Escherichia coli, Pseudomonas aeruginosa, and *Staphylococcus aureus* Attachment Patterns on Glass Surfaces with Nanoscale Roughness

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Abstract Attachment tendencies of *Escherichia coli*
K12, *Pseudomonas aeruginosa* ATCC 9027, and *Staphylococcus aureus* CIP 68.5 onto glass surfaces of different
degrees of nanometer-scale roughness have been studied.
Contact-angle and surface-charge measurements, atomic
force microscopy (AFM), scanning electron microscopy
(SEM), and confocal laser scanning microscopy (CLSM)
were employed to characterize substrata and bacterial
surfaces. Modification of the glass surface resulted in
nanometer-scale changes in the surface topography,
whereas the physicochemical characteristics of the surfaces
remained almost constant. AFM analysis indicated that the
overall surface roughness parameters were reduced by 60–
70%. SEM, CLSM, and AFM analysis clearly demonstrates
that although *E. coli*, *P. aeruginosa* and *S. aureus* present
significantly different patterns of attachment, all of the
species exhibited a greater propensity for adhesion to the
"nano-smooth" surface. The bacteria responded to the
surface modification with a remarkable change in cellular
metabolic activity, as shown by the characteristic cell
morphologies, production of extracellular polymeric substances, and an increase in the number of bacterial cells
undergoing attachment.

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Introduction

Bacterial attachment and the factors that influence the
process, together with the subsequent biofilm formation,
have been the focus of intensive study over the past few
decades [1, 3, 21, 22], mainly due to the ongoing effort to
design antibacterial surfaces or microtextured surfaces with
an "antifouling effect. The mechanisms that control bacterial adhesion have been addressed on various levels:
theoretical approaches such as the Derjaguin, Landau,
Verwey, Overbeek and thermodynamic theories have
revealed some of the basic physicochemical aspects of
bacterial adhesion [3, 4, 9] and cell studies have provided
useful information regarding the role that the cell surface
characteristics play in the attachment mechanism [16, 18].
Apart from the cell surface characteristics, it is now commonly accepted that a wide range of substratum surface
properties such as morphology, surface chemistry, roughness, and porosity can all exert a strong influence over the
tendency of bacteria to attach to different surfaces [22, 24].

One of the recently developed concepts is that of an
"attachment point" [14, 23]. According to this theory,
organisms smaller than the scale of the surface microtexture will attach in sufficient number and will have
greater adhesion strength because of the multiple attachment points on the surface when compared to
microorganisms that are of scale larger than the surface
roughness [8, 24]. They will also be well protected from
hydrodynamic shear forces in microscopic refuge shelters
on the textured surface [23]. A number of research projects studying the relationship between surface roughness
and attachment of organisms (such as barnacle cyprids
and algal spores) has supported the applicability of the
"attachment points" theory [20, 23]. On the other hand,
only a few studies have observed the effects of surface

topography on the adhesive behavior of smaller micro-organisms such as bacteria, despite the fact that they are believed to be the initial colonizers of many surfaces and to be necessary for further biofilm development and macrofouling colonization [23]. Some other studies have focused on physicochemical bacterial and substratum characteristics, such as surface wettability, tension and charge, or micrometer-scale roughness that is comparable to or greater than the cell size [11, 29]. In light of these current thoughts, the article aimed to further test our hypothesis as to whether surface roughness on the nanometer scale plays a role on the initial stage of bacterial attachment. We previously showed an impact of nanometer-scale surface roughness on the attachment strategy of a marine bacterium, *Pseudoalteromonas issachenkonii* KMM 3549[T], while interacting with glass surfaces [26]. In this study we investigate an impact of nanometer-scale surface roughness on the attachment strategy of three taxonomically unrelated medically important bacteria, *Escherichia coli* K12, *Staphylococcus aureus* CIP 68.5, and *Pseudomonas aeruginosa* ATCC 9027. The attachment pattern of these bacteria was tested on two types of glass surface, namely "as-received" and "modified" surfaces, where the latter were derived from the as-received material by means of chemical treatment.

Methods and Methods

Substratum Surface Analysis

Glass surfaces (7105-PPA premium glass slides Livingstone International) have been modified as described elsewhere [17, 28]. The surface wettability of both as-received and modified glass regions was measured with the FTA200 (First Ten Ångstroms Inc.) using the embedded-needle method [19, 21]. Droplets of nanopure water (18.2 м/cm) were deposited on the surfaces and movies (up to 35 s each), each delivering up to 100 images for analysis, were recorded. At least five measurements were conducted on each surface to obtain the average values.

A Solver P7LS instrument (NT-MDT Co.) was used to image the surface topography and to quantitatively measure and analyze the surface roughness on the nanometer scale. The analysis was performed in the semicontact mode, which reduces the interaction between tip and sample and thus allows the destructive action of lateral forces that exist in contact mode to be avoided. The carbon "whisker"-type silicon cantilevers (NSC05, NT-MDT) with a spring constant of 11 N/m, tip radius of curvature of 10 nm, aspect ratio of 10:1, and resonance frequency of 150 kHz were used to obtain good topographic resolution. Scanning was performed perpendicular to the axis of the cantilever at a typical rate of 1 Hz. Image processing of the raw topographical data was performed with first-order horizontal and vertical leveling, and the topography and surface profile of the samples were obtained simultaneously. In this way, the surface features of the samples were measured with a resolution of a fraction of nanometer and the surface roughness of the investigated areas could be statistically analyzed using the standard instrument software (LS7-SPM v.8.58).

Cell Surface Analysis

Bacterial sample preparation followed the procedure described in [17]. The physicochemical cell surface characteristics were determined by measuring the cell surface wettability and charge. The surface wettability of *E. coli, P. aerugionsa*, and *S. aureus* was evaluated from contact-angle measurements on lawns of bacterial cells using the sessile drop method in the FTA200 instrument. A bacterial cell suspension was prepared and applied to the glass substrates and measurements were performed, as described elsewhere [2, 9, 15]. Bacterial surface charge was inferred via measurement of the electrophoretic mobility (EPM) of the bacterial cells, followed by conversion into a zeta potential using Smoluchowski's approximation. The EPM was measured as a function of ionic strength in a buffered solution [10] using a zeta potential analyzer (ZetaPALS; Brookhaven Instruments Corp). The cell suspension was prepared and measurements were taken as explained elsewhere [10]. All measurements were carried out in triplicate, and for each sample, the final EPM represents the average of five successive ZetaPALS readings, each of which consisted of 14 cycles per run.

Scanning electron microscopy (SEM) images were obtained using a FeSEM instrument (SUPRA 40VP; Carl Zeiss SMT). Prior to imaging, the slides were gold-coated in a Dynavac CS300 coating unit. The extracellular polymeric substances (EPSs) produced by cells attaching to both regions of the glass substrates were labeled with Concanavalin A 488 (Molecular Probes Inc.) and observed using confocal laser scanning microscopy (CLSM) as described elsewhere [17, 28]. The number of attached cells observed in the SEM images (1000× magnification) was transformed into a number of bacteria per unit area as described elsewhere [26].

Statistical data processing was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Three independent *t*-tests were performed.

Results

Glass Surface Physicochemical Characteristics

It appeared that the glass surfaces were moderately hydrophilic, exhibiting a water contact angle (θ) of $\sim 45° \pm 4°$ for as-received and $42° \pm 3°$. Analysis of the chemical composition of both the as-received and modified glass surfaces revealed no significant differences, according to results obtained from X-ray fluorescence and X-ray photoelectron spectroscopy [17].

A detailed analysis of the surface roughness indicated that the modified glass surface appeared uniformly smoother and lacked the relatively prominent protrusions observed on the as-received glass surface (Table 1, Fig. 1). A statistical analysis of the surface roughness parameters confirmed this observed change in surface roughness for modified surfaces. All of the conventional roughness parameters [i.e., the average surface roughness (R_a, 2.1 nm vs. 1.3 nm), the root mean square (R_q, 2.8 nm vs. 1.6 nm), and the maximum roughness (R_{max}, 27.8 nm vs. 16.2 nm)] were found to be $\sim 50\%$ higher on the native glass surface. Taking into account that these parameters do not necessarily provide a satisfactory indication of the topographical differences, an alternative roughness measure has been employed [29] to provide additional information. The 10-point average roughness (R_z), is defined as the

difference in height between the average of the five highest peaks and the five lowest valleys along a profile [29]. Extending this concept to the five highest and lowest points in a scanned area, the modified surface (R_z = 4.8 nm) is approximately in the range of twofold smoother than the as-received surface (R_z = 12.2 nm). In summary, all four parameters (R_a, R_q, R_{max}, R_z) gave a strong indication that the as-received glass surfaces contain considerably greater surface roughness than the modified glass surfaces, albeit on the nanometer scale. Overall, the glass surface analysis clearly demonstrated that the chemical treatment modified the surface topography on the nanometer scale, whereas the other surface characteristics were not significantly affected by the etching process.
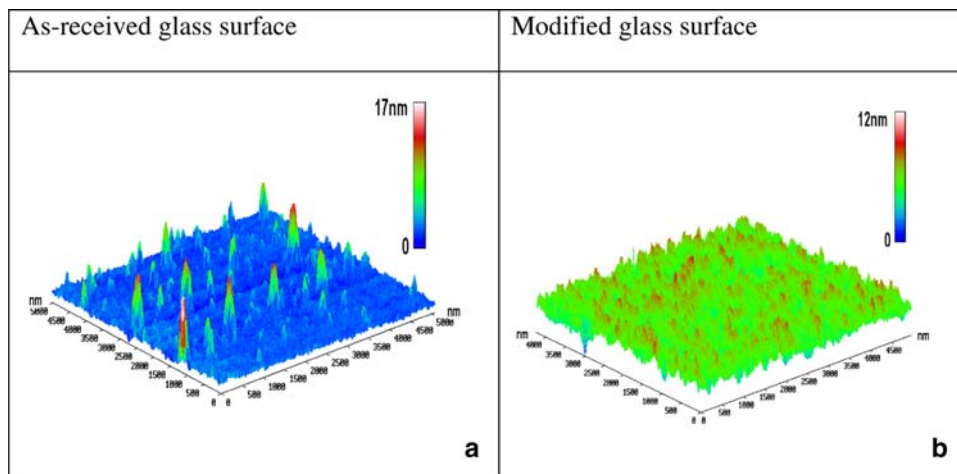
Bacterial Surface Characteristics

The bacterial surface characteristics, such as cell surface charge and wettability, are presented in Table 1. *E. coli* and *P. aeruginosa* cells were found to be moderately hydrophilic ($\theta = 34°$ and $\theta = 43°$, respectively) and *S. aureus* cells exhibited more hydrophobic characteristics ($\theta = 72°$). These contact-angle values are consistent with previously published data for various strains of *E. coli* [5] and *P. aeruginosa* [12]. The hydrohobic nature of *S. aureus* cells could be attributed to the presence of highly negatively charged and hydrophobic teichoic and lipoteichoic

Table 1 Surface characteristics and cell dimensions of *E. coli, P. aeruginosa,* and *S. aureus*

Strain	Water contact angle, θ (deg)	EPM [(μ/s)(V/cm)]	Zeta potential, ζ (mV)	Cell dimensions (μm)	
				As-received glass	Modified glass
E. coli	33.0 ± 4	−3.1 ± 0.6	−38.4 ± 0.3	1.7 × 1.0 × 0.20	2.1 × 1.3 × 0.25
P. aeruginosa	43.3 ± 8	−1.1 ± 0.1	−14.4 ± 0.7	2.1 × 1.1 × 0.18	2.4 × 1.8 × 0.25
S. aureus	72.2 ± 8	−2.8 ± 0.8	−35.2 ± 1.0	0.9 × 0.9 × 0.40	0.9 × 0.9 × 0.40

Fig. 1 Typical AFM images of as-received (**a**) and etched (**b**) glass surfaces. The chemical treatment has resulted in reduction of surface roughness on the nanometer scale. The imaged areas are approximately $5 \times 5 \ \mu m^2$

acid sites, which are some of the main constituents of *S. aureus* cell walls [7, 13].

The bacterial surface charge results are also listed in Table 1 and are in accordance with the notion that the majority of microbial cell surfaces are negatively charged [6, 27]. The least electronegative species was *P. aeruginosa*, with an EPM of $-1.12(\mu m/s)/(V/cm)$, followed by *S. aureus*, with a mobility of $\sim -2(\mu/s)(V/cm)$. The most electronegatively charged cells were those of *E. coli*, with an EPM above $-3(\mu/s)(V/cm)$. These values are within the range of previously reported values for *E. coli* [25], *P. aeruginosa* [12], and *S. aureus* [12].

Patterns of Bacterial Attachment

The attachment patterns of *E. coli, P. aeruginosa*, and *S. aureus* were examined by means of high-resolution SEM and atomic force microscopy (AFM) imaging (Fig. 2). In addition to characteristic differences between the attachment patterns of each species, inspection of the images reveals striking differences in the cell morphology, production of EPSs, and numbers of bacterial cells attached on the two surface regions (Figs. 2 and 3). Overall, the number of cells attached to the modified surface was approximately two times higher, depending on the strain, when compared to the number of cells attached to the as-received glass surface (Fig. 3). The density values have estimated errors of $\sim 10-15\%$ due to local variability in the surface coverage. No clear correlation between cell surface hydrophobicity and attachment density could be noted.

Changes in cell morphology were first observed in high-magnification SEM images and latter confirmed by AFM (Fig. 2). The cell dimensions presented in Table 1 indicate that cells of *E. coli* and *P. aeruginosa* increased in size while attaching to the modified glass surface. The average increase in the cell volume varied between 15% and 20%, depending on the strain. The increase in cell volume reported was found to be statistically significant ($p < 0.05$).

Notably, the difficulty experienced in accurately determining the cell size is probably due to large quantities of EPSs being produced by the cells on the modified glass surfaces. In contrast, *S. aureus* cells did not exhibit any significant difference in the cell morphology while attaching to the two surface types.

The attachment pattern of bacterial cells and the production of EPSs on the two regions of the glass surfaces after 12 h were confirmed using CLSM. It should be noted that cells of *E. coli* and *P. aeruginosa* deposited substantial quantities of EPSs on the modified surface (data not shown).

Discussion

Current theoretical predictions relating to the propensity of bacteria to adhere to different surfaces are based on physicochemical bacterial surface characteristics such as cell wettability and surface charge. According to this concept, both *E. coli* and *P. aeruginosa* cells should exhibit a greater propensity to adhere to hydrophilic glass surfaces than *S. aureus* [4, 14], in accordance with the thermodynamically predicted preference of hydrophilic cells for hydrophilic substrata and of hydrophobic cells for hydrophobic substrata [3, 4]. In general, the results of our study conform to this theory, because *S. aureus* was seen to attach less than the other bacteria to the hydrophilic glass surface. In terms of cell surface charge, it would be expected that the *E. coli* cells, being more electronegatively charged than *P. aeruginosa* and *S. aureus* with an EPM above $-3(\mu/s)(V/cm)$ (Table 1), would display the weakest (and *P. aeruginosa* display the strongest) attachment propensity toward the negatively charged glass surfaces. The results presented in Table 1 and Fig. 3 are consistent with this theory. They suggest that *P. aeruginosa* was the most proficient colonizer, particularly on the modified glass surfaces, where cells tended to form a multilayer biofilm, while producing greater quantities of EPS. Notably, all three of the species studied displayed an increased production of EPSs when attaching to the modified glass surfaces. This observation implies that bacteria might employ a somewhat similar strategy for attachment to nanosmooth surfaces by producing elevated amounts of EPSs in order to effect the attachment.

These results once again highlight the difficulty in understanding bacterial attachment behavior based solely on physicochemical cell surface characteristics. Although it might be expected that more electronegative cells would display greater hydrophilicity, it has been reported that the presence of an increased amount of EPS causes the cells to exhibit hydrophobic characteristics, mainly due to the dynamic motion of the outer surface proteins that can modify the apparent polarity or charge of the cell [26].

The results presented here indicate that nanometer-scale surface roughness influences bacterial adhesion. We have observed an $\sim 50\%$ increase in the number of Gram-negative bacteria attached to the smoother modified glass surface in contrast to the as-received surface. The variation in the number of *S. aureus* cells attached to the as-received and the modified glass was not as pronounced. This observation is in agreement with the "attachment point" theory that small sphere-shaped microorganisms (such as *S. aureus*) exhibit a different attachment pattern compared to large or elongated cells because of the different number of available access points [1].

Fig. 2 Representative *E. coli* (top row), *P. aeruginosa* (middle row), and *S. aureus* (bottom row) attachment patterns on the as-received (left column) and modified (right column) glass surfaces after 12 h of incubation. SEM images present an overview of the attachment pattern; insers: SEM images on the top corners and AFM images on the bottom corners show typical cell morphologies. Considerable amount of EPSs are observed on the modified glass surface (circled). Scale bars are 10 μm on low-magnification images and 2 μm on SEM inserted images. The AFM imaged areas range from ∼2.5 × 5 μm² to 7 × 7 μm²
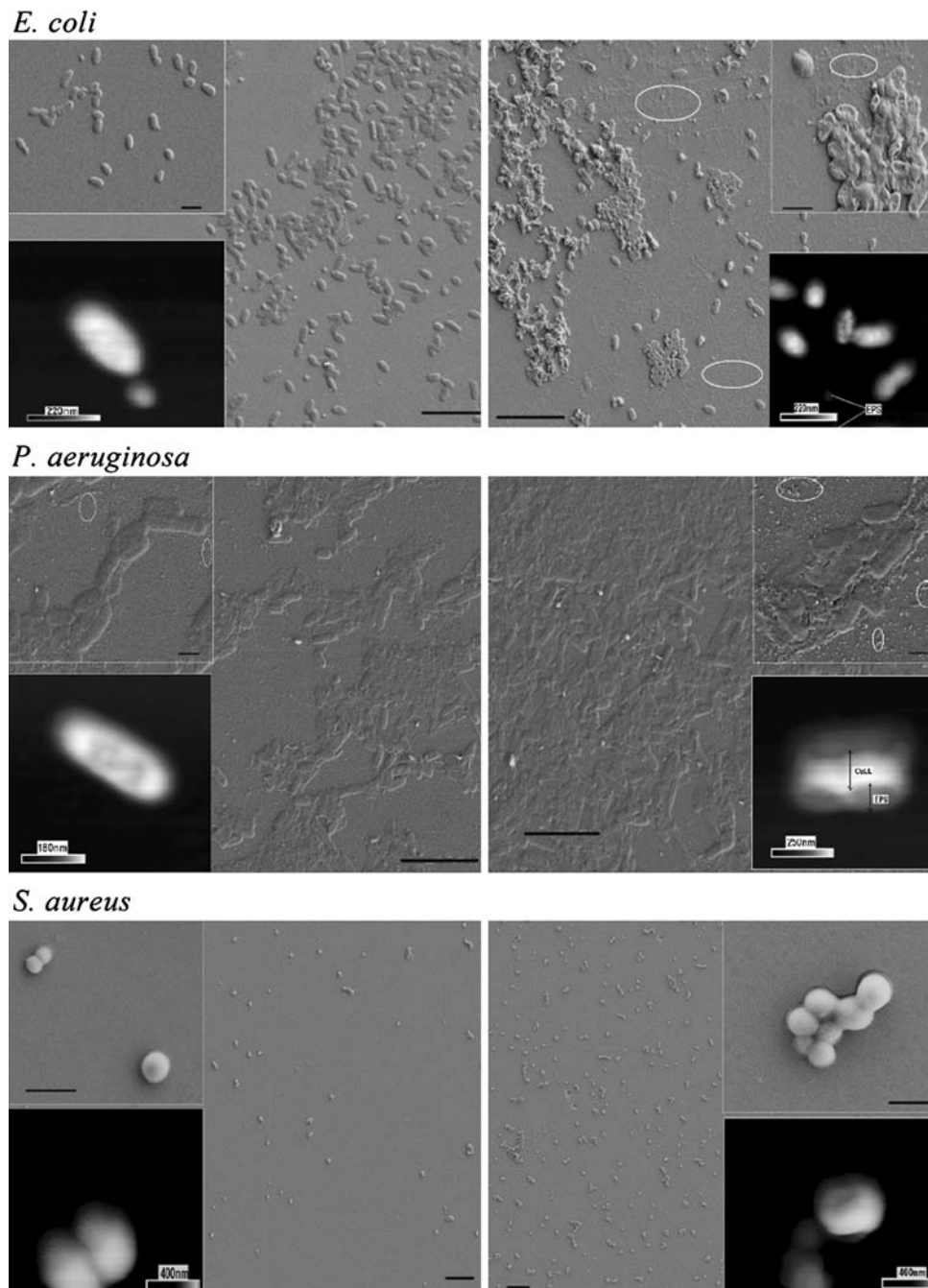
Notwithstanding individual species-specific patterns of adhesion, we were able to observe a consistent tendency for increased levels of bacterial adsorption onto the modified nanosmooth glass surfaces. These results are consistent with our recently reported study on the impact of surface nanotopography on attachment of the marine bacterium *Pseudoalteromonas issachenkonii* to nanosmooth glass surfaces [17], thus suggesting that nanoscale surface roughness might exert a greater influence on bacterial adhesion than previously believed and should therefore be considered as a parameter of primary interest alongside

other well-recognized factors that control initial bacterial attachment.

The suggestion that bacteria might be far more susceptible to nanoscale surface roughness casts serious doubt on the conventional wisdom that smoother surfaces represent a more repellent environment to bacteria. The effect of nanoscale surface roughness on bacterial adhesion has important implications for designing surfaces for use in surgical implants, the food industry, and sterile environments such as hospitals and pharmaceutical laboratories.
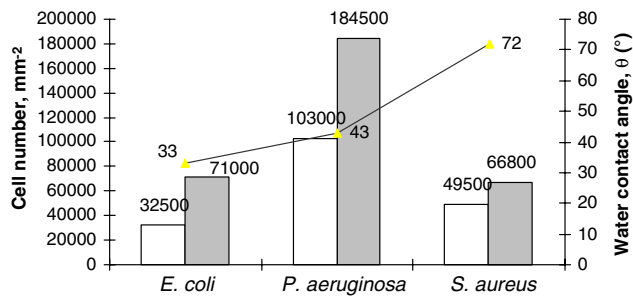
Fig. 3 *E. coli, P. aeruginosa*, and *S. aureus* attachment density on glass surfaces. The left column corresponds to the attachment density on the as-received glass surfaces; the right column corresponds to the attachment density on the modified glass surfaces. The line indicates the level of the cell surface's hydrophobicity according to water contact-angle measurements

References

1. Advincula MA, Petersen D, Rahemtulla F et al (2007) Surface analysis and biocorrosion properties of nanostructured surface sol-gel coatings on Ti6Al4v titanium alloy implants. J Biomed Mater Res B Appl Biomater 80:107–120
2. Bakker DP, Busscher HJ, van der Mei HC (2002) Bacterial deposition in a parallel plate and a stagnation point flow chamber: microbial adhesion mechanisms depend on the mass transport conditions. Microbiology 148:597–603
3. Bos R, van der Mei HC, Busscher HJ (1999) Physico-chemistry of initial microbial adhesive interactions: its mechanisms and methods for study. FEMS Microbiol Rev 23:179–230
4. Bruinsma GM, Rustema-Abbing M, van der Mei HC et al (2001) Effects of cell surface damage on surface properties and adhesion of *Pseudomonas aeruginosa*. J Microbiol Methods 45:95–101
5. Burks GA, Velegol SB, Paramonova E et al (2003) Macroscopic and nanoscale measurements of the adhesion of bacteria with varying outer layer surface composition. Langmuir 19:2366–2371
6. Busscher HJ, Norde W (2000) Limiting values for bacterial ζ potentials. J Biomed Mater Res 50:463–464
7. Canepari P, Boaretti M, Lleó MM et al (1990) Lipoteichoic acid as a new target for activity of antibiotics: mode of action of daptomycin (ly146032). Antimicrob Agents Chemother 34:1220–1226
8. Chae MS, Schraft H, Truelstrup Hansen L et al (2006) Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. Food Microbiol 23:250–259
9. Dong H, Onstotta TC, Kob C-HA et al (2002) Theoretical prediction of collision efficiency between adhesion-deficient bacteria and sediment grain surface. Colloids Surfaces B: Biointerfaces 24:229
10. Eboigbodin EK, Newton ARJ, Routh FA et al (2006) Bacterial quorum sensing and cell surface electrokinetic properties. Appl Microbiol Biotechnol 73:669–675
11. Emerson RJ, Bergstrom TS, Liu Y et al (2006) Microscale correlation between surface chemistry, texture, and the adhesive strength of *Staphylococcus epidermidis*. Langmuir 22:11311–11321
12. Gottenbos B, Grijpma DW, Van der Mei CH et al (2001) Antimicrobial effects of positively charged surfaces on adhering gram-positive and gram-negative bacteria. J Antimicrob Chemother 48:7–13
13. Gross M, Cramton SE, Gotz F et al (2001) Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. Infect Immun 69:3423–3426
14. Howell D, Behrends B (2006) A review of surface roughness in antifouling coatings illustrating the importance of cutoff length. Biofouling 22:401–410
15. Korenevsky A, Beveridge TJ (2007) The surface physicochemistry and adhesiveness of *Shewanella* are affected by their surface polysaccharides. Microbiology 153:1872–1883
16. Mandlik A, Swierczynski A, Das A et al (2008) Pili in gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends Microbiol 16:33–40
17. Mitik-Dineva N, Wang J, Mocanasu CR et al (2008) Impact of nano-topography on bacterial attachment. Biotechnol J 3:536–544
18. O'Toole GA, Kolter R (1998) Flagellar and twitching motility are necessary for *pseudomonas aeruginosa* biofilm development. Mol Microbiol 30:295–304
19. Öner D, McCarthy TJ (2000) Ultrahydrophobic surfaces. Effects of topography length scales on wettability. Langmuir 16:7777–7782
20. Petronis S, Berntsson K, Gold J et al (2000) Design and microstructuring of PDMS surfaces for improved marine biofouling resistance. J Biomater Sci Polym Ed 11:1051–1072
21. Quéré D, Lafuma A, Bico J (2003) Slippy and sticky microtextured solids. Nanotechnology 14:1109–1112
22. Riedewald F (2006) Bacterial adhesion to surfaces: the influence of surface roughness. PDA J Pharma Sci Technol 60:164–171
23. Scardino AJ, Harvey E, De Nys R (2006) Testing attachment point theory: diatom attachment on microtextured polyimide biomimics. Biofouling 22:55–60
24. Shellenberger K, Logan BE (2002) Effect of molecular scale roughness of glass beads on colloidal and bacterial deposition. Environ Sci Technol 36:184–189
25. Soni KA, Balasubramanian AK, Beskok A et al (2007) Zeta potential of selected bacteria in drinking water when dead, starved, or exposed to minimal and rich culture media. Curr Microbiol 56:93–97
26. Vadillo-Rodriguez V, Busscher HJ, Norde W et al (2004) Atomic force microscopy corroboration of bond aging for adhesion of *Streptococcus thermophilus* to solid substrata. J Colloid Interface Sci 278:251–254
27. van der Mei HC, Busscher HJ (2001) Electrophoretic mobility distributions of single-strain microbial populations. Appl Environ Microbiol 67:491–494
28. White JD, Stoddart RP (2005) Nanostructured optical fiber with surface-enhanced Raman scattering functionality. Opt Lett 30:598–600
29. Whitehead AK, Verran J (2006) The effect of surface topography on the retention of microorganisms. Food Bioprod Process 84:253–259