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Bacterial Footprints in Elastic Pillared Microstructures

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INTRODUCTION

The fabrication of materials that are sensitive to physical, chemical, or biological stimuli has opened opportunities for the development of a wide variety of technological applications such as switchable adhesion, mechanosensing, and stimuli-responsive materials. In particular, the design of biomimetic structures inspired by natural systems, has been a powerful tool in the implementation of smart, artificial systems. In this respect, the use of topographic surfaces is particularly interesting, with natural systems utilizing physical structures, from the nanoscale to the macroscale, to deliver functions such as superhydrophobicity, adhesion, and antibiofouling as demonstrated by the lotus leaf, shark skin, and gecko feet. There has been particular interest in developing mechanically responsive systems. An excellent example is the mechanical response of micropillar arrays upon drying of water (or water-based solutions). When water droplets evaporate on relatively soft elastic microstructured surfaces, capillary action can generate a significant force that is able to bend the soft micropillars. Depending on the geometry of the arrays, the capillary and elastic forces can form different pillar assemblies. The complexity of the assemblies varies with the pillar height and the interpillar distance. For example, large periodic chiral aggregates can be formed when the micropillars are higher and closer to each other. Each cluster of aggregates has a different potential to store elastic energy, embody information, enhance adhesion, or capture particles. 

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The demonstration of mechanically responsive topographic surfaces to bacterial stimuli during evaporation of small droplets is of great interest and has not been demonstrated before. Furthermore, the deflections seen in our systems are significant, leading to pillar aggregations into dimers, trimers, and higher order clusters. Recently, the formation of biofilm strings and networks between topographic pillars has been demonstrated in liquid media, however, the mechanical response of the pillars to bacterial presence upon evaporation is not observed. Chew and coauthors have shown small deflections of macropillared surfaces in response to the differential pressure exerted by biofilm growth within a growth chamber over a 24 h period, while Bias and Ng et al. have investigated the interaction of bacterial pili with pillared structures.

Here, we demonstrate how epoxy-made soft surfaces containing micropillar arrays interact with suspensions of different bacterial species. Our results suggest that the presence of motile bacteria with flagella drastically increases the mechanical response of the pillars, actively bending soft topographical substrates in the area contained within the contact line. In contrast, solutions containing nonmotile bacteria do not generate such responses. We attribute this to the ability of motile bacteria to interact with each other and with their topographical environment. Importantly, the response of the microarray is sensitive to the type and concentration of bacteria in the solution. These promising results could lay the foundation for the development of devices that are selectively responsive to specific microorganisms, paving the way to construct smart, fast, and cost-effective diagnostic tools.

RESULTS AND DISCUSSION

One of the key parameters in the mechanical response of soft micropillar arrays is the aspect ratio of a single pillar. We investigated the effect of the pillar aspect ratio by fabricating regular patterns of cylindrical pillars with a constant diameter (5 μm) and interspacing (5 μm) and with variable height (from 5 to 45 μm). The patterns were created on epoxy resin using a method described before based on casting uncured epoxy on a negative polydimethylsiloxane (PDMS) mold, followed by curing and mechanically removing of the mold. The micropatterns were transferred efficiently, with a high degree of fidelity, as shown by scanning electron microscopy (SEM) imaging (Figure 1 and Figure S1).

These microstructured substrates can be susceptible to elastocapillary forces in the presence of pure liquids. Therefore, we evaluated the effect of pure water over a surface decorated with micropillars with lengths varying from 5 to 45 μm (Figure 1) during the evaporation of water droplets (Figure 1). In these experiments, the liquid filled up the space between the pillars, resulting in an almost square-shaped droplet contour. Once the droplet spreads on the substrate, the liquid contact line is blocked by the pillared structure and remains immobilized (pinned) for the rest of the drying process.

Figure 1b shows that after complete evaporation, there is almost no trace of the droplet, except at the droplet contour, where lines of pillars were bent by capillary action at the contact line shown in Video S1. In the systems studied, the pillar lattice was kept constant (i.e., l = d = 5 μm), but different pillar heights (h) ranging from h = 5 to 45 μm were fabricated. Thus, a range of micropatterned surfaces were generated with different aspect ratios (i.e., h/d = 3 to h/d = 9). For large aspect ratio structures, we observed significant perturbation of the micropillars in the area within the contact line boundary. Imaging at low magnifications, or even examination by the naked eye, revealed that the inner part of the pattern was opaque, suggesting that the whole array of pillars inside the dried droplet perimeter was modified (Figure 1c). Higher magnification SEM imaging showed that this optical contrast

Figure 1. (a) Representative SEM image of pillared structure (H15), showing the topographic descriptors for the array. The pillars have a cylindrical shape and a height (h) of 15 μm and a diameter (d) of 5 μm forming a square lattice with an interpillar distance (l) = 5 μm. (b) Pure water droplet evaporating on the H15 substrate with micropillars leaving a distinct square-shaped contact line with no perturbation of pillars within this contour. (c) Pure water droplet evaporating on the H22 substrate with micropillars leaving a distinct shaped contact line pattern with significant modification of the micropillars within the contact line boundary. Time needed is represented in a dimensionless form as the ratio between the elapsed time (t) and the final evaporation time (te). (d–i) Pillared structures with constant (d = 5 μm) and different pillar heights (h) of (d) 15 μm (H15), (e) 22 μm (H22), (f) 28 μm (H28), (g) 33 μm (H33), (h) 38 μm (H38), and (i) 45 μm (H45). SEM images are presented for the different heights after evaporation of pure water droplets, probing the sensitivity of the structures to pure elastocapillary bending.
effect was caused by local bending of the micropillars (Figure 1d–i), with the pillars bent toward each other forming clusters and adopting complex geometries, e.g., dimer (white box), tetramer (blue box), hexamer (red box), octamer (yellow box), and nonamer (orange box). Similar effects have been reported before for larger pillar aspect ratios and were attributed to the elastocapillary coalescence of the flexible structures. In our experiments, as the aspect ratio decreased, the clusters contained lower numbers of aggregated pillars until a critical aspect ratio \( h/d = 3 \), for which no clusters were observed in the inner part of the droplet (Figure 1d).

The deformation of the pillars, upon water evaporation, is induced by the surface tension \( (\gamma) \) of the water/air meniscus connecting the pillars, and the corresponding force scales as \( F = \gamma r \), where \( r = d/2 \) is the pillar radius. The natural elasticity of the pillars resists deformation with an elastic force \( F_E = Elr^3/h^3 \), where \( E \) is the Young modulus and \( l \) the interpillar distance. This expression is analogous to the usual beam theory for slender objects, showing that the resistance to bending decreases strongly when the pillars height increases. If we define the pillar bending sensitivity as the ratio of capillary and elastic forces, \( F_c/F_E = \gamma/(Elr^3) \), we can conclude that it is directly proportional to the cubic power of the pillar aspect ratio \( h/r \), i.e., slender pillars are more prone to be bent by surface tension, while wide pillars tend to be more stable.

Under our experimental conditions, no pillar coalescence is observed in the area within the contact line boundary from pure water when the aspect ratio is below \( h/d = 3 \), suggesting that this is the critical aspect ratio threshold for which capillary action equals restoration mechanical stress on the micropillars. It is important to note that in this analysis, we are not considering the effect of the contact line. This effect is expected to have an enhanced deforming effect, but an accurate evaluation of this factor is beyond existing phenomenological modeling capabilities and will be the subject of future studies. Consequently, all of the results described below applies exclusively to the inner part of the dried pattern left by the droplet, ignoring possible contact line effects.

**Bacterial-Triggered Coalescence of Pillars.** From the elastocapillary assay discussed in the previous section, we identified the critical region within the topographic parameter space where the micropillared structure is able to resist capillary deformation in the presence of pure water droplets. Such a surface opens up the possibility to sense the presence of a second entity introduced into water (i.e., bacterial cells), which could induce a response in its own right. This critical structure corresponds to an aspect ratio \( h/d \approx 3 \) and pillar height \( h = 15 \mu m \) (H15, Figure 1d), as discussed in the previous section.

We, therefore, investigated the drying process of droplets containing different bacteria species over the H15 pillared structures. Similar to the case of pure water droplets, a pinned square drop shape is found. However, the patterns observed within the contact line formed after complete evaporation of the droplets were surprisingly different for some bacteria as clearly observed in Video S2.

Five different bacterial species, with a wide range of morphological and biological characteristics were investigated: **S. epidermidis**, L. sakei, P. aeruginosa, E. coli, and B. subtilis. The patterns formed after evaporation of droplets containing different bacteria on H15 pillar substrates (Figure 2) can be classified in two main groups: one group displaying significant bending of the pillars within the pattern (P. aeruginosa, E. coli, and B. subtilis) and another group that does not induce any responsive bending of the pillars in the center of the dried patterns (S. epidermidis and L. sakei). These distinct behaviors could be observed even by the naked eye in the form of a local change in contrast at the surface (Figure 2, S×). At higher magnifications, the difference is clearly revealed to be associated with the coalescence of adjacent pillars (Figure 2, 40× and SEM (100×)).

We attempted to correlate these results to the general characteristics of the bacterial species used in this work (Table 1). Atomic force microscopy (AFM) imaging confirmed the expected size and cell morphology for these bacteria: Gram-negative (−) P. aeruginosa and E. coli as well as Gram-positive (+) B. subtilis and L. sakei present a rod-like shape, while Gram-positive (+) S. epidermidis has a spheroidal shape (Figure S2). In addition, L. sakei and S. epidermidis are not motile (no flagella present), while the other three strains have flagella. From these considerations, we can conclude that the different pattern types showed in Figure 2 (bending vs nonbending) cannot be explained considering bacteria cell.
Table 1. General Characteristics of the Different Bacterial Strains Used in the Study*

<table>
<thead>
<tr>
<th>strain</th>
<th>gram</th>
<th>shape</th>
<th>$L \times W$ ($\mu m^2$)</th>
<th>flagella</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) P. aeruginosa</td>
<td>−</td>
<td>rod</td>
<td>1.4(±0.2) × 0.8(±0.2)</td>
<td>yes</td>
</tr>
<tr>
<td>(b) E. coli</td>
<td>−</td>
<td>rod</td>
<td>1.7(±0.2) × 0.9(±0.2)</td>
<td>yes</td>
</tr>
<tr>
<td>(c) B. subtilis</td>
<td>+</td>
<td>rod</td>
<td>1.8(±0.4) × 0.80(±0.2)</td>
<td>yes</td>
</tr>
<tr>
<td>(d) L. sakei</td>
<td>+</td>
<td>rod</td>
<td>1.5(±0.4) × 0.8(±0.2)</td>
<td>no</td>
</tr>
<tr>
<td>(e) S. epidermidis</td>
<td>+</td>
<td>spherical</td>
<td>1.3(±0.3) × 1.3(±0.3)</td>
<td>no</td>
</tr>
</tbody>
</table>

*AFM images of cells are presented in Figure S2.

No clear correlation was observed between bacterial species and the cluster symmetries obtained (e.g., dimer, trimer, tetramer, etc.). However, the data suggests that the assemblies emerge due to perturbation of the balance between capillary forces and elastic restoration forces in the presence of bacteria with flagella. In the next section, we discuss a possible mechanism for this distinctive behavior.

Possible Origin of Bacteria-Induced Coalescence.  In the previous sections, we determined the critical pillar aspect ratio, below which surface tension forces were not able to induce pillar coalescence in pure water. Interestingly, the responsivity is dramatically enhanced when the droplets contain flagellated bacteria. While the bending process at the perimeter of the contact line appears similar in both cases, coalescence within the central area is triggered at smaller aspect ratios by the presence of bacteria with flagella. This enhanced pillar bending effect results in characteristic patterns on the substrate, distinct for motile and nonmotile bacteria.

The possible origin of the enhanced pill bending may be related to the ability of the bacteria with flagella to adhere to more than one pillar (Figure S3), thus connecting adjacent pillars and inducing a mechanical deformation. In the presence of bacteria with flagella, we observed, at SEM, after drying, structures bridging bent pillars, while nonflagellated bacteria appeared attached to single pillars. The morphology of the single bacterial cells cannot be distinguished, probably due to distortions on the cell envelop after evaporation, in the absence of fixation.

These effects can also be understood by comparing the length scales of bacterial structures and pillar interspacing distances. The average size of the capsule for a single bacterial cell is below 2 $\mu m$ (Table 1), while flagella can reach tens of $\mu m$ beyond the outer cell membrane. Considering that in our microstructured surfaces the interpillar distance was 5 $\mu m$, bacteria without flagella will predominantly fall between the morphologies only. Similarly, the stiffness of the cell envelop does not appear to play a critical role, with rigid Gram-positive bacteria and softer Gram-negative bacteria distributed among both pattern groups.

Interestingly, the different response of the microstructures upon evaporation of the bacterial solutions correlates with the presence or absence of flagella. Bacteria with flagella clearly induce a bending response in the H15 pillars, while nonflagellated bacteria are unable to bend the pillars when used at the same bacterial concentration.

For the bacteria that induce a mechanical response, a concentration dependence is observed, with deformation of pillar clusters at the center of the dried droplet observed for bacteria concentrations between $10^7$ CFU/mL and $10^9$ CFU/mL, while none is observed for lower bacteria concentrations ($10^5$ CFU/mL). At low concentrations, only the perimeter near the corners of the dried square pattern presented coalescence of the pillars (Figure 3a–c). This can be attributed to the coffee-stain-like effect, able to drag bacterial cells toward the droplet contact line, increasing the local concentration of bacteria during evaporation.31 Interestingly, bacterial cells without flagella confirm the absence of responsivity at different cell concentrations (Figure 3d–f).
pills or strongly adhere to single pillars. On the other hand, bacteria with flagella, in which appendage sizes exceed the interpillar distance, can potentially interact with more than one pillar, leading to the observed pillar deformation. In support of this, we found evidence of bacterial matter residing between the bent pillars, after complete evaporation of droplets containing flagellated bacteria (Figure 4). Nonflagellated bacteria, on the other hand, are found attached to individual pillars only, forming nonconnecting structures (see Figures S4–S7).

Although a more detailed investigation of bacterial behavior during the actual drying process is necessary to confirm the hypothesis proposed, our results support the potential use of pillared soft substrates to discriminate between motile and nonflagellated bacteria using a cost-effective and immediate assay based on droplet-drying, which can be performed and quickly analyzed by the naked eye. In addition, discrimination of bacterial concentration is also possible, with only samples containing concentrations above a critical threshold producing an effective self-responsive surfaces for bacterial detection and differentiation.

CONCLUSIONS

We show that soft micropillared surfaces can be tailor-made during the actual drying process is necessary to confirm the hypothesis proposed, our results support the potential use of pillared soft substrates to discriminate between motile and nonflagellated bacteria using a cost-effective and immediate assay based on droplet-drying, which can be performed and quickly analyzed by the naked eye. In addition, discrimination of bacterial concentration is also possible, with only samples containing concentrations above a critical threshold producing an effective self-responsive surfaces for bacterial detection and differentiation.

EXPERIMENTS AND METHODS

The epoxy micropillars were fabricated by casting EPO-TEK OG142-13 from Epoxy Technology into a negative replica PDMS mold, as described. After the resin was casted, a 1.1 mm thick glass slide was placed over the mold and placed below an ultraviolet light for 20 min until the epoxy pillars was cured. The epoxy micropillars were mechanically removed from the mold. The SEM images of the epoxy pillars are shown in Figure S1. During the actual drying process is necessary to confirm the hypothesis proposed, our results support the potential use of pillared soft substrates to discriminate between motile and nonflagellated bacteria (Figure 4). Nonflagellated bacteria, on the other hand, are found attached to individual pillars only, forming nonconnecting structures (see Figures S4–S7).

**B. subtilis**

![B. subtilis SEM images](image1)

**E. coli**

![E. coli SEM images](image2)

Figure 4. Representative SEM images of H15 pillared structures after drying of bacterial suspensions, showing motile bacteria (**B. subtilis** and **E. coli**) bridging the bent pillars. The concentration of the different bacterial species is 10⁶ CFU/mL.

Transmission light microscopy images of the dried patterns were collected with a Zeiss 510 confocal microscope equipped with X10, X20, and X40 air objectives. AFM measurements from the Supporting Information were obtained using a Bruker Multimode 8 and a Keysights S500 instrument. Prior to AFM morphological analysis, a droplet of bacterial suspension (10⁷ CFU/mL) was deposited onto an epoxy surface. AFM measurements were performed for the H15 substrate with and without bacterial containing droplets only, the CA hysteresis was 50 ± 3°. No significant differences in CA and CA hysteresis were observed between water droplets and the deposited bacterial containing droplets. CA values are shown in Table S1.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsabm.8b00176.

SEM images of some of the pillared arrays fabricated; AFM images of bacterial cells dried on flat epoxy
surfaces; close-ups of E.coli dried over the H15 pillared substrate; additional SEM images of bacteria on H15 pillared structures; contact angle values for water and pillared suspensions on different pillared structures (PDF)

Video S1: droplet contour impalement (AVI)
Video S2: pillar bending by B. subtilis at the latest stages of evaporation (AVI)

Author Contributions
A.S.-A. and J.F.H.-S. contributed equally to this work

Notes
The authors declare no competing financial interest.

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