Role of hydrophobicity in bacterial adherence to carbon nanostructures and biofilm formation

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Role of hydrophobicity in bacterial adherence to carbon nanostructures and biofilm formation

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The role of cell and surface hydrophobicity in the adherence of the waterborne bacterium \textit{Mycobacterium smegmatis} to nanostructures and biofilm formation was investigated. Carbon nanostructures (CNs) were synthesized using a flame reactor and deposited on stainless steel grids and foils, and on silicon wafers that had different initial surface hydrophobicities. Surface hydrophobicity was measured as the contact angle of water droplets. The surfaces were incubated in suspensions of isogenic hydrophobic and hydrophilic strains of \textit{M. smegmatis} and temporal measurements of the numbers of adherent cells were made. The hydrophobic, rough mutant of \textit{M. smegmatis} adhered more readily and formed denser biofilms on all surfaces compared to its hydrophilic, smooth parent. Biofilm formation led to alterations in the hydrophobicity of the substratum surfaces, demonstrating that bacterial cells attached to CNs are capable of modifying the surface characteristics.

Keywords: bacterial adherence; \textit{Mycobacterium}; hydrophobicity; carbon nanostructures; surface roughness; biofilm

Introduction

Many nanotechnology applications require the assembly of nanostructures along with biological entities, such as cells, cellular substructures, and proteins and enzymes, for example, for chemical synthesis or use as biosensors. Nano- and microstructures can also occur as artifacts of manufacturing processes on the abiotic walls of catheters inserted into patients or in drinking water distribution systems. In all of these cases, the nanostructures can be colonized by bacteria, forming biofilms that alter the performance characteristics of these applications. In some anticipated applications it would be desirable to enhance bacterial adherence to nanostructures, eg when employing nanoscale drug-, protein-, or chemical-delivery systems for treating diseases and for detection of contaminants in drinking water. Therefore it is important to identify the factors that influence the adherence of bacteria to nanostructures.

Bacteria are living colloidal particles that range in size from about 0.2 \( \mu \text{m} \) to several micrometers in length and about 1 \( \mu \text{m} \) in diameter. Bacterial cells can possess relatively hydrophilic or hydrophobic surfaces (Rosenberg 1984). Bacterial attachment to a surface can occur through primary and reversible adhesion, secondary and irreversible adhesion, and biofilm formation (Dunne 2002). Nontuberculous mycobacteria including \textit{Mycobacterium smegmatis} used for these experiments are normal inhabitants of drinking water distribution systems and household plumbing and high numbers are found attached to pipe surfaces in biofilms (Falkinham et al. 2001; Torvinen et al. 2004). The bacteria can be mobile or immobile on the substratum during the adhesion process, which can be either reversible or irreversible (Boks et al. 2009). The initial reversible step involves weak forces, such as van der Waals, electrostatic, and hydrophobic interaction forces between the bacterial cell and the substratum surface (van Loosdrecht et al. 1987), whereas a successive irreversible step occurs through stronger covalent and hydrogen bonding forces, as well as through cellular surface structures, such as flagella and fimbrae (Goulter et al. 2009). Bacterial adhesion to a surface can depend upon the presence of suitable nutrients, surface charge and topology, and hydrophobicity (McLandsborough et al. 2006; Young 2006) as has been shown for \textit{Mycobacterium avium} (Carter et al. 2003) and other mycobacteria (Williams et al. 2009) so that different substrata can produce dissimilar biological responses (Diaz et al. 2007; Morozan et al. 2007). However, there is considerable disagreement in the literature over the past three decades regarding the adhesion mechanics and the influence of various factors on bacterial adhesion (Tang et al. 2009).
While some studies point to the importance of the substratum free surface energies in influencing bacterial adhesion (Absolom et al. 1983), for example, by increasing the surface roughness (Donlan 2002) and hydrophobicity (Ludwicka et al. 1984; Paulsson et al. 1993; Brydon et al. 1996; Faille et al. 2002), others find that although changing this energy by altering the surface hydrophobicity influences the adhesion dynamics (Boks et al. 2009), there is no clear effect on overall adhesion and biofilm formation (Vanpelt et al. 1985; Bayoudh et al. 2006; Oliveira et al. 2006). However, it is generally believed that microorganisms attach more rapidly to hydrophobic nonpolar surfaces than to hydrophilic materials (Fletcher and Loeb 1979; Pringle and Fletcher 1983; Bendinger et al. 1993; Donlan 2002) above a threshold surface roughness (Tang et al. 2009). Carbon nanostructures (CNs) can be deposited on surfaces to make them super-hydrophobic (Lau et al. 2003; Journet et al. 2005; Lin et al. 2006; Naha et al. 2007), but the literature is sparse on the response and adhesion of bacteria to them. Depositing nanostructures on an initially smooth surface creates microstructured unevenness that roughens it by changing the surface topology. Increasing roughness leads to increasing hydrophobicity due to a Cassie effect when the surface tension of a water droplet is supported by the rough bumps beneath it (Naha et al. 2007). The objective in the present study was to characterize the attachment of bacteria and the consequent formation of a biofilm on nanostructured surfaces of different hydrophobicity. CNs were deposited on a variety of surfaces and the adherence and biofilm formation of a representative waterborne mycobacterium, *M. smegmatis*, measured.

**Materials and methods**

**Synthesis and characterization of carbon nanostructures**

The base substrata used in the experiment were 10 mm × 10 mm square supports made from stainless steel (SS) AISI 304 (Fe/Cr18/Ni 10) mesh from 0.25 mm diameter wires, SS AISI 302 (Fe/Cr18/Ni 8) foils with a 0.1 mm thickness, and 4 mm × 10 mm silicon (Si) wafers (3″ N/Ph (100) 1–10 Ω-cm 380 μm SSP Prime). The CNs were deposited on these substrata using an inner annular flame reactor, a schematic of which is provided in Figure 1 (Sen and Puri 2004; Arana et al. 2005; Naha et al. 2007). A co-annular axisymmetric burner was used to generate a steady laminar flame burning ethylene (99%) and laboratory air in a 4:1 ratio. The substrata were placed 10 mm above the burner with an inner fuel flowrate of 0.269 l min⁻¹ and an outer airflow of 31.7 l min⁻¹. The exposure time to the flame in the reactor was 3 min for all samples. Since the width of the substrata was much smaller than that of the flame, only the inner flame core was responsible for the nanostructure deposition. High resolution Schottky field emission scanning electron microscope (FESEM) images of the nanostructures were obtained using a LEO (Zeiss) 1550 instrument at a 5 kV accelerating voltage in the conventional high vacuum mode.

**Bacterial cells and growth, adherence and biofilm measurements**

*M. smegmatis* strain mc²155 and its spontaneous rough mutant were used for the experiments. The rough colony phenotype was stable upon long term cultivation and did not revert to the parent's colony type. The strains were grown to exponential phase in 1/10-strength Brain Heart Infusion Broth (BHIB, Becton-Dickenson, Sparks, MD) at 37°C. BHIB was chosen rather than a standard mycobacterial medium, Middlebrook 7H9 containing oleic acid, because of possible changes in the surface characteristics of *M. smegmatis* due to oleic acid (Carter et al. 2003). Following growth, cells were pelleted by centrifugation (5000 × g for 20 min), the supernatant medium discarded, and cells suspended in the same volume of sterile Blacksburg tap water. Cell number was measured as colony-forming units (CFU) on 1/10-strength BHIB agar.

Multiple bare and CN-coated supports of all six types were placed in a sterile plastic Petri dish containing 20 ml of sterile Blacksburg tap water and inoculated with ~10⁷ CFU of each bacterial suspension. The Petri dish was then gently agitated on a
platform shaker at room temperature. After incubation for 1.5 h, the supports were transferred using alcohol-flamed tweezers to another dish containing 20 ml of sterile Blacksburg tap water and washed by gently swirling for 10 s. The washed support was transferred into a tube containing 1 ml of sterile Blacksburg tap water and vortexed at the highest speed for 60 s. The recovery of cells was approximately 90% as judged by microscopic examination of surfaces. The number of CFU in each suspension was measured by spreading 0.1 ml in triplicate of each suspension and a series of 10-fold dilutions. Following removal of supports for measurement of adherence, the remaining supports with and without CNs were incubated in the same cell suspension to permit biofilm formation. After 13 days incubation at 30°C, the supports were gently washed and adherent cells measured as described above. The results are reported as mean CFU cm⁻² of surface (calculated on the dimensions of the supports) of measurements from three supports of the same type where the standard errors were <10% of the values.

Measurement of bacterial cell and surface hydrophobicity

Hydrophobicity of M. smegmatis cells was measured as hexadecane adherence (Rosenberg 1984). Hexadecane (0.1 ml) was added to 3 ml of cells suspended in sterile Blacksburg tap water (≈10⁸ CFU ml⁻¹) and the suspension vortexed at the highest speed for 60 s. The aqueous and hexadecane layers separated after 60 min and the absorbance of the aqueous layer was measured at 540 nm. The percentage hexadecane adherence was calculated as the difference in absorbance of the untreated suspension minus the absorbance of the hexadecane-treated suspension divided by the absorbance of the untreated suspension. The results reflect the average (± standard deviation [SD]) of three independent measurements. Surface hydrophobicity was measured as the contact angles (both left and right) of water droplets on bare and CN-coated substrata, both before and after mycobacterial biofilm formation, using a Kruss DSA 100 surface analyzer (Kruss, USA). The results are reported as the mean ± the SD of the measured contact angles. This measurement was performed three times (ie the number of samples n = 3)

Results and discussion

Substratum surface characterization

FESEM images of the different coated substrata revealed uniformly distributed CNs, as shown in Figure 2 that compares representative bare Si and CN-covered substratum surfaces. The nanostructured coatings consisted of similarly agglomerated solid particles or nanobeads. Their deposition occurred
during combustion through gas phase fuel pyrolysis in the oxygen-deficient flame core. In this core, heavier hydrocarbons were transported into the stagnation layer adjacent to the relatively colder substratum surface and condensed upon it to form the nanostructures. The individual nanobeads ranged in size from 24 to 51 nm (Naha et al. 2007).

In general, CN deposition increased the substratum surface unevenness or roughness, thereby enhancing the surface hydrophobicity by inducing a Cassie equilibrium state. Water droplets in such a state sit on the deposited nanostructured rough surfaces while air is entrapped in the voids in the underlying structure and readily roll off the surface when disturbed (Naha et al. 2007). The contact angle measurements that characterize substratum surface hydrophobicity are summarized in Table 1. For a water droplet placed on an initially bare Si hydrophilic wafer, the contact angle $\theta \approx 39.3^\circ$. Once CNs were deposited on it, the surface became hydrophobic due to the enhanced surface roughness and $\theta$ increased to $\sim 135.4^\circ$. The SS mesh was intrinsically hydrophobic and became more so due to CN deposition with $\theta$ increasing from $\sim 102.2$ to $\sim 138.4^\circ$. This angle was roughly the same for both a bare ($\theta \approx 77.1^\circ$) and a CN-covered SS foil ($\theta \approx 79.3^\circ$).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\theta$ (°)</th>
<th>Observation</th>
<th>Characteristic</th>
<th>Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare Si wafer</td>
<td>39.3 ± 0</td>
<td>Droplet smears surface</td>
<td>Hydrophilic</td>
<td></td>
</tr>
<tr>
<td>CNs on Si wafer</td>
<td>135.4 ± 0.3</td>
<td>Almost spherical droplet rolls off readily when disturbed</td>
<td>Highly hydrophobic</td>
<td></td>
</tr>
<tr>
<td>Bare SS mesh</td>
<td>102.3 ± 0.5</td>
<td>Droplet rolls off readily when disturbed</td>
<td>Hydrophobic</td>
<td></td>
</tr>
<tr>
<td>CNs on SS mesh</td>
<td>138.3 ± 0.1</td>
<td>Almost spherical droplet rolls off readily when disturbed</td>
<td>Hydrophobicity increases</td>
<td></td>
</tr>
<tr>
<td>Bare SS foil</td>
<td>77.1 ± 1</td>
<td>Semicircular droplet</td>
<td>Hydrophobic</td>
<td></td>
</tr>
<tr>
<td>CNs on SS foil</td>
<td>79.3 ± 7</td>
<td>Semicircular droplet rolls of readily when disturbed</td>
<td>Hydrophobicity increases slightly</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Numbers of cells of *M. smegmatis* colony variants of wild type parent and rough mutant cells adhering to the substrata after 1.5 h and in biofilms after 13 days incubation.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Wild type CFU cm⁻²</th>
<th>Rough CFU cm⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 h</td>
<td>13 days</td>
</tr>
<tr>
<td>SS foil</td>
<td>Not detected</td>
<td>2.7 × 10⁴</td>
</tr>
<tr>
<td>SS foil + CNs</td>
<td>1.0 × 10³</td>
<td>6.6 × 10³</td>
</tr>
<tr>
<td>SS mesh</td>
<td>2.2 × 10³</td>
<td>3.6 × 10³</td>
</tr>
<tr>
<td>SS mesh + CNs</td>
<td>3.8 × 10⁴</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>Si</td>
<td>4.2 × 10³</td>
<td>1.0 × 10³</td>
</tr>
<tr>
<td>Si + CNs</td>
<td>2.8 × 10⁵</td>
<td>1.2 × 10³</td>
</tr>
</tbody>
</table>

Adherence of mycobacterial cells to nanostructures and biofilm formation

Two colony wild type and rough cell variants of *M. smegmatis* differed significantly in their cell surface hydrophobicity as reflected by their adherence to hexadecane in an aqueous medium, with wild type adherence as 32 ± 3% and rough adherence as 71 ± 5%. The wild type parent was significantly more hydrophilic towards water than the rough mutant (Student’s *t* test, *p* < 0.05). Both the wild type and rough mutant cells adhered to the different nanostructures upon short-term (1.5 h) exposure to cells in suspension as shown through Table 2. Starting with similar initial cell densities of 8.0 × 10⁵ wild-type cells ml⁻¹ and 6.5 × 10⁵ rough mutant cells ml⁻¹ in suspension, a higher number of the relatively hydrophobic rough cells adhered to the bare and CN-coated substrata. (Only for the CN-coated SS foil was the difference significant, *p* < 0.05 by Student’s *t* test, Table 2). However, far fewer bacteria of either type adhered to bare Si and SS foil surfaces as compared to the SS mesh and all three CN-coated surfaces. Whereas, the bacterial adherence in the latter cases was 10³ CFU cm⁻², it was only 10⁻¹0⁻² CFU cm⁻² for the bare Si and SS foil surfaces. More cells of either colony type adhered to the CN-coated surfaces compared to the uncoated surfaces, but rough cell attachment was typically higher than of the wild type cells. As the total surface areas of the substrata were likely different (e.g., foil vs. grid), comparisons are strongest between bare and CN-coated surfaces on the same supports. Comparisons between attachment of the colony variants to the different substratum surfaces showed that when substantial bacterial adherence (> 10⁵ CFU cm⁻²) occurred, the proportion of the more hydrophilic wild type parent cells that adhered to the surfaces increased with surface hydrophobicity although the population of the more hydrophobic rough cells was always higher.

The numbers of adherent cells were also measured after 13 days incubation at 30°C as is also shown in Table 2. The counts reflect the combined contributions of adherence and growth of cells on the surfaces since the CN-coated and uncoated surfaces were incubated in the presence of cells in suspension. The data show that substantial mycobacterial populations accumulated on all the surfaces forming biofilm, but there were significantly 10- to 100-fold higher numbers of cells of the hydrophobic rough mutant (*p* < 0.05, Student’s *t* test). In most cases, while the number of rough, hydrophobic mutant cells increased from 10³ to 10⁵ CFU cm⁻² over 1.5 h to 13 days, those of the hydrophilic wild type parent cells remained somewhat constant at 10³ CFU cm⁻².

The hydrophobicity of the substratum surfaces was again measured after these were covered with biofilms. Mycobacterial biofilm formation due to *M. smegmatis* cells substantially reduced the hydrophobicity of all three CN-coated surfaces (Schaumann et al. 2007), as shown in Table 3. Such a decrease in hydrophobicity would be expected to change the binding characteristics of CN-coated surfaces developed for various applications.

### Conclusions

The synthesis of hydrophobic CNs on different substrata allowed measurements of the adherence and biofilm formation by *M. smegmatis* cells. Surfaces coated with CNs bound higher numbers of mycobacterial cells in the short term and formed denser biofilms.
after incubation. The most striking result of the measurements was that biofilm formation resulted in substantial decreases in the hydrophobicity (characterized through the water-surface contact angle) of the CN-coated surfaces.

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**References**


