010). **Antimicrobial Activity And Biocompatibility of Polyurethane-Iodine Complexes.** Journal of Bioactive and Compatible Polymers, 25, 185-206.
2. Porteous NB, Luo J, Eskew EK, Vogt K, Sun Y. **A simple model simulating DUWL biofilm growth, reduction, and prevention**. Journal of Dental Research 89(Special Issue B):127,2010.