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MICROBIALS

A COMPARATIVE STUDY OF BACTERIAL ATTACHMENT TO HIGH-PURITY WATER SYSTEM SURFACES

Recent advances in science and technology have resulted in the use of a variety of stainless steels and plastics in high-purity water systems for the electronics and pharmaceutical industries. Generally, the selection of these materials is based upon cleanability, corrosion resistance, fabrication requirements, design specifications, and/or initial cost (1, 2), as opposed to microbial fouling potential. However, biofouling of these materials can cause significant and expensive long-term system performance problems.

It is generally understood that bacteria can attach to almost any surface in an aqueous environment and form a biofilm (3). High-purity water systems are not immune to this problem. Biofilms in high-purity water systems can be potential sources of microbial contamination (4). Several repercussions of biofilm formation are possible. The presence of any free-floating particles, including bacteria detached from biofilms, can be problematic in the semiconductor industry (5). Biofilms containing Gram-negative bacteria can be a source of endotoxins, a great concern to the pharmaceutical industry. Pyrogenic endotoxins (pyrogens) are lipopolysaccharide/lipoprotein complexes in Gram-negative bacteria cell walls (6). Routine sanitization/disinfection processes will not remove these pyrogens. Pyrogens are very heat-stable and have been shown to cause human death in extreme cases (7). Thus, the U.S. Food and Drug Administration (FDA) requires strict monitoring for pyrogens in any water for injection (WFI) system associated with pharmaceutical products (8). Although not commonly reported in high-purity water systems, microbially influenced corrosion of stainless steels also has the potential to be problematic when biofilms are present (9).

Biofouling has been reported at all stages, from treatment to distribution to point of use, on all types of piping surfaces (10). Once a biofilm has formed, removal of the attached bacteria from surfaces is very difficult. Attached bacteria growing as biofilms on surfaces have been shown to be more resistant to disinfection (11), presumably because the biofilm matrix creates a diffusion barrier for the biocide (12). This compounds the difficulty of maintaining low microbial contamination levels and nonpyrogenic conditions.

The study of initial attachment and colonization events is integral to understanding biofouling in high-purity water systems. If environmental conditions are conducive for growth after bacteria attach to a surface, a biofilm will ensue. Total organic carbon (TOC) levels have been correlated to biofilm growth in high-purity water systems (5). However, extremely low TOC levels (30 parts per billion [ppb]) still do not eliminate bacterial growth in these systems (13, 14). Oligotrophic conditions present in these systems result in thin biofilms (5). Thin biofilms, generally defined to be a few cell layers thick, are thought to be more reflective of initial events (attachment) than are thicker biofilms resulting from attachment and subsequent growth (15). Consequently, recent efforts have focused on elucidating bacterial attachment phenomena in relation to thin biofilms on stainless steel and plastic surfaces such as are typically found in high-purity water systems.

The majority of these studies on bacterial attachment have been done on stainless steel surfaces with the premise of elucidating contributions of surface chemistry and surface topography to the adhesion process. Initial colonization patterns have been described on electropolished and hand-polished 316L stainless steel under flowing conditions.

In one study on these materials, Camper et al. (16) reported statistically significant bacterial aggregation on the

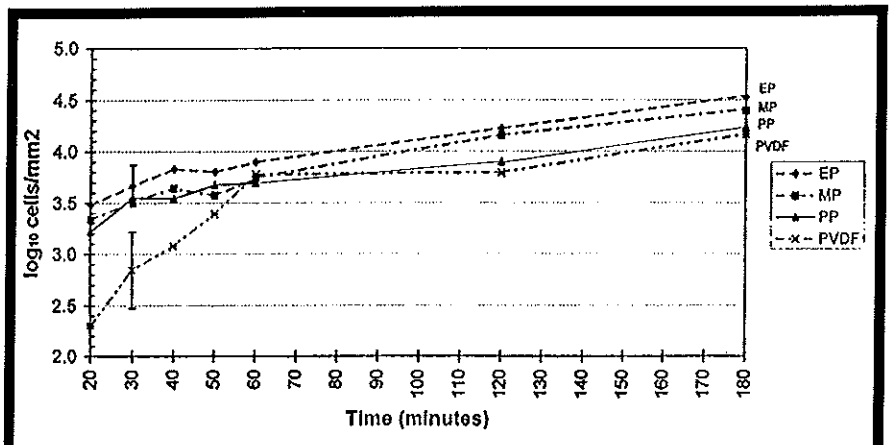

Figure 1. Log_{10} of areal density of P. aeruginosa (cells/mm²) attached to the representative high-purity water system surfaces versus time. Surfaces are abbreviated in legend is as follows: EP - electropolished 316L stainless steel, MP - mechanically polished 316L stainless steel, PP - polypropylene, PVDF - polyvinylidene fluoride. Each data point represents the mean of three experiments. Error bars represent ±1 standard error of the mean. These SEMs for EP, MP, and PP were the same (0.20, 0.19, 0.21, respectively), so only one is shown.

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Figure 2. Photomicrograph of Hoechst γ 33258-stained P. aeruginosa attached to mechanically polished 316L stainless steel surface using epifluorescent illumination at X 400 magnification. Mechanically polished 316L stainless steel surface after 180-minute incubation with P. aeruginosa suspension.

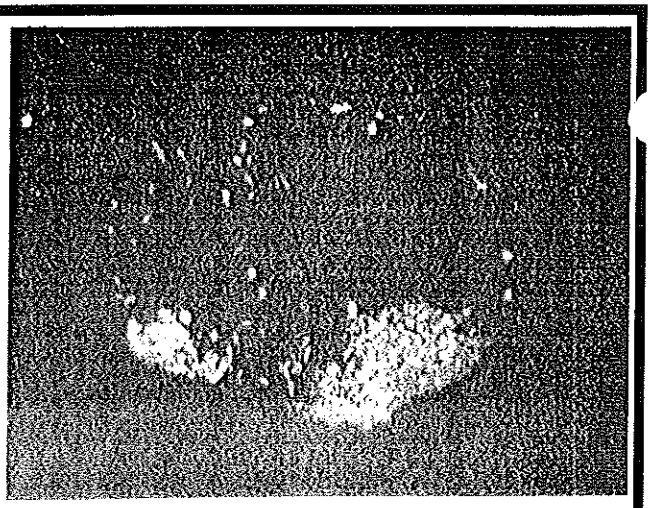

Figure 3. Photomicrograph of Hoechst γ 33258-stained P. aeruginosa attached to PVDF using epifluorescent illumination at X400 magnification. Photograph of PVDF after 60-minute incubation with P. aeruginosa suspension.

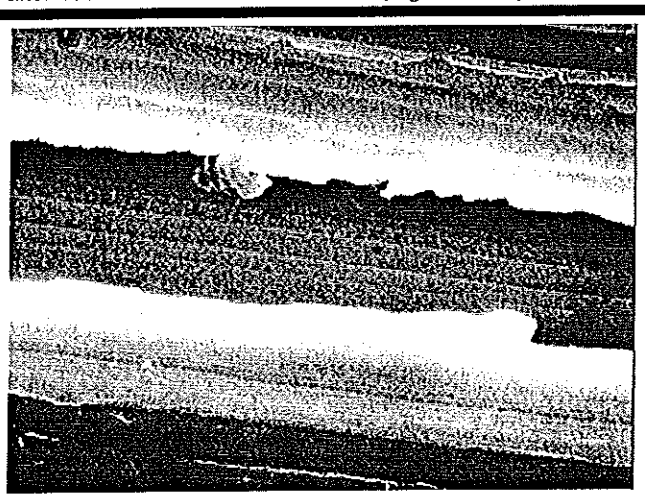

Figure 4. Scanning electron micrograph of uninoculated mechanically polished 316L stainless steel surface at X5,000 magnification. Note the large regular scratches in the surface.

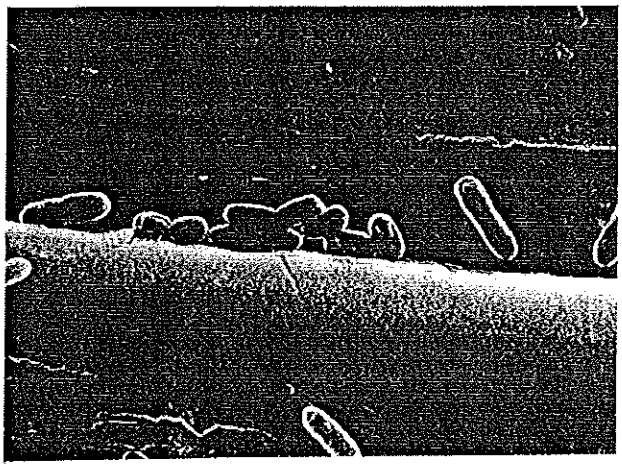

Figure 5. Scanning electron micrograph of mechanically polished 316L stainless steel surface after 180-minute incubation with P. aeruginosa suspension. Magnification = X5,000.

electropolished surface. Geesey et al. (9) reported significant influences of 316L stainless steel microstructure on initial bacterial attachment, with both surface topography and surface chemistry being influential factors. When comparing initial attachment on different surfaces (copper, silicon, glass, and hand-polished 316 stainless steel), Mueller et al. (17) concluded that the adsorption rate was positively correlated with surface free energy, surface roughness of the substratum, and the hydrophobicity of the cells. According to Vanhaeke et al. (18), bacterial cell surface charge may be of minor importance in adhesion to 304 and 316L stainless steel in static systems. However, growth phase, pH, and motility may be more significant factors influencing attachment onto 304 stainless steel (19).

In relation to studies on plastic surfaces, Lyklema et al. (20) studied initial attachment of specific bacteria to polystyrene. This work demonstrated that electrokinetic potential and hydrophobicity are important to the adhesion process.

It is generally recognized that bacterial attachment occurs much more readily during periods of low shear (21). Although a bacterium's transport to a surface may be enhanced during high-flow conditions, any stagnation periods (such as can occur in dead legs or during system shutdown for cleaning and repairs) may permit colonization that could persist during normal system operation. The present study was undertaken in an effort to characterize bacterial attachment on representative high-purity water system surfaces as may occur during periods of stagnation. Thus, for this study, quiescent cell suspensions were selected as a source of biofouling bacteria, and industrially relevant surfaces were selected as the substrata. The aim was to compare attachment of bacteria to different surfaces under identical initial conditions. The information gained from this study will be useful when considering the biofouling potential of one type of pipe or piping surface versus another.

Materials and Methods

Bacterial culture. *Pseudomona aeruginosa* Ps-4 was obtained from Dr. Martin Favero (Centers for Disease Control, Atlanta, Ga.). This culture had been

isolated from a hospital poloxamer-iodine solution and was found to have caused repeated contamination by fouling polyvinyl chloride (PVC) piping in a production plant (22). A 0.1-milliliter (mL) aliquot of stock culture frozen in glycerol-peptone was used to inoculate 100 mL of soy broth[1] and was incubated at room temperature for approximately 45 hours.

Cell density was monitored using a colorimeter[2] to establish growth curves and ensure that comparable population numbers were used in the adhesion assay. After incubation, a 10-mL sample of culture was centrifuged (10 minutes at 3,000X gram [g]) and resuspended in filtered (0.22-micron [µm]) autoclaved reverse osmosis (RO) water[3]. The suspension was vortexed, filtered, through a 5-µm nylon mesh to eliminate clumped bacteria.

To check cell density, a 10-mL sample of the final suspension was diluted to 10^{-4} in 10 mL of filtered, autoclaved RO water and fixed with 0.5 mL formalin for 5 minutes and stained with 0.1 mL 2% acridine orange. The stained culture was filtered onto a 0.22-µm black polycarbonate membrane[4] and enumerated by epifluorescent microscopy (23). Cells were counted at × 1,000 magnification with direct count reported as cells per milliliter (/mL) in the original suspension. Approximately 10^7 cells/mL were used for each adhesion assay. If necessary, the suspension was adjusted, usually via dilution, to achieve this cell density.

Viable counts were determined by diluting the suspension in sterile high-purity water, surface-plating 0.01-mL droplets (24) on R2A agar (25), and incubating at room temperature for 2 to 3 days. Direct and viable counts of the bacterial suspension were performed at 0, 75, and 150 minutes after exposure of coupons to bacteria.

Preparation of coupons. Coupons (25 millimeter [mm] by 6 mm) were cut from prepolished 316L stainless steel[5]. The surfaces, prepared via commercial procedures by the vendor, were either 240-grit mechanically polished followed by electropolishing (EPSS) or 180-grit mechanically polished only (MPSS). Coupons were washed in acetone, air-dried, then washed in 6.25% laboratory cleaning solution[6]. Coupons were transferred to fresh solution, boiled for 5 minutes, then insonated[7] for 5 minutes, followed by rinsing with sterile water until foaming ceased (approximately) 5 times), insonated again, and rinsed 3 times. The coupons were transferred to a vial containing 20 mL of high-purity water, autoclaved for 20 minutes, and stored at room temperature until use.

Similar-sized coupons were cut from extruded polypropylene (PP) and polyvinylidene fluoride (PVDF) plastics. These coupons were cut from samples of commercially extruded pipe[8]. The interior concave surfaces were used for the assays. The plastic coupons were washed in hexane and allowed to air-dry. They were then washed, rinsed, and sterilized as previously described for the stainless steel coupons.

Adhesion assay. Previously washed and sterilized coupons were placed into individual vials (one coupon per vial) containing 10 ml of the 10^7 cells/mL of

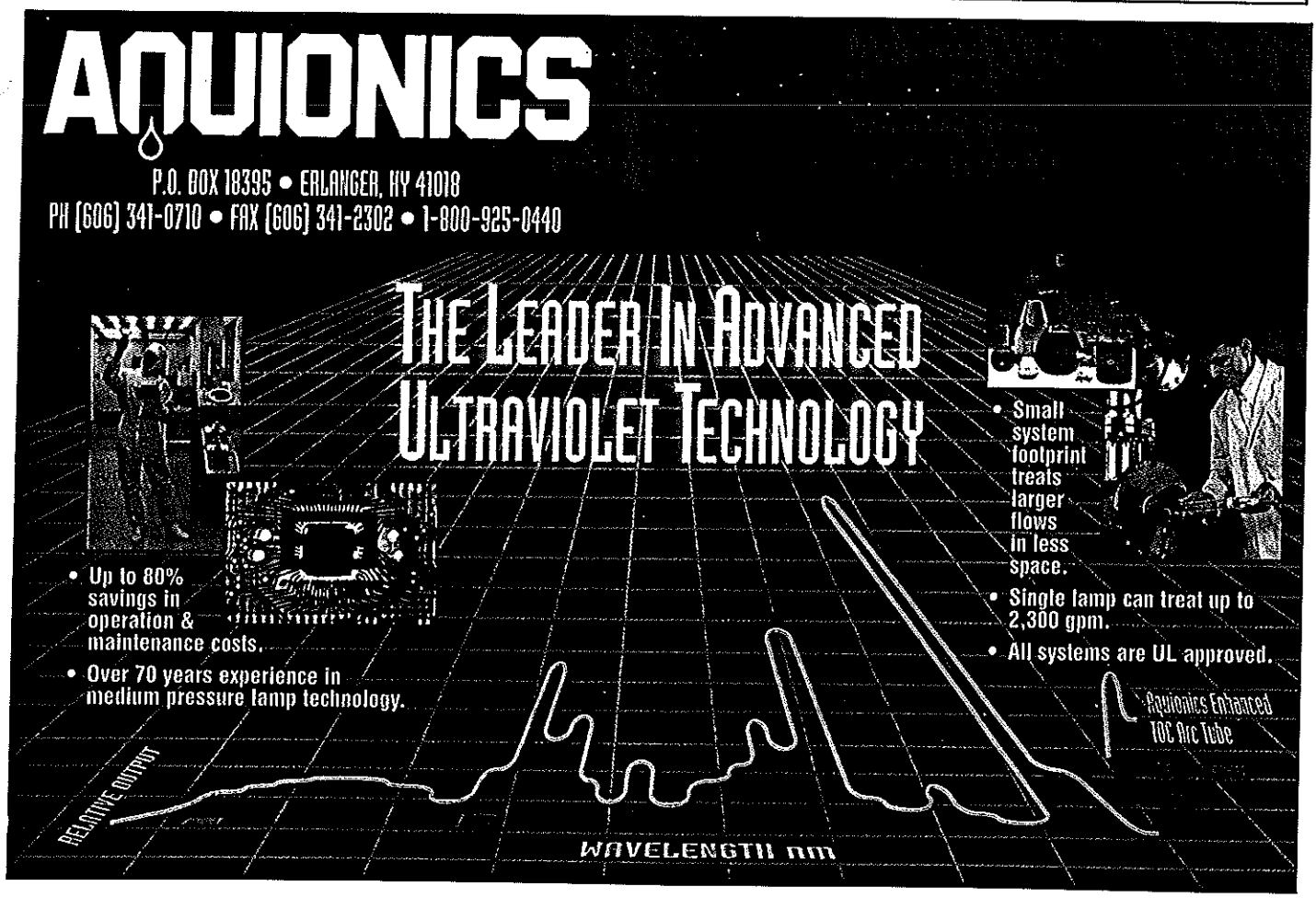

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TABLE A
Bacterial Adhesion Rates

Substratum	*Adhesion Rates (cells/mm²/min)*	*Relative Topography*	*Sloughing Potential*
Mechanically polished 316L SS	127.0	Rough	High
Electropolished 316L SS	178.8	Smooth	High
Extruded polypropylene	102.3	Smooth	Low
Extruded polyvinylidene fluoride	70.0	Smooth	Medium

Note: A comparison of bacterial adhesion rates, relative topography, and surface energy measurements of the four representative high-purity water system surfaces studied. Adhesion rates were areal densities of *P. aeruginosa* per min[-1] as averaged over 180-minute duration of each of three experiments per substratum type. Sloughing potentials were determined based on the polar component (γ_p) data presented in Table B.

Ps. aeruginosa suspension. Coupons were positioned in the bottom of the vials with the polished stainless surfaces or concave plastic surfaces facing upward. Since PP is less dense than water, coupons of this material were held submerged by inserting a sterile 12.7-mm hypodermic needle and 1-cm³ (cubic centimeter) syringe into the top surface near one end of the coupon. All vials were capped or covered with Parafilm[9] during bacterial exposure. One vial containing filtered, autoclaved RO water only was immediately fixed with 0.5 mL formalin (time zero negative control); while another vial containing filtered, autoclaved RO water was fixed near the end of the experiment (time 160-minute negative control).

Coupons were exposed to bacteria for 20, 30, 40, 50, 60, 120, and 180 minutes (min). After each time point, each exposed coupon was removed from the bacterial suspension, then rinsed to remove loosely attached cells. The rinsing was done by gently moving the exposed coupon from side to side 15 times in a sterile 250-mL beaker containing 200 mL of filtered, sterilized RO water. A second coupon, exposed to bacteria for 60 minutes, was not rinsed prior to enumeration in order to determine rinsing effects on areal cell densities. Each adhesion study was done in triplicate.

Staining and enumeration. All exposed and unexposed coupons were transferred into individual vials containing 9 mL of filtered, sterilized high-purity water and 0.5 mL of formalin. After 5 minutes, 0.1 mL of stain[10] was added to each vial to achieve a final concentration of 10 µM (26). Coupons were stained for 1 hour, then removed and secured to glass microscope slides for epifluorescent microscopic enumeration. The stainless steel coupons were mounted with the polished side upward, using rubber cement; while plastic coupons were mounted concave side upward, using double-sided tape.

Attached cells were enumerated via an epifluorescent microscope[11] at X400 magnification (365-nanometer [nm] excitation, 400-nm barrier, and dichroic mirror). Ten random fields were counted for each coupon at each time interval and data reported as areal cell densities in cells per square millimeter (/mm²). Microscopic counts were then averaged for each time interval on each surface for each of three experiments.

Adhesion rate calculations. Adhesion rates were calculated for each surface and reported as cells/mm²/min. This was performed by dividing the total number of attached cells per coupon area after each time interval by the corresponding time.

Critical surface tension. Diagnostic liquids of increasing surface tension were placed on the substrata and their advancing contact angle measured using a goniometer. Values for critical surface tension (gamma$_c$ [γ_c]) were derived graphically from least-squares fit of the relationship between the surface tension of the diagnostic liquids (x) and the cosine of the advancing contact angle (γ). When the contact angle is 0 (cosine = 1), the liquid just wets the surface and therefore represents the γ_c for the surface. Values for the dispersive (γ_d) and polar (γ_p) components of the composite surface free energies were calculated from γ_c (27).

Sloughing potentials of each surface were then reported as low, medium, or high relative to each other, based on the polar component (28). This information is reported in Table A.

Statistical analysis. Microscopic counts were averaged for each time interval on each surface for each of three experiments. Statistical analysis was performed on the $\log_{10}$ transformation of these counts. The means at each time for each surface are shown in Figure 1. Standard errors of the mean (SEM) are shown in Figure 1, with error bars denoting ±1 SEM. Bacterial adhesion rates were subjected to one-way analysis of variance to estimate the variance between replicates.

Photomicrographs. Photomicrographs were taken of the coupons using a camera[12] mounted onto the epifluorescent microscope[11] on Kodak Ektar 125 film. Scanning electron micrographs (also known as SEM) were taken using an electron microscope[13]. The SEM images were used to make qualitative conclusions about the surface topography. Ten images were taken of each uninoculated surface. Surfaces were determined to be either rough or smooth based on the ability to see surface anomalies at X5,000 magnification. This information is reported in Table A.

Results

Statistical analysis of the $\log_{10}$ direct and viable counts in the cell suspensions showed that there were no significant differences in bacterial numbers over the duration of the experiments. Thus, there was no bias of cell numbers that may have affected adhesion with time between surfaces. The arithmetic mean cell numbers were 2.18 ± 1.45 10^7 cells/mL by direct count, and $5.62 \pm 5.18 \times 10^6$ colony-forming units per mL (cfu/mL) by viable plate count methods.

Areal densities (cells/mm²) increased with time for all four surfaces (Figure 1). The rate of adhesion to the stainless steels was more linear than that to the plastics. Initial attachment to PVDF was an order of magnitude less than that for the other surfaces, but reached a density similar to that of the other surfaces

after 50 minutes. Bacterial adhesion rates to the four surfaces (Table A) were similar but not identical, with a significant difference between the stainless steels and the plastics ($p = 0.01$).

Visual analysis of the epifluorescent micrographs indicated the MPSS had polishing marks along which cells tended to congregate (Figure 2). Although no statistical spatial pattern analysis was done on the cell patterns (16), the distribution of cells on EPSS, PP, and PVDF appeared patchy. Bacteria attached to the PVDF had the most visually pronounced patchiness, as evidenced by the presence of numerous bacterial clumps (Figure 3). Electron micrographs of uninoculated stainless steel surfaces showed that the MPSS was visibly rough at X 5,000 magnification (Figure 4), thus giving it a designation of *rough* for relative topography in Table A. These scratches were large enough to harbor bacteria (Figure 5). The EPSS had only minor topographical variations, while the plastic surfaces had no visible surface anomalies at X 5,000 magnification (not shown). Based on this information, the EPSS, PP, and PVDF were reported to be smooth for relative topography as shown in Table A.

The surface energy measurements (Table B) show that while both the stainless steel surfaces had almost identical critical surface tension values, the plastics were much lower. The plastics had a lower polar component, with the PP having a lower polar component than did the PVDF. The higher values for the polar component of surface tension for the stainless steel surfaces indicated that the stainless steels were more wettable than the plastic surfaces, also indicating a higher potential for sloughing. The sloughing potentials of the surfaces were then rated based on this information, as reported in Table A.

TABLE B
Surface Energy Measurements

Substratum	γ_c (dynes cm^{-1})	γ_p (dynes cm^{-1})	γ_d (dynes cm^{-1})
Mechanically polished 316L SS	35.9	14.2	34.0
Electropolished 316L SS	35.7	15.0	34.6
Extruded polypropylene	29.1	3.4	26.0
Extruded polyvinylidene fluoride	29.2	10.5	26.9

Note: Surface energy measurements of the four representative high-purity water system surfaces studied. Values for the dispersive (g_d) and polar (g_p) components of the composite surface free energies were calculated from g_c (27). Critical surface tension values (g_c), polar component values (g_p), and dispersive component values (g_d) are given in dynes/cm.

Discussion

A variety of stainless steel and plastic surfaces are used for the piping and storage of high-purity water in both the electronics and pharmaceutical industries (1, 2). During periods of stagnation, as can occur in dead legs or during cleaning, repair, and other situations, all exposed surfaces are susceptible to bacterial adhesion and subsequent biofilm formation. As with most adhesion studies, there were two variables that influenced bacterial attachment in this study: surface topography, and surface chemistry.

Although the four surfaces examined in the present study were vastly different with respect to these two surface properties, bacterial adhesion was shown to occur on all four surfaces. The observed adhesion, however, was not similar between surfaces, indicating inherent differences in surface properties. These differences have been previously described for stainless steel in relation to bacterial adhesion (9, 16); however, adhesions to PP and PVDF have not been well characterized. The purpose of this study was not to quantitatively characterize these differences in surface chemistry and topography for the four surfaces studied, but rather to report some initial data from which qualitative comparisons could be made in relation to bacterial adhesion.

Comparing the four surfaces qualitatively based on surface topography using scanning electron micrographs indicated that the EPSS and two plastic surfaces were relatively smooth. Their finishes did not have irregularities of significant magnitudes in the same range as the size of a bacterial cell (of approximately 0.3 µm by 1.0 µm in size for *P. aeruginosa).* Surface topography of one sample of EPSS has been measured via atomic force microscopy with maximum surface topographical features of 0.0003 µm (29). This was not true of the MPSS, which contained regular scratches with bacteria aligned within.

Mandan (2) reported roughness averages (RA) of approximately 7 for an EPSS and approximately 85 for a 180-grit MPSS. These surface irregularities on MPSS may be large enough to protect cells from shear forces generated by surface cleaning processes used in production facilities (2). Such pockets of contamination, often containing large numbers of cells, could serve as sources of reinoculation for an ostensibly clean system. This is evident in the fact that regrowth of bacteria within high-purity water systems may occur as soon as 5 to 7 days after standard sanitization procedures have been performed (4). It is suspected that biofilms are the source of reinoculation or regrowth within these systems. The piping material may play a key role in the ability of biofilms to survive caustic procedures, thus contributing to regrowth problems.

Although all surfaces demonstrated the ability of bacteria to attach to them, initial bacterial adhesion rates varied considerably (Table A). As surface topography was relatively similar between the EPSS and plastic surfaces, adhesion rate differences between these surfaces may be attributed to differences in surface chemistry. Bacterial adhesion to PVDF appeared patchy and showed a lag of up to 1 hour compared to the other surfaces, for which attachment proceeded more linearly from 20 minutes (Figure 1). Although not investigated in this study, the considerable variability in the results for PVDF could have been related to the surface chemistry. This factor may have contributed to the appearance of patchy distribution of attached cells and the initial lag in adhesion. This supports the observations of Husted et al. (5) that patchy biofilm was observed on PVDF.

The lack of differences between adhesion rates for the surfaces after 2 hours was consistent with the values obtained

for the critical surface tensions. Surfaces with higher values for the polar component sometimes show short-term increases in adhesion rates as well as increased sloughing of adhered cells with time (28). This is demonstrated by the results for PVDF versus PP but not for the stainless steel surfaces.

Initial adhesion rates were related more to surface tension measurements than to surface topography, although bacteria did congregate in crevices in the MPSS. Although both plastic surfaces had low surface tension values, neither had values in the range of silicone (approximately 20 dynes/cm), thought to be a minimally adhesive material (27). The results suggest that initial adhesion to PVDF may be slower, and patchy distribution of cells may indicate some variation in the surface characteristics. A slower attachment rate may be in part caused by the slightly lower surface tension values when compared to the stainless steels (Table B). This may indicate that PVDF is advantageous under these specific conditions.

It should be noted that these differences in initial bacterial adhesion could be significant if the system is shut down for a short period of time. It should also be noted that once a biofilm has formed, regardless of the surface, other variables such as TOC may have a more significant long-term role in biofilm accretion (5). Pedersen (30) reported no differences in numbers of bacteria attached to stainless steel compared to polyvinyl chloride (PVC) after 167 days in drinking water.

High-purity water systems generally contain a mixture of stainless steel and plastic surfaces to which bacteria can stick. According to this study, during periods of stagnation, initial bacterial adhesion to PVDF may be slower and more patchy. This surface may be advantageous to minimize these interactions under these conditions, but it should be noted that higher levels of spontaneous detachment of biofilm flocs could occur (5). In lower-flow regions of a high-purity water system, it may be advantageous to use PVDF. Under high-pressure or high-temperature situations where the use of plastics is not practical, electropolished stainless steels are recommended over mechanically polished stainless steels. Although electropolished stainless steel may contribute to patchy distribution under flow conditions (16), its material strength and corrosion resistance make it superior under these conditions (2). It is important for designing and manufacturing teams to take these recommendations into account when building or repairing high-purity water systems.

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Endnotes

[1]Tryptic Soy Broth, Difco Laboratories, Detroit, Mich.
[2]Klett-Summerson colorimeter, Long Island, N.Y.
[3]Milli-RO 60 unit, Millipore Corp., Bedford, Mass.
[4]Poretics Corp., Livermore, Calif.
[5]Precision Stainless, Springfield, Mo.
[6]International Products Corp., Trenton, N.J.
[7]Sonogen Automatic Cleaner, Branson Instruments Inc., Stamford, Conn.
[8]Sanitech, Andover, N.J.
[9]American National Can, Greenwich, Conn.
[10]Hoeschst Cefanese Stain g 33258, Charlotte, N.C.
[11]Nikon epifluorescent microscope, Nikon USA, Melville, N.Y.
[12]Nikon camera model FM-2, Japan
[13]JEOL Model 100CX electron microscope, Peabody, Mass.

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