Thermodynamic Surface Analyses to Inform Biofilm Resistance

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SUMMARY

Biofilms, found on various surfaces, are the habitat of 95% of the earth’s bacteria and successfully protect bacteria from many antibiotics. By inhibiting biofilm formation, the propagation of infectious bacteria could be deterred. However, inhibiting biofilm formation is difficult in that it is a symbiotic, multi-determinant system involving the physical and chemical interaction of both substrate and bacteria. In our research group, we focus on the substrate surface and potential interactions with bacteria. Using a series of biofilm resistant phenyl acrylate monomer derivatives, we examined both physical and chemical properties of related homopolymers and copolymers. Atomic force microscopy (AFM) showed smooth surfaces often approximating surgical grade steel. Using surface analyses via goniometry, the van Oss-Chaudhury-Good equation was solved linear algebraically to determine the complete surface energy profile of each halogenated aryl homopolymer surface, two bacteria, and collagen, a representative bacterial adhesin. Induced biofilm growth of five separate bacteria on copolymer samples comprising varying concentrations of phenyl acrylate monomer derivatives evidenced differing degrees of biofilm resistance via microbial staining and optical microscopy. Copolymer samples were similarly analyzed after exposure to clarified raw sewage. Based on
the microscopy and surface energy profiles, a thermodynamic explanation for biofilm formation is posited.

INTRODUCTION

Current events regarding the coronavirus (SARS-CoV2) pandemic of 2020 has heightened public awareness of infectious pathogens and pathways. However, the public remains largely unaware of the biome that allows the vast majority of microorganisms – infectious or otherwise – to persist in our world of antibiotics and antivirals. That biome is biofilms, a complex, communicative aggregation of microorganisms in which 99% of all microorganisms persist (Flemming, 2002) Because of the ubiquitous nature of biofilms, their influence is similarly widespread – from biofouling of naval vessels, drinking and wastewater treatment facilities, medical implants and inserts to persistent pathogenic pathways in health care including nosocomial infections (Abbott et al., 2000; Cavitt et al., 2015; Cavitt et al., 2017; Cleaveland, 2005; Cooney et al., 1999; Flemming, 2002; Iwamoto et al., 2018; Kenawy et al., 2007; Monroe, 2007; Montanaro et al., 2007; O’Flaherty et al., 2004; Vertes et al., 2012). Likewise, biofilms are non-trivial in remediation methods and often require multiple modalities to simply reduce and impede biofilm growth. With the inherent difficulty of biofilm remediation, research is moving toward inhibiting biofilm growth and/or formation.

Developing methods to inhibit biofilm growth and/or formation requires an intimate knowledge of the growth mechanism which is parsed into five distinct categories: 1) primary colonization with reversible attachment, 2) aggregation and irreversible attachment, 3) growth and division, 4) maturation, and 5) dispersion (Monroe, 2007; Vertes et al., 2012). The most logical moment to disrupt biofilm formation is primary colonization in which microorganisms initially contact and reversibly adhere to a substrate’s surface. Primary colonization is a multi-determinant system based on the thermodynamic (i.e., static) and kinetic (i.e., dynamic) nature of both microorganism and substrate. To date, most research has focused on single determinants.

The most commonly investigated single determinant involves the concentration dependence of biofilm inhibition. At a critical concentration [i.e., minimum inhibitory concentration (MIC)], biofilm growth is inhibited without introducing a cidal mechanism. The MIC likely addresses the kinetic effect based on the reversible attachment at primary colonization. At larger critical concentrations [i.e., minimum biocidal concentration (MBC)], a thermodynamic, cidal effect introduces disruptors to basic cellular function and, over time, shifts the microbial equilibrium away from homeostasis. Most commonly, biofilm inhibitory concentrations are in excess of the MBC with the thought that both kinetic and thermodynamic effects would be simultaneously addressed. As a motile species, Pseudomonas aeruginosa (P. aeruginosa) is likely most affected by the MIC-related kinetic effect while the non-motile Staphylococcus aureus (S. aureus) would be more affected by the MBC-related thermodynamic effect. Therefore, one single determinant
cannot effectively address each microbial species’ biofilm formation; in other words, biofilm formation will likely still occur given the multi-organismal nature of biofilms.

Introducing antimicrobial components to the substrate is a very common method to inhibit biofilm formation; however, such focuses on the previously described single concentration determinant. Substrates are often functionalized with quaternary ammonium compounds (e.g., polymeric, polymer-grafted, etc.) in excess of MBC that penetrate the cell wall causing leakage and subsequent apoptosis (Kandiyote et al., 2019; Namivandi-Zangeneh et al., 2020; Valarikova et al., 2020). Upon apoptosis and in the absence of interfacial shear flow, the apoptotic fluids often remain associated with the substrate surface dynamically altering the nature of the substrate by concealing the quaternary ammonium under cellular debris. Furthermore upon cell death, subsequent activation of lysosomes, and release of proteases, putrefaction of cellular proteins will produce free amines which neutralize protonated quaternary ammonium salts used in some of the aforementioned examples. The use of phenolic compounds to denature proteins necessary for cellular function are also common; unfortunately, like protonated quaternary ammonium compounds, the phenolic compounds are biocidal for a time until cellular debris and/or neutralization by free amines render them inert pending surface treatment (Li et al.; 2020; Namivandi-Zangeneh et al., 2020). Other, more robust compounds are also used to disrupt biofilm growth such as sugar alcohols to prevent dental caries and substituted (e.g., fluoro, chloro, nitro, cyano, etc.) aryl hydrazones (Koljalg et al., 2020; Lu et al., 2020).

Using metals associated with the substrate to disrupt cellular function is also a single determinant system. Nanoparticles (e.g., silver) are of peculiar interest for thermodynamically-mediated biofilm inhibition and have enhanced efficacy when employed with a secondary antimicrobial component (Moola et al., 2019; Namasivayam et al. 2019). However, nanoparticles often cannot endure rigorous surface treatments to recondition surfaces after eventual biofilm formation and/or fouling. Metal salts [e.g., Ce(IV)] have been shown to disrupt saccharide-dependent biofilm formation during the kinetically controlled reversible adhesion of primary colonization (Bhatt et al., 2020). Again though, metal salts often do not persist long term in aqueous environments thereby rendering them inactive.

Because of 1) significant genetic differentiation between microorganisms and 2) the colloidal nature of microorganisms, even adhesion is a complex process that is phenotypically heterogeneous and not a single determinant process (Vissers et al., 2019). For example, the surface protein SdrC of *S. aureus* has been shown to use Ca\(^{2+}\)-mediated chelation of the N2 domains as a primary contributor to biofilm formation; the use of a metal salt associated with the substrate may disrupt the aforementioned chelation illustrating how metal salts effectively inhibit biofilm formation (Pi et al., 2020). To further illustrate the diversity in the adhesion process, McLay, *et al.* were able to genetically alter *Escherichia coli* (*E. coli*) to demonstrate that the amount of fimbriation contributes to adhesion of the bacterium (McLay et al., 2018). The concentration dependence of adhesion is probably a kinetic effect unique to each bacterium.
In a paper examining *P. aeruginosa* and its interfacial behavior, Deng, *et al.* noted that most bacteria align parallel to the oil-water interface (Deng et al., 2020). The parallel alignment is likely thermodynamically driven while non-parallel alignment is kinetically controlled. The kinetic (i.e., dynamic) component has been modeled using complex algorithms and applied theories to describe bacterial attachment (Conrad et al., 2018; McLay et al., 2018; Vissers et al., 2019).

In this paper, we primarily focus on the thermodynamic components that drive the interfacial interaction of substrates with bacteria present in primary colonization. Seven phenyl acrylate monomers, including six halogenated monomers, were synthesized and characterized. The substrate was coated with a formulation that included 5-20 weight percent of a phenyl acrylate derivative and subsequently polymerized. Using atomic force microscopy (AFM), the surface smoothness was determined for representative samples and compared to surgical grade steel. Induced single species biofilm growth and separate exposure to clarified sewage provided biological evidence of biofilm inhibition relative to each of the seven phenyl acrylate derivatives at varying concentrations. After solving the van Oss-Chaudhury-Good equation via a linear algebraic method for relevant samples, surface energy analyses and comparison of each polymerized phenyl acrylate derivative to collagen and two representative bacteria (e.g., *Pseudomonas aeruginosa* and *Staphylococcus aureus*) inform potential thermodynamic efficacy. Thereafter, a thermodynamic explanation for the observed behavior was posited based on the evidence gathered.

**RESULTS AND DISCUSSION**

**Development of Potential Biofilm Resistant Polymer Materials**

Given evidence that covalently bound halogenated moieties have demonstrated efficacy for biofilm resistance, we designed a series of monomers based on phenyl acrylate (an internal control) that are likely biofilm resistant candidates (Pickens, 2009). Table 1 details the reaction scheme and phenyl acrylate derivatives synthesized; impurities in acryloyl chloride (1), technical grade (70% purity), contributed in part to the low to moderate average isolated yields (Cavitt et al., 2015; Cavitt et al., 2017).
Table 1. Employing different phenols.

| Entry | R¹/R²/R³ (2)          | 3    | Yield (%)<sup>a</sup> |
|-------|-----------------------|------|-----------------------|
| 1     | H/H/H (2a)            | 3a   | 59                    |
| 2     | H/Cl/H (2b)           | 3b   | 33                    |
| 3     | H/H/Cl (2c)           | 3c   | 36                    |
| 4     | Cl/H/Cl (2d)          | 3d   | 30.                   |
| 5     | H/H/Br (2e)           | 3e   | 21                    |
| 6     | Br/H/Br (2f)          | 3f   | 39                    |
| 7     | H/H/I (2g)            | 3g   | <10                   |

Reaction conditions: 1 (55 mmol), 2 (50 mmol), Et<sub>3</sub>N (55 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (40 mL) at room temperature and under dry nitrogen atmosphere for 24 h.

<sup>a</sup> Yields refer to average isolated yields.

Monomers were shown to be stable upon exposure to broad spectrum ultraviolet (UV) radiation indicating that the aryl–halogen bonds persist upon irradiation thereto. Photo-differential scanning calorimetry (photo-DSC) was used to confirm that the aryl-halogen bond does not undergo homolytic cleavage to initiate polymerization at 10 weight percent of each monomer as compared to 1,6-hexanediol diacrylate. The aforementioned stability allowed for UV-curing of the monomer to form both homo- and copolymeric coatings on several substrates (e.g., stainless steel, glass slides, and plastic slides) for subsequent analyses. The monomers were incorporated at 5, 10, 15, and 20 weight percent into a standard formulation. Each liquid coating was manually drawn down to a wet thickness of 100 μm and polymerized thereafter.

Samples of the cured 20% monomer formulation were examined for methanol extractable monomer content using gas chromatographic (GC) – mass spectrometry (MS). No detectable monomer was observed in any of the samples to a detection limit of 100 μg/mL.
Atomic Force Microscopy

The average plate roughness \((R_a)\) and average peak-valley height \((R_z)\) was determined via atomic force microscopy (AFM), contact scanning mode. The two controls included the uncoated stainless steel and cured formulation with no additional phenyl acrylate derivative; both yielded \(R_z\) values of 0.819 \(\mu m\). The \(R_a\) and \(R_z\) was determined for each formulation at varying concentrations of representative monomers (3a, 3b, 3d, 3e, and 3f). With the exception of 3a, the smoothness as determined by the \(R_a\) and \(R_z\) generally increases as the concentration of the monomer increases due to the increased dipole-dipole interactions of the coating (Table 2). Scans of both controls and cured copolymers at 20 weight percent monomer incorporation are shown in Figure 1.

| Monomer | 5 wt % | 10 wt % | 15 wt % | 20 wt % |
|---------|--------|---------|---------|---------|
|         | \(R_z (\mu m)\) | \(R_a (\mu m)\) | \(R_z (\mu m)\) | \(R_a (\mu m)\) | \(R_z (\mu m)\) | \(R_a (\mu m)\) | \(R_z (\mu m)\) | \(R_a (\mu m)\) |
| 3a      | 0.7185 | 0.1532 | 2.5917 | 0.1562 | 4.4568 | 0.1575 | 5.1233 | 0.1567 |
| 3b      | 2.265  | 0.1575 | 1.6857 | 0.1383 | 1.1517 | 0.1070 | \textbf{0.8888} | 0.1077 |
| 3d      | 1.1553 | 0.1098 | 1.2905 | 0.1122 | \textbf{0.7758} | 0.1072 | \textbf{0.6703} | 0.105  |
| 3e      | \textbf{0.785} | 0.1532 | 1.3348 | 0.1552 | \textbf{0.819} | 0.1548 | 1.5117 | 0.1548 |
| 3f      | 1.4265 | 0.155  | 1.192  | 0.1545 | \textbf{0.7897} | 0.1537 | \textbf{0.7102} | 0.1528 |

Table 2. Surface roughness measured via AFM.

Based on cantilever deflection values measured during the contact scanning mode.

\(R_z\) is the average peak-valley height of the cured coating. Values meeting minimum surgical grade steel requisites are highlighted.

\(R_a\) is the average surface roughness of the cured coating.
Comparing $R_z$ of the cured coating formulations to the peak-valley requisite for surgical grade steel ($R_z \leq 1 \mu m$, 320 grit, electropolished), several of the cured coating formulations were well within the requisite value for surgical grade steel with 3a as a notable exception, providing evidence of smoothness capable of inhibiting many types of microbial growth by reducing the available surface area for attachment (Gillis et al., 1996; Mei et al., 2011).

**Single Species Biofilm Resistance Studies**

Each bacterium [e.g., *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*S. pneumoniae*), and *Salmonella typhimurium* (*S. typhimurium*)] was specifically chosen for its contribution to infectious pathways. *E. coli* is generally accepted as the causative agent of 12-50% of all nosocomial infections while *P. aeruginosa* causes 10% of nosocomial infections (Madappa et al., 2019; Qureshi, 2020). One of the most targeted species is *S. aureus* which is the most common cause of nosocomial pneumonia and operative wound infections and the second most common cause of nosocomial bloodstream infections (Baorto et al., 2019). *S. pneumoniae* is the most common cause of community-acquired pneumonia (CAP) and also significantly contributes to bacterial meningitis, bacteremia, and otitis media (Prado et al., 2018). *S. typhimurium*, the causative bacterium of typhoid, has also been linked to many cases of food poisoning (Gamarra et al., 2018; Owens et al., 2018).

Upon cultivating single species biofilms in the custom-built biofilm reactor, qualitative examination of coated plastic slides was performed via optical microscopy (100x magnification) to ascertain success of the biofilm resistant polymers after staining. Qualitative biofilm resistance [e.g., scale: 1 (excessive biofilm) – 3 (same as control) – 5 (minimal biofilm)] was determined when an appreciable difference in bacterial population and biofilm growth was observed between the uncoated portion of each slide and the corresponding coating containing varying weight percent of the halogenated monomers (Figures 2-6).

Having no halogenation, 3a was utilized as an internal standard having no inherent biofilm resistant structural component. For reference, we also compared the standard control coating (no compound 3) to the uncoated portion of the slide. The motile bacteria examined (e.g., *E. coli*, *P. aeruginosa*, and *S. typhimurium*) had nominal or increased biofilm development on the control coating while the non-motile *coci* had nominal or decreased biofilm development on the control coating relative to the uncoated portion of the slide.
Figure 2. Qualitative evaluation of *E. coli* biofilm resistance.

Efficacy compared to control (uncoated slide).

UV Control: UV-curable semi-gloss acrylic clearcoat (Allied Photochemical, KZ-7025-CL).

T = 38°C, t = 10 days, LB Miller agar broth, starvation conditions.

Some biofilm resistance was observed for monomers 3c-g; however, monomers 3a and 3b did not exhibit any appreciable *E. coli* biofilm resistance. While the lack of biofilm resistance for 3a was expected, that of 3b might indicate that *meta*-chlorination promotes limited biofilm resistance toward *E. coli*. With a maximum efficacy at 15 weight percent for 3c, 3f, and 3g as well as significant biofilm resistance of 3e, *para*-halogenation may increase biofilm resistance for *E. coli*. The brominated monomers were generally better biofilm inhibitors than either the chlorinated or iodinated derivatives.
Figure 3. Qualitative evaluation of *P. aeruginosa* biofilm resistance.

Efficacy compared to control (uncoated slide).

UV Control: UV-curable semi-gloss acrylic clearcoat (Allied Photochemical, KZ-7025-CL).

T = 38°C, t = 10 days, LB Miller agar broth, starvation conditions.

*P. aeruginosa* is among the best biofilm forming bacteria and was a logical choice for demonstrating biofilm resistance. For each concentration, the monohalogenated monomers (3b, 3c, 3e, and 3g) did not inhibit biofilm formation; yet, 3e may become biofilm resistant with increased concentrations based on the trend observed. Both dihalogenated monomers (3d and 3f) demonstrated some biofilm resistance at two or more concentrations. Interestingly, 3d was more efficacious at lower concentrations indicating that the biostatic effect inherent to a MIC may be a more important effect for this bacterium than ensuring a cidal effect via MBC. The aforementioned interplay of MIC and MBC may also be illustrated with 3f given that biofilm formation was reduced but not minimized.
Figure 4. Qualitative evaluation of *S. aureus* biofilm resistance.

Efficacy compared to control (uncoated slide).

UV Control: UV-curable semi-gloss acrylic clearcoat (Allied Photochemical, KZ-7025-CL).

$T = 38^\circ C$, $t = 10$ days, LB Miller agar broth, starvation conditions.

*S. aureus* did not exhibit biofilm resistance for any of the chlorinated monomers (e.g., 3b, 3c, and 3d). At increasing concentrations of the brominated monomers (3e and 3f), biofilm resistance increased noticeably indicating a direct concentration correlation. Furthermore, at 20 weight percent, 3g reduced biofilm formation. Thus, biofilm formation of *S. aureus* may be more sensitive to softer atoms such as bromine or iodine.
Figure 5. Qualitative evaluation of *S. pneumoniae* biofilm resistance.
Efficacy compared to control (uncoated slide).
UV Control: UV-curable semi-gloss acrylic clearcoat (Allied Photochemical, KZ-7025-CL).
\(T = 38^\circ C, t = 10\) days, blood base soy agar broth, starvation conditions.

Across the board, lower monomer concentrations (\(\leq 15\) weight percent) did not inhibit biofilm formation. The monochlorinated and iodinated derivatives (e.g., 3b, 3c, and 3g) also had limited utility thereof. However, at the highest tested concentration (20 weight percent), the dichlorinated monomer (3d) and both brominated derivatives (3e and 3f) demonstrated reduced biofilm formation with observable trends from low to high concentration. Thus, it is reasonable to conclude that multichlorinated and brominated monomer derivatives are likely resistant to *S. pneumoniae* biofilm formation.
Figure 6. Qualitative evaluation of *S. typhimurium* biofilm resistance.
Efficacy compared to control (uncoated slide).
UV Control: UV-curable semi-gloss acrylic clearcoat (Allied Photochemical, KZ-7025-CL).
T = 38°C, t = 10 days, LB Miller agar broth, starvation conditions.

Moderate biofilm inhibition was observed for the 15 weight percent concentration of 3b. *S. typhimurium* biofilms were most inhibited by the dichlorinated (3d) and brominated (3e and 3f) monomers at several concentrations with the multihalogenated derivatives outperforming the monohalogenated counterparts, especially at higher percent incorporation into the coating.

Overall, biofilm formation was observed on the uncoated portions, and the levels of growth clearly fall over a wide spectrum. As a non-halogenated internal standard, 3a did not exhibit any biofilm resistance. The monohalogenated derivatives (e.g., 3b, 3c, 3e, and 3g) showed some limited biofilm resistance especially at higher concentrations with 3e resisting biofilm formation best. Dihalogenated monomers (3d and 3f) seemed to impede biofilm formation better than their monohalogenated counterparts. Comparison of chlorinated, brominated, and iodinated derivatives, the brominated monomers were the most effective biofilm resistant monomers. Brominated coatings tended to perform better than chlorinated coatings with 40.% of the
brominated coatings passing our qualitative examination and 14% failing. Chlorinated coatings had a 15% pass rate with a 44% failure rate.

Multiple Species Biofilm Resistance Studies

Laboratory conditions using lab-grown bacteria, which may have reduced immune functionality from multi-generational reproduction, may not provide an adequate environment for evaluating biofilm resistance. Therefore, we evaluated the coatings’ biofilm resistance in raw clarified sewage at the Abilene (Texas) Wastewater Reclamation Plant. After preparing the slides similarly to the previous single species biofilm studies, the varying composition (3a-g) slides were glued onto a plastic plate and placed into a custom-built biofilm resistance apparatus (BRApp). The BRApp was inserted into a secondary clarifier and removed after two days’ exposure to raw sewage. The slides were prepared as before and then qualitatively evaluated for biofilm resistance [e.g., scale: 1 (excessive biofilm) – 3 (same as control) – 5 (minimal biofilm)] using optical microscopy at 100x magnification (Figure 7).

Figure 7. Qualitative evaluation of multiple species biofilm resistance in raw clarified sewage.
Efficacy compared to control (uncoated slide).
UV Control: UV-curable semi-gloss acrylic clearcoat (Allied Photochemical, KZ-7025-CL).
T = ambient outside, t = 2 days, V = 1.75 M gal/day.

Similar to the laboratory-based, single bacterium studies and after exposure to 3.5 million gallons of raw clarified sewage, the coatings incorporating the brominated monomers (3e and 3f) were most efficacious as biofilm resistant materials. Likewise, the dihalogenated (3d and 3f) compounds seemed to also be more effective biofilm resistant monomers than the monohalogenated monomers of which 3g exhibited some biofilm resistance unlike the monochlorinated monomers (3b and 3c). Thus, the individual single and multiple species biofilm resistance studies yielded comparatively consistent results. Interestingly, visible algae growth was restricted solely to the BRApp (and not the slides) thereby indicating cursory resistance to algae growth.

**Normalized Aggregate Biofilm Resistance Studies**

To evaluate the monomers and concentrations for optimal biofilm resistance, all biofilm resistance data (single and multiple species biofilm resistance studies) were aggregated and normalized relative to the equivalent of no biofilm resistance differential between uncoated control and coating. (Figure 8).
Figure 8. Normalized aggregated qualitative evaluation of multiple species biofilm resistance.
Normalized relative to no biofilm resistance differential between uncoated control and coating. Reduced biofilm resistance relative to the control is negative. Increased biofilm resistance relative to the control is positive.

The normalized data seem to indicate that biofilm resistance is directly related to concentration of the monomers with the exception of the internal control (3a). The normalized data also demonstrates that monomers 3b, 3c, and 3g are not biofilm resistant at the incorporated amounts in the coating. 3d is nominally biofilm resistant at higher concentrations (20 weight percent) while 3e is significantly more efficacious as a biofilm resistant monomer as evidenced by the efficacy at both 15 and 20 weight percent. However, 3f as a dibrominated derivative seems to be the best biofilm resistant monomer of the data set with burgeoning biofilm resistance beginning at 10 weight percent.
Surface Energy Analyses

Surface energy analyses may be accomplished via many methods; however, we chose a goniometric method for its simplicity and affordability. Using three fully characterized liquids to obtain statistical contact angle averages, the van Oss-Chaudhury-Good equation (1) was solved linear algebraically for the following surface energy components: nonpolar ($\gamma_s^{LW}$), acid ($\gamma_s^+$), and base ($\gamma_s^-$) components (van Oss et al., 1987).

$$ (1 + \cos \theta) \gamma_i^{tot} = 2 \left( \sqrt{\gamma_s^{LW}\gamma_s^{LW}} + \sqrt{\gamma_s^+\gamma^-} + \sqrt{\gamma_s^-\gamma^+} \right) $$

(1)

The polar component ($\gamma_{sAB}^+$) and overall surface energy ($\gamma_s^+$) was then calculated via Equations 2 and 3, respectively (van Oss et al., 1988).

$$ \gamma_{AB} = 2\sqrt{\gamma^+\gamma^-} $$

(2)

$$ \gamma^{tot} = \gamma^{LW} + \gamma_{AB} $$

(3)

Table 3 tabulates the surface energy profiles for each polymerized phenyl acrylate derivative (3a-g).

| Substrate | $\gamma_s$ | $\gamma_s^{LW}$ | $\gamma_{sAB}$ | $\gamma_s^+$ | $\gamma_s^-$ |
|-----------|------------|-----------------|----------------|--------------|--------------|
| 3a        | 34.48      | 28.72           | 5.76           | 1.75         | 4.74         |
| 3b*       | 56.25      | 36.48           | 19.77          | 4.29         | 22.78        |
| 3c        | 25.56      | 25.04           | 0.52           | 0.01         | 6.37         |
| 3d        | 37.66      | 30.12           | 7.53           | 0.646        | 21.98        |
| 3e        | 28.85      | 24.14           | 4.70           | 0.591        | 9.36         |
| 3f        | 36.81      | 28.12           | 8.69           | 0.790        | 23.91        |
| 3g        | 30.85      | 30.19           | 0.66           | 0.35         | 0.31         |

Table 3. Surface energy profile of derivatized phenyl acrylate polymers.
All units are mJ/m².
Calculations based on contact angles from bromonaphthalene, formamide, and water.
* Obtaining a smooth coating without smearing or orange peeling was difficult and may have contributed to an anomalous/inaccurate surface energy profile; however, for completeness, the surface energy profile for 3b was included in the dataset.
In order to compare the surface energy profiles of the polymerized phenyl acrylate derivatives (3a-g), the surface energy profile was similarly obtained for collagen (insoluble and soluble), *S. aureus*, and *P. aeruginosa* (Table 4).

| Substrate                  | $\gamma_s$ | $\gamma_s^{LW}$ | $\gamma_s^{AB}$ | $\gamma_s^+$ | $\gamma_s^-$ |
|----------------------------|------------|------------------|------------------|--------------|--------------|
| Collagen, insoluble$^a$    | 31.25      | 27.44            | 3.82             | 0.44         | 8.27         |
| Collagen, soluble$^b$      | 33.45      | 33.25            | 0.20             | 0.00083      | 11.61        |
| *S. aureus*$^c$            | 39.12      | 33.54            | 5.58             | 0.12         | 67.60        |
| *P. aeruginosa*$^c$        | 43.55      | 39.57            | 3.97             | 0.054        | 73.32        |

Table 4. Surface energy profile of various biologic materials.
All units are mJ/m$^2$.

$^a$ Calculations based on contact angles from bromonaphthalene, formamide, and water for insoluble collagen (100 $\mu$g/mL).

$^b$ Calculations based on contact angles from bromonaphthalene, formamide, and water for soluble collagen (100 $\mu$g/mL) in phosphate buffer solution (1x, pH = 7.4).

$^c$ Calculations based on contact angles from bromonaphthalene, dimethylsulfoxide, and water.

The surface energy component values for collagen (e.g., $\gamma_s^{LW}$, $\gamma_s^{AB}$, and $\gamma_s$) were comparable to established literature values with additional values for the acid ($\gamma_s^+$) and base ($\gamma_s^-$) components (Lewandowska et al., 2017; Skopińska-Wiśniewska et al., 2009). Generally, easy release substrates have a significantly diminished $\gamma_s$ relative to tight substrates (i.e., those that have significant interfacial interactions). Often a quick comparison of the overall surface energy ($\gamma_s$) of two interacting materials has been used to establish a degree of interfacial interaction between two materials. However, upon plotting the overall surface energy ($\gamma_s$) for all phenyl acrylic coatings, collagens, and bacteria, no clear trend is apparent (Figure 9).
Figure 9. Overall surface energy comparison ($\gamma_s$) of phenyl acrylic coatings, collagens, and bacteria.

All units are mJ/m$^2$.

a Calculations based on contact angles from bromonaphthalene, formamide, and water for insoluble collagen (100 $\mu$g/mL).

b Calculations based on contact angles from bromonaphthalene, formamide, and water for soluble collagen (100 $\mu$g/mL) in phosphate buffer solution (1x, pH = 7.4).

c Calculations based on contact angles from bromonaphthalene, dimethylsulfoxide, and water.

* Obtaining a smooth coating without smearing or orange peeling was difficult and may have contributed to an anomalous/inaccurate surface energy profile; however, for completeness, the surface energy profile for $3b$ was included in the dataset.

Therefore, each individual component was examined for all phenyl acrylate monomer derivatives, collagens, and bacteria. The most interesting individual component comparisons involved the polar ($\gamma_s^{AB}$) and base components ($\gamma_s^-$) plotted in Figures 10 and 11, respectively.
Figure 10. Surface energy polar component ($\gamma_s^{AB}$) comparison of phenyl acrylic coatings, collagens, and bacteria.

All units are mJ/m$^2$.

a Calculations based on contact angles from bromonaphthalene, formamide, and water for insoluble collagen (100 $\mu$g/mL).

b Calculations based on contact angles from bromonaphthalene, formamide, and water for soluble collagen (100 $\mu$g/mL) in phosphate buffer solution (1x, pH = 7.4).

c Calculations based on contact angles from bromonaphthalene, dimethylsulfoxide, and water.

* Obtaining a smooth coating without smearing or orange peeling was difficult and may have contributed to an anomalous/inaccurate surface energy profile; however, for completeness, the surface energy profile for 3b was included in the dataset.

Excepting 3a (internal control) and 3b (inaccurate profile), the substrates with the most similar $\gamma_s^{AB}$ include 3d, 3e, 3f, insoluble collagen, P. aeruginosa, and S. aureus. Focusing on the 3d, 3e, 3f, and bacteria, the similarities in the polar component ($\gamma_s^{AB}$) likely indicates a more significant thermodynamic interaction. The single species biofilm resistance studies seem to qualitatively support a more significant interaction between 3d, 3e, 3f, and bacteria. Because the magnitude of the acid components ($\gamma_s^+$) is comparatively small, the base components ($\gamma_s^-$) should be the most significant interaction as shown in Figure 11.
Figure 11. Surface energy base component ($\gamma_s^-$) comparison of phenyl acrylic coatings, collagens, and bacteria.
All units are mJ/m$^2$.

a Calculations based on contact angles from bromonaphthalene, formamide, and water for insoluble collagen (100 $\mu$g/mL).
b Calculations based on contact angles from bromonaphthalene, formamide, and water for soluble collagen (100 $\mu$g/mL) in phosphate buffer solution (1x, pH = 7.4).
c Calculations based on contact angles from bromonaphthalene, dimethylsulfoxide, and water.

* Obtaining a smooth coating without smearing or orange peeling was difficult and may have contributed to an anomalous/inaccurate surface energy profile; however, for completeness, the surface energy profile for 3b was included in the dataset.

3d is qualitatively less efficacious as a biofilm resistant substrate perhaps due to the larger nonpolar component which may obfuscate the relatively hard (i.e., charge dense) chlorine atoms, especially when bound in an amorphous, crosslinked polymer matrix with little polar directionality. Furthermore, biofilm formation of S. aureus seemed to be more significantly inhibited by softer halogens.

Based on Figure 8, 3e and 3f were clearly the most biofilm resistant substrates examined in this study and, based on Figure 11, have large base components ($\gamma_s^-$). A significant intermolecular (base-base) repulsion may be a causative agent of biofilm resistance. Qualitatively, S. aureus, a non-motile bacterium, was most affected by 3e while both 3e and 3f equally inhibited biofilm
formation of the motile *P. aeruginosa*. With surface interactions being diffusion controlled, *S. aureus* adhesion is thermodynamically controlled. The parallel movement of *P. aeruginosa* along a surface interface would contribute a competing kinetic effect to the thermodynamic driving force for surface adhesion. Due to kinetic competition, the biofilm resistance of 3e and 3f are slightly diminished for *P. aeruginosa* relative to *S. aureus* as observed. The increased thermodynamic biofilm resistance may be twofold. First as previously stated, the interacting base components ($\gamma_s^-$) of a stationary substrate with a diffusing bacterium would have an increasing intermolecular charge repulsion as the distance between substrate and bacterium decreases. Such would especially be present in the non-chelated N2 domain of the surface protein SdrC of *S. aureus*. Second and likely to a lesser degree, a polarizable soft atom (e.g., bromine) or other polarizable moiety could allow the bacterium to remain associated with the substrate in the absence of reversible adhesion during primary colonization. The latter reasoning is used to explain, in part, limited bacterial attachment onto superhydrophilic substrates (Noorisafa et al., 2016; Yuan et al., 2017).

**CONCLUSIONS**

Seven monomers (i.e., phenyl acrylate and halogenated derivatives thereof) were successfully synthesized through a standard laboratory synthesis. Each monomer was added at variable concentrations (e.g., 5, 10, 15, and 20 weight percent) to a compatible industrial formulation that was subsequently UV-cured onto various substrates. Examination via atomic force microscopy illustrated that several of the cured coating formulations, including those of 3e and 3f, yielded exceptionally smooth coatings with limited surface areas evidenced by average peak to valley heights ($R_z$) less than 1.0 $\mu$m and very low roughness ($R_a$) measurements. 3f exhibited an inverse relationship of both $R_z$ and $R_a$ as concentration increased. The coatings were then analyzed for single (e.g., *E. coli*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, and *S. typhimurium*) and multiple (e.g., clarified raw sewage) species biofilm resistance. After normalizing the biofilm resistance studies, coatings incorporating the brominated phenyl acrylate monomers 3e and 3f exhibited the most biofilm resistance. Because biofilm resistance is a symbiotic, multi-determinant system involving the physical and chemical interaction of both substrate and bacteria, we also examined the surface energies of the polymerized phenyl acrylate derivatives, collagen, and two representative bacteria (e.g., *P. aeruginosa* and *S. aureus*). Comparative analysis of each surface energy component demonstrated that the polar component ($\gamma_s^{AB}$), specifically the base component ($\gamma_s^-$), is likely the primary thermodynamic contributor to the observed biofilm resistance. Repulsive intermolecular interactions between base components of both the substrate and bacteria prevent intimate bacterial association with the substrate. Secondarily, we posit that the presence of soft atoms (e.g., bromine) and/or polarizable moieties in the coating may allow
bacterial association while inhibiting adhesion and biofilm formation during primary colonization.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found attached to the end of this article.

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Synthesis, A.R.D., R.A.F., T.C.G., P.R.H., J.R.L.; Atomic Force Microscopy, D.S.; Surface Energy Analyses of coatings, J.G.C., V.N.G., D.S.O.; Surface Energy Analyses of bacteria, T.B.C., E.B.H., W.W.; Biofilm Studies, R.A.F., P.R.H. C.J.H., J.A.L.; Writing – Original Draft & Review & Editing, T.B.C.; Conceptualization & Project Administration, T.B.C.; Supervision, T.B.C.
DECLARATION OF INTERESTS

The authors declare no competing interest.

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SUPPLEMENTAL INFORMATION

Thermodynamic Surface Analyses to Inform Biofilm Resistance

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Data S1. Spectra of Products: Related to Table 1.
Data S2. Product Characterization: Related to Table 1.

Phenyl Acrylate (3a).

![Phenyl Acrylate](image)

A colorless oil; average isolated yield = 59%; $n_D^{25} = 1.5354$; $\varepsilon_{266\text{ nm}} = 587.18\ M^{-1}\ cm^{-1}$; $\varepsilon_{313\text{ nm}} = 11.33\ M^{-1}\ cm^{-1}$; $\varepsilon_{365\text{ nm}} = 4.00\ M^{-1}\ cm^{-1}$; $^1\text{H NMR (60 MHz, NEAT)} \delta 6.93-7.61$ (m, 5H), 5.83-6.59 (m, 3H); IR ($\nu$, cm$^{-1}$) 1740.3, 1591.5, 1197.9, 982.8, 920.9, 883.3, 691.3.

3-Chlorophenyl Acrylate (3b).

![3-Chlorophenyl Acrylate](image)

A pale yellow oil; average isolated yield = 33%; $n_D^{25} = 1.5359$; $\varepsilon_{266\text{ nm}} = 761.75\ M^{-1}\ cm^{-1}$; $\varepsilon_{313\text{ nm}} = 18.74\ M^{-1}\ cm^{-1}$; $\varepsilon_{365\text{ nm}} = 12.06\ M^{-1}\ cm^{-1}$; $^1\text{H NMR (60 MHz, NEAT)} \delta 6.71-7.45$ (m, 4H), 5.83-6.68 (m, 3H); IR ($\nu$, cm$^{-1}$) 1747.3, 1589.9, 1209.4, 982.4, 877.1, 778.1, 678.0.

4-Chlorophenyl Acrylate (3c).

![4-Chlorophenyl Acrylate](image)
A pale yellow oil; average isolated yield = 36%; \( n^D_{25} = 1.5550 \); \( \varepsilon_{266\ nm} = 454.64\ M^{-1}cm^{-1} \);  
\( \varepsilon_{313\ nm} = 0.42\ M^{-1}cm^{-1} \);  
\( \varepsilon_{365\ nm} = 0.00\ M^{-1}cm^{-1} \);  
\( ^1H\ NMR\ (60\ MHz,\ NEAT)\ \delta\ 6.92-7.00\ (m, 4H), 5.88-6.91\ (m, 3H);\ IR\ (\nu,\ cm^{-1})\ 1745.0, 1635.4, 1203.0, 982.1, 899.2, 807.2.\)

2,4-Dichlorophenyl Acrylate (3d).

A yellow-orange oil; average isolated yield = 30.%;  
\( n^D_{25} = 1.5900 \);  
\( \varepsilon_{266\ nm} = 901.33\ M^{-1}cm^{-1} \);  
\( \varepsilon_{313\ nm} = 0.00\ M^{-1}cm^{-1} \);  
\( \varepsilon_{365\ nm} = 0.00\ M^{-1}cm^{-1} \);  
\( ^1H\ NMR\ (60\ MHz,\ NEAT)\ \delta\ 6.71-7.53\ (m, 4H), 5.97-6.61\ (m, 3H);\ IR\ (\nu,\ cm^{-1})\ 1751.1, 1583.9, 1218.2, 982.0, 875, 866.0\ cm^{-1}, 810.9\ cm^{-1}.\)

4-Bromophenyl Acrylate (3e).

A yellow-orange oil; average isolated yield = 21%;  
\( n^D_{25} = 1.5580 \);  
\( \varepsilon_{266\ nm} = 465.11\ M^{-1}cm^{-1} \);  
\( \varepsilon_{313\ nm} = 5.37\ M^{-1}cm^{-1} \);  
\( \varepsilon_{365\ nm} = 0.34\ M^{-1}cm^{-1} \);  
\( ^1H\ NMR\ (60\ MHz,\ NEAT)\ \delta\ 6.61-7.73\ (m, 4H), 5.84-6.58\ (m, 3H);\ IR\ (\nu,\ cm^{-1})\ 1747.9, 1635.4, 1200.5, 981.8, 898.5, 804.5.\)

2,4-Dibromophenyl Acrylate (3f).
A yellow-orange oil; average isolated yield = 39%; $n_D^{25} = 1.5998$; $\varepsilon_{\text{266 nm}} = 764.62 \text{ M}^{-1}\text{cm}^{-1}$; $\varepsilon_{313 \text{ nm}} = 0.00 \text{ M}^{-1}\text{cm}^{-1}$; $\varepsilon_{365 \text{ nm}} = 0.00 \text{ M}^{-1}\text{cm}^{-1}$; $^1$H NMR (60 MHz, NEAT) $\delta$ 7.25-7.81 (m, 3H), 5.91-7.23 (m, 3H); IR ($\nu$, cm$^{-1}$) 1752.1, 1636.1, 1214.3, 981.2, 895.0, 883, 796.9.

4-Iodophenyl Acrylate (3g).

A pale yellow oil; average isolated yield < 10%; $n_D^{25} = 1.5229$; $\varepsilon_{\text{266 nm}} = 3434.39 \text{ M}^{-1}\text{cm}^{-1}$; $\varepsilon_{313 \text{ nm}} = 0.00 \text{ M}^{-1}\text{cm}^{-1}$; $\varepsilon_{365 \text{ nm}} = 0.00 \text{ M}^{-1}\text{cm}^{-1}$; $^1$H NMR (60 MHz, NEAT) $\delta$ 6.65-8.00 (m, 4H), 5.81-6.64 (m, 3H); IR ($\nu$, cm$^{-1}$) 1746.6, 1634.0, 1201.5, 981.5, 898.7, 802.3.
Transparent Methods

A. General Information

Materials and Instrumentation.

Most chemicals used in the monomer syntheses and testing, including the phenolic precursors, triethylamine (Et₃N), acryloyl chloride, and acetonitrile, were purchased from Sigma-Aldrich. The dichloromethane also used in the syntheses was purchased from Pharmacia. The material used for the coating formulations was obtained from Allied Photochemical and is a proprietary formulation. Cytec Specialty Chemicals provided the 1,6-hexanediol diacrylate (HDODA) used in the photo-DSC. Albemarle Corporation donated the photoinitiator, 2,2-dimethoxy-2-phenylacetophenone (DMPA). The uncoated, polished stainless-steel plates were purchased from Q Panel Products. Methyl ethyl ketone (MEK) for the double rub test was purchased from The Paint Center. Cytology fixative spray was obtained from Andwin Scientific. Nitrogen gas was provided by Airgas.

The bacteria were stained using Hema-diff solution 3-thiazine dye from Anapath. Bacteria were obtained from Carolina Biological Supply. The Luria-Bertani, Miller (LB Miller) nutrient agar was provided by Fisher Scientific. The Trypticase Soy Agar and Bacto Blood Agar Base, Dehydrated were obtained from Difco. BBL SIM Medium was obtained from BBL Microbiology Systems.

Characterization of monomers and polymers was conducted using multiple machines. The NMR Spectrometer Eft-60 was provided by Anasazi Instruments Inc. The Infrared Spectrophotometer was obtained from Perkin Elmer (1600 Series). The rotary evaporator (Rotovap), collegiate model, was provided by Heidolph LABORTA. Sargent-Welch Scientific Company provided the Welch DuoSeal Vacuum Pump, Model 1400. Mel-Temp Electrothermal melting point apparatus (Model 1201D) was provided by Barnstead/Thermolyne. Refractometer was obtained from Thermo Electron Corporation (Model 334610). Photo-Differential Scanning Calorimeter (DSC 822°) was obtained from Mettler Toledo and the ultraviolet spot light source was the Lightningcure 200 by Hamamatsu. The Ultraviolet-Visible Spectrometer (HP 8453) was obtained from Hewlett-Packard. Light Microscope (Model M404DP) was provided by Swift Instruments International. AMSCO provided the autoclave model AMSCO 3021 Gravity. Incubation was performed in a GI200A-1 model incubator from Thermo Electron Corporation.

UV-Vis Spectroscopy.

10.0 mL acetonitrile was measured out in a graduated cylinder then added to scintillation vials containing 0.05 g of the monomer. UV-Vis spectra were taken of each monomer then diluted
with acetonitrile as necessary [until absorbance was measured between 0.75-1.0]. Measurements were taken of absorbance at specific wavelengths of the monomers using UV Vis and used to determine the extinction coefficient at wavelengths of 266, 313, and 365 nm.

**Coating Production.**

*Application of Formulation to Unpolished Stainless-Steel Plates.* The plates and draw down bar were washed with acetone. The drawdown bar was set in the position demarking four mils (100 µm), and the formulation was applied to the plate along the top edge of the drawdown bar. The drawdown bar was then drawn at uniform speed to evenly apply the coating. If streaking or orange peel occurred, the drawdown was repeated until the formulation was evenly applied.

*Application of Formulation to Plastic Slides.* Six one-inch by three-inch (1 in. x 3 in.) plastic (optically clear vinyl) slides were prepared per coating formulation. Six slides were laid horizontally together to form a solid plastic surface with the sides taped down. The drawdown bar was placed on the slides and formulation was applied to the top-most slides. The drawdown bar was observed to be in the four mil position and drawn down at uniform speed to evenly coat half of the surface of each of the six slides. If streaking or orange peel occurred, the drawdown was repeated until the formulation was evenly applied.

*Application of Formulation to Glass Slides.* One, one-inch by three-inch (1 in. x 3 in.) glass slide was prepared per formulation. Four glass slides were laid down vertically to form two columns of two slides each far enough apart to fit a fifth glass slide in the middle (the one to be coated) and to allow the edges of the draw down bar to rest on them. In this manner a uniform coating depth was achieved for the targeted glass slide because the drawdown bar rested not on the experimental surface, but on glass slides of the same size as the target. When the drawdown bar was placed on the supporting glass slides and observed to be in the four mil position, formulation was applied to the target glass slide. The drawdown bar was drawn at a uniform speed to spread the formulation evenly; however, if streaking or orange peel occurred, the drawdown was repeated.

*Polymerization of phenyl acrylate monomer derivatives.* Multiple passes under a Fusion UV Systems, Inc. LC-6/F300S equipped with a H-bulb cured the formulations (one weight percent DMPA dissolved in monomer) in air at 20 feet per minute and were confirmed via a traditional thumb-twist test. The coatings were cured to metal plates, plastic slides, and glass slides to conduct various physical and biological tests.

*Biologic sample preparation for contact angle measurements.* Bovine collagen (purchased from Aldrich) coatings were prepared according to established literature protocols (Hansen et al., 2011). For consistency, insoluble and soluble collagen concentrations were measured to be 100
μg/mL. Soluble collagen was dissolved in a phosphate buffer system (1 x PBS, pH = 7.4) purchased from Aldrich.

Both S. aureus and P. aeruginosa were rehydrated according to established procedures from the supplier and transferred aseptically onto two 10 mL Luria-Bertani (LB), Miller, nutrient agar plates. The inoculated plates were placed in an incubator at 37°C for 72 hours. After incubation, the specimens were placed in a refrigerator and stored at 6°C until use.

Atomic Force Microscopy (AFM).

Atomic force microscopy (AFM) contact scanning curves of each coating were obtained at two locations that were an approximate 80 μm x 80 μm area (6,400 μm²) to eliminate the effects of interference on the roughness measurements. Both X and Y roughness calculations were averaged to yield the average three-dimensional surface roughness (Ra) for each location (Equation 4) (Raposo et al., 2007).

\[
Ra(M, N) = \frac{1}{NM} \sum_{x=1}^{N} \sum_{y=1}^{M} [z(x, y) - \bar{z}(x, y)]
\]  

(4)

Three locations were arbitrarily selected for each plate whereupon the roughness and peak-valley height (Rz) were determined (Equation 5) (Raposo et al., 2007).

\[
R_z = \frac{1}{n} \left( \sum_{i=1}^{3} R_{p,j} + \sum_{i=1}^{3} R_{v,j} \right)
\]  

(5)

The roughness values and peak-valley height for the three locations were then all averaged for an overall average plate roughness and average peak-valley height. The average peak-valley height was then compared to that of surgical grade steel (Rz ≤ 1 μm).

Extraction Studies.

Extraction studies were performed for all cured formulations at 20 weight percent active monomer incorporation using gas chromatography (GC) – mass spectrometry (MS). Each cured coating (0.5 g) was scraped from the steel plates, powdered, placed into 10 mL of methanol in a capped vial, and allowed to soak for one week at which point one milliliter of the supernatant was placed into a GC sample vial. The GC-MS was then run for each of the samples whereupon the percent extractables were calculated. The lower limit of detection is 100 μg/mL. The GC used a 30 m (0.1 mm inside diameter) nonpolar column with a 250°C injection temperature, 150°C oven temperature, and 280°C interface temperature.

Electronic copy available at: https://ssrn.com/abstract=3624435
Photo-Differential Scanning Calorimetry (Photo-DSC).

The monomers (i.e., phenyl acrylate derivatives, 3a-g) were formulated at ten weight percent as shown in Table 3, and then two microliters (2 µL) of each formulation was measured into crimped, aluminum sample pans. The light intensities were measured using black body absorbers. The calorimetric measurements were performed using a Mettler-Toledo DSC 822e modified with a Hamamatsu Lightning Cure 200 UV-spot, equipped with a high-pressure mercury lamp. The sample cell was kept at a constant 20°C by a Julabo FT 100 intercooler. The sample was purged with nitrogen for two minutes prior to beginning the run and continued through the completion of the run. The polymerization rates of each monomer were compared to that of NEAT HDODA and to a HDODA sample photoinitiated by a standard Norrish Type I photoinitiator (e.g., DMPA).

| Compound | Mass Run 1 (g) | Mass Run 2 (g) | Mass Run 3 (g) |
|----------|---------------|---------------|---------------|
| HDODA    | 8.9           | 9.9           | 9.0           |
| Monomer  | 1.0           | 0             | 1.0           |
| DMPA     | 0.1           | 0.1           | 0             |

Table 3. Photo-DSC formulations.
2 µL sample; 2 min N₂ purge; full arc; high-pressure mercury lamp.

Single Species Biofilm Resistance Studies.

The ability of microorganisms to form biofilms on the coatings was tested through the cultivation of five different bacteria and subsequent exposure of these microorganisms to the coatings.

Cultivation. All specimens were rehydrated according to established procedures from the supplier. The rehydrated bacteria (Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, and Pseudomonas aeruginosa) were transferred aseptically into four test tubes containing 10 mL LB, Miller, nutrient agar slants. The inoculated test tubes were placed in an incubator at 37°C for 72 hours. After incubation, the specimens were placed in a refrigerator and stored at 6°C until use.

Streptococcus pneumoniae was cultivated within an agar composed of 45% Trypticase™ Soy Agar, 30% Blood Base Agar, and 25% BBL SIM agar. Due to the fastidious nature of S. pneumoniae, S. pneumoniae was the only specimen cultivated anaerobically in six plates composed of 10mL each of the aforementioned custom agar and in four test tube slants containing 10 mL of the custom agar. The inoculated samples were placed in an incubator at 37°C for 72 hours and afterward stored in a refrigerator at 6°C.
**Induction of Biofilm Formation.** A biofilm reactor was built to characterize the biofilm resistance of coatings within a bacteria rich environment. A fish tank (30 inches in length, 12.25 inches wide, and 12.5 inches high) was divided into five equally sized sections using custom cut poly(methyl methacrylate) sheets and sealed with waterproof sealant to prevent cross contamination. An evaporative cooler pump was placed in each compartment to circulate approximately three liters of bacterial broth in each compartment. The solution was composed of 3000 mL sterilized water with three grams of LB, Miller, nutrient agar. Each bacterial strain was cultivated in 100 mL of water and 0.3 g of LB, Miller, nutrient agar at 37°C for 24 hours. After 24 hours the inoculated broths were poured into corresponding sections of the biofilm reactor. Plastic slides containing each of the coating formulations were placed in the reactor on a holding apparatus built to allow for a flow assay to measure biofilm growth. Each plastic slide was divided into an uncoated side (internal control) and a coated side (measuring biofilm growth). Over the course of 10 days, 500 mL of broth was replaced with 500 mL sterilized water each day. After this 10 day period, the holding apparatus and all slides were removed. The unattached bacteria and any other materials were rinsed from the slides with sterile, deionized water. The slides were sprayed with a cytology fixative [poly(ethylene glycol)-based]. After the fixative was air dried, the slides were rinsed with deionized water, and stained with methylene blue/Azure A. The excess dye was removed with sterile water leaving behind any residual stained bacteria on the slide. Representative stained samples are provided in Figure 12.

| Sample                  | E. coli | S. aureus | P. aeruginosa | S. typhimurium | S. pneumoniae |
|-------------------------|---------|-----------|---------------|----------------|---------------|
| Control coating (uncoated) | ![Image](https://example.com/image1.png) | ![Image](https://example.com/image2.png) | ![Image](https://example.com/image3.png) | ![Image](https://example.com/image4.png) | ![Image](https://example.com/image5.png) |
| Control coating         | ![Image](https://example.com/image6.png) | ![Image](https://example.com/image7.png) | ![Image](https://example.com/image8.png) | ![Image](https://example.com/image9.png) | ![Image](https://example.com/image10.png) |
| 3a control (uncoated)   | ![Image](https://example.com/image11.png) | ![Image](https://example.com/image12.png) | ![Image](https://example.com/image13.png) | ![Image](https://example.com/image14.png) | ![Image](https://example.com/image15.png) |
| 3a                      | ![Image](https://example.com/image16.png) | ![Image](https://example.com/image17.png) | ![Image](https://example.com/image18.png) | ![Image](https://example.com/image19.png) | ![Image](https://example.com/image20.png) |
| 3b control (uncoated)   | ![Image](https://example.com/image21.png) | ![Image](https://example.com/image22.png) | ![Image](https://example.com/image23.png) | ![Image](https://example.com/image24.png) | ![Image](https://example.com/image25.png) |
3b

3d control (uncoated)

3d

3e control (uncoated)

3e

3f control (uncoated)

3f

Figure 12. Stained plastic slides (100x magnification) after biofilm reactor incubation. Representative monomers (i.e., 3a, 3b, 3d, 3e, and 3f) at 20 weight percent coating incorporation.

Multiple Species Biofilm Resistance Studies.

A multiple species biofilm resistance study for each coating was performed by immersion into sedimented (i.e., clarified) raw sewage in the secondary clarifiers at the Abilene Wastewater Reclamation Plant in Abilene, Texas. The aforementioned plant configuration is represented in Figure 13 where the secondary clarifier used for our testing is highlighted.
Figure 13. Schematic of the Abilene (Texas) Wastewater Reclamation Plant where the secondary clarifier used for our testing is highlighted.

Each cured slide was hot glued to a poly(methyl methacrylate), PMMA, sample sheet obtained from a local home improvement store. The sample sheet was placed into another custom-built apparatus resembling a metal cage, termed the biofilm resistance apparatus (BRApp), in order to protect the samples from mechanical processes that could remove either the coating or the grown biofilm (Figure 14).

Figure 14. Biofilm resistance apparatus (BRApp) submerged in clarified raw sewage.

Then, the BRApp was taken to the Abilene Wastewater Reclamation Plant and submerged into the secondary clarifier which allows the aerated raw sewage to grow existing microbes, some of which consume a portion of the raw sewage materials. It is important to note that the bulk of the solid sewage was removed via sedimentation in the primary clarifiers prior to aeration. Each secondary clarifier is capable of handling 1.75 million gallons of raw sewage each day. The BRApp was left in the secondary clarifier for two days (3.5 million gallons of exposure) at about
a six foot depth, just above the paddle arm that mixes the contents at a rate of six revolutions per hour.

The BRApp was removed and transported back to the lab in a plastic bag whereupon the PMMA sheet was removed, rinsed with deionized water, and treated with an ethanol spray to kill the microbes attached to the sheet and samples. The microbes were then fixated with a poly(ethylene glycol) cytological spray and allowed to dry. Then the slides were stained with a methylene blue/Azure A solution. Each stained slide was qualitatively evaluated by comparing each coating relative to the uncoated portion of the slide both with the naked eye and through an optical microscope (100x) in three different locations on the coating.

B. Representative Procedures

General Procedure for Synthesis of 3.

The phenolic derivative was dissolved in a slight molar excess of triethylamine (TEA) and then added to a 250 mL round bottom flask with 40 mL dichloromethane. Acryloyl chloride (equimolar amount compared to TEA) was added dropwise to the mixture with stirring (Table 1). After the flask was provided with a nitrogen atmosphere the mixture was stirred for 24 hours and thereafter suction filtered to remove the TEA hydrochloride salt. The resulting solution was washed in a separatory funnel 15 times with 15 mL deionized water then anhydrous magnesium sulfate (MgSO₄) was added until clumping stopped. The mixture was suction filtered to remove the anhydrous MgSO₄ and thereafter placed on the Rotovap for solvent volume reduction. The remaining liquid acrylate was dried via vacuum for 24 hours.

NOTE: 3g was a difficult synthesis and required multiple scaled-up reactions to acquire an adequate amount of the compound for testing; therefore, the corresponding reported average isolated yield is low and not comparative to the other syntheses.

C. Surface Energy Calculations

While surface energy analyses have many forms, we have chosen one that is arguably the simplest analyses and has historically been used to describe biological systems (Owens et al., 1969; Schrader, 2002; van Oss et al., 1987; van Oss et al., 1988). According to Equation 6, the change in Gibbs energy of an interface (ΔG_{interface}) is directly related to the surface energy of an interacting material (γ_m) (van Oss et al., 1988).
\[ \Delta G_{\text{interface}} = \left(1 + \cos \theta_{\text{interface}}\right) \gamma_m \]  \hspace{1cm} (6)

Equation 3 defines \( \gamma_m \) (generically represented as \( \gamma^{\text{tot}} \)) to be the sum of the material’s individual nonpolar (\( \gamma^{\text{LW}} \)) and polar (\( \gamma^{\text{AB}} \)) components where \( \gamma^{\text{AB}} \) is defined to be the geometric mean of the separate acid (\( \gamma^+ \)) and base (\( \gamma^- \)) components (Equation 5) (van Oss et al., 1988).

\[
\gamma^{\text{tot}} = \gamma^{\text{LW}} + \gamma^{\text{AB}} \hspace{1cm} (3)
\]

\[
\gamma^{\text{AB}} = 2 \sqrt{\gamma^+ \gamma^-} \hspace{1cm} (2)
\]

Using two fully characterized liquid materials, the Owens-Wendt equation (7) allows the determination of multiple surface energy components of a substrate (e.g., \( \gamma_s^{\text{LW}} \), \( \gamma_s^{\text{AB}} \), and \( \gamma_s \)) via contact angle (\( \theta_{sl} \)) (Owens et al., 1969; Schrader, 2002).

\[
(1 + \cos \theta_{sl}) \gamma_i^{\text{tot}} = 2 \left( \sqrt{\gamma_i^{\text{LW}} \gamma_i^{\text{LW}}} + \sqrt{\gamma_i^{\text{AB}} \gamma_i^{\text{AB}}} \right) \hspace{1cm} (7)
\]

The van Oss-Chaudhury-Good (OCG) equation (1) expands Equation 6 to similarly delineate the substrate’s separate acid (\( \gamma_s^+ \)) and base (\( \gamma_s^- \)) components using three fully characterized liquids. Both \( \gamma_s^{\text{AB}} \) and \( \gamma_s \) can be determined sequentially via Equations 2 and 3 (van Oss et al., 1987; van Oss et al., 1988).

\[
(1 + \cos \theta_{sl}) \gamma_i^{\text{tot}} = 2 \left( \sqrt{\gamma_i^{\text{LW}} \gamma_i^{\text{LW}}} + \sqrt{\gamma_i^{\text{AB}} \gamma_i^{\text{AB}}} + \sqrt{\gamma_s^+ \gamma_s^-} \right) \hspace{1cm} (1)
\]

Using a linear algebraic method to simultaneously solve for components, we used Equation 7 to determine the surface energy profile (e.g., \( \gamma_s^{\text{LW}} \), \( \gamma_s^{\text{AB}} \), \( \gamma_s^+ \), \( \gamma_s^- \), and \( \gamma_s \)) of each polymerized halogenated monomer.

**Contact angle measurements.**

The sessile drop method was utilized where a 2 \( \mu \text{L} \) droplet was placed on a surface and allowed to equilibrate for one minute. Following equilibration, the drop was photographed using a mounted second generation iPad Mini equipped with a macro lens. Contact angle measurements were obtained via a protractor app (Photo Protractor). To maintain quality control, the contact angle photographs were printed and secondarily validated manually via a physical protractor. Statistical averages for each contact angle measurement \( (N \geq 8) \) were obtained after omitting the statistical outliers. We used bromonaphthalene, dimethylsulfoxide, formamide, and/or water to obtain contact angles used to determine the surface energy profiles.
Linear algebraic determination of surface energy profiles.

A linear algebraic approach could be used to solve Equation 7 as given below using the complete characterization of the solvents used. After obtaining the contact angle measurements, Equation 7 was rearranged to yield Equation 8:

$$\frac{1}{2} \left[ (1 + \cos \theta_{sl}) \gamma_{l}^{tot} \right] = \sqrt{\gamma_{LW}^{LW} \gamma_{s}^{LW}} + \sqrt{\gamma_{l}^{+} \gamma_{s}^{-}} + \sqrt{\gamma_{l}^{-} \gamma_{s}^{+}}$$

(8)

We then represent the experimentally determined or known values as \(a\), \(b\), \(c\), and \(d\) and the unknown substrate values as \(x\), \(y\), and \(z\) (Equation 9).

\[d = (a \cdot x) + (b \cdot y) + (c \cdot z)\]

(9)

where the corresponding contact angle measurement for a peculiar liquid is related to its characterized surface energy profile. Furthermore, \(d\) is defined to be \(\frac{1}{2} \left[ (1 + \cos \theta_{sl}) \gamma_{l}^{tot} \right]\), \(a\) is \(\sqrt{\gamma_{l}^{LW}}\), \(x\) is \(\sqrt{\gamma_{s}^{LW}}\), \(b\) is \(\sqrt{\gamma_{l}^{+}}\), \(y\) is \(\sqrt{\gamma_{s}^{-}}\), \(c\) is \(\sqrt{\gamma_{l}^{-}}\), and \(z\) is \(\sqrt{\gamma_{s}^{+}}\). We can now combine all values in matrix form.

\[
\begin{pmatrix}
\text{bromonaphthalene} & d \\
\text{formamide OR} & a & b & c & x \\
\text{dimethylsulfoxide} & h & e & f & g . y \\
\text{water} & l & i & j & k & z
\end{pmatrix}
\]

The 3x1 vector \(dhl\) (i.e., \(d\)), 3x3 matrix (i.e., \(A\)), and 3x1 vector \(xyz\) (i.e., \(x\)) can then be represented more simply as Equation 10.

\[d = A \cdot x\]

(10)

After performing an allowed matrix inversion, we can solve for \(x\) which gives us values for the substrate’s heretofore unknown surface energy components: \(\gamma_{s}^{LW}\), \(\gamma_{s}^{+}\), and \(\gamma_{s}^{-}\) (Equation 11).

\[x = A^{-1} \cdot d\]

(11)

Using the calculated component values, the substrate’s acid-base component \((\gamma_{s}^{AB})\) and overall surface energy \((\gamma_{s})\) can be determined using Equations 2 and 3, respectively.