# The Effectiveness of Antibiotic Activity of Penicillin Attached to Expanded Poly(tetrafluoroethylene) (ePTFE) Surfaces: A Quantitative Assessment

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Recent studies identified and established a platform of polymer surface modifications allowing the attachment of penicillin (PEN) to expanded poly(tetrafluoroethylene) (ePTFE) surfaces. The effectiveness of this approach was accomplished by creating surfaces with chemically attached PEN that prevent the proliferation of microbes. In this study, quantitative assessments of PEN effectiveness attached to ePTFE were conducted. Using variable-angle attenuated total reflectance (ATR-FTIR) spectroscopy, the volume concentration changes of PEN were determined as a function of depth from the ePTFE surface. At depths ranging from 0.2 to 1.2 $\mu$m from the surface, PEN concentration levels decrease from 8.85 to 3.33 $\mu g/m^3$. Assessments of concentration levels of the colony forming units (CFUs) of *Staphylococcus aureus* bacteria as a function of contact time with the penicillin-polyethylene glycol spacer separated by maleic anhydride ePTFE (PEN-PEG-MA-ePTFE) surfaces showed profound effectiveness of PEN in preventing microbial proliferation. Hydrolytic stability of PEN-PEG-MA-ePTFE surfaces revealed that even with a 32% loss of PEN due to the cleavage of the ester linkages between PEN and PEG spacer, antimicrobial activity is still maintained.

## Introduction

It is well known that adhesion of bacteria to surfaces of medical implants and devices results in the formation of biofilms and often leads to the outbreak of detrimental infections. According to the U.S. Centers for Disease Control (CDC), approximately 90 000 people annually die in the U.S. alone from infections unrelated to their medical conditions.[1] To alleviate this serious problem, one approach is to disrupt the proliferation of microbes on surfaces, and a number of studies[2−7] have investigated and developed surface modifications with the goal of eliminating harmful microbial films. The recent Roundtable on Biomedical Engineering Materials and Applications (BEMA) at the National Academy of Sciences (NAS) manifests the need for further advances in this field.[8]

Although a number of polymer surface modification reactions have been offered ranging from grafting-from[9−13] and grafting-to[14−16] approaches, considering the fact that the majority of polymeric substrates are hydrophobic and inert, it is often difficult to develop a universal surface modification that would provide a platform for further reactions of a variety of species leading to antimicrobial, antifouling, or anticoagulating surface properties. To add to this effort, we developed solventless, clean, and ultrafast microwave plasma surface reactions that utilize maleic anhydride, which, upon hydrolysis, forms COOH groups on poly(dimethylsiloxane) (PDMS),[17−20] poly(vinylidene fluoride) (PVDF),[21] and expanded poly(tetrafluoroethylene) (ePTFE).[22] The presence of COOH groups serves as a platform for further surface reactions shown in Figure 1, whereby the attachment of penicillin (PEN) was one of the recent developments that tested positive in inhibiting the proliferation of microbes.[22] It should be also noted that the effectiveness of these surface reactions in preventing microbial film formation is not only accomplished by the presence of PEN, but one of the key components is a polyethylene glycol (PEG) spacer[23] that was placed between COOH surface groups and terminal PEN. This approach facilitates the mobility of PEN molecules to become effective in preventing the proliferation of microbes, which we believe is a critical component to achieve effectiveness against infections. Thus, a crucial component is the length of a PEG spacer and its lengths dispersity, which results in sub-nano level surface roughness resulting in antimicrobial activity as well as minimized protein adsorption.

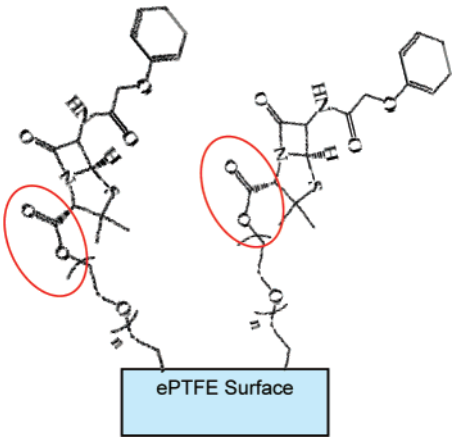


**Figure 1.** Molecular structure of PEN-PEG attached to the surface of MA-ePTFE.

In view of the previous finding and our limited knowledge regarding the quantitative effectiveness of antimicrobial activity of PEN-PEG-MA-ePTFE surfaces, this study extends the scope

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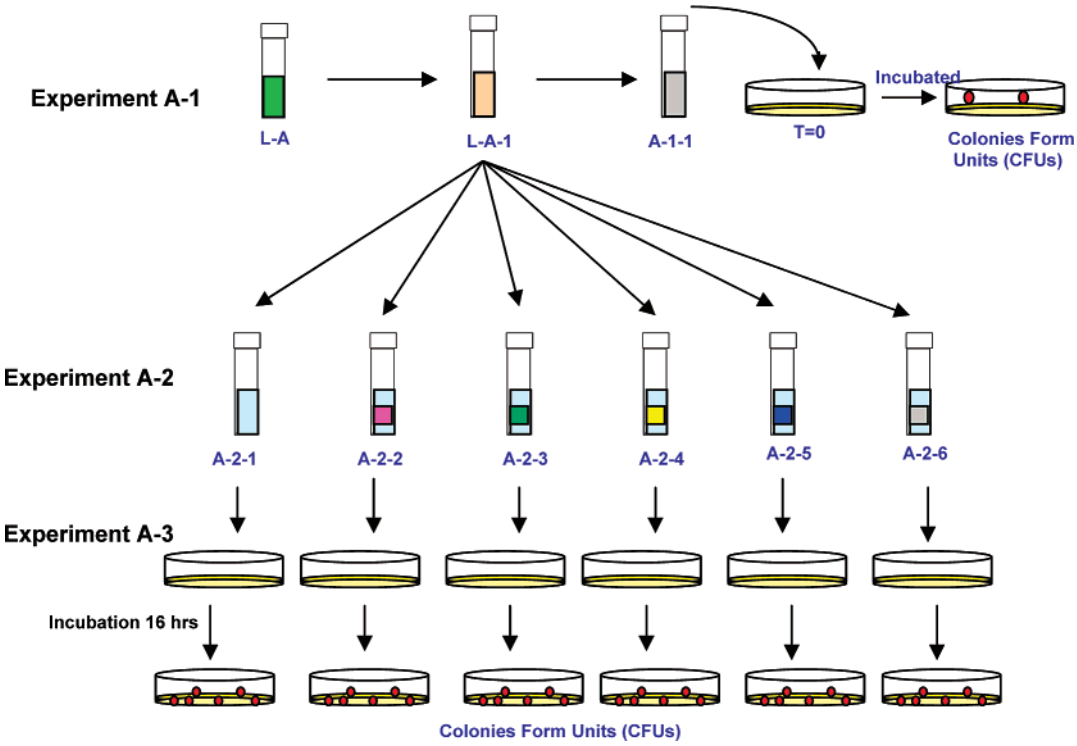
**Scheme 1.** Schematic Diagram of Experimental Sequences To Determine Colony-Forming Units (CFU)




of the previous findings and focuses on a quantitative assessment of antibiotic effectiveness of these surfaces.

## Experimental Section

The sequence of quantitative assessments of the antimicrobial activity of PEN-PEG-MA-ePTFE surfaces as a function of time is outlined in Scheme 1. In experiment A-1, 20 $\mu$L of the *Staphylococcus aureus* overnight culture (label L-A) was combined with 15 mL of fresh Luria−Bertani (LB) broth. This culture is labeled L-A-1. Next, 2 $\mu$L of the liquid culture from L-A-1 was extracted and mixed with 3 mL of LB broth. This culture is labeled A-1-1. Next, 10 $\mu$L of the A-1-1 liquid culture was extracted and diluted 5 times to reduce the amount of bacteria. This culture was spread over an agar plate to grow colonies (time = 0). Colony forming units (CFUs) at time = 0 were determined by counting the colonies formed after incubating the agar plate for 16 h at 37 °C. The concentration at time = 0 is used as an initial concentration. In experiment A-2, 2 $\mu$L of the liquid culture from L-A-1 was placed into six test tubes containing 3 mL of fresh LB broth. The content of the test tube A-2-1 was the positive control, whereas solutions A-2-2 through A-2-6 contained ePTFE, MA-ePTFE, PEG-MA-ePTFE, PEN-PEG-MA-ePTFE, and ePTFE exposed in PEN solution (PEN-ePTFE), respectively. Typical size of each polymer specimen was 7 × 7 × 1.5 mm. The reason of exposing ePTFE in PEN solution was to examine if PEN physisorbs on the surface of ePTFE and becomes effective in antimicrobial activity. After ePTFE immersion in PEN, ePTFE was washed in methylene chloride and dried in a desiccator. All test tubes were incubated at 37 °C for 3, 6, 9, 12, and 24 h. In experiment A-3 shown in Scheme 1, aliquots of the liquid culture from each test tube were extracted at 3, 6, 9, 12, and 24 h time intervals, diluted 6, 7, 8, and 9 orders of magnitude, respectively, and spread on agar plates to grow the colonies. After incubating the plates for 16 h, CFUs in each aliquot were determined. Each datum represents an average of three measurements.

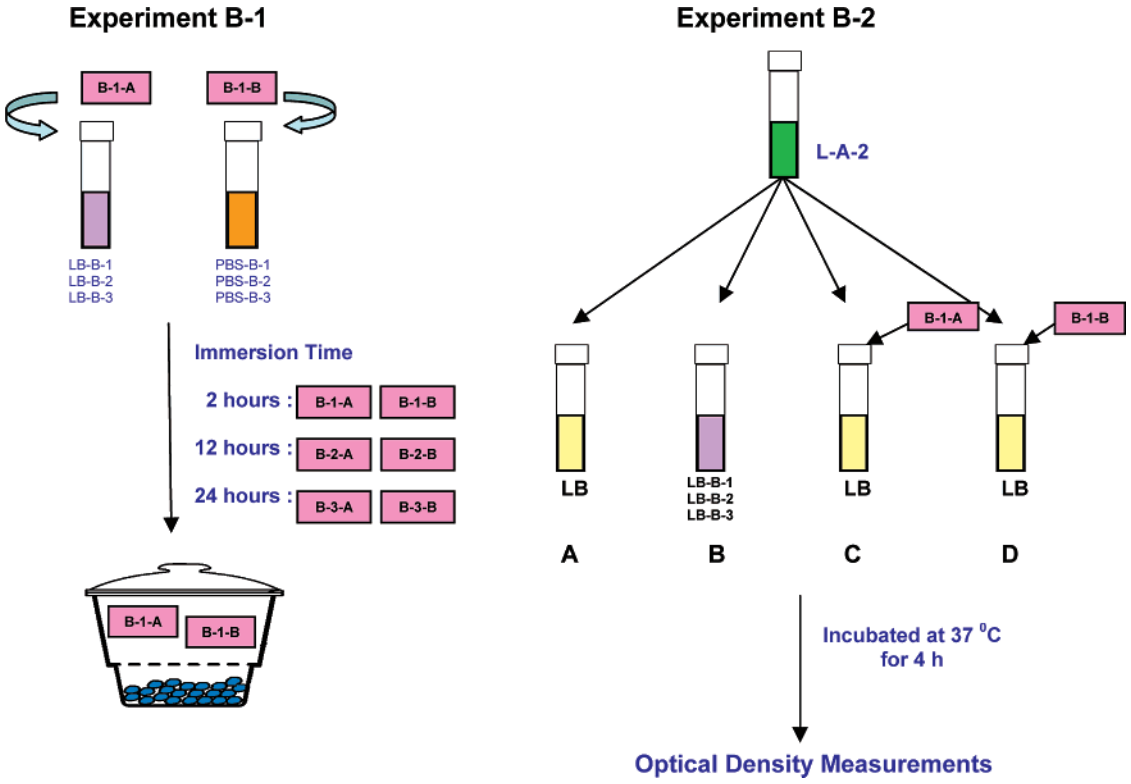
Hydrolytic stability of PEN-PEG-MA-ePTFE surfaces was conducted by immersing a specimen in a phosphate-buffered saline (PBS) solution and incubating it at 37 °C. After 0, 1, 2, 3, 6, 9, 12, 18, and 24 h exposure times, each specimen was dried and analyzed using ATR-FTIR spectroscopy.

Another set of experiments was performed to determine stability of PEN attached to ePTFE. This is depicted in Scheme 2. In experiment B-1, PEN-PEG-MA-ePTFE specimens were immersed in the LB broth and PBS buffered solution for 2 (labeled B-1-A and B-1-B in Scheme 2), 12 (labeled B-2-A and B-2-B in Scheme 2), and 24 h (labeled B-3-A and B-3-B in Scheme 2). After exposure, all specimens were dried in a desiccator for 24 h, and LB that was used to immerse all specimens LB-B-1 (2 h), LB-B-2 (12 h), and LB-B-3 (24 h) were used again in experiment B-2. In this case, 2 $\mu$L solution of the *S. aureus* culture that was incubated for 16 h (L-A-2) was placed into four separate test tubes labeled A, B, C, and D. Test tube A contained fresh LB, whereas B contained LB-B-1, LB-B-2, and LB-B-3, tube C contained fresh LB and specimen B-1-A from experiment B-1, and test tube D contained fresh LB and the specimen B-1-B from experiment B-1. All test tubes were incubated for 4 h, followed by solution optical density measurements using a UV−vis spectrometer (Beckman DU-600).

Modified ePTFE surfaces (PEN-PEG-MA-ePTFE) were prepared as previously described.[22] Attenuated total reflectance Fourier transform infrared (ATR FTIR) spectra were collected using a Bio-Rad FTS-6000 FT-IR single beam spectrometer set at a 4 $cm^{-1}$ resolution equipped with a deuterated triglycine sulfate (DTGS) detector and a 45° face angle Ge crystal. Each spectrum represents 400 co-added scans ratioed against a reference spectrum obtained by recording 400 co-added scans of an empty ATR cell. All spectra were corrected for spectral distortions using Q-ATR software.[24] Variable-angle ATR was employed to determine the volume concentration of newly formed species after each step of the reaction as a function of depth by using both Ge and KRS-5 50 × 20 × 3 mm crystals and angles varying from 35° to 60°.

Because quantitative ATR-FTIR depth profiling requires knowledge of the extinction coefficient for each of these bands,[24−26] a series of PEG/chloroform and PEN/methylene chloride solutions of known concentrations were prepared, and plots of the absorbance of the 1105 $cm^{-1}$ C−O−C and 1780 $cm^{-1}$ C═O stretching bands as a function of concentration were generated. The results are shown in Figure 2A and B, and the extinction coefficients of the bands due to PEG and PEN are 778.58 and 944.68 L/mol·cm, respectively, whereas the 1710 $cm^{-1}$ extinction coefficient of C═O vibrations of COOH groups was previously determined to be 544.32 L/mol·cm.[17,21] Using double

**Scheme 2.** Schematic Diagram of Experiments Examining the Stability of PEN-PEG-MA-ePTFE Surfaces




Kramers−Kronig transformation (KKT) and previously developed algorithm for quantitative analysis using ATR-FTIR spectroscopy,[24] concentration levels of COOH groups resulting from the microwave plasma modifications, PEG reactions, and PEN attachment were determined.

## Results and Discussion

As a first step in this study, we used ATR-FTIR spectroscopy to quantitatively analyze the volume concentration levels of surface species resulting from MA, PEG, and PEN reactions on ePTFE surfaces. Details regarding surface measurements were published elsewhere.[17,21] Here, we focused on the characteristic IR bands at 1710, 1105, and 1780 $cm^{-1}$, which were used to determine COOH, PEG, and PEN concentrations as a function of depth from the surface, respectively. Figure 3A−C illustrates volume concentration changes for COOH, PEG, and PEN as a function of depth. As seen, in all cases there is a decay in concentrations while probing from approximately 0.2 to 1.2 $\mu$m into the bulk. The volume concentration of the COOH groups decreases from 23.6 to 9.71 $\mu g/m^3$ when going from 0.2 to 1.2 $\mu$m, and similar trends were observed for PEG and PEN, with the decrease from 12.8 to 7.84 $\mu g/m^3$ and 8.85 to 3.33 $\mu g/m^3$, respectively. An estimated accuracy of the volume concentration measurements are $\pm 0.1$ $\mu g/m^3$.

These data will be used later to determine the antimicrobial effectiveness of these reactions after a fraction of PEN molecules were hydrolyzed.

While one aspect of quantitative analysis is the determination of surface concentration levels, another aspect is the response

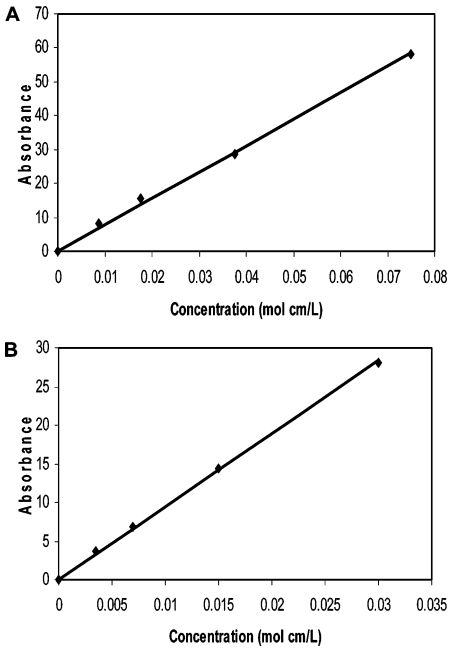



**Figure 2.** Absorbance plotted as a function of concentration for PEG (A) and PEN (B) dissolved in chloroform and methylene chloride, respectively.

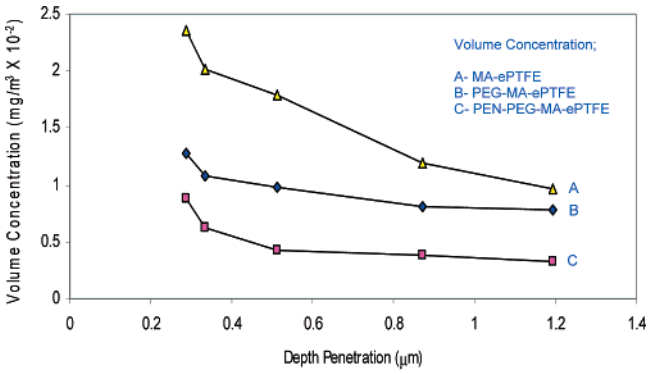



**Figure 3.** The volume concentration plotted as a function of depth from the surface for (A) MA-ePTFE, (B) PEG-MA-ePTFE, and (C) Pen-PEG-MA-ePTFE.


**Figure 4.** (A) Photographs of colony-forming units (CFU) of *S. aureus*/LB broth liquid culture (A-2-1), *S. aureus*/LB broth liquid culture containing ePTFE (A-2-2), *S. aureus*/LB broth liquid culture containing MA-ePTFE (A-2-3), *S. aureus*/LB broth liquid culture containing PEG-MA-ePTFE (A-2-4), *S. aureus*/LB broth liquid culture containing PEN-PEG-MA-ePTFE (A-2-5), and *S. aureus*/LB broth liquid culture containing PEN-ePTFE (A-2-6). (B) Concentration plotted as a function of time for the experimental sequence depicted in Scheme 1: control (A-2-1), ePTFE (A-2-2), MA-ePTFE (A-2-3), PEG-MA-ePTFE (A-2-4), PEN-PEG-MA-ePTFE (A-2-5), and PEN-ePTFE (A-2-6).

of bacteria solutions in contact with PEN-modified surfaces. To determine how each step of surface modification alters bacterial growth, solution L-A-1 was utilized to conduct a series of experiments A-2 in Scheme 1. Antimicrobial effectiveness of PEN-PEG-MA-ePTFE surfaces was examined by exposing ePTFE, MA-ePTFE, PEG-MA-ePTFE, PEN-PEG-MA-ePTFE,

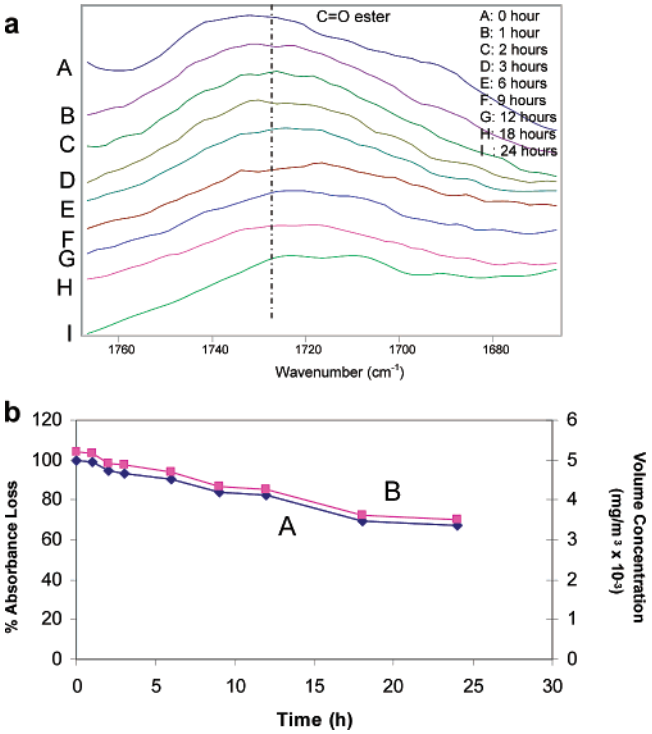



**Figure 5.** (a) ATR-FTIR spectra in the C═O region recorded during 0−24 h exposure to PBS buffer solution: (A) 0; (B) 1 h; (C) 2 h; (D) 3 h; (E) 6 h; (F) 9 h; (G) 12 h; (H) 18 h; and (I) 24 h. (b) (A) Percent absorbance loss of the C═O ester plotted as a function of exposure time of PEN-PEG-MA-ePTFE to PBS solution; (B) percent loss of PEN volume concentration as a function of exposure time of PEN-PEG-MA-ePTFE to PBS solution.

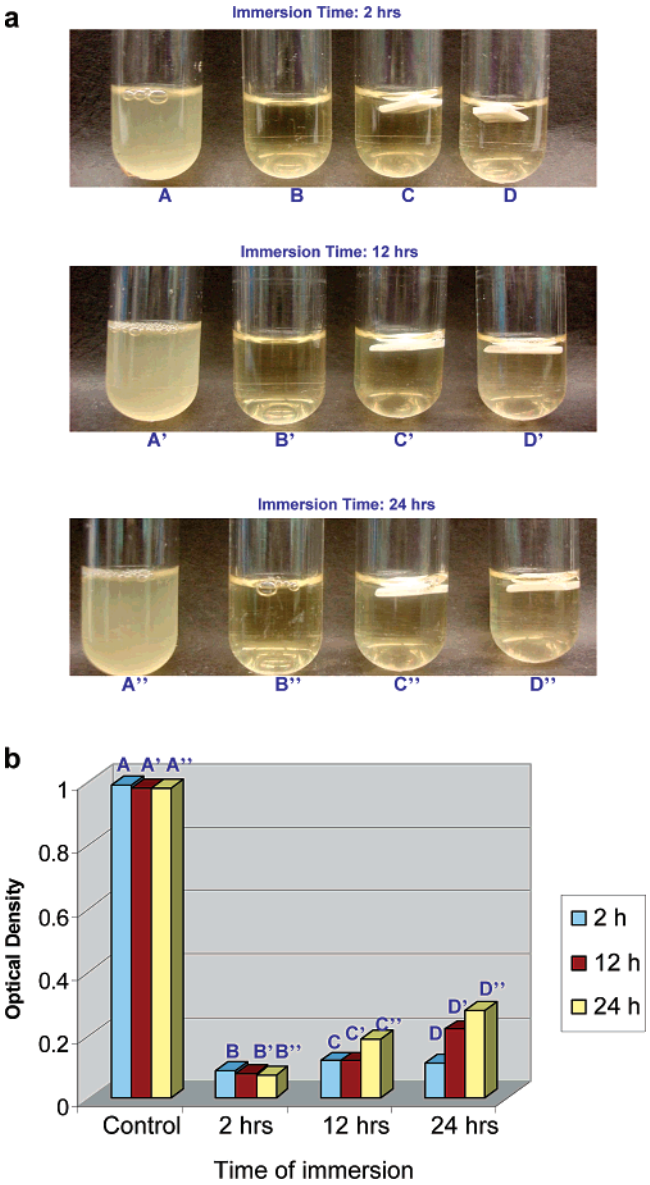



**Figure 6.** (a) Photographs of solutions containing cultures of *S. aureus* exposed to PEN-PEG-MA-ePTFE for 2, 12, and 24 h: A, A′, and A″ are solutions of positive control containing fresh LB and *S. aureus* culture; B, B′, and B″ are LB-B-1, LB-B-2, LB-B-3 and *S. aureus* culture; C, C′, and C″ are fersh LB, *S. aureus*. culture, and B-1-A, B-2-A, B-3-A specimens; D, D′, and D″ are fresh LB, *S. aureus* culture, and B-1-B, B-2-B, B-3-B specimens. (b) Relative optical density plotted as a function of time for A, B, C series solutions.

and PEN-ePTFE to *S. aureus*/LB broth liquid culture, followed by the determination of the number of colony forming units (CFUs) as a function of time (experiment A-3). While CFU represents the number of live bacteria in the culture, Figure 4A illustrates photographs of agar plates with the cultures from A-2-1 through A-2-6 specimens after incubation for 3 h (experiments A-2 and A-3). As seen, photograph A-2-1 is a positive control, whereas A-2-2, A-2-3, A-2-4, A-2-5, and A-2-6 represent the bacteria growth after immersion of ePTFE, MA-ePTFE, PEG-MA-ePTFE, PEN-PEG-MA-ePTFE, and PEN-ePTFE, respectively. As seen, only when PEN-PEG-MA-ePTFE is exposed into the culture (A-2-5), formation of bacteria colonies is inhibited, as manifested by the low number of colonies shown in photograph A-2-5.

To estimate CFU concentration levels in each of the experiments discussed above (experiment A-3), the colonies were counted and their CFU/mL are plotted as a function of the exposure time. This is illustrated in Figure 4B. As seen, in all cultures except the one containing PEN-PEG-MA-ePTFE (A-2-5), the bacteria continue to multiply. In the presence of PEN-PEG-MA-ePTFE, the bacteria population diminishes, and after 24 h no live bacteria are presented.

While these data undeniably show the effectiveness of PEN-PEG-MA-ePTFE surface modifications, one question that needs to be addressed is the stability of PEN-PEG linkages to hydrolysis. This is particularly important in view of the fact that these surfaces are exposed to aqueous environments and PEN is attached to the PEG spacer via an ester linkage known to have limited hydrolytic stability (Figure 1). To determine hydrolytic stability of this linkage, we exposed PEN-PEG-MA-ePTFE specimen to PBS buffered solution and monitored the intensity changes of the C═O group (circled) as a function of exposure time. Figure 5a illustrates a series of ATR-FTIR spectra in the C═O region and shows that the ester linkage between the PEG spacer and PEN diminishes. However, after 24 h of exposure, the band is still detected. The plot illustrated in Figure 5b (a) shows the % absorbance loss (curve A) of PEN as a function of time. As seen, approximately 30% decrease of the C═O bond intensity is observed after 24 h of incubation at 37 °C. To illustrate the actual concentration levels after 24 h of exposure, we utilized quantitative surface analysis shown in Figure 3 and converted the C═O intensity changes after hydrolysis to the actual PEN volume concentration changes at the surface of ePTFE.[21] This is illustrated in Figure 5b (curve B), which shows that the actual volume concentration loss of PEN at 0.2 $\mu$m from the surface is 32% with respect to its initial concentration.

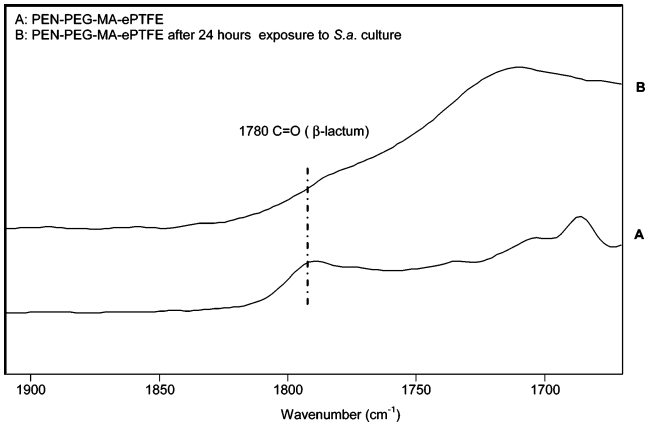



**Figure 7.** ATR-FTIR spectra of the PEN-PEG-MA-ePTFE surface: (A) before and (B) after exposure to *S. aureus* culture for 24 h.

These data prompted us to conduct further experiments to determine the effectiveness of the remaining PEN attached to PEG-MA-ePTFE surfaces. The sequence of experiments conducted in this part of the study is shown in Scheme 2, and Figure 6a illustrates photographs of the A, B, and C series of solutions containing cultures of *Staphylococcus aureus* exposed to PEN-PEG-MA-ePTFE that have been immersed in PBS and LB broth for 2, 12, and 24 h, respectively. Solutions A, A′, and A″ shown in Figure 6 are positive controls containing fresh LB and *S. aureus* culture, whereas solutions B, B′, and B″ are LB-B-1, LB-B-2, and LB-B-3 and *S. aureus* culture. However, solutions labeled C, C′, and C″ are fresh LB, *S. aureus* culture containing B-1-A, B-2-A, and B-3-A specimens, respectively. Solutions D, D′, and D″ are also fresh LB, *S. aureus* culture containing B-1-B, B-2-B, and B-3-B specimens, respectively. As seen in Figure 6a, solutions A, A′, and A″ are turbid, which is indicative of the bacteria growth. In contrast, solutions B′, B′, and B″ are clear and similar to those of C, C′, and C″ and D, D′, and D″, thus manifesting that the bacteria proliferation has been minimized even after about a 32% PEN loss during prior exposure and results from the hydrolysis of the ester linkage. To quantify these data, optical density of each culture was measured at 600 nm, which is illustrated in Figure 6b and plots the normalized optical density for each solution. As seen, B and C series give low relative absorbance values as compared to A, which is a positive control, indicating that the effectiveness of antimicrobial activity for PEN-PEG-MA-ePTFE specimens still remains significant, even after 32% loss of the PEN molecules from the surfaces. It should be noted that PEN is effective toward gram positive bacteria and paralleled experiments conducted in the presence of gram positive and gram negative bacteria confirmed its effectiveness toward gram positive. This is illustrated in Figure S-1 of the Supporting Information.

The final set of experiments involved the analysis of the remaining active $\beta$-lactam ring of PEN in the surface of PEN-PEG-MA-ePTFE. After exposure to the *S. aureus.* bacteria culture at 37 °C for 24 h, a PEN-PEG-MA-ePTFE specimen was washed with PBS buffered solution three times, dried in a desiccator, and ATR-FTIR analysis was conducted. Figure 7 shows ATR-FTIR spectra recorded from the PEN-PEG-MA-ePTFE surfaces before and after contact with the bacteria. As expected, the band at 1780 $cm^{-1}$ attributed to the C═O of $\beta$-lactam ring disappears after contact with bacteria, which is attributed to the ring opening of the $\beta$-lactam ring in reactions that inhibits cell wall synthesis preventing the cross-linking of peptide chains in the peptidoglycan layer.[27,28]

## Conclusions

These studies illustrate that antibacterial effectiveness of PEN-PEG-MA-ePTFE modified surfaces remains significant even after 24 h of exposure to *S. aureus* gram positive bacteria. Despite the loss of 32% of PEN volume concentration, these surfaces are still effective in preventing proliferation of microbes, and the weakest link responsible for the loss of PEN from the surface is the ester linkage between PEN and PEG molecules. Using variable-angle ATR-FTIR, volume concentration of PEN attached to the surface decreases from 8.85 $\mu g/m^3$ at 0.2 $\mu$m to 3.33 $\mu g/m^3$ at 1.2 $\mu$m.

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**Supporting Information Available.** Exposure of PEN-PEG-MA-ePTFE specimens to *S. aureus* gram positive and *P. aeruginosa* gram negative bacteria. This material is available free of charge via the Internet at http://pubs.acs.org.

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