ACS APPLIED MATERIALS & INTERFACES

FORUM ARTICLE

www.acsami.org

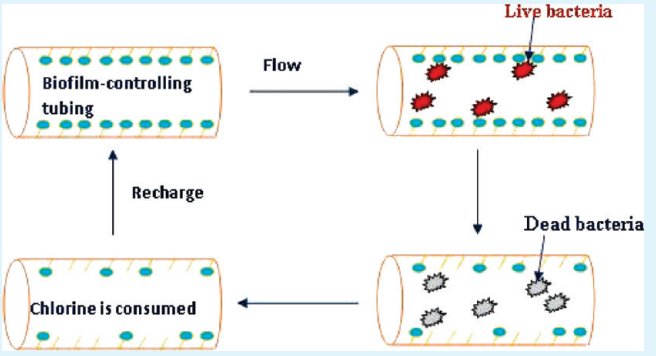
Rechargeable Biofilm-Controlling Tubing Materials for Use in Dental Unit Water Lines

Jie Luo,[†] Nuala Porteous,[‡] and Yuyu Sun*[,†]

[†]Biomedical Engineering Program, University of South Dakota, Sioux Falls, South Dakota 57107, United States

[‡]Department of Comprehensive Dentistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229-4404, United States

ABSTRACT: A simple and practical surface grafting approach was developed to introduce rechargeable *N*-halamine-based antimicrobial functionality onto the inner surfaces of continuous small-bore polyurethane (PU) dental unit waterline (DUWL) tubing. In this approach, tetrahydrofuran (THF) solution of a free-radical initiator, dicumyl peroxide (DCP), flowed through the PU tubing (inner diameter of 1/16 in., or 1.6 mm) to diffuse DCP into the tubing's inner walls, which was used as initiator in the subsequent grafting polymerization of methacrylamide (MAA) onto the tubing. Upon chlorine bleach treatment, the amide groups of the grafted MAA side chains were transformed into acyclic *N*-halamines. The reactions were confirmed with attenuated total reflectance infrared (ATR) spectra and iodometric titration. The mechanical properties of the tubing were not significantly affected by the grafting reactions. The biofilm-controlling function of the new *N*-halamine-based PU tubing was evaluated with *Pseudomonas aeruginosa* (*P. aeruginosa*), one of the most isolated water bacteria from DUWLs, in a continuous bacterial flow model. Bacteria culturing and SEM studies showed that the inner surfaces of the new *N*-halamine-based PU tubing completely prevented bacterial biofilm formation for at least three to four weeks. After that, bacteria began to colonize the tubing surface. However, the lost function was fully regenerated by exposing the tubing inner surfaces to diluted chlorine bleach. The recharging process could be repeated periodically to further extend the biofilm-controlling duration for long-term applications.



KEYWORDS: polyurethane, tubing, surface grafting, *N*-halamine, biofilm-controlling, rechargeable

INTRODUCTION

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 handpiece. However, because of the large surface-area-to-volume ratio, low flow rates, and frequent quiescent periods, the inner surfaces of dental unit waterline (DUWL) tubing are particularly susceptible to biofilm formation.[1−3] Biofilms are populations of microorganisms growing on a surface and enclosed in an exopolysaccharide matrix.[4] Once formed, biofilms are very difficult to destroy. Protected by the polysaccharides, microbes living in a biofilm are up to 1000 times more resistant to disinfection, causing serious problems including medical/dental device-related infections and healthcare-associated infections.[5−8]

Since the existence of adherent microbial biofilms in DUWL tubing was first reported in 1963,[9] there has been growing concerns of the potential health impact caused by biofilms and contaminated dental water. The Centers for Disease Control and Prevention (CDC) recommended that dental patient treatment water should be consistent with the Environmental Protection Agency (EPA) Drinking Water Standard (less than 500 colony-forming units per milliliter, or CFU/mL, of microorganisms).[10] The American Dental Association (ADA) currently recommends that dental offices should comply with the 2003 CDC Guidelines although they originally set a goal of no more than 200 CFU/mL of heterotrophic, mesophilic bacteria in unfiltered output water by the year 2000.[11,12] Unfortunately, the delivery of patient treatment water with ≤500 CFU/mL is difficult to achieve in general practice. Because of biofilm formation and subsequent sloughing off of bacteria from the inner surfaces of the tubing, dental water can be heavily contaminated with microorganisms, and microbial populations ranging from 1000 to160 million CFU/mL have been cited.[1−3] These microorganisms pose a potentially significant risk to dental-care workers and patients, particularly those who are medically compromised or immuno-compromised.[3]

Since the vast majority of microorganisms in DUWLs are caused by biofilms on plastic tubing, several methods have been proposed to control biofilms in order to improve the quality of dental unit water. These methods include the use of sterile water

Received: May 9, 2011
Accepted: June 14, 2011
Published: July 01, 2011

dx.doi.org/10.1021/am200576q | *ACS Appl. Mater. Interfaces* 2011, 3, 2895–2903

delivery systems, UV-treatment, independent water systems, intermittent or continuous chemical treatments, filters, etc.[13−17] However, all of these methods have limitations. For example, sterile water delivery systems can largely prevent the formation of biofilms, but the cost is high and the system is inconvenient to operate. Other methods cost less, but most of them cannot eliminate biofilms; after treatments, microbial populations rapidly rise to their original levels.

It is widely viewed that if the inner surfaces of tubing can inactivate microorganisms upon contact, the formation of biofilms in DUWL would be inhibited and the quality of dental unit water would be significantly improved. This approach has been used for the antimicrobial treatment of catheters to control biofilms and catheter-related infections.[18,19] In one of our previous studies, we successfully developed a rechargeable antimicrobial approach by which *N*-halamine compounds were covalently bound onto the inner surfaces of tubing to provide biofilm-controlling functions.[20] *N*-halamines are compounds containing one or more nitrogen-halogen covalent bonds that are normally formed by the chlorination of imide, amide, or amine groups. As broad-spectrum food and water disinfectants, *N*-halamines have antimicrobial efficacy similar to that of hypochlorite bleach, one of the most widely used disinfectants in dental and hospital settings, but *N*-halamines are more stable, less corrosive, and have much less tendency to generate halogenated hydrocarbons .[21−23] Our previous treatment strategy consisted of three steps: hydroxylation of plastic surfaces with potassium persulfate at elevated temperature, grafting methacrylamide (MAA) onto the hydroxylated surface using ceric(IV) ammonium nitrate(CAN) as an initiator, and chlorination of the grafted polymethacrylamide (PMAA) side chains to produce *N*-halamines. The resulting *N*-halamine-based tubing demonstrated potent, durable, and rechargeable biofilm-controlling functions.[20]

Nevertheless, activation with potassium persulfate at high temperature and initiation with CAN could be difficult to perform in general practice. Thus, in this study, we developed a simple and practical surface grafting approach using preabsorbed dicumyl peroxide (DCP) as initiators to graft MAA onto the inner surface of continuous small-bore PU tubing, one of the most widely used dental tubing materials. The biofilm-controlling efficacy of the resulting *N*-halamine-based PU tubing was evaluated with continuous flow of 1×10^4 to 1×10^5 CFU/mL of *Pseudomonas aeruginosa* (*P. aeruginosa*), one of the most cited water bacteria that are responsible for DUWL biofilm formation.[12,24,25]

EXPERIMENTAL SECTION

Materials. PU tubing for DUWL (O.D.: 1/8 in.; I.D.: 1/16 in.) was obtained from A-dec (Newberg, OR). The tubing was washed with acetone to remove possible impurities. Methacrylamide (MAA) was provided by VWR International, Inc. (West Chester, PA), and was purified by recrystallization from distilled water. Dicumyl peroxide (DCP) 98% was obtained from Sigma-Aldrich (St. Louis, MO). Other chemicals were analytical grade and used as received.

Instruments. Attenuated total reflectance infrared (ATR) spectra of the samples were obtained on a Thermo Nicolet Avatar 370 FT-IR spectrometer with ATR accessory (Woburn, MA). The mechanical properties of the tubing samples were evaluated by a MTS Insight I Electromechanical Testing System (Eden Prairie, MN). The MTS tensile testing method was followed with 2 in. of grip separation and 10 in./min of test speed. Each test was repeated 6 times.

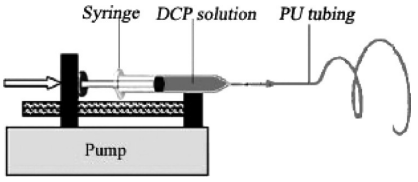


Figure 1. Experimental setup for DCP absorption into the inner walls of PU tubing.

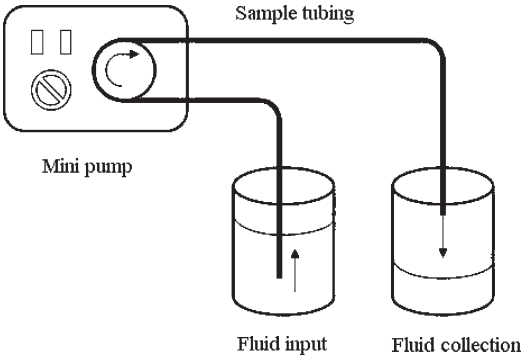


Figure 2. Experimental setup for treatments of the inner surfaces of the tubing.

Preabsorption of the Initiator DCP into the Inner Walls of PU Tubing. The THF solution of 20 wt % DCP flowed through the small-bore PU tubing using a syringe pump (Chemyx Inc. Stafford, TX) at a dispense rate of 8 mL/h for 160 min, as schematically shown in Figure 1. The resulting tubing was dried in open air for 60 min, and then the inner surface was blown with dry N_2 gas for 30 min to remove THF.

Grafting MAA onto the Inner Surfaces of the DCP-Containing PU Tubing (MAA-grafted-PU). The grafting polymerization of MAA onto the inner surfaces of PU tubing preabsorbed with DCP was performed using a variable-speed mini pump (Control Company, Friendswood, TX).[20,26,27] In this study, the PU tubing was immersed in a water bath maintained at 90 °C-95 °C. An aqueous solution containing 10 wt % MAA was pumped through the PU tubing at 1.0−1.5 mL/min (1 min on, 19 min off) for 3 h. After the reaction, the grafted PU tubing was washed with distilled water at 90 °C for an hour to remove possible homopolymers and unreacted MAA, followed by flowing with circulating ethanol to remove the residual DCP. The resulting tubing was dried at 40 °C for 24 h. The thickness of grafted MAA polymer layer was measured with a precision digital caliper (Carrera Precision, CP7906).

Chlorinating the inner Surfaces of the MAA-Grafted-PU Tubing. The inner surfaces of the MAA-grafted-PU tubing were chlorinated at room temperature using the variable-speed mini pump, as shown in Figure 2.[20] In this treatment, the input fluid (0.6 wt % sodium hypochlorite solution containing 0.005 wt % Triton X-100) was pumped through the MAA-grafted-PU tubing at room temperature for 2 h. The pH value of the flowing solution was adjusted to pH 4 with acetic acid to improve chorination efficacy,[27] and the flow rate was kept at 1.0−1.5 mL/min. After chlorination, the input fluid was changed to distilled water, which washed the tubing for 1 h to remove any residual free chlorine (the washing water was tested with KI/starch to ensure that most of the free chlorines were washed away). The resulting tubing was air-dried to reach constant weights.

The active chlorine contents of the inner walls of the chlorinated MAA-grafted-PU tubing were determined by an iodometric titration

method reported previously.[28] Briefly, 8 cm sections (the inner surface area was around 4.0 cm^2) of the chlorinated MAA-grafted-PU tubing were cut into small pieces, which were treated with 1 g KI in 10 mL of distilled water containing 0.5 mL of acetic acid at room temperature under constant stirring for 50 min, followed by the addition of 30 mL ethanol, with further stirring for 20 min. The formed I_2 was titrated by 0.001 mol/L of standardized sodium thiosulfate ($Na_2S_2O_3$) solution. The same length of unchlorinated MAA-grafted-PU tubing sample was also titrated using the same method as controls. Available active chlorine content on the chlorinated MAA-grafted-PU tubing was calculated according to eq 1

$$[Cl] = \frac{35.5}{2} \times \frac{(W_{Cl} - W_0)}{S_{Cl}} \quad (1)$$

Where [Cl] was the active chlorine content ($\mu g/cm^2$), and W_{Cl} and W_0 were the weight (g) of the $Na_2S_2O_3$ solutions consumed in the titration of the chlorinated and unchlorinated sample tubing, respectively; and S_{Cl} was the inner surface area of the chlorinated tubing sample (cm^2). Each test was repeated 3 times.

Bacterial Initial Adhesion. The challenging bacteria *P. aeruginosa* (ATCC 10145) was purchased from American Type Culture Collection (ATCC, Manassas, VA). In the microbial studies, the bacteria were grown in nutrient broth at 37 °C for 24 h. The bacterial suspensions were centrifuged, washed with sterile phosphate buffered saline (PBS), and then resuspended in PBS to predetermined densities (CFU/mL).[29] To ensure lab safety, all the microbial studies followed the guidelines provided by the U.S. Department of Health and Human Services.[30]

To test the initial adhesion of the bacteria onto the PU tubing samples, a series of 2-cm sections of the original PU tubing, unchlorinated MAA-grafted-PU tubing, and chlorinated MAA-grafted-PU tubing were immersed individually in vials containing 10 mL of 10^6-10^7 CFU/mL of *P. aeruginosa* suspension in PBS. The vials were shaken gently at 37 °C for 1 h in a water bath to allow microbial adhesion. After shaking, each section was taken out of the bacterial suspension with sterile forceps, and gently washed 3 times with sterile PBS to remove any nonadherent bacteria. Half of the washed tubing sections were sonicated individually for 10 min using an Ultrasonic cleaner (Branson 1510) to transfer the adherent bacteria into sterile PBS.[20] The resulting solution was then serially diluted, and 0.1 mL of each diluent was placed onto nutrient agar plates. After overnight incubation at 37 °C, the colony forming units on each agar plate were counted, the level of adherent bacteria on each tubing section was calculated, and the results were presented as CFU/cm^2.[2,20,31,32] Each test was repeated at least 3 times.

At the same time, the other half of the washed tubing sections were fixed in 2.5% of glutaraldehyde in 0.1 M sodium cacodylate buffer and stored at 4 °C overnight. At the end of the fixation, the samples were washed 3 times with PBS, followed by gradual dehydration with 25, 50, 70, 95, and 100% ethanol (10 min at each concentration).[33] The resulting samples were dried, sputter-coated with gold, and observed with a Quanta 450 SEM (FEI Company).

***In vitro* Biofilm-Controlling Activity in a Continuous Bacterial Flow Model.** To simulate biofilm growth on DUWL tubing, an in vitro continuous bacterial flow model was used to evaluate the biofilm-controlling functions of the new *N*-halamine-based PU tubing. The setup of this experiment was very similar to that of the chlorination treatment, as shown in Figure 2. Freshly prepared bacteria suspensions containing 1×10^4 to 1×10^5 CFU/mL of *P. aeruginosa* in 10% sterile PBS (to avoid bacteria sudden lysing) were used as the source water. On weekdays, bacterial flow continued for 8 h per day at a rate of 1.0−1.5 mL/min. At nights and on weekends, the pump was shut off. The bacteria solution was changed weekly. After different periods of bacterial flow, a series of 2 cm sections were cut from the tubing. Those sections were gently washed 3 times with PBS to remove nonadherent bacteria. Half of the sections were sonicated for 10 min to determine the level of recoverable adherent bacteria, and the other half was subjected to fixation and SEM observation, using the same procedures as described in the section above. Meanwhile, the active chlorine contents on the inner walls of the new *N*-halamine-based PU tubing after different periods of bacterial flow were determined with iodometric titration, as described before. The original untreated PU tubing was tested under the same conditions to serve as controls. Each test was repeated at least 3 times.

Rechargeability of the Biofilm-Controlling Activity on the Inner Surface of the New *N*-Halamine PU Tubing. After a certain period of bacterial flow, the source water was changed to diluted chlorine bleach (0.30 wt % of sodium hypochlorite solution containing 0.005 wt % of Triton X-100), and the *N*-halamine-based PU tubing (the sample) and the untreated original PU tubing (the control) were rechlorinated using the same procedure as described before. The biofilm-controlling functions of the rechlorinated sample and control tubing were reevaluated. The rechlorinating and reevaluating processes were repeated 3 times to fully characterize the rechargeability of the biofilm-controlling function of the new *N*-halamine-based PU tubing.

RESULTS AND DISCUSSION

Preparation and Characterization of Chlorinated-MAA-Grafted-PU Tubing. PU is one of the most widely used dental/medical device materials because of its wide availability, good mechanical properties, biocompatibility, and low cost.[34,35] However, like most conventional polymeric materials, PU is susceptible to microbial adhesion, colonization and biofilm formation, particularly in small-bore tubing applications because of the large surface-area-to-volume ratio, low flow rates, and frequent quiescent periods.[1−3] Although considerable efforts have been devoted to PU surface modification,[36−39] little has been done for functionalization of the inner surfaces of small-bore PU tubing (e.g., inner diameter of 1/16 in., or 1.6 mm).

Here, we report a simple and practical surface grafting technique to introduce *N*-halamine-based rechargeable antimicrobial functionality onto the inner surfaces of continuous small-bore PU tubing. As schematically shown in Figure 3, the new approach consists of three steps: (1) preabsorption of the initiator dicumyl peroxide (DCP) into tubing inner walls, (2) surface grafting methacrylamide (MAA) from an aqueous solution via the preabsorbed DCP, and (3) chlorination of the inner surfaces of the MAA-grafted-PU tubing with diluted chlorine bleach to transform the amide groups of the grafted MAA side chains into acyclic *N*-halamines, which showed potent biocidal effects against Gram-positive bacteria, Gram-negative bacteria, fungi, and viruses in our previous studies.[27]

We choose DCP, one of the most widely used dialkyl peroxides, as the initiator rather than other classes of initiators because our screening tests demonstrated that keeping other conditions constant, DCP led to a much higher MAA grafting yield than other widely used water-soluble inorganic (e.g., potassium persulfate, ceric ammonium nitrate, etc.) or oil-soluble organic (e.g., azobisisobutyronitrile, benzoyl peroxide, etc.) initiators (data not shown). This was because unlike the water-soluble initiators, DCP had a high solubility in PU and very low solubility in aqueous solutions. Thus, during the MAA grafting process, instead of entering the MAA aqueous solution to initiate MAA homopolymerization, DCP mainly located on the PU tubing inner surfaces to initiate grafting polymerization. On the other hand, unlike other classes of oil-soluble organic initiators, during MAA grafting, DCP generated alkoxy radicals, which had a much higher tendency to abstract hydrogen atoms from PU to

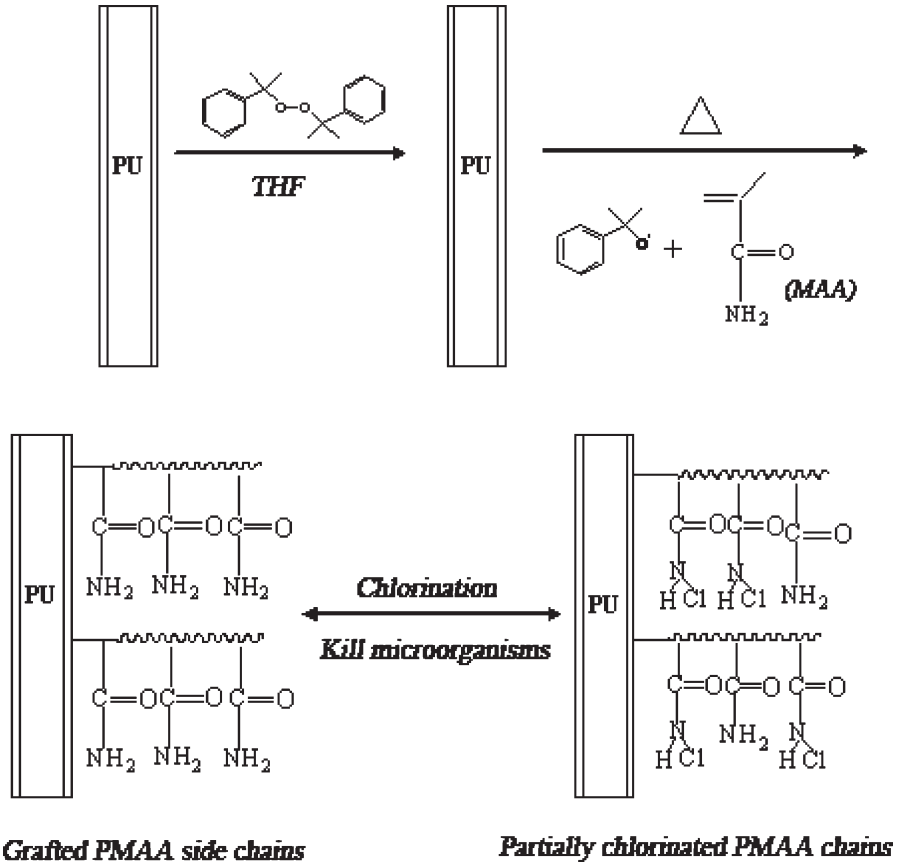


Figure 3. Preparation of the chlorinated MAA-grafted-PU tubing to achieve rechargeable biofilm-controlling functions.

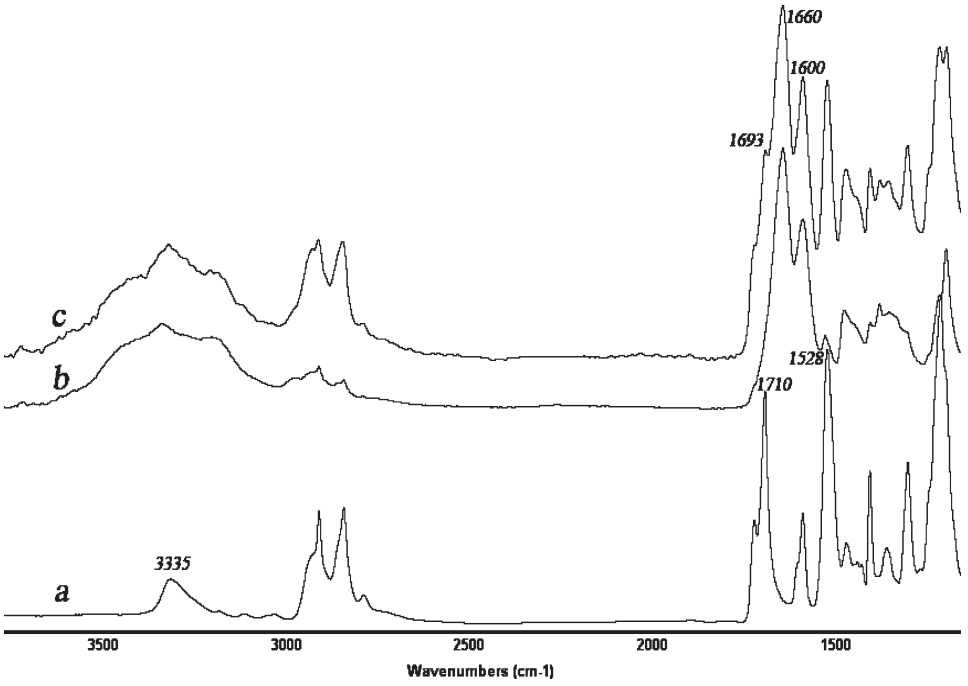


Figure 4. ATR spectra of the inner surfaces of (a) original PU tubing, (b) MAA-grafted-PU tubing, and (c) chlorinated MAA-grafted-PU tubing.

generate PU macro-radicals than the benzoyloxy radicals generated by benzoyl peroxide or the cyanoalkyl radicals generated by azobisisobutyronitrile.[40] PU macro-radicals were the main sites that could initiate MAA grafting onto the tubing's inner surfaces.

Table 1. Mechanical Properties of the Original PU Tubing and the Chlorinated-MAA-Grafted PU Tubing[a]

mechanical properties	original PU tubing	chlorinated-MAA-grafted PU tubing
tensile strength (MPa)	28.8 ± 3.4	21.2 ± 1.0
elongation at break (%)	301.5 ± 36	320.2 ± 17

[a] Grip separation was 2 in. and the test speed was 10 in./min.

Our new grafting approach readily grafted a layer of MAA polymers on the inner surfaces of small-bore PU tubing. The content of the grafted MAA on the tubing could be easily controlled by controlling the grafting conditions (e.g., MAA content in the solution, grafting time, etc.). Under our reported conditions, a layer of ca. 0.2 mm of grafted MAA-based polymer could be formed on the tubing inner surfaces, which contained around 50−60 $\mu g/cm^2$ of active chlorines after chlorination.

The presence of grafted MAA polymer chains on the inner surfaces of PU tubing was further confirmed with ATR studies, as shown in Figure 4. In the spectrum of the original PU tubing (Figure 4a), the urethane group showed absorption at 3335 and 1528 cm^{-1}. The band at 1710 cm^{-1} was designated to the carbonyl groups in the esters of the soft segments of the PU.[41] After the surface grafting polymerization of MAA, in the spectrum of MAA-grafted-PU tubing (Figure 4b), the PU characteristic signals were hardly detectable. The signals at 1660 and 1600 cm^{-1} were attributable to the amide I and amide II bands of the amide groups, respectively,[42] suggesting that the grafted MAA chains were the major component of the resulting tubing surfaces. Figure 4c presented the ATR spectrum of the chlorinated MAA-grafted-PU tubing, which was very similar to that of spectrum b (unchlorinated MAA-grafted-PU). However, in addition to the strong 1660 cm^{-1} peak, spectrum c showed a weak shoulder at 1693 cm^{-1}, which was assigned to the C═O stretching vibration of the chlorinated amide groups (−CONHCl),[27,43] indicating that upon chorine bleach treatment, acyclic *N*-halamines were formed on the tubing surfaces.

The effects of the grafting and chlorination reactions on mechanical properties were summarized in Table 1. Compared with the original untreated PU, the tensile strength of the chlorinated-MAA-grafted PU was slightly decreased, which could be caused by the acetone wash/grafting/chlorination process, but the elongation at break was almost unchanged. These results suggested that the new *N*-halamine-based PU tubing had comparable mechanical properties of the original tubing, which could be readily used in real applications.

Bacterial Initial Adhesion. The inner surfaces of the new PU tubing were grafted with MAA-based acyclic *N*-halamines, whose biocidial activities against a wide range of microorganisms have been confirmed in our previous study.[27] It was thus expected that the chlorinated MAA-grafted-PU tubing could effectively prevent or reduce bacterial adhesion, the first step of biofilm formation,[44−46] by killing the incoming bacteria upon contact.[47] Table 2 presented the level of recoverable adherent bacteria from the tubing after contacting with 1×10^6 to 1×10^7 CFU/mL of *P. aeruginosa* for 1 h. From the original PU tubing and the unchlorinated-MAA-grafted-PU tubing, as high as 1×10^4 CFU/cm^2 of adherent *P. aeruginosa* were recovered by sonication, indicating that *P. aeruginosa* had strong abilities to adhere onto those tubing surfaces, which could lead to biofilm formation.

Table 2. Levels of Recoverable Bacteria from the Original PU Tubing, MAA-Grafted-PU Tubing, and Chlorinated MAA-Grafted-PU Tubing in Bacterial Initial Adhesion Test[a]

tubing sample	recoverable adherent bacteria (CFU/cm^2)
original PU tubing	$(5.1 \pm 1.8) \times 10^4$
unchlorinated MAA-grafted-PU tubing	$(6.0 \pm 2.3) \times 10^4$
chlorinated MAA-grafted-PU tubing[b]	0

[a] *P. aeruginosa* concentration was 1×10^6 to 1×10^7 CFU/mL and the adhesion time was 1 h. [b] The active chlorine content of the chlorinated MAA-grafted-PU tubing was 53 $\mu g/cm^2$.

However, after chlorination, acyclic *N*-halamines structures were formed on the surfaces of chlorinated-MAA-grafted-PU tubing. The positive chlorines of the chlorinated MAA side chains could be directly transferred to appropriate receptors in the bacteria cells, and this reaction could effectively destroy or inhibit the enzymatic or metabolic process of the cells, leading to the expiration of the bacteria.[21,47] As a result, from the surfaces of the chlorinated MAA-grafted-PU tubing, no adherent *P. aeruginosa* could be recovered by sonication, demonstrating that the new *N*-halamine-based PU tubing had potent antimicrobial/antiadherent effects against the test microorganisms.

SEM observations further confirmed the recoverable adherent bacteria results, as shown in Figure 5. On the surfaces of the original PU tubing (Figure 5a), a large number of adherent rod-shaped *P. aeruginosa* could be observed after 1 h of contact with 1×10^6 to 1×10^7 CFU/mL of *P. aeruginosa* (see arrows in the figure). On the surfaces of the chlorinated MAA-grafted-PU tubing (Figure 5b), however, almost no adherent bacteria could be detected.

Biofilm-Controlling Function in the Continuous Bacterial Flow Model. The potent antimicrobial/antiadherent activities of the chlorinated MAA-grafted-PU tubing pointed to effective biofilm-controlling function. To evaluate this, an *in vitro* continuous bacterial flow model was used to challenge the inner surfaces of the *N*-halamine-based PU tubing, using the original PU tubing as controls. Shown in Figure 6 were the SEM results of the tubing inner surfaces after 1−6 weeks of bacteria flow. After 1 week of flow (Figure 6, A), scattered rod-shaped *P. aeruginosa* adhered to the original PU tubing surfaces, which gradually developed into layered biofilms in the following weeks (Weeks 2−6; Figure 6, B−F). On the *N*-halamine-based PU tubing, however, almost no adherent bacteria could be observed in Week 1−4 (Figure 6G−J). Even after 5−6 weeks of flow, only scattered adherent bacteria could be observed (see arrows in Figure 6K−L), and no biofilms were formed, suggesting that the new *N*-halamine-based PU tubing inner surfaces had potent biofilm-controlling functions against *P. aeruginosa*, one of the most cited water bacteria that are responsible for biofilms in DUWLs.

To provide further information on the biofilm-controlling functions of the new *N*-halamine-based PU tubing, Table 3 presented the level of recoverable adherent *P. aeruginosa* from the original PU tubing and chlorinated-MAA-grafted PU tubing after different periods of bacterial flow. The original PU tubing inner surfaces showed much higher level of recoverable bacteria: after one week of flow, the mean of adherent bacteria recovered from sonication was 2.0×10^2 CFU/cm^2; with longer flowing time, this level gradually increased, and at Week 6, as high as

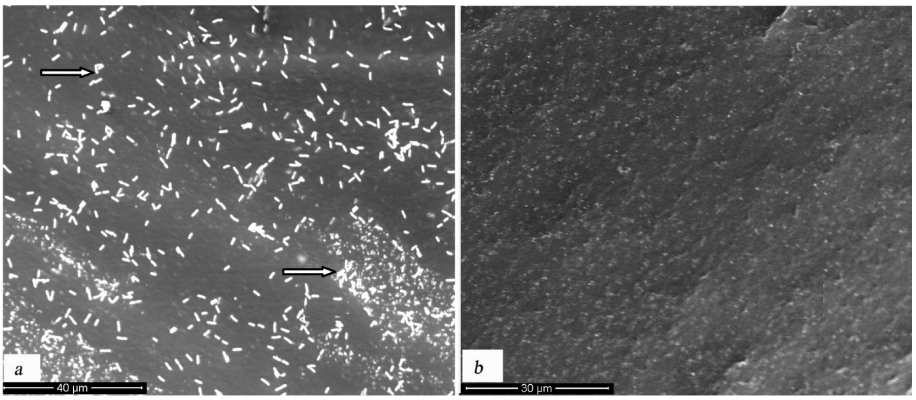


Figure 5. SEM results of bacterial initial adhesion on (a) the original PU tubing, and (b) chlorinated MAA-grafted-PU tubing.

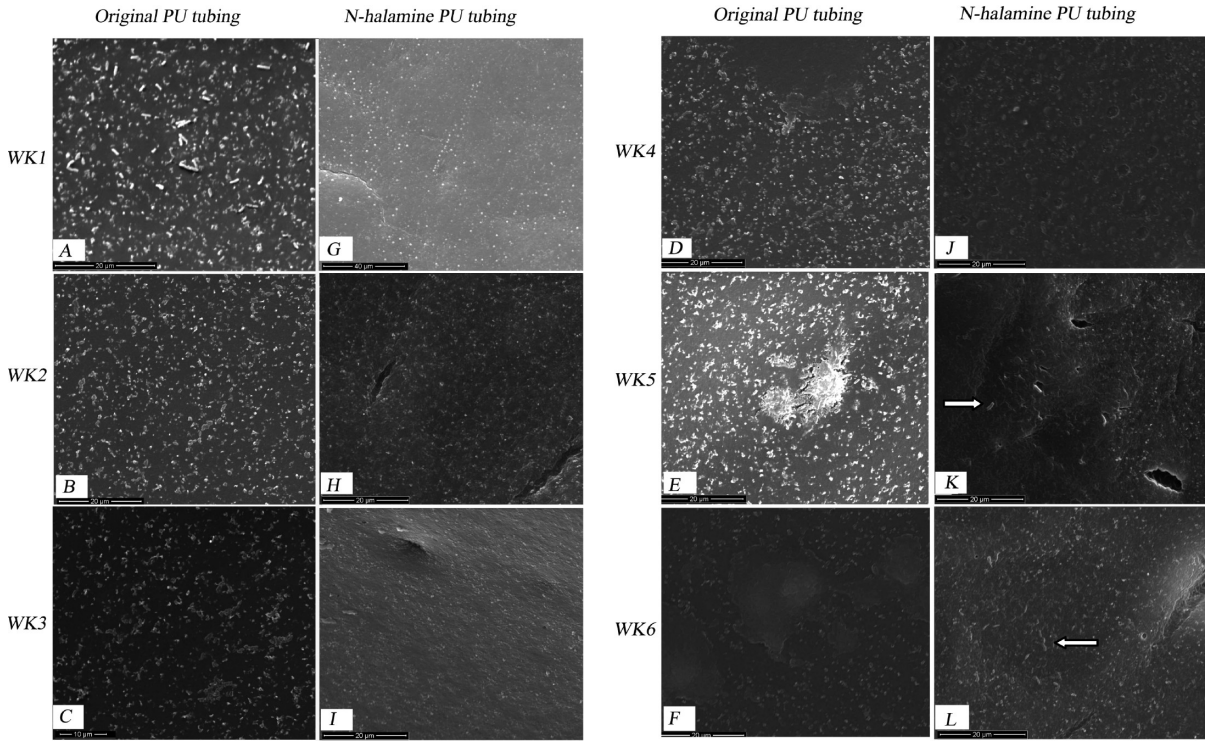


Figure 6. SEM images showing bacteria adhesion taken throughout the 6-week bacterial flow study from (A−F) the original PU tubing, and (G−L) the chlorinated-MAA-grafted PU tubing.

Table 3. Level of Recoverable *P. aeruginosa* from the Original PU Tubing and *N*-Halamine PU Tubing after Different Periods of Bacterial Flow[a]

	recoverable adherent *P. aeruginosa* after different periods of bacterial flow (CFU/cm^2)					
tubing samples	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
original PU tubing	$(2.0 \pm 1.2) \times 10^2$	$(1.6 \pm 0.9) \times 10^3$	$(4.1 \pm 1.7) \times 10^3$	$(5.5 \pm 2.7) \times 10^4$	$(1.4 \pm 0.6) \times 10^5$	$(2.4 \pm 1.9) \times 10^5$
N-halamine PU tubing	0	(8.2 ± 2.1)	$(3.5 \pm 1.5) \times 10$	$(1.2 \pm 0.9) \times 10^2$	$(2.7 \pm 1.3) \times 10^2$	$(1.1 \pm 0.8) \times 10^3$

[a] The *P. aeruginosa* concentration in the flowing solution was 1×10^4 to 1×10^5 CFU/mL. The active chlorine content of the chlorinated MAA-grafted-PU tubing was 53 μg/cm^2.

1×10^5 CFU/cm^2 of *P. aeruginosa* were recovered from the tube's inner surfaces. However, on the chlorinated MAA-grafted-PU tubing inner surfaces, because of the presence of acyclic *N*-halamines, no bacteria could be recovered from the surfaces in Week 1. In Week 2, the level of recoverable cells was in the range of only several CFU/cm^2, and in Week 4, the recoverable level

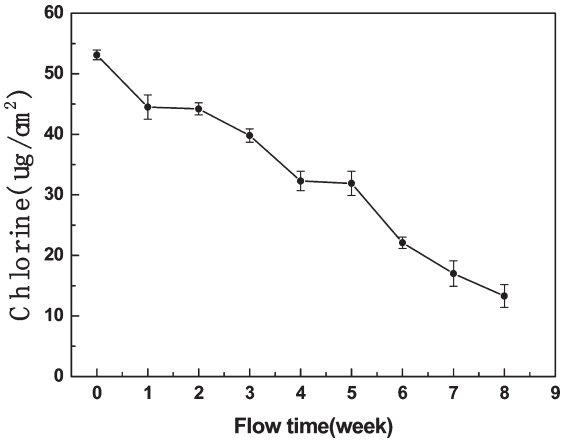


Figure 7. Effects of bacterial flow time on the active chlorine content of the inner surfaces of the *N*-halamine PU tubing.

was in the lower range of 1×10^2 CFU/cm^2. Even after 6 weeks of bacterial flow, only 1×10^3 CFU/cm^2 of adherent bacteria could be recovered from the *N*-halamine PU tubing surfaces, more than 100 times lower than the level from the original PU tubing.

The SEM and bacteria culturing results suggested that under the current experimental conditions, the chlorinated MAA-grafted-PU tubing surfaces could effectively prevent bacterial adhesion and the subsequent biofilm formation for at least 3 to 4 weeks. After that, this effect began to decrease. This trend could be explained by the fact that the biofilm-controlling effects of the *N*-halamine PU tubing surfaces would consume the covalently bound chlorines; when the active chlorine content decreased to lower than a certain limit, bacteria began to adhere to the surfaces, but the level of the adherent bacteria was much lower than the level on the original PU because the residual covalently bound chlorines on the surfaces could still provide antimicrobial/antiadherent functions. To test this hypothesis, we determined the active chlorine contents on the inner surfaces of the *N*-halamine PU tubing weekly by iodometric titration during the *P. aeruginosa* flowing test, and the results were shown in Figure 7. The freshly chlorinated surfaces contained 53 μg/cm^2 of active chlorine. Increasing flowing time decreased the chlorine content: after 2 weeks of flow, the surfaces had 41 μg/cm^2 of active chlorine; at Week 3 and Week 4, the chlorine content was 39 and 32 μg/cm^2, respectively; and when the flowing time was extended to 8 weeks, the chlorine content further decreased to 11 μg/cm^2. These results suggested that chlorine content was the determining factor of the biofilm-controlling functions of the *N*-halalmine-based PU tubing materials, and under our experimental conditions, 30−39 μg/cm^2 was the range of minimum chlorine content on the new PU tubing's inner surfaces for complete prevention of *P. aeruginosa* adhesion/colonization.

Rechargeability of the Biofilm-Controlling Functions of the *N*-Halamine PU Tubing. Although longer flowing time could decrease the biofilm-controlling activities of the new *N*-halamine-based PU tubing, the function could be regenerated by a simple diluted chlorine bleach treatment. To determine the effects of rechlorination on biofilm-controlling activities, in the biofilm-controlling studies (see the section above), after 3 weeks of bacterial flow, the *N*-halamine PU tubing (the sample) and the original PU tubing (the control) were rechlorinated with diluted bleach (0.30 wt % of sodium hypochlorite solution), using the same procedure as in the preparation of the first-generation *N*-halamine PU tubing. The biofilm-controlling functions of the rechlorinated control and sample tubing were reevaluated following the same experimental setup as described above. The rechlorination and reevaluation processes were repeated three times.

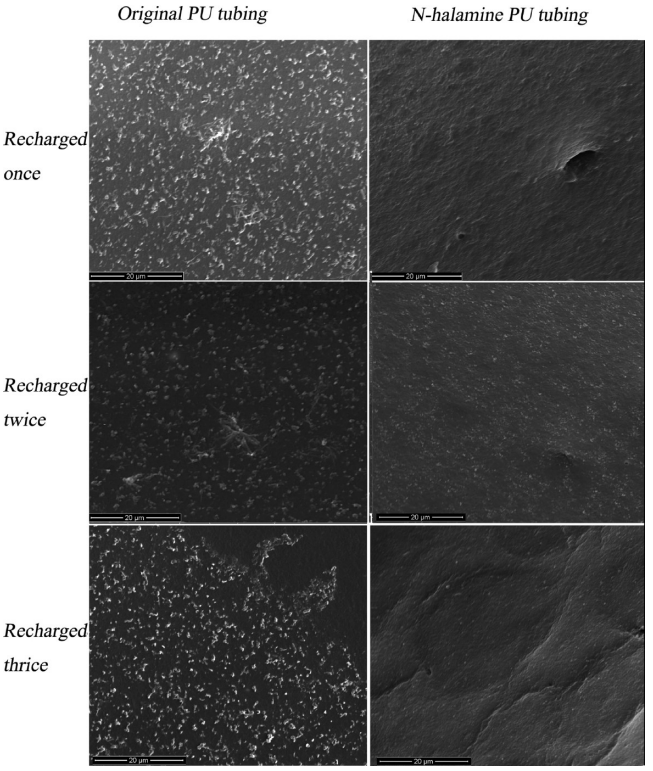


Figure 8. SEM results of the biofilm-controlling functions of the rechlorinated original PU tubing (left panel), and the rechlorinated *N*-halamine PU tubing (right panel). After each rechlorination, bacterial flow continued for 3 weeks, and SEM images were taken.

The SEM results of the biofilm-controlling functions of the control (rechlorinated original PU) tubing and the sample (rechlorinated MAA-grafted-PU) tubing were presented in Figure 8. On the control tubing (Figure 8, left panel; rechlorinated 1, 2, and 3 times, respectively), after each rechlorination treatment, 3 weeks of continuous bacterial flow led to bacteria clusters with slimes on the tubing inner surfaces. On the rechlorinated sample tubing (Figure 8, right panel; rechlorinated 1, 2, and 3 times, respectively), however, no adherent bacteria could be detected after 3 weeks of bacterial flow following each rechlorination treatment, suggesting that the biofilm-controlling functions of the chlorinated MAA-grafted-PU tubing were fully rechargeable.

Presented in Table 4 was the level of recoverable adherent bacteria from the control tubing and the sample tubing after each rechlorination cycle. After one rechlorination treatment, the mean of recoverable adherent bacteria from the control tubing was 7.0×10^3 CFU/cm^2 after 3 weeks of bacterial flow; after two recharging treatments, the level of recoverable adherent bacteria was 2.2×10^4 CFU/cm^2; and after three recharging treatments, as high as 3.1×10^4 CFU/cm^2 of adherent bacteria could be recovered from the same tubing surfaces after 3 weeks of bacterial flow. This increasing trend in the mean of recoverable adherent bacteria after each rechlorination treatment might imply that during the rechlorination treatment, although diluted bleach

Table 4. Level of Recoverable Adherent *P. aeruginosa* from the Original PU Tubing and the *N*-Halamine PU Tubing after Different Rechlorination Treatments[a]

	level of recoverable *P. aeruginosa* (CFU/cm^2)		
tubing	rechlorinated once	rechlorinated twice	rechlorinated thrice
original PU tubing	$(7.0 \pm 3.3) \times 10^3$	$(2.2 \pm 0.8) \times 10^4$	$(3.1 \pm 1.2) \times 10^4$
N-halamine PU tubing	$(6.7 \pm 0.5) \times 10$	$(3.2 \pm 1.7) \times 10$	$(1.6 \pm 3.7) \times 10^2$

[a] *P. aeruginosa* concentration in the flowing solution was 1×10^4 to 1×10^5 CFU/mL; the bacteria flow time after each rechlorination was 3 weeks.

could inactivate most of the adherent bacteria, some of the exopolysaccharide glycocalyx polymers and other residues secreted by the adherent bacteria might still attach to the tubing surface, making it more accessible for *P. aeruginosa* colonization on the control tubing in the subsequent bacterial flow tests.

On the recharged *N*-halamine PU tubing, however, the means of recoverable bacteria after 3 weeks of bacteria flow were 100−1000 times lower than those of the rechlorinated original PU tubing, further suggesting that the chlorinated MAA-grafted-PU tubing had potent and rechargeable biofilm-controlling functions against *P. aeruginosa*.

CONCLUSIONS

In this study, a simple and practical surface grafting technique was developed to introduce *N*-halamine-based rechargeable antimicrobial functionality onto the inner surfaces of small-bore PU tubing to control biofilm formation. The new *N*-halamine-based PU tubing could effectively prevent bacterial initial adhesion, the first step of biofilm buildup. *In vitro* continuous *P. aeruginosa* flow test demonstrated that the *N*-halamine PU tubing completely prevented biofilm formation for at least three to four weeks. After that, the biofilm-controlling activity began to decrease because of the consumption of covalently bound chlorines. However, the lost activity could be repeatedly regenerated by chlorine bleach treatment to further extend biofilm-controlling duration for long-term effects. These unique features make the new tubing materials attractive candidates to control biofilms in dental unit waterlines and a wide range of other related medical, environmental, industrial, and institutional applications.

AUTHOR INFORMATION

Corresponding Author

*Phone: (605) 367-7776; E-mail: yuyu.sun@usd.edu.

ACKNOWLEDGMENT

This study was sponsored by NIH, NIDCR (Grant R01DE018707).

REFERENCES

(1) Mills, S. E. *J. Am. Dent. Assoc.* **2000**, *131*, 1427–1441.
(2) Depaola, L. G.; Mangan, D.; Mills, S. E.; Costerton, W.; Barbeau, J.; Shearer, B.; Bartlett, J. *J. Am. Dent. Assoc.* **2002**, *133*, 1199–1206.
(3) Williams, J. F.; Johnston, A. M.; Johnson, B.; Huntington, M. K.; Mackenzie, C. D.; Path, M. R. C. *J. Am. Dent. Assoc.* **1993**, *124*, 59–65.
(4) Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. *Nat. Rev. Microbiol.* **2004**, *2*, 95–108.
(5) Van Loosdrecht, M. C. M.; Lyklema, J.; Norde, W.; Zehnder, A. J. B. *Microbiol. Rev.* **1990**, *54*, 75–87.
(6) Davey, M. E.; O'toole, G. A. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 847–867.
(7) Ramagea, G.; Tunneyb, M. M.; Patricka, S.; Gormanb, S. P.; Nixonc, J. R. *Biomaterials* **2003**, *24*, 3221–3227.
(8) Gorman, S. P.; McGovern, J. G.; Woolfson, A. D.; Adair, C. G.; Jones, D. S. *Biomaterials* **2001**, *22*, 2741–2747.
(9) Blake, G. C. *Br. Dent. J.* **1963**, *115*, 413–416.
(10) Centers for Disease Control and Prevention Guidelines. *MMWR* **2003**, *52*, RR-17.
(11) American Dental Association Statement on Dental Unit qWaterlines. July 2004. Available at: http://www.ada.org/1856.aspx. qAccessed May 9, 2011.
(12) ADA council on scientific affairs. *J. Am. Dent. Assoc.* **1999**, *130*, 1653-1664.
(13) Robert, H. W.; Karpay, R. I.; Mills, S. E. *J. Am. Dent. Assoc.* **2000**, *131*, 179–183.
(14) Karpay, R. I.; Plamondon, T. J.; Mills, S. E.; Dove, S. B. *J. Am. Dent. Assoc.* **1999**, *130*, 957–965.
(15) Linger, J. B.; Molinari, J. A.; Forbes, W. C.; Farthing, C. F.; Winget, W. J. *J. Am. Dent. Assoc.* **2001**, *132*, 1287–1291.
(16) Ozcan, M.; Kulak, Y.; Kazazoglu, E. *J. Oral. Rehabil.* **2003**, *30*, 290–294.
(17) Porteous, N. B.; Cooley, R. L.; Lau, C. A. *Gen. Dent.* **2003**, *51*, 472–476.
(18) Pai, M. P.; Pendland, S. L.; Danziger, L. H. *Ann. Pharmacother.* **2001**, *35*, 1255–1263.
(19) Raad, I.; Hachem, R.; Zermeno, A.; Dumo, M.; Bodey, G. P. *Biomaterials* **1996**, *17*, 1055–1059.
(20) Luo, J.; Sun, Y. Y. *J. Biomed. Mater. Res., Part A* **2008**, *84A*, 631–642.
(21) Worley, S. D.; Williams, D. E. *CRC. Crit. Rev. Environ. Control* **1988**, *18*, 133–175.
(22) Worley, S. D.; Kim, J.; Wei, C. I.; Williams, J. F.; Owens, J. R.; Wander, J. D.; Bargmeyer, A. M.; Shirtliff, M. E. *Surf. Coat. Int., Part B* **2003**, *86*, 273–277.
(23) Worley, S. D.; Burkett, H. D.; Price, J. F. *Water Resour. Bull.* **1984**, *20*, 369–371.
(24) Barbeau, J.; Gauthier, C.; Payment, P. *Can. J. Microbiol.* **1998**, *44*, 1019–1028.
(25) Whiteley, M.; Bangera, M. G.; Bumgarner, R. E.; Parsek, qM. R.; Teitzel, G. M.; Lory, S.; Greenberg, E. P. *Nature* **2001**, *413*, 860–864.
(26) Hu, S.; Brittain, W. J. *Macromolecules* **2005**, *38*, 6592–6597.
(27) Luo, J.; Sun, Y. Y. *J. Polym. Sci., Part A: Polym. Chem.* **2006**, *44*, 3588–3600.
(28) Braun, M.; Sun, Y. Y. *J. Polym. Sci., Part A: Polym. Chem.* **2004**, *42*, 3818–3827.
(29) Benson, D. E.; Burns, G. L.; Mohammad, S. F. *ASAIO J* . **1996**, *42*, M655–660.
(30) *Biosafaty in Microbiological and Biomedical Laboratories*, 4th ed.; Richmond, J. Y., McKinney, R. W., Eds; U.S. Government Printing Office: Washington, D.C., 1999; p 32.
(31) Yorganci, K.; Krepel, C.; Weigelt, J. A.; Edmiston, C. E. *Intensive Care Med.* **2002**, *28*, 438–442.
(32) Tollefson, D. F.; Bandyk, D. F.; Kaebnick, H. W.; Seabrook, G. R.; Towne, J. B. *Arch. Surg.* **1987**, *122*, 38–43.

(33) Cen, L.; Neoh, K. G.; Kang, E. T. *J. Biomed. Mater. Res.* **2004**, *74A*, 70–80.
(34) Griesser, H. J. *Polym. Degrad. Stab.* **1991**, *33*, 329–354.
(35) Coury, A. J; Slaikeu, P. C.; Cahalan, P. T.; Stokes, K. B; Hobot, C. M. *J. Biomater. Appl.* **1988**, *3*, 130–179.
(36) Rhodes, N. P.; Williams, R. L. *Biomaterials* **2003**, *24*, 5069–5081.
(37) Weibel, D. E.; Vilani, C.; Habert, A. C.; Achete, C. A. *J. Membr. Sci.* **2007**, *293*, 124–132.
(38) Tan, K. T.; Obendorf, S. K. *J. Membr. Sci.* **2007**, *289*, 199–209.
(39) Pulat, M.; Babayigit, D. *J. Appl. Polym. Sci.* **2001**, *80*, 2690–2695.
(40) Russell, K. E. *Prog. Polym. Sci.* **2002**, *27*, 1007–1038.
(41) Guan, J. J.; Gao, C. G.; Feng, L. X.; Sheng, J. C. *J. Biomater. Sci.: Polym. Ed.* **2000**, *11*, 523–536.
(42) Hass, H. H.; MacDonald, R .L. *J. Polym. Sci.. Part A-1: Polym. Chem.* **1971**, *9*, 3583–3593.
(43) Lin, J.; Cammarata, V.; Worley, S. D. *Polymer* **2001**, *42*, 7903–7906.
(44) Dunne, W. M., Jr. *Clin. Microbiol. Rev.* **2002**, *15*, 155–166.
(45) Baumgartner, J. N.; Yang, C. Z.; Cooper, S. L. *Biomaterials* **1997**, *18*, 831–837.
(46) Jansen, B.; Kohnen, W. *J. Ind. Microbiol.* **1995**, *15*, 391–396.
(47) Sun, Y. Y.; Chen, T.; Worley, S. D.; Sun, G. *J. Polym. Sci.. Part A: Polym. Chem.* **2001**, *39*, 3073–3084.