Langmuir
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Reduced Bacterial Deposition and Attachment by Quorum-Sensing Inhibitor 4-Nitro-pyridine-*N*-oxide: The Role of Physicochemical Effects

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Received April 2, 2010. Revised Manuscript Received May 6, 2010

Surface-attached chemical groups that resist protein adhesion are commonly characterized as being hydrophilic, H-bond acceptors, non-H-bond donors, and electrically neutral. Quorum-sensing (QS) inhibitor 4-nitropyridine-*N*-oxide (4-NPO) that previously was found to decrease *Pseudomonas aeruginosa* biofilm formation possesses all of these characteristics, making this molecule an ideal antiadhesive compound. It was hypothesized that once 4-NPO adsorbs to either the solid surface or bacteria, resultant changes in the physical−chemical surface properties of the solid surface and bacteria will reduce the extent of bacterial adhesion. These physical−chemical effects take place prior to the commencement of already well-established QS biofilm-inhibition mechanisms. Bacterial adhesion experiments to silica conducted in quartz crystal microbalance with dissipation (QCM-D) and parallel plate flow cells demonstrated that 4-NPO reduces bacterial adhesion to silica-coated surfaces by the adsorption of 4-NPO to the silica surface as well to the outer membrane of both gram-negative *P. aeruginosa* PAO1 and gram-positive *Staphylococcus aureus*. 4-NPO effectively neutralizes both the bacterial and silica surface charge, and it is proposed that this neutralization of local surface charge heterogeneities by 4-NPO adsorption is the mechanism responsible for decelerating rates of bacterial deposition.

Introduction

Bacterial deposition on solid surfaces is the first step in the biofilm formation process that in some cases can be qualitatively described by the interaction energies between a bacterial cell surface and a solid surface using Derjaguin−Landau−Verwey−Overbeek (DLVO) theory.[1−4] The DLVO theory summarizes the total interaction energies of attractive van der Waals and repulsive electrostatic interactions. When repulsive forces are suppressed, DLVO predicts that there is no energy barrier for deposition, which implies irreversible attachment of a bacterium to a negatively charged surface in a deep primary minimum. In some cases, discrepancies between DLVO calculations and experimental data can be overcome by the extended DLVO (X-DLVO) calculations[5,6] that also take into account thermodynamics that include interaction forces, such as Lewis acid−base interactions, hydrophobic attraction, and hydrophilic repulsion.[7] Other discrepancies between X-DLVO and DLVO theories and bacterial deposition in practice are attributed to the outer membrane of bacteria that is a "soft" ion-penetrable layer in which chemical functional groups as well as variety of molecular appendages such as fimbriae, flagella, and lipopolysaccharides are located. Specific chemical interactions between a small proportion of sites on the microbial and solid surfaces can control bacterial adhesion, including interactions promoted by cell-surface polymers and appendages. Bos et al.[7] demonstrated how even negatively charged appendages can extend beyond the electrostatic energy barrier as predicted by classical DLVO theory. Physical and chemical heterogeneities of the surface are also an important reason for the failure to predict bacterial adhesion: intrinsic nanoscale surface charge heterogeneities were shown to affect colloidal and bacterial attachment in a way that very small portion of sites on the surface controlled the adhesion process.[8,9] Hence, in many cases the prediction of bacterial adhesion is impossible even when the X-DLVO approach is being carried out.

When the goal is to reduce initial bacterial adhesion and hence the subsequent biofilm formation, both physiological and physicochemical approaches can be taken. Physiological approaches include the application of antimicrobials and QS inhibitors. For example, adhesion can be inhibited by pillicides, a broad class of antimicrobials targeting pili formation.[10] Also, reducing bacterial attachment and biofilm formation is achieved by interfering with the bacterial QS circuits that are key regulators of virulence expression.[11−13] A novel class of antimicrobials are known today to inhibit the QS system efficiently and eventually reduce bacterial attachment.[12,14] The best-studied system is the acylhomoserine lactone (AHL) signal-mediated system that is expressed by most

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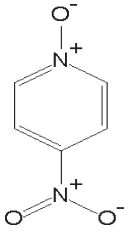

Figure 1. Chemical structure of 4-nitropyridine-*N*-oxide (4-NPO). It is an ideal antiadhesion compound: hydrophilic, H-bond acceptor, non-H-bond donor, and electrically neutral.

gram-negative bacteria.[15,16] QS inhibitors act in three modes of attack: blocking the signal production (LuxI homologue), inactivating the signal molecules, and interfering with receptor function (LuxR homologue).[12] Alternatively, physicochemical approaches for reducing bacterial deposition and attachment are also a promising alternative via modification of the substrata, making it an antifouling or antimicrobial surface. Luk and co-workers found that a polyhydroxyl-terminated monolayer (an uncharged and hydrophilic layer) created a protein-resistant surface.[17] On the basis of the study of Luk et al[17] and others,[18,19] the surface-attached chemical groups that resist protein adhesion are characterized as being hydrophilic, H-bond acceptors, non-H-bond donors, and electrically neutral.[20] Recently, the development of surfaces that prevent nonspecific protein adsorption and microbial biofilm formation has emerged for biomaterials, medical devices, a variety of biotechnological applications, and membranes for the water-treatment sector.[18,21,22] Examples for nonfouling materials that have been extensively studied are poly(ethylene glycol) (PEG) and phosphorylcholine (PC) as well as recent improvements of polyampholytes with homogeneous charge balance.[18]

4-NPO, a QS inhibitor,[12,23] was found to decrease the biofilm formation of *Pseudomonas aeruginosa* through the blockage of its QS machinery.[24] 4-NPO has two functional groups in its molecular structure, NO_2 and NO (Figure 1). 4-NPO has proton-accepting ability on the oxygen atoms of the NO and the NO_2 groups that is strongly correlated to the charge of the oxygen atom on the NO group.[25] 4-NPO cannot serve as a hydrogen bond donor, it is hydrophilic, and it is overall electrically neutral (Figure 1). Because of its interesting physicochemical properties, 4-NPO was selected as a biofilm control agent candidate that may act via a dual mechanism of altering the physicochemical properties of the bacterial and solid surfaces as well as blocking the QS machinery leading to reduced biofilm formation. Combining these two physicochemical and physiological characteristics makes this compound an outstanding candidate for bacterial biofilm formation reduction.

Therefore, the effect of 4-NPO on bacterial attachment was tested in the present project, and the physicochemical mechanisms involved were investigated. It was hypothesized that once 4-NPO adsorbs to either a solid surface or bacteria, a decrease in bacterial adhesion will take place by physicochemical mechanisms in addition to its already established QS inhibition effects. To test this hypothesis, quartz crystal microbalance with dissipation monitoring (QCM-D) technology and direct microscopy were utilized. The effects of 4-NPO adsorption to quartz surfaces and to gram-positive and gram-negative bacteria, *Staphylococcus aureus* and *P. aeruginosa* PAO1, respectively, upon cell attachment to the surface were also analyzed. After changes in bacterial attachment that are attributed to 4-NPO adsorption were delineated, physicochemical characterization of the quartz and bacterial surfaces was carried out and the physicochemical mechanisms involved with cell adhesion and 4-NPO were clarified.

Materials and Methods

Bacterial Deposition Experiments in a Parallel Plate Flow Cell. Bacterial suspension flow rate into the parallel plate flow cell was set to 2 mL/min. Parallel plate flow channel dimensions were 47.5 mm long by 12.7 mm wide by 1.6 mm high (cross-sectional area of 12.7 mm $\times$1.6 mm). For each deposition expertiment, the cell concentration of the injected culture was counted in a Burker-Turk cytometer chamber (Marienfield Laboratory Glassware, Germany) using a phase-contrast microscope (Zeiss, ZX10). Prior to each experiment, concentrated cell suspensions were washed two times and then diluted with the electrolyte solution of interest. From a single cell-deposition experiment, the cell-transfer rate coefficient was calculated as the ratio between the cell deposition flux and the initial cell bulk concentration. The cell deposition flux was the observed deposition rate of bacteria as normalized by the camera viewing area. The deposition experiments were performed at different ionic strength solutions with and without 4-NPO. The ionic strengths of the solutions used were 15, 50, 150, and 1500 mM (adjusted with NaCl) with and without 100 mM 4-NPO. During the first 20 min, background solution was injected, then the stationary-phase (24 h of growth after 1/100 dilution) bacterial suspension was injected for 1 h at a diluted concentration of OD_{600} 0.01, then the flow cell was washed with bacteria-free background solution. Afterwards, the number of deposited cells per hour was counted by taking between 15 and 20 pictures at different locations on the slide with the same camera zoom and under 40$\times$ magnification in all experiments. The number of deposited cells in different areas was normalized to a unit of area and to the initial cell concentration in each experiment separately. {For *P. aeruginosa* and *S. aureus*, the cell concentrations were $(4.97 \pm 0.36) \times 10^6$ and $(5.04 \pm 0.68) \times 10^6$ cells/mL, respectively}. The bacterial deposition experiments were carried out in duplicate.

Bacterial Deposition Experiments in a QCM-D Flow Cell. The QCM-D measurements were performed with AT-cut quartz crystals mounted in an E1 system (Q-sense AB, Gothenburg, Sweden). The crystals, with a fundamental resonance frequency of around 5 MHz, were coated with amorphous silica by vapor deposition. All QCM-D experiments were performed under flow-through conditions using a digital peristaltic pump (IsmaTec Peristaltic Pump, IDEX) operating in pushing mode for the studied solutions that were injected into the sensor crystal chamber at 150 μL/min. Before each measurement, the crystals were soaked in a 5 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 11.5) for 1 h, rinsed thoroughly with double distilled water, dried with pure N_2 gas, and treated for 10 min in a UV/O_3 (BioFORCF nanoscience) chamber. The bacterial deposition experiments in the QCM-D were carried out using similar conditions, order, and background solutions to those in the parallel plate flow cell. Prior to each experiment, a stable baseline with double distilled water was acquired until a smaller frequency change of 0.01 Hz/min was measured. Data were collected for 20 min in each stage of the experiment. For an analysis of the

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effect of 4-NPO on bacterial deposition, solutions were added to the QCM-D system in the following order: (1) background NaCl solutions with or without 100 mM 4-NPO (2) a bacterial suspension in the studied background solution, and (3) a similar solution with or without 4-NPO for washing planktonic and loosely bound bacteria. The variations in frequency (Δf, Hz) were measured for the five overtones ($n = 5, 7, 9, 11$, and 13). Data are presented for the ninth overtone, and for comparison purposes of the 4-NPO effects on bacterial deposition, changes in the frequency shift were calculated by subtracting the decrease in the frequency due to the background solution without bacteria. For an analysis of the effect of conditioning the silica surface of the QCM-D crystals with 4-NPO on bacterial deposition, solutions were added to the QCM-D system in the following order: (1) a 150 mM NaCl solution with 100 mM 4-NPO, (2) a 150 mM NaCl solution without 4-NPO, (3) a bacterial suspension in 150 mM NaCl without 4-NPO, and (4) a 150 mM NaCl solution without 4-NPO for washing planktonic and loosely bound bacteria from the collector surface.

Hydrodynamic Calculations. Bacterial suspension velocities in the QCM-D and in the parallel plate flow cells were adjusted to achieve similar orders of magnitude for shear stress values that the bacteria would face either in the parallel plate flow cell or in the QCM-D flow cell. For the laminar developed flow of a Newtonian fluid, the velocity profile between two parallel plates can be described by the following equation

$$v = \frac{3}{2} V_m \left[1 - \left(\frac{b - z}{b} \right)^2 \right]$$

where V_m is the mean fluid velocity, b is half the channel height, and z is the distance coordinate perpendicular to the surface ($z = b$ in the middle of the channel and $z = 0$ at the surface). Therefore, the maximum liquid velocity ($V_{max} = {}^3/_2\, V_m$) occurs in the middle of the channel and is equal to 0 near the surface. The shear stress profile $G_{sh}(z)$ in a thin channel can be calculated as dv/dz and is equal to the following equation:[26]

$$G_{sh} = \frac{3V_m}{b^2}(b - z)$$

The highest shear rate that the bacteria experience will be at $z = 0$. In the parallel plate flow cell at an injected flow rate of 2 mL/min, $V_m = 0.16$ cm s^{-1} and the corresponding maximum value of the shear stress, G_{sh}, is equal to 6 s^{-1}. For the QCM-D flow cell, a complicated numerical calculation of the shear stress is required for an approximation because of the unequal distribution of the fluid velocities at various locations in the channel. Therefore, an estimation of a parallel plate channel was applied to the circular QCM channel with a height of approximately 0.06 cm (i.e., $b \approx 0.03$ cm) and a diameter of 1.4 cm (disk diameter value, Q-Sense, Sweden). By neglecting the different geometries, V_m was estimated according to an average cross-sectional area, which was assumed to be the maximum cross-sectional area of 0.084 cm^2 (in the middle of the channel) divided by 2. For this estimated V_m of 0.06 cm s^{-1}, a maximum G_{sh} value of 6 s^{-1} was calculated (similar to the maximum G_{sh} of the original parallel plate flow cell). On the basis of the dimensions and flow rates employed in the parallel plate and QCM-D flow cells, the *Pe* number was also calculated.[27] The *Pe* number for both flow cells was 0.001, indicating that the bacteria within both systems effectively experienced the same diffusion-dominated flow regime.[28]

DLVO Calculations. DLVO theory was utilized to predict the interaction energy between the cell and collector surfaces.[29] Total interaction energies existing between the pathogen and sand particle (assuming sphere−plate interaction) were quantified as the sum of van der Waals[30] and electrostatic interactions with constant surface potential,[31] both of which decay with a separation distance.[27]

Bacterial Physicochemical Characterization: Relative Hydrophobicity and Zeta Potential. Cultures of *P. aeruginosa* PAO1 and *S. aureus* were incubated in an LB medium at 150 rpm and 30 °C and collected in the stationary growth phase for an analysis of their relative hydrophobicity and electrophoretic mobility. Surface characterization was repeated either three or four times in different experiments. The bacterial suspension was centrifuged and washed three times with the relevant medium in which the deposition experiments were performed. Measurements of bacterial electrophoretic mobility were performed with a zeta potential analyzer (ZetaPlus 1994, Brookhaven Instruments Co., Holtsville, NY) according to de Kerchove and Elimelech.[32] All cultures were washed in the relevant solution tested and diluted to an $OD_{600\ nm}$ of 0.1 prior to analysis. In fact, 40 measurements were used to get an average of electrophoretic mobility because for each analysis 10 measurements were taken. Electrophoretic mobility measurements were converted into zeta potentials by using the Smoluchowski equation. This equation was applicable because of the relatively large cells and the ionic strengths used.[27] The relative levels of hydrophobicity of the *P. aeruginosa* PAO1 and *S. aureus* strains with and without 4-NPO were measured using the microbial adhesion to hydrocarbons (MATH) test with *n*-dodecane (Sigma-Aldrich, Israel).[33] This analysis was repeated four times. Hydrophobicity is defined here as the fraction of total cells partitioned into the *n*-dodecane phase.

Results and Discussion

The effect of 4-NPO on the deposition of *P. aeruginosa* and *S. aureus* on a quartz surface in a parallel plate flow cells was assessed under various ionic strength conditions. For *P. aeruginosa*, ionic strength conditions included 15, 50, and 150 mM, and for *S. aureus* experiments, the ionic strengths were 15, 150, and 1500 mM adjusted with NaCl. 4-NPO was supplemented at a concentration of 100 mM in the NaCl bacterial suspensions for all ionic strength conditions unless mentioned otherwise.

The deposition results indicate clearly that the bacterial mass-transfer rate of both *P. aeruginosa* and *S. aureus* significantly decreases when the bacterial suspension is supplemented with 4-NPO (Figure 2A,B). For *P. aeruginosa* at ionic strengths of 15, 50, and 150 mM and for *S. aureus* at ionic strengths of 15 and 150 mM, a significant decrease in cell deposition was observed. At an ionic strength of 1500 mM, the opposite behavior was observed for *S. aureus*. Under this condition, a higher deposition rate onto the quartz surface was observed in the presence of 4-NPO (Figure 2B). Similar trends for bacterial deposition and inhibition by 4-NPO were observed in the QCM-D flow cell. Similar aquatic and hydraulic conditions were applied to the deposition processes in the QCM-D as in the parallel plate flow cells when the bacteria was facing a maximum shear stress value of 6 s^{-1} and a *Pe* of 0.001 in both types of flow cells. Figure 2C,D shows that the frequency shift due to bacterial adsorption was significantly higher without 4-NPO in all cases except for the deposition of *S. aureus* at 1500 mM. Hence, all QCM-D experiments were able to confirm the deposition results from the parallel plate flow cells. When the suspension was supplemented with 4-NPO, there was

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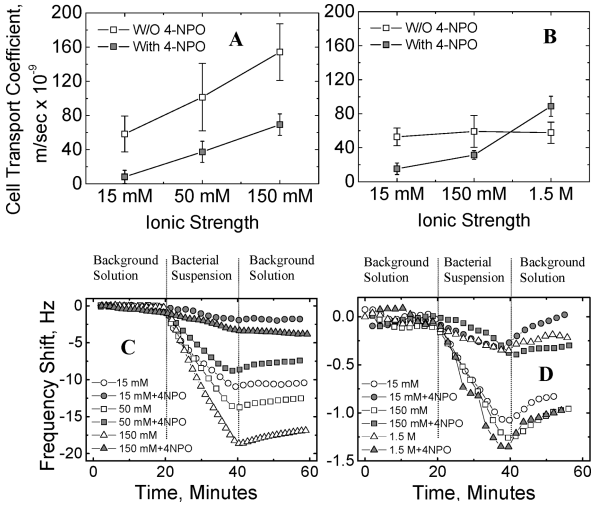

Figure 2. Bacterial transport and deposition analysis in the presence and absence of 4-NPO at ionic strength ranging from 0.015 to 1.5 M NaCl (pH of 6.1 ± 0.2 and 25 ± 2 °C). Mass-transfer rate coefficients for (A) *P. aeruginosa* PAO1 and (B) *S. aureus* in parallel plate flow cells and frequency shifts for (C) *P. aeruginosa* PAO1 and (D) *S. aureus* in the QCM-D. Frequency shifts values caused by changes in ionic strength alone were subtracted from the plots. The mass-transfer rate coefficients in the parallel flow cell were statistically analyzed for their significance with p value that was lower than 0.001.

reduced cell attachment except for the case of *S. aureus* at 1500 mM, when cell attachment increased significantly. The biological quorum-sensing capacity of 4-NPO is not expected to be a factor because of the low density of bacteria in this experiment ($O.D._{600\ nm}$ of 0.01). This result of a higher deposition rate for *S. aureus* at 1500 mM in the presence of 4-NPO is further evidence that 4-NPO is not playing a biological role under this condition, but rather is significant in later stages of biofilm formation when the cell density is much higher.

The addition of 4-NPO to both types of flow cells, the parallel plate and the QCM-D, was just prior to the onset of injection of the bacterial suspension. The instantaneously observed reduction in bacterial deposition by 4-NPO in both the parallel plate and QCM-D flow cells implies that physicochemical changes in the bacterial cell surface or in the quartz/silica surface take place. To show that 4-NPO had no physiological effect but rather has a physicochemical effect on the cells to which the reduction of attachment is attributed, the effect of surface conditioning with 4-NPO on the deposition of *P. aeruginosa* cells that were not exposed to 4-NPO was tested. *P. aeruginosa* cells were washed in a 150 mM NaCl solution without 4-NPO. Then, prior to the bacterial deposition experiment in the QCM-D, the silica-coated crystal was conditioned with 100 mM 4-NPO in a 150 mM NaCl solution for 20 min. After the crystal was conditioned with 4-NPO, the bacterial suspension in 150 mM NaCl solution was injected for another 20 min and the associated frequency shift observed was significantly lower than in the case without conditioning the silica surface (Figure 3). Interestingly, *P. aeruginosa* attachment was significantly reduced by conditioning the silica surface with 4-NPO. This suggests that the adsorbed phase of 4-NPO, which does not affect cell physiology, reduces the deposition of bacteria to the silica surface. A frequency shift due to bacterial adsorption to the nonconditioned silica surface of −18 Hz was detected versus ca. −2.5 Hz for the 4-NPO-conditioned silica-coated crystal (Figure 3). Furthermore, washing with double distilled water completely restored the conditioned sensor

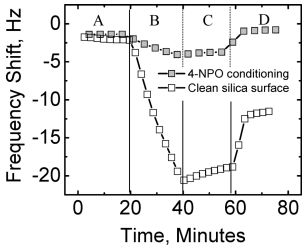

Figure 3. Effect of conditioning the silica surface of the QCM-D sensor with 4-NPO on *P. aeruginosa* attachment: clean silica and a silica surface that was exposed to 4-NPO are being compared. Prior to the experiment, silica-coated crystal was exposed for 20 min to 100 mM 4-NPO in a 150 mM NaCl solution (■), followed by a 20 min 4-NPO-free electrolyte (NaCl) rinse (stage A). The preconditioning step was skipped in the case of clean silica (□). The bacterial suspension was injected in the absence of 4-NPO (stage B), followed by another 20 min 4-NPO-free electrolyte rinse (stage C). The final stage involved a 20 min injection of double distilled water (stage D). Experimental conditions included a solution with ionic strength of 150 mM adjusted with NaCl (except for stage D), a pH of 6.1 ± 0.2, a temperature of 25 ± 2 °C, and a constant flow rate of 150 μL/min.

frequency to its original clean level (i.e., nearly all cells were released from the surface) versus a residual frequency measurement of ca. −10 Hz that was attributed to attached cells being irreversibly attached to the nonconditioned sensor.

To further investigate the physicochemical changes in the bacteria and silica surfaces that impact cell deposition, the zeta potentials of the bacteria and quartz surfaces were acquired in solutions with different ionic strengths. The electrokinetic measurements show that both bacteria and the quartz surface carry a negative charge that becomes less negative in higher ionic strength solutions (Figure 4) because of shielding of the electric double layer by excess sodium cations. The measured electrophoretic mobility values for the bacterial cells were converted to zeta potential values using the Smoluchowski equation.[27] When supplementing the NaCl solutions with 4-NPO, the zeta potential becomes less negative in all cases and in high ionic strength solutions neutrality is achieved (Figure 4). The only exception was that at an ionic strength of 1500 mM, when the solution was supplemented with 4-NPO, *S. aureus* becames positively charged (Figure 4 C). In all cases, the presence of 4-NPO in 100 mM NaCl solutions results in favorable *P. aeruginosa*−quartz interactions based upon DLVO calculations. This is also the case for *S. aureus*−quartz interactions at ionic strengths of ≥150 mM. Only under lower ionic strength conditions (< 100 mM) does an energy barrier still exist for interactions (for either cell type) in the presence of 4-NPO. However, this is also the case in the absence of 4-NPO, suggesting that the contribution of 4-NPO to cell−quartz interactions is independent of DLVO-type interactions. Surface-charge heterogeneities of both bacteria and substrata can lead to significant interaction energies.[8] As further delineated in this study, the results presented strongly suggest that 4-NPO shields both the substrata and the bacterium charge heterogeneities that eventually reduce attachment.

The cell and quartz surface hydrophobicity were also analyzed in the presence and absence of 4-NPO. It was found that for both bacterial strains analyzed at high ionic strength (1500 mM) no significant change was detected for the percentage of cell partitioning in *n*-dodecane (Table 1). At lower ionic strength (at and below 150 mM), significant changes in cell partitioning are detected. Interestingly, at ionic strengths ≤150 mM, 4-NPO makes a distinct contribution to cell hydrophocity−hydrophilic bacteria such as *P. aeruginosa*, cells become more hydrophobic,

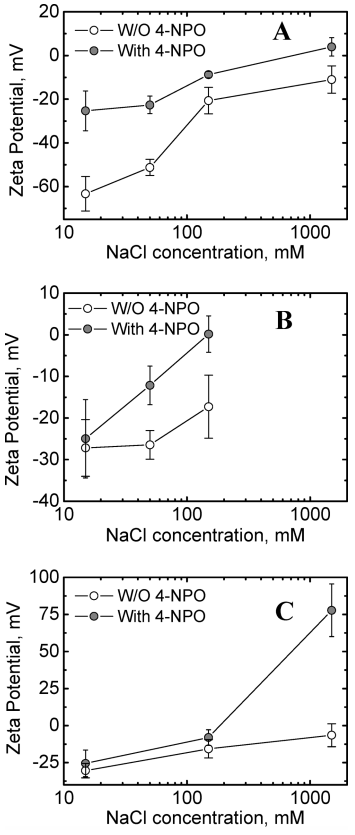

Figure 4. Zeta potential of quartz particles and cells and in the presence and absence of 4-NPO under various ionic strength conditions adjusted with NaCl: (A) ultrapure quartz particles, (B) *P. aeruginosa* PAO1, and (C) *S. aureus*. Zeta potential values were statistically analyzed for their significance with a p value that was lower than 0.001.

Table 1. Relative Hydrophobicity of *P. aeruginosa* PAO1 and *S. aureus* Analyzed by the MATH Test in the Presence and Absence of 4-NPO

	P. aeruginosa PAO1		*S. aureus*	
ionic strength, mM	with 4-NPO	w/o 4-NPO	with 4-NPO	w/o 4-NPO
15	29.7 ± 2.8	11.1 ± 0.81	74.7 ± 9.0	91.0 ± 9.5
50	14.8 ± 0.66	8.1 ± 1.3	ND	ND
150	5.0 ± 1.6	5.5 ± 0.2	66.1 ± 9.0	83.9 ± 3.3
1500	ND	ND	94.7 ± 5.5	94.0 ± 2.2

and hydrophobic bacteria such as *S. aureus* become more hydrophilic (Table 1). Because 4-NPO effectively reduced cell deposition for all ionic strength conditions tested, except for *S. aureus* at an ionic strength of 1500 mM, no correlation was found between cell hydrophobicity and the reduced cell deposition by 4-NPO (Table 1 and Figure 2). Also, no change in the silica surface hydrophobicity was observed using a nitrogen bubble contact angle technique. A contact angle of 0° was detected for all water chemistries being studied, confirming the highly hydrophilic nature of the silica surface (results not shown).

However, under all conditions except for *S. aureus* at an ionic strength of 1500 mM, bacterial cell and silica surface charge neutralization correspond to reduced cell deposition. Note that in accordance with the deposition experiments using either the QCM-D or the parallel plate flow cells, the positive charge of *S. aureus* achieved at an ionic strength of 1500 mM with 4-NPO leads to a more favorable degree of cell−surface interactions for cell deposition as compared to the same solution without 4-NPO.

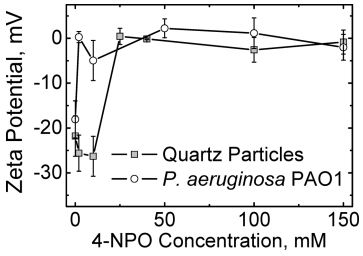

Figure 5. Zeta potential of *P. aeruginosa* PAO1 and quartz particles at different 4-NPO concentrations in 150 mM NaCl solution. pH values for all measurements of quartz and bacterial suspensions in 150 mM NaCl solution were 5.80 ± 0.22 and 5.77 ± 0.4, respectively.

As observed by Ostuni et al.,[20] surface neutrality is a good precondition for antifouling surface behavior. 4-NPO adsorption to the bacterial and silica surfaces can be attributed to hydrogen bonding with carboxyl functional groups on both bacterial surface and silanol residues on the silica surface. Additionally, electrostatic attraction may promote 4-NPO adsorption to counter charges located on both the bacterial and silica surfaces. Specific bacteria−surface interactions are, in most cases, promoted by electrostatic attractive forces. The deposition and attachment of bacteria can occur on either negatively or positively charged surfaces because bacteria will interact with oppositely charged functional groups that are heterogeneously distributed on the cell outer membrane and the substrata.[8] Corroborating with Ostuni et al.,[20] the neutralization of both bacterial and substratum surfaces by 4-NPO reduced bacterial adhesion. It appears that altering electrostatic attraction by neutralizing the localized charges on the bacterial outer membrane and the silica surface with 4-NPO reduced bacterial attachment. The results in our study are distinctly opposite from the DLVO predictions (calculations not shown). Because DLVO theory accounts for only homogeneous surfaces, the results observed here support our claim of surface charge heterogeneity in that 4-NPO is shielding and reducing adhesion.

The zeta potential of the bacterial and quartz surfaces is presented in Figure 5, and from this data, the influence of 4-NPO concentration can be observed. Both quartz and bacterial cell surfaces were neutralized at a 4-NPO concentration > 10 mM. It is possible that lower concentrations of 4-NPO solution (ca. 2 mM) are sufficient to neutralize the *P. aeruginosa* cell surface compared to the silica surface (Figure 5) because of the higher surface charge heterogeneity of the bacteria, which can provide more sites for 4-NPO adsorption. Considering this adsorption phenomenon, a lower bulk concentration of 4-NPO will be required to neutralize the bacteria sufficiently as compared to the quartz particles.

Concluding Remarks

Under all conditions applied in this study, charge neutralization was accompanied by a significant reduction in cell attachment. Notably, hydrophobic interactions did not play a role in cell attachment. *P. aeruginosa* cells were more hydrophobic after treatment with 4-NPO and *S. aureus* cells were more hydrophilic after treatment with 4-NPO, and in both cases, bacterial adhesion was reduced. Zwitterions such as 4-NPO and mixed-charge macromolecules have been reported to bind water molecules via electrostatic interactions whereas neutral and hydrophilic polymers bind water molecules via hydrogen bonding.[34] Zwitterions are capable of binding a significant number of water molecules

(34) Chen, S.; Zheng, J.; Li, L.; Jiang, S. *J. Am. Chem. Soc.* **2005**, *127*, 14473–14478.

and therefore are potentially excellent candidates for creating low fouling materials. Thus, binding 4-NPO residues on surfaces appears to be a good choice for reducing bacterial adhesion via physicochemical interactions. The low zeta potential attributed to 4-NPO adsorbed to surfaces reduced the electrostatic attractive forces that would otherwise promote specific interactions between bacteria and the surface. Also, simultaneous inhibition of the QS machinery in bacteria due to the presence 4-NPO is an attempted physiological solution for biofouling control[35] because no selective pressure is induced by quorum-sensing inhibitors but rather is induced by antimicrobial compounds. This combination of mechanisms leads to a significantly reduced level of cell adhesion, either gram negative or positive. However, future work must identify the specifics of how the adsorption of these compounds alters bacterial cells and the substratum surfaces as well as QS properties so that the mechanism can be delineated beyond a single bacterial, substrate, or zwitterion type and a broader-spectrum anti-fouling agent can be found.

(35) Yeon, K.-M.; Cheong, W.-S.; Oh, H.-S.; Lee, W.-N.; Hwang, B.-K.; Lee, C.-H.; Beyenal, H.; Lewandowski, Z. *Environ. Sci. Technol.* **2008**, *43*, 380–385.