Air-directed attachment of coccoid bacteria to the surface of superhydrophobic lotus-like titanium

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Superhydrophobic titanium surfaces fabricated by femtosecond laser ablation to mimic the structure of lotus leaves \textit{(Nelumbo nucifera)}, cabbage leaves \textit{(Brassica oleracea)}, taro leaves \textit{(Colocasia esculenta)}, butterfly wings \textit{(Papilionidae ulysse)} and cicada wings \textit{(Psaltoda claripennis)} have been shown to exhibit a similar range of properties, including self-cleaning, directed wetting, anti-reflection and drag reduction (Barthlott and Neinhuis 1997; Koch et al. 2009; Liu et al. 2010; Bhushan and Jung 2011; Webb et al. 2011). It has been established that surface superhydrophobicity is directly linked to the presence of hierarchical surface roughness (Lafuma and Quéré 2003; Otten and Herminghaus 2004; Shirtcliffe et al. 2010) and that on flat surfaces, regardless of the fact that surface chemical nature water contact angles ($\theta_W$) $> 120^\circ$ cannot be achieved (Qu et al. 2008). Two theoretical models are widely used to explain the phenomenon of superhydrophobicity, ie Wenzel’s model describes the effect of surface roughness upon surface hydrophobicity (Wenzel 1949) and Cassie and Baxter’s model explains how the portions of the surface that are occupied by air increase the overall surface heterogeneity and induce hydrophobicity (Cassie and Baxter 1944). These two models, which can explain topography-induced hydrophobicity, are fundamental in the fabrication of superhydrophobic surfaces. The combination of surface heterogeneity and roughness is the major factor responsible for superhydrophobicity and the self-cleaning characteristics widely found on natural surfaces, such as lotus leaves (Marmur 2004; Gao and McCarthy 2006).

The knowledge generated from studying naturally superhydrophobic surfaces is the basis for the reproduction of their unusual properties in the development of biomaterial surfaces (Toes et al. 2002; Sun et al. 2005; Zhao et al. 2007; Yang et al. 2010; Webb et al. 2011). One of the approaches employed for the fabrication of superhydrophobic surfaces is to mimic those seen in naturally superhydrophobic surfaces (Genzer and Efimenko 2006; Koch et al. 2009; Bhushan and Jung 2011; Webb et al. 2011). Recently, femtosecond laser ablation was employed for the fabrication of titanium (Ti) surfaces with two tier microscale and nanoscale quasi-periodic self-organised structures, mimicking the surface of a \textit{Nelumbo nucifera} lotus leaf (Fadeeva et al. 2011). The first tier of the fabricated surfaces consisted of large grain-like convex features between 10 to 20 $\mu$m in size. The

\textbf{Keywords:} coccoid bacteria; superhydrophobic titanium surfaces; femtosecond laser ablation; microtopography and nanotopography

\textbf{Introduction}

Since the first description and explanation of a natural superhydrophobic surface in the 1940s (Fogg 1944; Cassie and Baxter 1945), a large number of natural surfaces such as lotus leaves \textit{(Nelumbo nucifera)}, cabbage leaves \textit{(Brassica oleracea)}, taro leaves \textit{(Colocasia esculenta)}, butterfly wings \textit{(Papilionidae ulysse)} and cicada wings \textit{(Psaltoda claripennis)} have been shown to exhibit a similar range of properties, including self-cleaning, directed wetting, anti-reflection and drag reduction (Barthlott and Neinhuis 1997; Koch et al. 2009; Liu et al. 2010; Bhushan and Jung 2011; Webb et al. 2011). It has been established that surface superhydrophobicity is directly linked to the presence of hierarchical surface roughness (Lafuma and Quéré 2003; Otten and Herminghaus 2004; Shirtcliffe et al. 2010) and that on flat surfaces, regardless of the fact that surface chemical nature water contact angles ($\theta_W$) $> 120^\circ$ cannot be achieved (Qu et al. 2008). Two theoretical models are widely used to explain the phenomenon of superhydrophobicity, ie Wenzel’s model describes the effect of surface roughness upon surface hydrophobicity (Wenzel 1949) and Cassie and Baxter’s model explains how the portions of the surface that are occupied by air increase the overall surface heterogeneity and induce hydrophobicity (Cassie and Baxter 1944). These two models, which can explain topography-induced hydrophobicity, are fundamental in the fabrication of superhydrophobic surfaces. The combination of surface heterogeneity and roughness is the major factor responsible for superhydrophobicity and the self-cleaning characteristics widely found on natural surfaces, such as lotus leaves (Marmur 2004; Gao and McCarthy 2006).

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second tier existed on the surface of the microscale features as 200 nm (or less) wide irregular undulations. One of the peculiar features of these Ti surfaces was their differential ability to retain bacteria, ie \textit{S. aureus} was shown to be capable of colonizing the lotus-like Ti surfaces, while the adhesion propensity of \textit{Pseudomonas aeruginosa} cells was limited (Ivanova et al. 2011a). This finding provided evidence that superhydrophobic surfaces may not be anti-biofouling despite their self-cleaning ability, even though some research groups have recently reported the possibility of superhydrophobic surfaces inhibiting bacterial colonization (Genzer and Efimenko 2006; Magin et al. 2010; Banerjee et al. 2011; Scardino and de Nys 2011). As yet, the correlation between superhydrophobicity and anti-biofouling has not been clearly demonstrated.

In this study the bacterial cell–superhydrophobic surface interactions between four coccoid bacteria, \textit{S. aureus} CIP 65.8\textsuperscript{T}, \textit{S. aureus} ATCC 25923, \textit{S. epidermidis} ATCC 14990\textsuperscript{T} and \textit{Planococcus maritimus} KMM 3738, and Ti surfaces mimicking the lotus leaf (\textit{Nelumbo nucifera}) were investigated, in order to determine whether different strains of coccoid bacteria were capable of sustainable adhesion on superhydrophobic lotus-like Ti surfaces, and to confirm that superhydrophobic surfaces are not necessarily inherently anti-biofouling. Further studies were conducted to examine the adhesion mechanism employed by \textit{S. aureus} CIP 65.8\textsuperscript{T} to colonise superhydrophobic lotus-like Ti surfaces.

Materials and methods

Fabrication of superhydrophobic Ti surfaces

Commercially pure Ti (Grade 2) disc samples with diameter of 10 mm and thickness of 2 mm were used in this study. The samples were mechanically polished and further cleaned with acetone followed by methanol. Structuring of the Ti surfaces was performed with a commercially available amplified Ti:Sapphire femtosecond laser system (Femtopower Compact Pro, Femtolasers Produktions GmbH, Austria) as illustrated in Figure S1 [Supplementary material is available via a multimedia link on the online article webpage.] The system delivers sub-30 fs pulses at 800 nm central wavelength with a pulse energy of up to 1 mJ, and a repetition rate of 1 kHz. An x–y motorised translation stage (Physik Instrumente GmbH, Germany) was used for sample positioning and translation. A computer controlled LCD element was used for laser pulse energy setting (Fadeeva et al. 2011). For fabrication of hierarchical superimposed nano- and microstructures, Ti surfaces were uniformly irradiated with 50 femtosecond laser pulses at fluences of 100 J cm\textsuperscript{-2}. The samples were processed under ambient air conditions. Following femtosecond laser fabrication, samples were cleaned with acetone using an ultrasonic bath. Ti sample surfaces ablated by femtosecond laser will be referred to as ‘lotus-like’ and non-ablated samples will be referred to as ‘as-received’.

Ti surface topography

The Ti surface topographies were evaluated using an Innova atomic force microscope (Veeco, Bruker, USA). Three samples of each type of surface type were briefly scanned to evaluate the overall homogeneity of the surface and the topographical profiles were studied in detail at several different locations. Imaging was performed under tapping mode, utilising phosphorus doped silicon probes (MPP-31120-10, Veeco, Bruker) with a spring constant of 0.9 N m\textsuperscript{-1}, a tip radius of curvature of 8 nm and a resonance frequency of \(\sim 20\) kHz. Scanning was carried out perpendicular to the axis of the cantilever at 1 Hz. To visualise the three-dimensional topography of the AFM scanning images, raw data files were processed by Avizo\textsuperscript{®} Standard v6.3 (Visualization Science Group, France).

To assess the micro/nanotopography of both the as-received and the lotus-like Ti surfaces, two scanning dimensions of 10 \(\mu\text{m} \times 10 \mu\text{m}\) and 5 \(\mu\text{m} \times 5 \mu\text{m}\) were analysed using the instrument software (SPM Lab Analysis v.7.11, Veeco, Bruker). All the roughness data presented in this study are an average of four scans. The roughness parameters including the average roughness \(S_{A}\), the root-mean-square (rms) roughness \(S_{q}\), the maximum height difference \(S_{\text{max}}\), skewness \(S_{\text{sk}}\), kurtosis \(S_{\text{ku}}\) and the developed surface area ratio \(S_{\text{dr}}\), were used to describe the surface morphology at the micro/nanoscale (Webb et al. Forthcoming 2012).

Raman microspectroscopic mapping of entrapped air

The air retained on the lotus-like Ti surfaces after immersion in water for 18 h was observed using a WiTEC Raman micro-spectrometer with a 532.1 nm laser wavelength \((\hbar\nu = 2.33\) eV). A water-immersion \(60 \times\) objective (numerical aperture = 0.9) was used to observe the lotus-like Ti surfaces under 5 ml of MilliQ water. A grid of 50 spectra \(\times\) 50 spectra was acquired of a scanning area of 20 \(\mu\text{m} \times 20 \mu\text{m}\). The integration time for a single spectrum was 2.5 s. Scanning was repeated independently twice on two different samples.

In situ small-angle x-ray scattering (SAXS): quantification of entrapped air

The amount of retained air on the lotus-like Ti surfaces after immersion in water was quantified using
transmission small-angle x-ray scattering measurements conducted at the Australian Synchrotron SAXS/WAXS beamline. All measurements were done with an x-ray wavelength of 1.512 Å with beam energy of 15 keV and a camera length set at 7 m. The scattering vector \( q \) has units of Å\(^{-1}\) and is given by:

\[
q = 4\pi \sin(\varphi/2)/\lambda
\]

where \( \varphi \) is the scattering angle and \( \lambda \) is the irradiating wavelength (Glatter and Kratky 1982).

Four custom-built fluid cells that allowed remote fluid injection through a peristaltic pump and a reservoir of MilliQ water were used for the \textit{in situ} measurements. The cell was constructed from two sheets of 50 \( \mu \)m-thick kapton separated and sealed by a 1.4 mm-thick neoprene gasket. In each fluid cell, a small hole was cut in one of the kapton sheets and the Ti substrata were mounted over the hole with the laser-ablated surface on the inward-facing side using thermosetting adhesives, forming a watertight seal. The cell could then be filled with fluid without changing its position relative to the incident x-ray beam. As the scan position and the physical morphology is not altered between scans, any change in scattering over time can only be due to a change in the nature of the interface between the coating and liquid.

Variations on the lotus-like Ti surfaces can lead to significant differences in scattering signal from one point on the substratum to the next. To mitigate this, six SAXS measurements were made on different points on each sample, and the average used as a representative SAXS profile for each sample. The x-ray beam irradiated a spot approximately 200 \( \mu \)m \( \times \) 100 \( \mu \)m, with each sampling point spaced 500 \( \mu \)m apart. Ample distance between each measured point prevented overlap between irradiated points.

To measure the amount of entrapped air at the interface, SAXS measurements were first made on dry Ti surfaces. MilliQ water was then introduced into the fluid cell, and SAXS measurements were made at each point every 3 min, up to 60 min. To obtain a SAXS profile of immersed titanium surfaces in the fully wetted regime, the fluid within the cell was exchanged with ethanol, followed by water. The ethanol was used as a wetting agent, to remove air from the interface and thereby forcing the immersed Ti surfaces into a wetted Wenzel state when water was reintroduced.

\textbf{Bacterial sample preparation}

\( S. \) \textit{aureus} CIP 65.8\( ^{T} \), \( S. \) \textit{aureus} ATCC 25923, \( S. \) \textit{epidermidis} ATCC 14990\( ^{T} \) and \( P. \) \textit{maritimus} KMM 3738 were employed to study their adhesion on the Ti samples. Prior to each experiment, fresh bacterial suspensions of OD\( _{600} \) = 0.3 in nutrient broth (Oxoid, UK) and marine broth (Difco, USA) for \( P. \) \textit{maritimus} were prepared as previously described (Mitik-Dineva et al. 2009a, 2009b). Bacterial cells were collected in the logarithmic phase of growth as confirmed by growth curves (data not shown) as previously described (Mitik-Dineva et al. 2009a).

Approximately 5 ml of the suspension were poured into a sterile Petri dish where the Ti specimens were completely immersed and left for 18 h (unless specified otherwise) incubation at room temperature (ca 22°C, 52% humidity). After incubation, specimens were washed with a copious amount of MilliQ water (Millipore, USA) and left to dry at room temperature (ca 22°C). This approach allowed the experiments for bacterial adhesion to be performed under identical conditions for each Ti sample.

\textbf{Cell surface analysis}

The physico-chemical cell surface characteristics were determined by measuring the cell surface wettability and charge. The surface wettabilities of all four strains were evaluated from contact angle measurements on lawns of bacterial cells using the sessile drop method. Measurements were performed using an FTA1000c equipped with a nanodispenser (First Ten Angstrom Inc., USA).

The cell surface charge was approximated by measuring zeta potentials, which are an indication of the overall net surface charge (de Kerchove and Elimelech 2005; Eboigbodin et al. 2006). Bacterial cell suspensions were prepared as described above, and zeta potential measurements were performed as described elsewhere (Bakker et al. 2002; Dong et al. 2002; Korenevsky and Beveridge 2007). Briefly, the electrophoretic mobility of each strain was measured as a function of ionic strength using a zeta potential analyser (ZetaPALS, Brookhaven Instruments Corp.). 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 consists of 14 cycles per run.

\textbf{Imaging techniques}

To observe the bacterial cells by scanning electron microscopy (SEM), the Ti discs were sputter-coated with gold using a Dynavac CS300 device using a previously developed protocol (Mitik-Dineva et al. 2008, 2009a; Ivanova et al. 2010; Truong et al. 2009). High-resolution images of the Ti discs with the retained bacterial cells were taken using a FESEM (ZEISS SUPRA 40VP) at 3 kV with 1000 \( \times \), 5000 \( \times \).
and 15,000 \times  magnifications. The lower detection limit was estimated as $1.1 \times 10^3$ cells mm$^{-2}$ according to the method set out by Morono et al. (2009) using the following formula:

\[
    n = \frac{T_{\text{fov}}}{C_{\text{fov}}} \ln(1 - p)
\]

where $n$ is the number of cells required giving a probability $p$ ($p = 0.95$) of detecting a cell, $T_{\text{fov}}$ is total area of fields of view, $C_{\text{fov}}$ is the number of fields of view, and total area is 314 mm$^2$.

In order to visualise the viable bacterial cells, images of the cells attached to Ti surfaces and the EPS produced by bacterial cells were recorded with a confocal laser scanning microscope (CLSM, Olympus Fluoview FV1000 Spectroscopic Confocal System). The system included an inverted microscope Olympus IX81 (with $20 \times$, $40 \times$ (oil), $100 \times$ (oil) UIS objective lenses) and was operated with multiple Ar, He and Ne laser lines (458, 488, 515, 543, 633 nm). The 488 nm laser was used to image the SYTO® 9 Green (Molecular Probes™, Invitrogen) fluorescent dye and the 633 nm laser was used to image the concanavalin A Alexa® 633 dye. The procedures for staining cells were followed as previously reported (Truong et al. 2010; Fadeeva et al. 2011; Ivanova et al. 2011b). The imaging software Fluoview FV 7.0 was employed to process the CLSM images.

To quantify 3D CLSM image stacks, the computer software COMSTAT was used (Heydorn et al. 2000). Six typical areas of a given bacterial strain on each type of Ti surface were exported as a stack of grey-scale 8-bit images by Fluoview FV 7.0. As previously described, biovolume is used to approximate the level of secreted exo-polysaccharides (Heydorn et al. 2000; Fadeeva et al. 2011).

**Results and discussion**

**Attachment response of coccoïd bacterial cells on lotus-like Ti**

Quantification of the level of cell attachment via SEM and CLSM revealed that all four strains were capable of adhering to lotus-like Ti. However, strain-specific differences in the number of adhered cells and EPS production were observed (Figure 1, Table S1). [Supplementary material is available via a multimedia link on the online article webpage.] The number of adhered cells ranged from $1.1 \times 10^6$ cells mm$^{-2}$ for *S. aureus* CIP 65.8$^T$ down to $5.0 \times 10^4$ cells mm$^{-2}$ in the case of *P. maritimus*. Attachment levels of *S. aureus* have been previously reported to range from $3.8 \times 10^4$ cells mm$^{-2}$ to $2.2 \times 10^6$ cells mm$^{-2}$ on a variety of Ti substrata (Ivanova et al. 2010; Truong et al. 2009, 2010; Ivanova et al. 2011b; Singh et al. 2011).

However, no direct comparisons can be made to the current work, as this is the first report on the attachment of coccoïd bacteria to lotus-like Ti.

The theoretical approaches typically used for explaining such differences in cell adhesion behaviour on the same surface are based primarily on bacterial surface characteristics such as surface charge and wettability (Bos et al. 1999, 2000). According to the theory, bacterial cells with large negative surface charge will experience more difficulty when attempting to adhere to the negatively-charged Ti surfaces due to electrostatic repulsive forces (Ponsonnet et al. 2003; Truong et al. 2009, 2010; Fadeeva et al. 2011). Also, in terms of bacterial surface wettability, bacteria with more hydrophobic surfaces are thermodynamically favoured for adhesion on superhydrophobic lotus-like Ti surfaces. The results of this study do not conform to these predictions particularly well, for example, while the most negatively charged strain tested (*P. maritimus*, $\zeta = -23.1$ mV) did exhibit the lowest propensity for adhesion, the least negatively charged (*S. epidermidis*, $\zeta = -13.6$ mV) did not show the highest level of attachment (Figure 1). Similarly, the most hydrophobic cells, *S. aureus* CIP 65.8$^T$ adhered to the Ti surfaces to the highest degree. However, *P. maritimus* was the next most hydrophobic and adhered the least. This suggests that in the case of these four bacteria, cell surface charge and wettability may not be the only contributing factors in determining the adhesion response. Substratum topography is known to be a contributing factor in most cases, eg increased surface area leads to greater contact area between cells and surfaces, favouring better adhesion (Ponsonnet et al. 2003; Mitik-Dineva et al. 2008; Ivanova et al. 2010), and is likely to be the case here also. In the case of lotus-like Ti surfaces, it would be expected that the considerable increase in surface area ($S_{dr} = 50.1\%$, Table S2 [Supplementary material is available via a multimedia link on the online article webpage]) in the comparison with as-received Ti surfaces ($S_{dr} = 0.24\%$, Table S2) would allow for much greater contact between the surfaces and the cells. The cells might be expected to lodge between the nanotopographic features, as this would afford them maximum contact area with the surfaces (Figure 2).

**Localisation of cell adhesion**

In order to determine if the bacterial cells were becoming lodged within the nanostructure of the lotus-like Ti, each of the four strains adhering to the surface were imaged using SEM (Figure 3). The apparent density of cells that could be seen in the resulting micrographs appeared to agree quite
well with the cell adhesion numbers presented in Figure 1; large numbers of both *S. aureus* CIP 65.8^T^, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 14990^T^ and *P. maritimus* KMM 3738 were visible in electron micrographs, and very few visible cells of *P. maritimus*. Some of the variation in adhesion density may be attributed to the different culture medium required for the marine bacterium *P. Maritimus*. However, despite these variations a common observation was made between each of the four strains, viz. all four of the coccoid bacteria tested were found to be localised almost exclusively in the crevices between the microscale topographical features. The more exposed areas at the top of the microstructures were essentially free of adhering cells. This seems counter-intuitive, as the nanostructure on the upper regions of the microscale features significantly enhances the surface area, which would be
expected to increase their favourability for cell attachment. This suggests the existence of some phenomenon that prevents the bacteria from making contact in these regions.

The explanation for this behaviour may lay in the nature of the surface. Surfaces with hierarchical topographical structures are frequently highly hydrophobic due to their enhanced ability to trap air. As a result, a large proportion of the surface that would otherwise be exposed to particles in solution is occupied by air (Cassie and Baxter 1944). Small particles with low mass, such as bacteria, are often unable to cross an air-water interface due to the effects of surface tension. Based on this theory, a scenario is proposed whereby the coccoid bacteria tested were unable to make contact with the lotus-like Ti due to occupation of

Figure 2. Typical three-dimensional (3D) AFM images of as-received and lotus-like Ti surfaces over 10 μm × 10 μm scanning areas.

Figure 3. Representative SEM images of adhesion patterns of *S. aureus* CIP 65.8\(^T\), *S. aureus* ATCC 25923, *S. epidermidis* ATCC 14990\(^T\) and *P. maritimus* KMM 3738 on control and lotus-like Ti surfaces after incubation for 18 h.
Figure 4. Proposed mechanism by which bacterial cells accumulate at the tri-phase interface on immersed lotus-like Ti surfaces. Bacterial cells slide across nanoscale bubbles trapped within the nanotopographical features. Cells are unable to cross the air-water interface and begin to accumulate in the regions that provide the best shelter from water turbulence.

Figure 5. Mapping the presence of air retention on lotus-like Ti surfaces after immersion for 18 h under water. The top image is the distribution map of retained air detected using Raman microspectroscopy. The presence of retained air is depicted by the darker colour. The arrows indicate two typical Raman spectra in which there is retained air (dark colour) and no retained air (brighter colour). In the spectra, a Raman shift ($\nu$) from 3100 cm$^{-1}$ to 3600 cm$^{-1}$ indicates the presence of –OH groups belonging to water molecules.
the surface by nano-sized bubbles (Figure 4). The cells then ‘skate’ across the nanobubbles until they come to rest in the larger crevices, which provide them with better shelter from water flows.

**Location of trapped air using Raman microspectroscopy**

In order to test the proposed hypothesis, a Raman microspectroscopic analysis was performed in an attempt to detect the presence of air on the lotus-like Ti surfaces when immersed in solution. By mapping the intensity of the spectral peak at 3100–3600 cm\(^{-1}\) corresponding to the O–H bonds of water molecules, sections of the surface lacking water, ie sections occupied by air bubbles could be identified. Air in the resulting spectral maps could be identified as darker regions (Figure 5). Microscale pockets of air on the surfaces of the lotus-like Ti were clearly observed in Raman spectral maps. However, nanoscale bubbles could not be resolved. The spatial resolution of Raman microspectroscopy is limited by the spot size of the laser, which is dependent on the diffraction limit. This means that numerous nanoscale bubbles can fit within the smallest spot size possible, making them impossible to resolve using this technique. Despite the experimental limitations of Raman microspectroscopy, evidence supporting the scenario proposed in Figure 4 was obtained, as it was determined that the lotus-like Ti surfaces do trap detectable quantities of air upon submersion, even over relatively long immersion periods.

Quantification of trapped air using in situ synchrotron transmission SAXS

Transmission SAXS measurements based on the technique described previously (Zhang et al. 2007; Scardino et al. 2009) were used to measure the immersed Ti surfaces over a scattering \(q\)-range of 0.003 < \(q\) < 0.1, where the scattering momentum \(q\) is a function of the x-ray scattering angle \(\varphi\) and x-ray wavelength \(\lambda\), given by Equation (1). To avoid the effects of fluid compressibility at high \(q\) and resolution limit at extremely low \(q\), the data analysis range was narrowed to between 0.005 < \(q\) < 0.05.

The intensity \(I_{AB}\) of x-ray scattering into a given solid angle from a rough interface between two media with average electron densities \(\rho_A\) and \(\rho_B\) is proportional to the square of the difference between the densities. In the condition of a dry Ti surface, x-ray scattering arises from the electron density difference between air and the Ti surface. It has been shown previously that as wetting progresses on an immersed superhydrophobic surface, x-ray scattering intensity decreases as the air/surface interface is replaced by a water/surface interface (Zhang et al. 2007).

In all immersed lotus-like Ti surfaces, the scattering profile at high \(q\) can be described using the Porod slope:

\[
I(q) = Bq^{-4}.
\]  

(3)

This equation holds a special significance when the exponent of \(q\) is equal to \(-4\), which is the case for all measured surfaces in this study. The pre-factor \(B\) is directly proportional to the total amount of interface that is illuminated by the x-ray beam. As air/Ti interface is replaced with water/Ti interface during wetting, the pre-factor \(B\) can be used to directly quantify the percentage of surface that remains dry (Zhang et al. 2007).

In the case of the surface/air/water interface of immersed lotus-like Ti surfaces, the SAXS profile of a wetted substratum is lower in scattering intensity than a dry substratum, as shown in Figure 6. The SAXS profiles obtained suggest that immersed lotus-like Ti
surfaces exhibit a degree of resilience against wetting as the surface is still not fully wetted after immersion for 50 min. Figure 6 also shows the percentage of dry interface remaining on an immersed lotus-like Ti surface as a function of time. The percentage of dry interface is calculated by dividing the normalised

Figure 7. Adhesion kinetics of *S. aureus* CIP 65.8<sup>T</sup> onto lotus-like Ti surfaces. Adhesion patterns of *S. aureus* CIP 65.8<sup>T</sup> are shown using SEM (cells are in pink, first row) and CLSM (cells are in green, second row). EPS distribution maps were reconstructed using COMSTAT analysis (third row). The graph demonstrates that cell attachment and EPS production both increase as the proportion of air trapped on the surface in the form of microbubbles decreases over time.
pre-factor \( B \) at time \( t \) by the pre-factor \( B \) when the interface is completely dry.

\[
\text{% dry interface} = \frac{B_t}{B_{\text{dry}}} \tag{4}
\]

From the data presented in this figure, it can be seen that a rapid decrease in the dry interface percentage occurred within the first 6–9 min of immersion. This is indicative of the rapid decline in the amount of air still present at the interface due to wetting phenomena. After 10 min, a constant, gradual decline in the air/surface interface was observed, up to 50 min where \( \sim 45\% \) of the interface remained dry.

**Bacterial adhesion kinetics and metastability of substratum superhydrophobicity**

While it has previously been reported that the proportion of air trapped on the lotus-like Ti surface decreases over a 1 h period until it reaches an equilibrium state in which \( \sim 6\% \) of the surface is occupied by air, it was found in this study that this proportion is closer to 45\%. This is attributed to the limitation in the technique used in the previous study where dry interfaces at the nanoscale could not be resolved. The results suggest that within the first hour, many of the microscale bubbles would have disappeared due to wetting phenomena, whereas the remaining dry interface is due to the presence of nanoscale bubbles. This agrees with traditional capillary wetting models, where more work is required to wet nanoscale pores than microscale ones (de Gennes 1985; Truong and Wayner 1987).

According to the model proposed in Figure 4, the observed replacement of trapped air by the aqueous medium would result in increased attachment levels. To test this, the adhesion characteristics of *S. aureus* CIP 65.8T over a 10, 30 and 60 min incubation periods were investigated, and correlated with previous data on air replacement (Figure 7, Figure S2, Table S3 [Supplementary material is available via a multimedia link on the online article webpage]). It was found that the number of cells that were able to adhere to the surface of the lotus-like Ti increased substantially as the proportion of air trapped on the surface in the form of microbubbles fell below 35\%. In addition, the location and size of air trapped on the surface plays an important role in the adhesion behaviour of bacteria. As proposed in Figure 4 and observed in Figure 3, bacterial adhesion favours the crevices between microscale asperities, but its peaks are relatively free of bacteria. This is in agreement with the wetting model proposed above, where microwetting occurs first, displacing the microscale bubbles which are located in the crevices, followed by nanowetting. Thus, bacteria would first colonise and settle at the crevices where rapid disappearance of trapped air is occurring, whereas air trapped within the nanofeatures prevents bacterial colonization and adhesion. This is consistent with the proposed model; as the air is replaced by water, a larger proportion of the surface becomes accessible for the bacterial cells. The replacement of air bubbles can be explained by the thermodynamically unstable state of trapped air on the surface. The hydraulic pressure of the system was able to overcome the surface tension of water-air interface, and subsequently the air bubbles were displaced by water. This phenomenon can be modelled using the transition from Cassie–Baxter to Wenzel wettability models (Lafuma and Quére´ 2003). This model evaluates the stability of Cassie–Baxter state of superhydrophobic surfaces based on the area fraction \( (f_1) \) of the surface and the Wenzel roughness factor, according to:

\[
\cos \theta_c = \frac{f_1 - 1}{r - f_1} \tag{5}
\]

where \( \theta_c \) is the critical contact angle of the heterogeneous surface, \( f_1 \) is the area fraction of surface component of titanium and \( r \) is the Wenzel roughness. The critical contact angle is the threshold defining the boundary between the Wenzel and Cassie–Baxter models; ie if the apparent contact angle \( (\theta) \) is \( > \theta_c \), the system is in the Cassie–Baxter state, and if \( \theta < \theta_c \), the system is in the Wenzel state. As the air on the lotus-like Ti surface is replaced by water, \( f_1 \) increases, which leads to an increase in the value of \( \theta_c \). Once \( \theta_c \) exceeds \( \theta \) the system can be considered to have transitioned into the Wenzel state (Zheng et al. 2005; Forsberg et al. 2011; Sheng and Zhang 2011).

**Conclusions**

Despite their different propensities for adhesion, all four of the tested coccoid bacteria were found to attach primarily in the crevices between the microscale features on the lotus-like Ti surfaces. A model was proposed whereby the attachment of the spherical cells is directed by the presence of air bubbles trapped on the surface. Using Raman microspectroscopy, it was confirmed that the lotus-like Ti surfaces trap air bubbles within their features, and that after immersion for 18 h the remaining air is located between the microscale surface features. Attachment of *S. aureus* CIP 65.8T cells was shown to increase substantially over a period of 1 h, which corresponds with the period of maximum replacement of trapped air by the incubation medium. Replacement of air increases the surface area available for cells to make contact.
with the Ti surface. This work provides strong evidence supporting the hypothetical model of air-directed attachment of bacteria proposed here.

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References
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.

Banerjee I, Pangule RC, Kane RS. 2011. Antifouling coatings: recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms. Adv Mater 23:690–718.

Barthlott W, Neinhuis C. 1997. Purity of the sacred lotus, or escape from contamination in biological surfaces. Planta 202:1–8.

Bhushan B, Jung YC. 2011. Natural and biomimetic artificial surfaces for superhydrophobicity, self-cleaning, low adhesion, and drag reduction. Prog Material Sci 56:1–108.

Bos R, Van der Mei HC, Busscher HJ. 1999. Physicochemistry of initial microbial adhesive interactions – its mechanisms and methods for study. FEMS Microbiol Rev 23:179–229.

Bos R, Van der Mei HC, Gold J, Busscher HJ. 2000. Retention of bacteria on a substratum surface with micro-patterned hydrophobicity. FEMS Microbiol Lett 189:311–315.

Cassie ABD, Baxter S. 1944. Wettability of porous surfaces. Trans Faraday Soc 40:546–551.

Cassie ABD, Baxter S. 1945. Large contact angles of plant and animal surfaces. Nature 155:21–22.

de Gennes PG. 1985. Wetting: statics and dynamics. Rev Modern Phys 57:827–863.

de Kерchovе AJ, Elimelech M. 2005. Relevance of electrokinetic theory for “soft” particles to bacterial cells: implication for bacterial adhesion. Langmuir 21:6462–6472.

Dong H, Onstotta TC, Kob C-HA, Hollingsworth AD, Brown DG, Mailloux BJ. 2002. Theoretical prediction of collision efficiency between adhesion-deficient bacteria and sediment grain surface. Colloids Surf B – Biointerfaces 24:229–245.

Eboigbodin KE, Newton ARJ, Routh FA, Biggs AC. 2006. Bacterial quorum sensing and cell surface electrokinetic properties. Appl Microbiol Biotechnol 73:669–675.

Fadeeva E, Truong VK, Stiesch M, Chichkov BN, Crawford RJ, Wang J, Ivanova EP. 2011. Bacterial retention on superhydrophobic titanium surfaces fabricated by femtosecond laser ablation. Langmuir 27:3012–3019.

Fogg GE. 1944. Diurnal fluctuation in a physical property of leaf cuticle. Nature 154:515.

Forsberg P, Nikolajeff F, Karlsson M. 2011. Cassie–Wenzel and Wenzel–Cassie transitions on immersed superhydrophobic surfaces under hydrostatic pressure. Soft Matter 7:104–109.

Gao L, McCarthy TJ. 2006. The “lotus effect” explained: two reasons why two length scales of topography are important. Langmuir 22:2966–2967.

Genzer J, Elimenko K. 2006. Recent developments in superhydrophobic surfaces and their relevance to marine fouling: a review. Biofouling 22:339–360.

Glatter O, Kratky O. 1982. Small angle x-ray scattering. New York (NY): Academic Press. 515 pp.

Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Erboll BK, Molin S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146:2395–2407.

Ivanova EP, Truong VK, Webb HK, Baulin VA, Wang JY, Mohammadi N, Wang F, Fluke C, Crawford RJ. 2011a. Differential attraction and repulsion of Staphylococcus aureus and Pseudomonas aeruginosa on molecularly smooth titanium films. Sci Rep 1:165–172.

Ivanova EP, Truong VK, Webb HK, Baulin VA, Wang JY, Mohammadi N, Wang F, Fluke C, Crawford RJ. 2011b. Differential attraction and repulsion of Staphylococcus aureus and Pseudomonas aeruginosa on molecularly smooth titanium films. Sci Rep 1:1:no. 165.

Ivanova EP, Truong VK, Wang JY, Bendt CC, Jones RT, Yusuf II, Peake I, Schmidt HW, Fluke C, Barnes D, et al. 2010. Impact of nanoscale roughness of titanium thin film surfaces on bacterial retention. Langmuir 26:1973–1982.

Kosh K, Bhushan B, Jung YC, Barthlott W. 2009. Fabrication of artificial lotus leaves and significance of hierarchical structure for superhydrophobicity and low adhesion. Soft Matter 5:1386–1393.

Korenovsky A, Beveridge TJ. 2007. The surface physicochemistry and adhesiveness of Sheewanella are affected by their surface polysaccharides. Microbiology 153:1872–1883.

Lafuma A, Quéré D. 2003. Superhydrophobic states. Nature Mater 2:457–460.

Liu K, Yao X, Jiang L. 2010. Recent developments in bio-inspired special wettability. Chem Soc Rev 39:3240–3255.

Magin CM, Cooper SP, Brennan AB. 2010. Non-toxic antifouling strategies. Mater Today 13:36–44.

Marmur A. 2004. The lotus effect: superhydrophobicity and metastability. Langmuir 20:3517–3519.

Mitik-Dineva N, Wang J, Mocanasu CR, Stoddart PR, Crawford RJ, Ivanova EP. 2008. Impact of nanotopography on bacterial attachment. Biotechnol J 3:536–544.

Mitik-Dineva N, Wang J, Truong VK, Stoddart P, Malherbe F, Crawford RJ, Ivanova EP. 2009a. Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus attachment patterns on glass surfaces with nanoscale roughness. Curr Microbiol 58:268–273.

Mitik-Dineva N, Wang J, Truong VK, Stoddart PR, Malherbe F, Crawford RJ, Ivanova EP. 2009b. Differences in colonisation of live marine bacteria on two types of glass surfaces. Biofouling 25:621–631.

Morono Y, Terada T, Masui N, Inagaki F. 2009. Discriminative detection and enumeration of microbial life in marine subsurface sediments. ISME J 3:503–511.

Otten A, Herminghaus S. 2004. How plants keep dry: a physicist’s point of view. Langmuir 20:2405–2408.

Ponsonnet L, Reybier K, Jaffrezic N, Comte V, Lagneau C, Lissac M, Martelet C. 2003. Relationship between surface properties (roughness, wettability) of titanium and titanium alloys and cell behaviour. Mater Sci Eng C 23:551–560.
Qu M, Zhao G, Wang Q, Cao X, Zhang J. 2008. Fabrication of superhydrophobic surfaces by a Pt nanowire array on Ti/Si substrates. Nanotechnology 19:no. 055707.

Scardino AJ, de Nys R. 2011. Mini review: Biomimetic models and bioinspired surfaces for fouling control. Biofouling 27:73–86.

Scardino AJ, Zhang H, Cookson DJ, Lamb RN, de Nys R. 2009. The role of nano-roughness in antifouling. Biofouling 25:757–767.

Sheng X, Zhang J. 2011. Air layer on superhydrophobic surface underwater. Colloids Surf A 377:374–378.

Shirtcliffe NJ, McHale G, Atherton S, Newton MI. 2010. An introduction to superhydrophobicity. Adv Colloid Interface Sci 161:124–138.

Singh AV, Vyas V, Patil R, Sharma V, Scopelliti PE, Bongiorno G, Podestà A, Lenardi C, Gade WN, Milani P. 2011. Quantitative characterization of the influence of the nanoscale morphology of nanostructured surfaces on bacterial adhesion and biofilm formation. PLoS One 6: e25029.

Sun T, Tan H, Han D, Fu Q, Jiang L. 2005. No platelet can adhere – largely improved blood compatibility on nanostructured superhydrophobic surfaces. Small 1:959–963.

Toes GJ, Van Muiswinkel KW, Van Oeveren W, Suurmeijer AJH, Timens W, Stokroos I, Van den Dungen JJAM. 2002. Superhydrophobic modification fails to improve the performance of small diameter expanded polytetrafluoroethylene vascular grafts. Biomaterials 23: 255–262.

Truong JG, Wayner PC. 1987. Effects of capillary and van der Waals dispersion forces on the equilibrium profile of a wetting liquid: theory and experiment. J Chem Phys 87: 4180–4188.

Truong VK, Lapovok R, Estrin Y, Rundell S, Wang JY, Fluke CJ, Barnes DG, Crawford RJ, Ivanova EP. 2010. The influence of nanoscale surface roughness on bacterial adhesion to ultrafine-grained titanium. Biomaterials 31: 3674–3683.

Truong VK, Rundell S, Lapovok R, Estrin Y, Wang JY, Berndt CC, Barnes DG, Fluke CJ, Crawford RJ, Ivanova EP. 2009. Effect of ultrafine-grained titanium surfaces on adhesion of bacteria. Appl Microbiol Biotechnol 83:925–937.

Webb HK, Hasan J, Truong VK, Crawford RJ, Ivanova EP. 2011. Nature inspired structured surfaces for biomedical applications. Curr Med Chem 18:3367–3375.

Webb HK, Truong VK, Hasan J, Fluke C, Crawford RJ, Ivanova EP. Forthcoming 2012. Roughness parameters for standard description of surface nanoarchitecture. Scanning.

Wenzel RN. 1949. Surface roughness and contact angle. J Phys Coll Chem 53:1466–1467.

Yang Y, Lai Y, Zhang Q, Wu K, Zhang L, Lin C, Tang P. 2010. A novel electrochemical strategy for improving blood compatibility of titanium-based biomaterials. Colloids Surf B – Biointerfaces 79:309–313.

Zhang H, Lamb RN, Cookson DJ. 2007. Nanowetting of rough superhydrophobic surfaces. Appl Phys Lett 91: 254106.

Zhao N, Zhang XY, Li YF, Lu XY, Sheng SL, Zhang XL, Xu J. 2007. Self-organized polymer aggregates with a biomimetic hierarchical structure and its superhydrophobic effect. Cell Biochem Biophys 49:91–97.

Zheng QS, Yu Y, Zhao ZH. 2005. Effects of hydraulic pressure on the stability and transition of wetting modes of superhydrophobic surfaces. Langmuir 21:12207–12212.