# Investigation on bacterial adhesion and colonisation resistance over laser-machined micro patterned surfaces

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Micro–nano patterns created directly over solid surfaces to combat microbial activity help in preventing hospital-acquired infections. This Letter is focused on defining surface topologies by laser patterning over solid surfaces. Studies on designing surface topologies and bacterial culture have been carried out and the feasibility of micro scale features in restricting bacterial growth has been investigated. The effects of the engineered roughness index and contact angle are discussed. Contact angle measurement over patterned surfaces using a novel computer vision-based technique is demonstrated and the effect of contact angle on bacterial adhesion has been presented. The results obtained show that the designed micro scale geometries can effectively reduce the growth of bacteria on the said surfaces.

**1. Introduction:** Hospital-acquired infections (HAIs) that account for about 30% of the infections globally, have become a significant healthcare issue, culminating in extended hospitalisation and hefty expenditures. Most of these infections result because of bacterial build-up that manifests itself in various forms. Very often, surgical removal is the only solution to bacterial infections caused by the prolonged usage of biomedical devices like implants, intravascular devices such as joint prostheses, heart valves, catheters, contact lenses and dentures [1]. Similarly, marine biofouling that refers to the unwanted accumulation of organic molecules and living organisms on the submerged hulls of ships [2], results in higher fuel consumption, consequently raising both environmental and economic concerns. Biofouling is also responsible for the introduction of non-indigenous marine species, thereby disturbing the ecological balance. Understanding the processes associated with the attachment of bacteria is very complex and is the fundamental requirement to the development of novel strategies to control fouling [3]. Current technologies such as coatings, bacteria-repellent surfaces, antimicrobial films and antibiotics being deployed to curtail bacterial activity, have many disadvantages. Tin and lead paints peel off with time, exposing the surface underneath to microbial attacks, as in the case of ship hulls. Antimicrobial films and bacteria repellent surfaces produced by incorporating photocatalytic materials, titanium oxide, nano particle coating, carbon nanotubes, bioactive glass and other metal oxide nano particles avoid the formation of bacterial colonies [4]. However, most of these are highly toxic and their disposal is an environmental threat. The use of antibiotics in the healthcare sector to control the growth of bacteria leads to the evolvement of a new generation of antibiotic-resistant bacteria.

Recent literature indicates that nano or micro patterning of surfaces is the most feasible solution to inhibit bacterial colonisation owing to changes in the cellular responses to nano and micro scale topographies. Adhesion, detachment and physiological properties of bacteria are influenced by the wettability of the surfaces, the surface roughness, Reynolds number, van der Waal forces and surface energy. The influence of micro–nano scale topologies on these properties has not yet been fully understood; prompting the authors to pursue the current research. This Letter is focused on identifying the challenges in direct laser patterning over solid surfaces in micro–nano scale. It also explores the efficacy of using such patterned surfaces in preventing colonisation and migration of one of the most common pathogens, *Escherichia coli*. The relations between various parameters such as engineered roughness index (ERI) and contact angle and the growth of bacteria on the μm-patterned, laser-machined surfaces have also been studied and will be presented in the following Sections.

**2. Related work:** PDMS (structured through replica moulding using patterned samples) is the most widely used substrate for antimicrobial studies performed in research laboratories. There have been multiple processes and substrate materials utilised for development of these patterned surfaces. The most commonly used techniques include photolithography, micro moulding and focused ion beam (FIB) machining. The majority of these processes would become a hurdle when looking for alternatives to produce micro–nano scale patterns of desired geometries directly over the surfaces of metals, ceramics and other solid substrates, while designing new generation products imbibed with antibacterial properties.

On the other hand, the process capabilities and maturity level of micro patterning techniques vary significantly in terms of geometric complexity, minimum feature size, range of materials, repeatability, etc., and are summarised in Table 1. For example, lithography-based processes need a mask; thus, they are ineffective for patterning non-planar surfaces and for three-dimensional features. Replica

**Table 1** Comparative evaluation of various micro–nano patterning techniques

| Process | Feature resolution | Material compatibility | Maturity level |
|---|---|---|---|
| photolithography | ~3 μm | resists, functional organics | in production |
| optical soft lithography | ~90 nm | resists, functional organics | research |
| embossing | ~1 nm | mouldable resists | in production |
| hard imprint lithography | <10 nm | organics, mouldable resists, | early production |
| soft imprint lithography | <10 nm | organics, mouldable resists, | research |
| laser machining | ~5 μm | organics, semiconductors, conductors | early production |




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moulding uses softer material and it needs an already patterned mould that restricts its use to laboratory experiments. On the other hand, FIB machining is relatively versatile, but is highly expensive and a slow process that makes it unsuitable for bulk integration. Therefore there is a lot of emphasis on the integration of mechanical based micro–nano fabrication techniques such as scratching, milling, electro-discharge machining (EDM) and pulsed laser processing to produce desired micro–nano patterns directly over a wide range of materials. However, many of these process capabilities leave a lot to be desired.

To understand the fabrication issues of micro patterned surfaces for bacteria-inhibiting properties, the mechanism of bacterial adhesion must be observed. When a bacterium initially comes into contact with a substrate surface it binds reversibly through non-specific interactions. This eventually leads to an irreversible binding through the formation of a protein–ligand interaction. Therefore the adhesion of bacterium, and most other cell types, is dependent on the formation of a protein layer on the surface of the substrate.

The effect of a microscopic physical surface modification to produce a sharklet-like surface shows 47% reduction in colony-forming units (CFUs) and bacterial area coverage plus 77% reduction in colony size studied for uropathogenic *E. coli* in tryptic soy broth and artificial urine [5]. The incidence of *E. coli* migration was reduced by more than 80% for the Sharklet transverse patterned rods compared with the unpatterned control rods. A study undertaken by Schumacher *et al.* [3] reported an increase of fouling deterrence for both zoospores and cyprids with enhanced aspect ratio (feature height/feature width) of topographical features engineered in PDMS. In another study, a predictive model for the attachment of spores of the green alga Ulva on patterned topographical surfaces was developed using a constant refinement approach. It was predicted that spores attached in fewer numbers when the area of feature tops increased or when the number of distinct features in the design increased. The size and motility of the bacterial cells and algal spores in relation with the Reynolds number (Re) of the cells were plotted. The results showed a negative linear correlation of normalised transformed attachment density for both organisms with ($ERI_{II} \times$ Re). These studies demonstrate that organisms respond in a uniform manner to a model, which incorporates surface energy and the Reynolds number of the organism.

Similarly, nano-sized structures such as square-shaped cavities and protrusions with intermittent hydrophobicity and hydrophilicity, or channels or dots of different surface characteristics, can elicit responses in bacteria including differential cell distribution or orientation [6]. The nano shell arrays made by lithographic techniques have shown intrinsic low solid/liquid interface areas reducing the bacterial binding [7]. On the other hand, customised pattern shapes created in micro–nano scale over the surgical needle edge minimise the tissue piercing force with the increase in the surface area [8].

In summary, the need for direct patterns over a solid surface and better understanding of the cell–pathogen surface interface phenomenon to impart improved physiological and antibacterial properties is continuously progressing. This demands a trade-off in the definition of optimised antibacterial surface topologies and manufacturing protocols. The results of bacterial studies performed by the authors, particularly on laser machined micro–nano patterned surface topologies, are presented in the following Sections.

## 3. Methodology:

In this study, the focus is on the design of different surface topologies and the evaluation of their antibacterial properties. The methodology adopted for investigation involves five components: (1) designing the micro-patterned geometries, (2) direct patterning over PDMS samples using a $CO_2$ laser, (3) contact angle measurement using image processing algorithms, (4) culturing bacteria over smooth and patterned surfaces immersed in LB–agar media to assess the colonisation, and (5) testing the growth of bacteria over different incubation time intervals. The key aspects under each technique are discussed next.

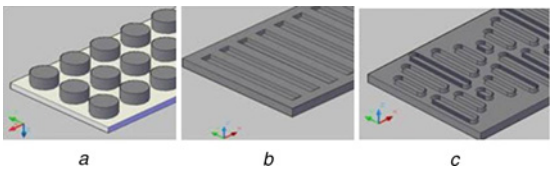


**Figure 1** *Typical patterned topologies*
*a* Pillars
*b* Ridges
*c* Sharklet

### 3.1. Design of surface topologies:

In this study, three surface topologies consisting of sharklet, circular pillars and ridges shown in Fig. 1 have been designed to establish a relation between the surface topology and motility of the bacterial cells. These geometries are inspired from natural surfaces such as lotus leaf, shark scales, etc., which are known to possess nano and micro patterns thereby imparting antimicrobial properties. Although there are several control factors that alter the surface–microbial interactions such as contact angle, zeta potential, Reynolds's number, etc., these are interrelated. In these designs, the ERI has been chosen as a variable, change in contact angle will in turn determine the bacterial adhesion or inhibition properties which will be used as a control parameter to assess the impact of a particular geometry on antibacterial properties.

The ERI is a dimensionless ratio based on Wenzel's roughness factor ($r$), the depressed surface area fraction ($1-F$), and the degrees of freedom (df) of the pattern. Wenzel's roughness factor ($r$) is the ratio of the actual surface area to the projected planar surface area. The actual surface area includes the surface area of the feature tops, sides and depressed surface area between features. The depressed surface fraction ($f_D$) is the ratio of the depressed surface area between features and the projected planar surface area. The depressed surface fraction is equivalent to $1-f_1$, where $f_1$ is the solid–liquid interface term of the Cassie-Baxter equation for wetting.

The degree of freedom for movement relates to the tortuosity of the surface and refers to the ability of a bacterium to follow recesses (i.e. grooves) between features within the topographical surface. If the recesses form a continuous and intersecting grid, movement in both the $X$ and $Y$ coordinates is permitted and the degree of freedom is 2. Alternatively, if the grooves are individually isolated (e.g. as in channel topographies) then movement is only allowed in one coordinate direction and the degree of freedom is 1 [9]. In this work the authors adopted modified ERI, that is, $ERI_{II}$ to include additional topographical designs. The df term was replaced with $n$, which is the number of unique features in each topography. For example, the ridges topography has an $n$-value of one. $ERI_{II}$ is represented as

$$ERI_{II} = \frac{(r \times df)}{(f_D)} \tag{1}$$

$$r = \frac{\text{actual surface area}}{\text{projected planar surface area}} \tag{2}$$

$$f_D = \frac{\text{depressed surface area between features}}{\text{projected planar surface area}} \tag{3}$$

For example, ridges topology width is 60 μm, height is 20 μm with spacing is 170 μm, the calculated $r$ is 1.17; $f_D$ is 0.739; and df is 1. Therefore $ERI_{II}$ is 1.58.

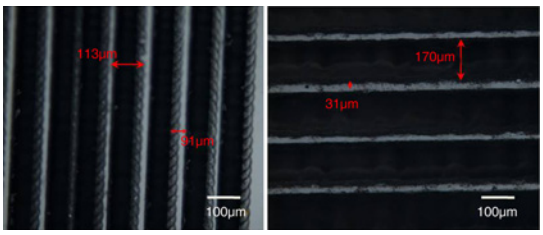


**Figure 2** *Ridges profile created over PDMS substrate using $CO_2$ laser*

3.2. Fabrication of topologies

3.2.1 Laser patterning: The authors have created micro scale patterns by direct patterning on PDMS substrate using a $CO_2$ laser. The $CO_2$ laser machine Model: VLS2.30 of Universal Laser Systems was used to create the patterns directly over PDMS substrate. The lasing operation was performed at 3 W power, with spot size of 100 µm and 40% spot overlap to create a 170 µm slot between the two consecutive ridges. Micro scale ridges of the order of 30–90 µm width and with a pitch of 170 µm as shown in Fig. 2 could be fabricated using $CO_2$ laser machining using the above-stated process conditions. For bacterial culture studies, the authors used ridge shaped patterned surfaces in two sizes (60 and 90 µm wide, height 20 µm and spacing of 170 µm).

3.3. Contact angle measurement: The laboratory setup shown in Fig. 3 was developed by the authors. The heat shield was used to remove any side effects of the heat produced by the lamp and also to ensure uniform distribution of light. The micro patterned sample was placed on the work table. A small droplet was placed over the sample using a needle and syringe pump arrangement.

Current techniques to identify contact angles, that is, goniometry and the Wilhelmy plate method have certain drawbacks like user interaction and user-induced errors such as parallax, etc., which can be reduced to a great extent by the use of this algorithm.

For instance, contact angle measurement by fitting a curve to the extracted drop edge gets rid of the size constraints imposed by previous methods. Multiple points on the drop edge are selected from the images and a curve is fitted to these profile points.

The algorithm for computing the contact angle of a water droplet placed on the samples was implemented using MATLAB (Fig. 4). An image of the droplet placed on the given sample captured by the high-speed camera is taken as input. Next, contrast limited adaptive histogram equalisation (CLAHE) is done followed by binarisation. Morphological operations are followed by boundary extraction to isolate the droplet boundary. Curve fitting is then done on the droplet boundary based on the two contact points identified by the user. A tangent is drawn to the curve and the contact angle is computed. The contact angle for the ridge patterns 60 and 90 µm wide with a 170 µm pitch were 105° and 107°, respectively. The results obtained were compared with a wide range of standard contact angle values and validated. The angles obtained were accurate with a tolerance of less than 1°. In this work, authors have produced various patterns namely square and circular pillars, sharklet, channels and ridges at different dimensional scales. Table 2 shows

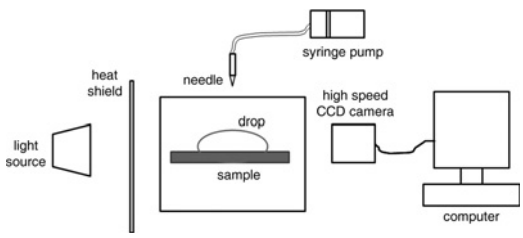


**Figure 3** *Experimental set-up for contact angle measurement*

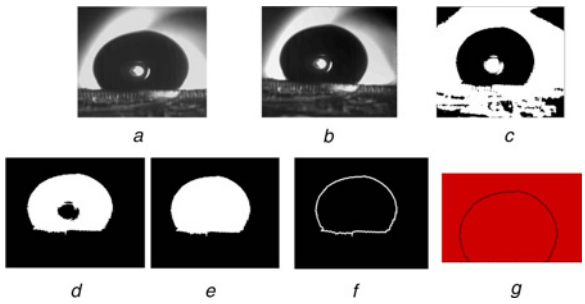


**Figure 4** *Stages of contact angle computation for ridges pattern*
*a* Droplet placed on the patterned sample
*b* After CLAHE
*c* Binary image
*d* and *e* After morphological operations
*f* Boundary extracted
*g* After cropping substrate

**Table 2** Contact angles for various geometries

| Features | Height, µm | Spacing, µm | Width, µm | Contact angle, $\theta$ |
|---|---|---|---|---|
| circular pillars | 10 | 10 | 50 | 118 |
| square pillars | 10 | 20 | 20 | 128 |
| channels | 10 | 60 | 30 | 119 |
| sharklet | 10 | 20 | 20 | 124 |
| ridges | 20 | 170 | 60 | 105 |
| ridges | 20 | 170 | 90 | 107 |

the pattern geometries of different shapes with varying contact angles confirming their influence on wetting properties.

3.4. Bacterial culture studies: Bacterial culture studies had been performed on the ridge-shaped samples of two different dimensions in order to evaluate them for antibacterial properties. The first sample has 60 µm ridges of height 20 µm at 170 µm spacing between two consecutive ridges. The second sample has larger ridges of 90 µm, while other dimensions were kept constant. These studies have been carried out in both solid as well as liquid media to obtain a comparative analysis. Although certain geometries have been reported to respond to certain types of bacteria positively and negatively to certain other types, the authors have performed studies on the response of the ridges pattern to the common *E. coli*. Studies have been carried out in both solid and liquid media and the surfaces have displayed antibacterial properties in both the media. This shows that these surfaces can be deployed in both solid as well as liquid media.

3.4.1 In LB–agar media: The patterned surfaces were challenged with gram negative *E. coli* in LB media to assess the extent of colonisation on each surface type. The growth was quantified using two measurement parameters – CFU enumeration and optical

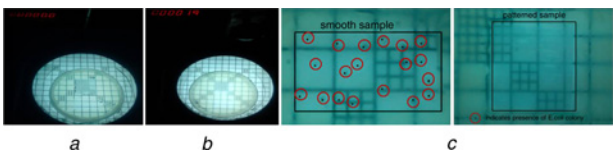


**Figure 5** *Bacterial studies carried out on smooth and ridges pattern*
*a* Initial colony count
*b* Colony count after 36 h
*c* Enlarged view of colonies on smooth and ridges sample




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**Table 3** Antimicrobial behavioural results of ridge-shaped patterned surfaces

| Ridge specifications | | | $ERI_{II}$ | Contact angle, ° | Bacteria culture studies | | |
|---|---|---|---|---|---|---|---|
| Width, μm | Height, μm | Spacing, μm | | | Colonies formed (solid media) | | Optical density (liquid media) |
| | | | | | After 24 h | After 36 h | |
| 60 | 20 | 170 | 1.58 | 105 | 1 | 4 | 2.1545 |
| 90 | 20 | 170 | 1.63 | 107 | 1 | 2 | 1.9848 |

density. Samples were cleaned with ethanol, autoclaved and sterilised under UV light in a laminar air flow and then bonded to Petri Dishes and immersed in LB–agar media; to be incubated at 37°C and 120 rpm. The samples were removed for enumeration at different intervals: 1 day, 2 days, 3 days and 7 days; similar to the procedure followed by Reddy *et al* [5]. The number of colonies on the smooth PDMS sample and the ridge-patterned sample were counted using Colony Counter.

Although the smooth PDMS sample (25 × 10 mm) showed 14 colonies, the two patterned samples (25 × 25 mm – 60 μm and 90 μm wide ridges) showed only one colony each after 24 h. After incubation for the next 12 h, the smooth sample showed 19 colonies, whereas the ridge-patterned samples still showed only four and two colonies, respectively (Fig. 5). After 7 days of incubation, the patterned samples were visibly less populated compared with the high number of colonies on the smooth sample. Cell colonies were found to respond to these micro scale features by altering their shape, such as elongating along the grooves.

3.4.2 In liquid media: Liquid media tests were performed to test the adherence properties of the bacteria to the patterned and smooth surfaces. The colonies are no longer distinct in liquid media but get dissolved. Therefore the rate of growth of bacteria was tested by measuring the optical densities of the respective liquid media used for both the samples. In this study, the PDMS samples were then bonded to Petri Dishes and immersed in the inoculum suspension and incubated at 37°C and 70 rpm. After 24 h, the optical densities of the LB media were measured using a spectrophotometer. For culture studies carried out in liquid media, the 25 × 25 mm patterned samples (60 and 90 μm wide ridges) showed an optical density of 2.1545 and 1.9848, respectively, while the smooth 25 × 10 mm PDMS sample showed an optical density of 1.7945 after 24 h.

In summary, the bacterial culture experiments demonstrated significant reduction of colony number, bacterial area coverage and bacterial migration on patterned PDMS surfaces. This clearly indicates that the growth of *E. coli* on laser machined micro patterned surfaces is highly restricted compared with the growth on smooth surfaces. It has also been observed that ridges with 90 μm width show a better antimicrobial behaviour compared with 60 μm ridges at the same spacing and ridge height (Table 3). This may be attributed to higher values of ERI and contact angle, thereby proving that altering the wetting properties can have a strong influence on bacterial growth.

**4. Conclusions:** This study investigated the opportunities for the integration of mechanical based processes for large area and bulk scale micro–nano patterning. Direct laser patterning on solid substrates in micro scale for antibacterial properties has been done and the results obtained are indicative of antibacterial properties displayed by the samples. Existing techniques can fabricate only on a certain set of substrate materials. However, using laser patterning, a wide variety of substrates may be machined in micro scale. The results obtained also show that antibacterial properties may be observed even at higher sizes of geometries (about 60–90 μm). This is not only convenient from the manufacturability point of view but is also highly economical. The preliminary experiments show that higher values of ERI and contact angle have a strong influence on resistance to bacterial growth. The computer vision-based algorithm for computing the contact angle is yet another feature that has been explored in this work. It has also been realised that imparting antibacterial properties over any new class of biomedical devices is a trade-off between the definition of geometries and the selection of manufacturing protocol. It could be envisaged that identification of the dominating bacteria environment for a specific application and defining patterns to resist their colonisation and growth rate would become a reality in the future. On successful development at commercial scale, direct-patterned antibacterial surfaces may potentially be used for multiple purposes such as in the case of mobile phones, touch screens, laptops, medical, and hospital equipment such as catheters, hospital beds, as well as items of daily use such as tables, cutlery, etc.

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