Antifouling Properties of a Self-Assembling Glutamic Acid-Lysine Zwitterionic Polymer Surface Coating

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ABSTRACT

There is a need for the development of antifouling materials to resist adsorption of biomacromolecules. Here we describe the preparation of a novel zwitterionic block copolymer with the potential to prevent or delay the formation of biofouling. The block copolymer comprised of a zwitterionic (hydrophilic) section of alternating glutamic acid (negatively-charged) and lysine (positively-charged) units and a hydrophobic polystyrene section. First, we characterized the self-assembly of the block-copolymer in aqueous solutions and onto polystyrene (hydrophobic) surfaces. As extracellular polymeric substances (EPS) represent a formidable challenge for anti-biofouling, we then analyzed the adsorption of EPS biomacromolecules onto polystyrene surfaces with and without the block co-polymer surface coating. Cryo-TEM and dynamic-light-scattering (DLS) results showed that on average, the block co-polymer self-assembled into 7 nm micelles in aqueous solutions (0 to 100 mM NaCl, pH 6). Quartz crystal microbalance with dissipation monitoring (QCM-D), atomic force microscopy (AFM), and contact angle measurements demonstrated that the block co-polymer self-assembled into a brush-like monolayer on polystyrene surfaces, exhibiting an average distance, $d$, of approximately 4 - 8 nm between each block co-polymer molecule. Once the brush-like monolayer self-assembled, it reduced EPS adsorption onto the polystyrene surface by 70% (mass). QCM-D results revealed that the EPS molecules penetrate between the chains of the brush and adsorb onto the polystyrene surface. Additionally, AFM analyses showed that the brush-like monolayer prevents the adhesion of large ($>d$) hydrophilic colloids onto the surface via hydration repulsion; however, molecules or colloids that are small enough to fit in between the brush polymers ($<d$) can still adsorb onto the surface via van der Waals interactions. Overall, we found that the penetration of extracellular organelles, as well as biopolymers of microorganisms through the brush is critical for the failure of the anti-fouling coating, and likely could be prevented through tuning of the brush density.

KEYWORDS: biofouling, extracellular polymeric substances, polymer brush, quartz crystal microbalance with dissipation, atomic force microscopy
1. INTRODUCTION

There is a significant need for the development of novel antifouling materials to resist nonspecific adsorption of bio-macromolecules including proteins, polysaccharides, and lipids as well as control of microbial cell adhesion. There is enormous demand for these materials for a variety of applications, including medical devices, drug delivery carriers, biosensors, water filters and membranes, and marine coatings.\(^1\)\(^{-2}\) Therefore, creating surfaces that are initially resistant to biofouling has been a subject of many academic and industrial research and development efforts described by plethora of publications.\(^1\)\(^{-2}\)

Principal strategies for preparing antifouling coatings that resist the adhesion, degrade, or kill bio-contaminants include the use of polymer immobilization techniques such as poly(ethylene glycol) (PEG) on surfaces, photo-activated self-cleaning coatings, incorporation of biocidal agents (silver, antibiotics, nanoparticles, polycations, enzymes, and antimicrobial peptides), and the use of structured surfaces.\(^1\) Surface chemistries need to have certain characteristics to allow for decreased fouling in aqueous media. These surface characteristics include the combination of hydrophilic nature, electrical neutrality, and the absence of H-bond-donating groups with H-bond-accepting groups allowed.\(^2\)\(^{-4}\)

Overall, low-fouling capacity surfaces should be kosmotropic.\(^5\)

The ‘hydration repulsion’ of the antifouling coating was a proposed mechanism for the decrease in protein adsorption to materials grafted with polymer chains upon the surface.\(^6\) In addition, when protein approaches a surface, the compression of the polymer brush decreases the entropy, which then causes steric repulsion.\(^7\) Low-fouling polymer coatings, which rely on steric repulsion mechanism, depend on the surface packing of the polymer coating, chain flexibility of the long-chain polymers and the interplay between hydration and these two factors along the material surface.\(^8\)\(^{-9}\)

Polyethylene glycol (PEG) or oligoethylene glycol (OEG), polysaccharides, polyamides and a number of other low-fouling or non-fouling polyhydrophilic materials have been found to share some common structural and chemical properties.\(^2\) However, for some applications, PEG’s hydrolytic and oxidative stability may be insufficient as it has a tendency to auto-oxidize and form aldehydes and ethers in the presence of oxygen.\(^10\) As an alternative to the less-stable PEGs, zwitterionic blocks of polybetaines have been suggested. Zwitterionic blocks are stoichiometric complexes of alternating
positively and negatively charged polyelectrolytes which in turn are electrostatically neutral overall. The inclusion of positive and negative charges within the zwitterionic polymer increases the hydrophilicity while maintaining a neutral charge, which are two key components identified above for effective anti-biofouling characteristics of surfaces. The hydration of zwitterionic polymers due to ionic solvation,\textsuperscript{11} chain flexibility along with their ease of applicability and availability of functional groups,\textsuperscript{2} and the extension of a sufficient number of zwitterionic polymers from the surface can create a hydrated brush structure, which offers an additional physical obstruction to biological materials depositing on the surface.\textsuperscript{12-13} The morphological structure of this brush, however, depends on the concentration of monovalent salts present in solution which stabilize the positive and negative charges of the polymer branches and therefore cause the brush to swell.\textsuperscript{12} Previous studies evaluating polyelectrolyte brushes found dramatic differences in brush structure and adhesion properties when multivalent ions were present.\textsuperscript{14}

Polyzwitterionic materials can be classified into two types, polybetaines and polyampholytes. Polybetaines carry a positive and negative charge on the same monomer unit while polyampholytes carry an equal ratio of positive and negative moieties on different monomer units. Due to their strongly hydrated structures, a few commonly-used examples of polybetaine zwitterionic blocks are sulfobetaines (SB), carboxybetaines (CB), phosphonate-betaines (PB), and carboxybetaine methacrylate (CBMA).\textsuperscript{2} Both SB and CB demonstrated very low fouling capacities in single protein solutions,\textsuperscript{15-16} undiluted blood plasma and serum,\textsuperscript{15-16} and in a variety of water and wastewater applications.\textsuperscript{17-19}

In addition to the use of longer-chain zwitterionic polymers, several studies have shown the potential of using small zwitterionic ligands, e.g., peptides, for membrane surface modification.\textsuperscript{20-21} Based on the design idea of uniformly mixed charges and the choice of suitable amino acid combinations, zwitterionic peptides were created to display significant resistance to nonspecific protein adsorption. For example, Chen et al.\textsuperscript{11} and Nowinski et al.\textsuperscript{22} created ultra-low-fouling peptides with self-assembled monolayers consisting of alternating negatively and positively charged amino acids, glutamic acid (E) and lysine (K) - two amino acids which are popularly used for the creation of low-fouling materials.\textsuperscript{11, 22} While the antifouling capabilities of self-assembled zwitterionic brushes
have been proven for a variety of applications, there are still considerable uncertainties regarding the mechanisms in charge of the antifouling performance of these zwitterionic polymers. There are few studies, which focus on zwitterionic peptides and their coating techniques.\textsuperscript{11, 22-26} These studies however focused only on the application of the peptides onto gold surfaces,\textsuperscript{11, 22-24} or to other materials doped into polymer film coatings,\textsuperscript{25-26} restricting the ease of application. Hence, a universal method for immobilizing zwitterionic peptides on a diverse range of materials is desirable for the preparation of low-fouling surfaces.

In this study, we aimed to fundamentally analyze a self-assembled brush-layer made from a block copolymer (BCP) of polystyrene and a zwitterionic peptide comprised of repeated units of lysine-glutamic acid (Figure 1). The self-assembly of the BCP was observed in real time using quartz crystal microbalance with dissipation (QCM-D) and verified with contact angle measurements. The antifouling performance of the BCP layer was then evaluated with QCM-D, employing real biofoulant materials, and with atomic force microscope (AFM), emplying bio-representative probes. The decrease in the performance of engineered systems and contamination of medical devices brought by fouling of bacterial extracellular polymeric substances (EPS) represents a formidable challenge, since bacterial biofilms acquires from the EPS mechanical stability and strong adhesion to the surface.\textsuperscript{27} Therefore, QCM-D testing was conducted to elucidate interactions between the BCP brush and EPS extracted from \textit{Pseudomonas aeruginosa} biofilms. Critical surface characteristics such as the adsorption of hydrated ions onto the brush, hydrophilicity, topography, and interactions of bio-representative AFM probes with different sizes – were explored and characterized to elucidate the antifouling mechanisms of the zwitterionic peptide brush presented in this study.
2. EXPERIMENTAL SECTION

2.1 Synthesis of peptides and peptide-polystyrene conjugation. Fmoc-(L)Lysine(Boc)-OH and Fmoc-(L)Glutamic Acid(OtBu)-OH acids were purchased from Protein Technologies (Protein Technologies, Tucson, AZ, USA). Zwitterionic peptides were synthesized on Rink amide resin using standard Fmoc solid phase peptide synthesis strategy\textsuperscript{28-29} on an automated peptide synthesizer, model PS3 Benchtop Peptide Synthesizer (Protein Technologies). Carboxyl-end-capped polystyrene was synthesized through living anionic polymerization technique, carbon dioxide gas (CO\textsubscript{2}) was bubbled into solution of living polystyryllithium during the termination step, producing well-defined carboxylic acid-functionalized polystyrene.\textsuperscript{30} The Fmoc groups on the Lysines’ N-terminus were cleaved with 20% piperidine in Dimethylformamide (DMF). The free amine groups were then conjugated with 4X equivalent molar (with respect to the concentration of the peptides) of carboxylic acid functionalized polystyrene, 8X equivalent molar DIPEA and 3.95X equivalent molar HATU in DMF\textsuperscript{31} by shaking (Simethicone Wrist Action Shaker Model 85-AA, Burrell Scientific, Pittsburgh, PA) for 24 hours at room temperature. Then the cleavage from the resin was performed using a trifluoroacetic acid (TFA): triisopropylsilane: water (95:2.5:2.5) solution (Sigma Aldrich, St. Louis, MO). The peptide-polystyrene was precipitated and washed with ice-cold ether several times, and dissolved in DI water.

![Figure 1](image_url)

**Figure 1.** Molecular schematic of the combination of hydrophobic polystyrene (2) to the hydrophilic peptide polymer (1) to form the zwitterionic peptide-polystyrene block copolymer (3).
The BCP was next purified by reverse phase preparative high performance liquid chromatography (RP-HPLC, Prominence, Shimadzu Corp., Columbia, MD) with a C₈ Waters column (Waters Corp., Milford, MA) at 50°C using 0.1% trifluoroacetic acid in water/acetonitrile (ACN) mixture as mobile phase. The flow rate of RP-HPLC was set to 10 mL/min from 10% (H₂O/ACN) to 100% (ACN) within 45 min and 100% ACN for 10 more min. Product identity was confirmed using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Supplemental Information - Figure S1.1) (Ultraflextreme MALDI-Tof-Tof, Bruker Corp., Billerica, MA, USA).

2.2 Dynamic light scattering size characterization. The distribution of hydrodynamic radius values for 100 mg/L BCP dissolved in DI water and in 100 mM NaCl was collected using a Dynamic Light Scattering (DLS) system at 22 ± 0.2 °C, with 30 second read times (ALV/CGS-3 Goniometer, ALV/LSE-5004 Correlator, ALV/GmbH, Langen, Germany).

2.3 Contact Angle Measurements. The contact angle results presented correspond to captive air bubble measurements using a dedicated instrument (DataPhysics Instruments, Filderstadt, Germany). Polystyrene (PS, average mw 192 kDa, Sigma-Aldrich, Israel) was spin coated on silicon wafer with 1 μm thermaly grown silica top-layer (University Wafer, MA, USA). 100 μL drop of 0.1% PS dissolved in DCM (Bio-Lab, CN: 001379052100), filtered through a 0.22 μm filter (Millipore, PVDF, CN: SLVH033RS) was added to the middle point and then rotated at 2700 rpm for 1 min (Laurell Tech. Co., Model WS-400BZ-6NPP/LITE) Then, the polystyrene-coated wafer was submerged in either DI water or 100 mM NaCl and allowed to equilibrate for at least one hour. A 5 μL air bubble was then deposited on the polystyrene surface with or without the BCP brush-like monolayer from below and an image of the air bubble was captured at 5 different locations on the sample. To measure the contact angle, the air bubble images were processed using OCA-20 software (DataPhysics).

2.4 EPS preparation and extraction. For EPS extraction, a microbial biofilm from Pseudomonas aeruginosa PAO1 was grown in a flow-through column according to Ferrando et al. In brief, the 100 mL packed bed column (~2.5 cm in diameter) contained acid washed glass beads (425-600 μm in diameter, cat. #G8772 Sigma-Aldrich, St. Louis, MO). The column was wet-packed, sterilized with 70% ethanol and washed with
sterilized deionized water prior to inoculation with a 100 mL of the stationary phase culture for 50 minutes (2 mL/min). At the end of the inoculation stage, pure Luria-Bertani (LB) media was injected into the column for 48 hours to allow biofilm growth on the beads. The EPS extraction from three biological replicates of the *P. aeruginosa* biofilms was carried out according to the method described in Liu and Fang. For a brief description of the modification of this method, the column was disassembled after 48 hours of biofilm growth and the biofilm-covered beads were gently washed twice with 0.145 M NaCl. The beads were immersed in 50 ml of 0.145 NaCl and 0.3 ml of 36% formaldehyde and then they were cooled to 4°C. After 1 hour incubation at 4°C under gentle shaking, 20 ml of 1 M NaOH was added and incubation continued for an additional three hours. The liquid portion of the mixture was then centrifuged for 30 minutes at 4°C and the supernatant was filtered through 0.22 μm hydrophilized polyvinylidene fluoride (PVDF) filters (Millipore, Billerica, MA, USA). The supernatant was then dialyzed with a 3500 Dalton membrane (Spectrum Laboratories, Rancho Dominguez, TX, USA) against DI water for approximately two days until the conductivity of the solution dropped below 1 μS/cm. The total organic carbon (TOC) was measured using an Apollo 9000 combustion TOC analyzer (Teledyne Tekmar, Mason, OH, USA). The EPS was then diluted using DI water to 15 mg/L as TOC.

The range of the EPS molecular weight was analyzed using gel permeation chromatography (GPC) (Supplemental Information - Figure S2.1). One mg of lyophilized EPS was dissolved in 1 ml of double distilled water. The AQUITY advanced polymer chromatography (APC) system (Waters) is comprised of APC isocratic solvent manager (p-ISM) pump, UV (at 254 nm) and Ultrahydrogel columns, which cover wide MW range of 100 Da to 4 MDa. We employed polystyrene sulfonate standards (1 mg/ml) with peak MW of 29.5 kDa, 10.2 kDa, 4.21 kDa, 1.67 kDa and 0.208 kDa and polydispersity index (PDI) less than 1.2 (Polymer Standards Service, Mainz, Germany) (2 mg/ml for standard of 0.208 kDa). The injection volume for samples was 25 μl with 0.8 ml/min flow rate and the eluent which delivers the injected sample through the gel columns was ammonium buffer (0.01M NH₄Cl, 0.033 M NaCl and NH₄OH at pH 10.5).

**2.5 Block co-polymer coating, characterization and EPS fouling.** The deposition, background solution responses, and EPS fouling behavior of the BCP coatings were
monitored using an E4 QCM-D system (Q-sense AB, Gothenburg, Sweden). First, the adsorption of BCP at different concentrations onto polystyrene-coated QCM-D sensors (Q-sense AB) was monitored. This was done using a peristaltic pump (IsmaTec, Wertheim, Germany) set at a flow rate of 150 µL/min and via the implementation of the following program using a robotic liquid controller: (a) DI water baseline for 20 min; (b) BCP in DI water for 30 min; (c) DI water wash for 30 min. BCP concentrations of 1, 10 and 100 mg/L were tested. To monitor the effect of ions concentration on the brush-like BCP monolayer, QCM-D sensors with and without BCP monolayer were subjected to alternating background solutions of DI and 100 mM NaCl (30 min each) while the changes in frequency and dissipation were measured by the QCM-D.

Fouling experiments with EPS were conducted on polystyrene sensors with a brush-like BCP monolayer (100 mg/L BCP coating) and using polystyrene controls without any BCP. Four conditions were tested: (i) BCP coating in a DI water background solution; (ii) BCP coating immersed in a NaCl background solution; (iii) without a BCP coating immersed in a DI water background solution; and (iv) without a BCP coating immersed in a NaCl background solution. The following program was then implemented: (a) background solution baseline for 20 min; (b) EPS in background solution for 30 min; (c) background solution wash for 30 min. In the fouling experiments, the same background solutions were used throughout the entire experiment, using either the DI water or a 100 mM NaCl solution.

2.6 Atomic force microscopy (AFM) used for topography imaging and for measuring the adhesion forces between silica and polystyrene surface. Atomic force microscopy (AFM) topography and force measurements were conducted using a NanoWizard 4 (JPK, Berlin, Germany) in DI water and in 100 mM NaCl at a pH 6 ± 1. The topography and adhesion force images were captured using ‘quantitative imaging’ mode, where an array of 256 x 256 force vs. piezo-displacement curves were captured and analyzed. The probe used was a HYDRA6V-100NG (AppNano, Mountain View, CA, USA) with a nominal radius approximately 7 nm and a cantilever sprint constant of 0.3 N/m. For measuring the force between the silica colloid(s) and the surface, a 5 µm diameter silica probe attached to 0.06 N/m cantilever was used (Novascan, Ames, IA, USA).
3. RESULTS AND DISCUSSION

3.1 BCP self-assembles into micelles in aqueous solution (DI water to 100 mM NaCl). The self-assembly of Glu-Lys-b-Polystyrene BCP in DI water and in 100 mM NaCl was studied using cryo-TEM and dynamic light scattering. The results are summarized in Figure 2 and show that Glu-Lys-b-Polystyrene BCP self-assembled into approximately 7 nm diameter micelles in DI water and in 100 mM NaCl, at concentrations of 1-100 mg BCP/L. Figure 2 demonstrates how the hydrophilic (Glu-Lys) section of the BCP interacts with the water, while the hydrophobic (polystyrene) section has a greater affinity for the hydrophobic sections of other BCP units, shielded from the water. Micellization of the Glu-Lys-b-Polystyrene BCP in both DI water and 100 mM NaCl is demonstrated in Figure 2D by a sharp peak in the DLS size profile ($d'$) at ~7 nm. A cryo-TEM image in Figure 2C visualizes dispersed micelles in solution, confirming approximately the size measured using DLS. Figure 2B illustrates how the polystyrene section of the BCP forms the core of the micelle and the alternating glutamic acid and lysine units extend into the bulk liquid.
Figure 2. Lysine, glutamic acid and styrene-based BCP presented as: (A) molecular schematic of components; (B) DLS-characterized hydrodynamic radius of micelles in DI water and 10 mM NaCl; (C) cryo-TEM image of micelles and (D) illustration of the micelle structure.

3.2 The BCP self-assembles into a brush-like structure on hydrophobic surfaces. As discussed in Section 3.1, while formation of micelles can better stabilize the BCP in aqueous solutions, encountering a hydrophobic surface, such as polystyrene, results in the self-assembly of a brush-like monolayer. The van der Waals forces ‘hold’ the hydrophobic (polystyrene) section of the BCP to the hydrophobic surface (e.g., polystyrene), while the zwitterionic (Glu-Lys) section favors the interaction with the water molecules. The fundamental concept behind QCM-D is that attaching mass to an oscillating sensor reduces the frequency of the oscillation. Monitoring the deposition of the BCP molecule onto the surface with QCM-D allowed us to estimate the surface coating density, i.e., the average distance between each BCP molecules on the surface.
Figure 3A presents the change in frequency corresponding to the 5th overtone. Changes in frequency at the 7th, 9th and 11th overtones are presented in Supplemental Information Figure S3.1. When a rigid layer is formed on the surface, the change in frequency at each overtone normalized to the overtone number should equal the same value.\(^{34}\) In the case of the BCP monolayer produced from the 100 mg/L BCP coating solution, the change in frequency normalized to overtone number for the 5th, 7th, 9th and 11th overtones were -4.1, -2.8, -1.0 and -1.6 Hz, respectively. The differences in these values indicate that our BCP is not a rigid layer, but rather it exhibits viscoelastic characteristics.

The changes in oscillation frequency caused by deposition of molecules onto the sensor is used to estimate the mass of the adsorbed molecules. The Sauerbrey equation describes this relationship quantitatively for rigid even-distributed surface coatings.\(^{35}\) We note that in the case of viscoelastic layer on the surface, such as the case of the BCP
molecules on the polystyrene surface, estimation of the adsorbed molecular mass on the
surface by the Sauerbrey equation, is not fully accurate. A viscoelastic film that will not
fully couple to the oscillation of the sensor will not fully contribute to the decrease of the
frequency and Sauerbrey equation will underestimate the adsorbed mass. Concurrently,
however, the viscoelastic characteristics of the brush-like layer, and the increase in
surface roughness and surface area, will cause energy losses caused by the viscous
friction and can lead to an overestimation of the mass calculated through the Sauerbrey
equation. However, applying the Sauerbrey equation to our system provides a useful
approximate value. Applying the Sauerbrey equation of the monolayer coatings from 1,
10 and 100 mg/L BCP concentrations using the 5th overtone yields respective masses of
19, 39 and 72 ng per cm$^2$ of sensor surface. Combining these values with the known
composition of our BCP permits an understanding of BCP orientation and density on the
surface.

Each BCP molecule consists of three different monomers (Figure 2A): lysine (Lys, 129 g/mol), glutamic acid (Glu, 128 g/mol), and styrene (104 g/mol). With 15 pairs of
Lys-Glu and 19 monomers of styrene, the molecular weight of BCP is 5862 g/mol, thus
each BCP molecule weighs $9.7 \times 10^{-12}$ ng. Using the QCM-D results, as discussed above,
we can then estimate the packing density on the surface to be $2.0 \times 10^{12}$, $4.0 \times 10^{12}$ and
$7.4 \times 10^{12}$ BCP molecules per cm$^2$ of surface areas for BCP monolayers from 1, 10 and
100 mg/L BCP bulk concentrations, respectively. These densities can then be used to
g eo metrically calculate average center-to-center distance between individual BCP units
($d$) of approximately 8, 5 and 4 nm for BCP monolayers from coating concentrations of
1, 10 and 100 mg/L BCP, respectively (Figure 3D). The positioning of BCP on the
surface is illustrated in Figures 3C and 3D, estimating the length of the fully stretched
hydrophilic section ($l$) to be 11 nm and a surface roughness range of 10 nm (roughness of
the polystyrene surface) as measured by AFM (Figure S4.1 and S4.2). This maximum 11
nm span was estimated by combining the known molecular composition (Figure 1A) with
standard values for bond lengths.$^{36}$
Figure 3. The mass of the adsorbed BCP brush-like monolayer on a polystyrene surface was measured by QCM-D. (A) Shows the change of the sensor oscillation frequency during adsorption of BCP for 1, 10 and 100 mg/L BCP concentrations in DI water; (B) shows contact angles, as measured by ‘captive-bubble’ on polystyrene surface, and on polystyrene with BCP brush-like monolayer, which was adsorbed from different BCP bulk concentrations $C_{BCP}$; (C) and (D) illustrations depict the BCP monolayer on the polystyrene surface.

Apart from QCM-D, the self-assembly of the BCP on a polystyrene surface was studied by contact angle measurements. Polystyrene samples (see Section 2.3) were soaked in 1 and 100 mg/L BCP solutions for 30 min. In these measurements, the BCP-with and without the BCP brush-like monolayer is inverted in DI water or in a 100 mM NaCl solution. Then a 5 μL air bubble was added to the surface from below. The contact angle on the water side ($\theta$) was then measured using a microscope and camera (Figure 3B). The results demonstrate increasing hydrophilicity (lower contact angle) with
increasing BCP bulk concentration, with angles of $73 \pm 4^\circ$, $69 \pm 3^\circ$, and $45 \pm 2^\circ$, after coating in BCP bulk concentrations of 0, 1 mg/L and 100 mg/L, respectively. We note that the contact angle measurements were done in DI water (similar results were observed for 100 mM NaCl, data not shown) without BCP in the solution.

Using the Cassie-Baxter equation, the contact angle, $\theta$, for chemically heterogeneous surfaces – having hydrophobic and hydrophilic microscopic areas – is given by:

$$\cos \theta = \frac{A_{PB}}{A_{PB} + A_{PL}} \cos \theta_{PB} + \frac{A_{PL}}{A_{PB} + A_{PL}} \cos \theta_{PL}$$

(1)

where $\theta_{PB}=73^\circ$, is the measured contact angle of water in air on polystyrene (the subscript ‘PB’ stands for hydrophobic), $\theta_{PL}\sim0^\circ$ is an estimate of the contact angle of water in air on the hydrophilic area (the subscript ‘PL’ stands for hydrophilic), $A_{PB}$ is the hydrophobic area of a single BCP molecule with its surrounding polystyrene surface, and $A_{PL}$ is the hydrophilic area of a single BCP molecule.

To estimate $A_{PB}$ and $A_{PL}$, we considered a single BCP molecule surrounded by polystyrene, as illustrated in Fig. 3C and D. Based on the BCP surface coating density, as estimated by QCM-D, each single BCP occupies a hydrophobic surface area, $A_{PB}$, of $\sim64$ nm$^2$ (8 nm spacing) and $\sim16$ nm$^2$ (4 nm spacing) for BCP monolayers corresponding to coating concentrations of 1 mg/L and 100 mg/L, respectively. The hydrophilic area of a single BCP molecule, $A_{PL}$, was estimated by modeling a hydrophilic block of the BCP as a cylinder of $\sim27$ nm high ($\sim11$ nm of the main chain of the hydrophilic section of the BCP + $\sim16$ nm of the branches of the hydrophilic section of BCP molecule, Fig. 2A) and with a radius of $\sim0.12$ nm (C=O and N=O bond lengths), which yields $A_{PL} \approx 2\pi \times 0.12 \times 27 = 20$ nm$^2$. Based on Equation 1, the contact angles for 1 and 100 mg/L BCP coated surfaces are $62^\circ$ (measured $69 \pm 3^\circ$) and $46^\circ$ (measured $45 \pm 2^\circ$). The small discrepancy between the measured and the calculated contact angles is another indication that it is reasonable to model the self-assembly of the BCP molecule as a brush-like monolayer.
3.3 Ion-adsorption of BCP brush-like monolayer. Figure 4 shows an analysis of the change in resonance frequency ($\Delta f$) of the pristine QCM-D sensor (covered with polystyrene), and QCM-D sensor coated with the BCP brush-like monolayer (on top of the polystyrene) while alternating between DI water and 100 mM NaCl solution. Figure 4A provides representative data for one cycle of this response. For sensors with and without the BCP brush-like monolayer, $\Delta f$ was positive (mass was released from the surface) when 100 mM NaCl solution was replaced by DI water (Figure S4), and $\Delta f$ was negative (mass adsorbed to the surface) when DI water was replaced by 100 mM NaCl solution.

![Figure 4](image_url)

Figure 4: (A) Represent the changes in QCM-D frequency ($\Delta f$) in response to alternating background solutions between 100 mM NaCl and DI water and (B) Illustrates the ion adsorption to the Glu-Lys section of the BCP in presence of NaCl. Number of replicates = 3.

The sign of $\Delta f$ was similar for pristine QCM-D sensors and for sensors with brush-like monolayer: however, the absolute value of $\Delta f$ increased with the BCP surface concentration. At the 5th overtone, $\Delta f$ was $2.5 \pm 0.14$ (SD), $2.8 \pm 0.20$, $2.8 \pm 0.46$ and $4.6 \pm 0.40$ Hz measured by QCM-D for sensors that were exposed to 0, 1, 10 and 100 mg/L BCP solutions, respectively. There are two mechanisms that could contribute to these changes in frequency. First, looking at the uncoated polystyrene sensor alone, the
decrease in frequency in NaCl could be attributed to elevation in the density and viscosity of the medium as well as interactions with the electrolytes. The second effect, however, operates on the BCP layer and the ability of the BCP to hydrate and possibly, change its conformation.

In the case of the 100 mg/L BCP coating, we estimated there to be $1.12 \times 10^{14}$ pairs of Glu-Lys per cm$^2$ of surface area. This is 15 times the estimate for the density of BCP units (according to the QCM-D and similar to the contact angle measurements), as each BCP contains 15 Glu-Lys pairs. If we assume that each charged group attracts a corresponding ion, this demand is then $1.12 \times 10^{14}$ molecules of NaCl per cm$^2$. Subtracting the change in frequency observed for the uncoated sensor from the change in frequency of the coated sensor lets us define the difference of only the BCP layer. In the case of 100 mg/L coating this corresponds to 2.1 Hz. Using the Sauerbrey equation, this is converted to 7.43 ng per cm$^2$, which would correspond to $7.6 \times 10^{13}$ molecules of NaCl. This estimate of NaCl molecules derived from the NaCl/DI water cycling is comparable to the estimate of the Glu and Lys pairs on the sensor ($\sim 1.12 \times 10^{14}$ pairs of Glu-Lys). Hence, the difference in frequency attributed only to the presence of the BCP layer is derived mainly from the mass of additional hydrated Na$^+$ and Cl$^-$, both of which would interact strongly with the Glu and Lys charges, respectively (Figure 4B).

In addition to the mass of hydrated cations interacting with the layer, the zwitterionic Glu–Lys chain could either collapse onto itself or extend out into the bulk liquid. This extension can be driven by the charged groups of the BCP interacting with the hydrated ions of the NaCl instead of interacting with other charged groups within the BCP itself.$^{38-40}$ Analyzing the change in frequency attributed to the Glu-Lys end of the BCP may support the occurrence of this conformational change, extension in NaCl, and collapse in DI water. However, such possible conformational changes were not differentiated from the main suggested mechanism of ion adsorption to the zwitterionic chain, in this study.

### 3.4 The BCP brush-like monolayer reduces the adhesion of EPS to hydrophobic surface via hydration/undulation repulsion.

EPS extracted from microbial biofilms has been utilized extensively as a model indicator of biological fouling potential.$^{41-43}$ Figure 5 depicts the changes in frequency ($\Delta f$) and the energy dissipation ($D$) as the QCM-D sensors were exposed to EPS, followed by a washing step in a solution without EPS. To
isolate the effect of the BCP brush-like monolayer from the effects of the NaCl concentration on EPS adsorption to the surface, pristine QCM-D sensors and sensors with the BCP brush-like monolayer were each tested for EPS adsorption in both DI water and 100 mM NaCl. The background solution was maintained throughout each experiment, so the baseline and washing steps matched the background solution in which the EPS was immersed in when adsorbed to the surface. The largest mass of EPS adsorption (fouling) occurred in the NaCl background solution without the BCP brush-like monolayer, corresponding to a reduction in QCM-D frequency of 10.4 Hz at the 5th overtone. Adding a BCP brush-like monolayer to the sensor surface reduced the EPS adsorption by ~70% (Δf = -3.1 Hz). In DI water, lower EPS adsorption (less fouling) was observed than in NaCl, with 1.51 and 1.24 Hz reductions, with and without BCP brush-like monolayer, respectively. The corresponding estimated masses of EPS adsorbed to the sensor according to Sauerbrey equation were 11 and 37 ng/cm² with and without BCP in NaCl and 5.3 and 4.4 ng/cm² with and without BCP in DI water.

The molecular weight distribution of the EPS extracted from the P. aeruginosa biofilm provided an average size of 6 kDa as analyzed using gel permeation chromatography (GPC), which is equivalent to $1.0 \times 10^{-11}$ ng per molecule. (Figure S2). To estimate the surface area that each EPS molecule occupied, a two-dimensional projection was mapped out on graph paper for the PSL polysaccharide, which is present in P. aeruginosa. This projection was constructed using standard bond length values. A ratio of total mass to occupied surface area allowed us to estimate that a 6 kDa molecule of EPS would occupy a surface area of about 17 nm². Assuming a circular shape for each 17 nm² EPS molecule yields a diameter of around 4.6 nm. Assuming the EPS molecules were homogenously dispersed over the surface, the average center-to-center distances between EPS molecules were 16 and 6 nm on surfaces without BCP in DI and NaCl, respectively, as depicted in Figure 5 panels A and B. As the 4.6 nm diameter of each EPS molecule is less than the 16 and 6 nm center-to-center spacing of individual BCP molecules, we concluded that the EPS was a monolayer (and not multilayered). This monolayer is likely formed by the adsorption of individual EPS molecules to unoccupied regions (no BCP molecules) of the polystyrene surface due to van der Waals forces. In addition, electrostatic repulsion may force negatively charged EPS molecules apart from...
each other. This repulsion between EPS molecules is dramatically increased in DI water, with the Debye Length increasing from about 1 nm in 100 mM NaCl solution, to more than 100 nm in DI solution (See SI section S4). This explains the greater distances between EPS molecules on the surface when EPS deposition is conducted in DI water vs. 100 mM NaCl (Figure 5 A and B). Without the BCP brush-like monolayer, the EPS occupies 7.5 and 63% of the total surface area in DI water and 100 mM NaCl, respectively. With the BCP brush-like monolayer, the EPS occupies 9.1 and 18% of the total surface area in DI water and 100 mM NaCl, respectively. With these results, it is expected that further increasing the BCP surface coating density would further limit EPS fouling onto the polystyrene surface.

Our QCM-D EPS adsorption experiments do indicate that interactions exist between the EPS and the BCP. This is apparent in the changes in dissipation observed during EPS adsorption. In Figure 5A, we see an expected behavior, that as we add a non-rigid layer to the sensor, the viscoelastic character of the added layer increases the dissipation factor. In figure 5B, we would also expect an increase in dissipation factor, however of lower magnitude, due to lower mass of EPS attached. On the other hand, in the two cases with BCP coating (Figure 5C and D), we see the dissipation factor decreases with EPS attachment along with the decrease of the frequency. An increase in dissipation factor while mass is added onto the sensor occurs when the added mass increases the rigidity of the layer. The forces that prevent EPS attachment to the BCP are primarily hydration repulsion between the hydrophilic section of the BCP and the hydrophilic EPS. We believe the EPS that does attach to the surface and penetrates the void space between BCP molecules (Figure 5C); however, this EPS may still contribute a small degree of repulsion between the BCP molecules. It is this repulsion and restriction that reduces the dissipation of the sensor by effectively making the BCP more rigid. Interestingly, the dissipation factor was recovered after the washing step with BCP in NaCl (Figure 5C), however it did not recover when exposed to BCP in DI water (Figure 5D). These results may be attributed to either (i) the hydration of the zwitterionic chain when exposed to NaCl and/or (ii) an extended conformation of the Gly-Lys portion of the BCP in the NaCl and the collapsed conformation in DI water. The hydration of the BCP brush-like monolayer in the presence of NaCl will likely enhance the separation between the EPS
and the zwitterionic chains; it is this way by which the dissipation factor can be recovered upon washing with a NaCl solution.

![Figure 5. EPS adsorption monitored using QCM-D. Panels A and B present the changes in frequency and dissipation of polystyrene surfaces when exposed to EPS fouling and conducted in 100 mM NaCl and DI water, respectively. Parts C and D present the changes in frequency and dissipation of polystyrene surfaces previously coated in 100 mg/L BCP solution and conducted in 100 mM NaCl and DI water, respectively.](image)

3.5 The adhesion energy between silica and polystyrene covered by the BCP brush-like monolayer. Silica surfaces and the EPS extracted mixture are hydrophilic and negatively-charged in DI water and in a 100 mM NaCl aqueous solution. Therefore, the silica-polystyrene interactions in DI water and in a 100 mM NaCl solution, with and without a BCP brush-like monolayer were used to mimic the interactions between EPS molecules and the pristine polystyrene as well as the polystyrene with the BCP brush-like monolayer. In this section, we used AFM to study the topography and the adhesion force \( F_\perp \), between silica and polystyrene with and without the BCP brush-like monolayer.
The adhesion energy was then calculated using the Derjaguin approximation, \( W = \frac{F_\perp}{2\pi R} \), where \( R \) is the radius of the AFM probe. The topography imaging and force measurements were done using a probe with a nominal curvature of ~7.5 nm (see Materials and Methods). In addition, force measurements were also conducted using a colloidal silica probe of 5 \( \mu \)m diameter.

The topographies of the polystyrene with and without the brush-like BCP monolayer were qualitatively similar (Supplemental Information - Figures S4.1 A and B). However, as can be seen in Figure 6A and B (also in Figure S4.1 C and D), the adhesion force (and energy) between the silica AFM probe and the surface decreases by 40% in the presence of the BCP brush-like monolayer due to the hydration repulsion forces (discussed below).

Figure 6 shows two typical adhesion energy versus separation distance curves, \( W(D) \), that were measured between the AFM probe and the polystyrene without (panel A) and with (panel B) BCP brush-like monolayer. In the absence of the brush-like BCP monolayer, as the AFM probe contacts the polystyrene surface, (panels C and D) the van der Waals (VDW) interactions dominated the system. The measured adhesion energy, \( W = \frac{F_\perp}{2\pi R} = -25.6 \pm 5.3 \text{ mJ/m}^2 \), is in agreement with the prediction of the Extended DLVO model that combines VDW, double layer, and hydration-repulsion interactions (see Supplemental Information Section S5 and Figure S5.1).

On the other hand, when the BCP brush-like monolayer was self-assembled onto the polystyrene surface, the average adhesion energy between the AFM and the surface was significantly smaller (\(-13.3 \pm 2.7 \text{ mJ/m}^2\)) than the adhesion energy without the BCP monolayer. This was likely due to hydration-repulsion\(^{47-49}\) between the BCP zwitterionic hydrophilic chains and the hydrophilic silica AFM probe (Figure 6 E and F). Since the topography of polystyrene with and without the BCP brush-like monolayer appeared qualitatively similar (Supplemental Information Figure S4.1 and S4.2), we concluded that the AFM probe penetrated the hydrophilic brush-like monolayer. Once the AFM probe penetrated the brush, it contacted the polystyrene and adhere to it via van der Waals interactions. However, since parts of the AFM probe were covered by the hydrophilic blocks (Figure 6E and F), the hydration repulsion between the hydrophilic blocks and the
polystyrene decreased the adhesion energy between the AFM probe and the polystyrene (when compared to the pristine polystyrene surface).

Figure 6. The effect of the BCP brush-like monolayer on the adhesion energy between the 15 nm diameter silica AFM probe and the surface.

Force analysis using a similar probe (silica), but two orders of magnitude larger (diameter of 5 μm versus curvature diameter of 7.5 nm) was conducted to gain further insight of the fouling prevention mechanisms at the micron scale. Figure 7 shows two plots of adhesion energy versus separation distance, $W(D)$, between spherical a silica colloid ($R=2.5$ μm) and polystyrene (panel A) and between the spherical silica colloid ($R=2.5$ μm) and polystyrene covered by brush-like monolayer (panel B). In agreement with the AFM nano probe measurements (shown in Fig. 6), van der Waals adhesion forces governed the interactions between silica and polystyrene. Note that the adhesion energy between the colloidal probe ($\sim -1.0$ mJ/m$^2$) was significantly smaller than the adhesion energy between the AFM probe and the polystyrene ($\sim -26$ mJ/m$^2$). The weaker adhesion between the colloidal probe and surface was likely due to the roughness of the polystyrene which reduced the contact area between the colloidal probe and the surface.
In contrast to the results with the AFM nano probe, in the case of the brush-like BCP monolayer on surface, no adhesion was measured between the colloidal probe and the surface and the large colloidal probe did not penetrate the hydrophilic brush. This indicated that hydration repulsion governed the system because the zwitterionic peptide brush could prevent contact between the probe and the polystyrene surface.

Figure 7. The effect of the BCP layer on the adhesion energy of a 5 µm diameter silica probe to the polystyrene surface.

4. CONCLUSIONS

We synthesized a novel block co-polymer (BCP) molecule which is comprised of hydrophilic (zwitterionic peptide) and hydrophobic (polystyrene) blocks. We showed that these BCP molecules self-assemble into a brush-like monolayer on hydrophobic (polystyrene) surfaces from aqueous solutions with different BCP concentrations (1 to 100 mg/L). The brush-like monolayer demonstrated a great potential for preventing or slowing-down organic fouling. The main mechanism responsible for the antifouling behavior can be described as follows: (1) The layer(s) of water molecules that hydrate the hydrophilic section of the BCP prevents the van der Waals adhesion between the hydrated EPS and the hydrated section of the BCP molecules; (2) the several nanometer scale of the hydrated brush on the polystyrene surfaces keeps the EPS and large hydrophilic colloids apart from the polystyrene surface, which diminishes the van der Waals adhesion between the EPS (or hydrophilic colloids) and the polystyrene.

Nevertheless, the BCP brush-like monolayer cannot completely prevent the adhesion of EPS to the polystyrene surface. By combining QCM-D analyses, contact angle
measurements, and AFM force spectroscopy, we concluded that the adsorption of foulants (e.g., EPS) onto the surface takes place in the voids between individual BCP molecules covering the surface. We estimated the average diameter of the void to be in the order of ~4 to 8 nm apart, for the BCP surface densities tested. Based on our results, we conclude that the BCP brush-like monolayer might be more effective for reducing attachment of large biological foulants, such as bacteria, in comparison to smaller components such as their associated EPS matrix. However, a denser brush (smaller $d$, see Fig. 3D) is expected to improve its antifouling performance for a variety of bacteria, EPS and other biomacromolecules. The BCP coating method presented in this paper is based on a simple coating procedure, which shows great promise for ease-of-application onto a variety of polymeric surfaces.

AUTHOR INFORMATION

Notes
The authors declare no competing financial interest.

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