Evaluation of Surface Microtopography Engineered by Direct Laser Interference for Bacterial Anti-Biofouling

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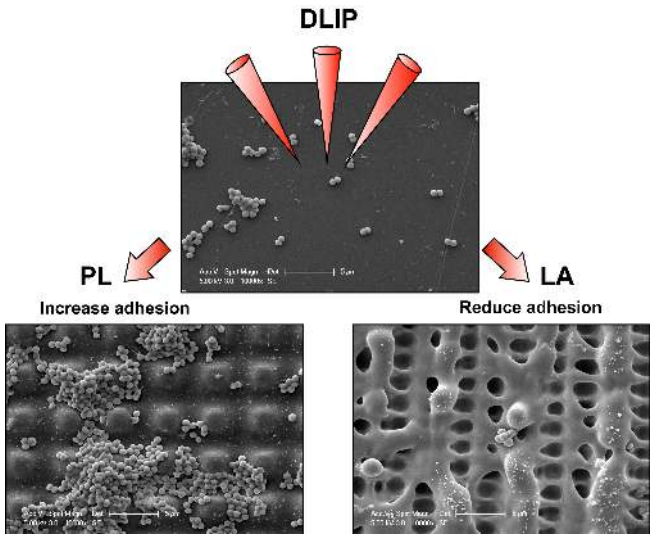
Abstract

Biofilm formation by bacterial pathogens on the surface of medical and industrial settings is a serious health problem. Modification of the biomaterial surface topography is a promising strategy to prevent bacterial attachment and biofilm development. However, fabrication of functional biomaterials at large scale with periodic network-topology is still problematic. In this study, we use direct laser interference patterning (DLIP), an easily scalable process, to modify polystyrene surface (PS) topography at sub-micrometer scale. The resulting structure surfaces were interrogated for their capacity to prevent adhesion and biofilm formation of the major human pathogen *Staphylococcus aureus*. The results revealed that three-dimensional micrometer periodic structures on PS have a profound impact on bacterial adhesion capacity. Thus, line- and pillar-like topographical patterns enhanced *S. aureus* adhesion, whereas complex lamella microtopography reduced *S. aureus* adhesion both in static and continuous flow culture conditions. Interestingly, lamella-like textured surfaces retained the capacity to inhibit *S. aureus* adhesion both when the surface is coated with human serum proteins *in vitro* and when the material is implanted subcutaneously in a foreign-body associated infection model. Our results establish that the DLIP technology can be used to functionalize polymeric surfaces for the inhibition of bacterial adhesion to surfaces.



1. Introduction

One of the major challenges of the materials engineering discipline is to generate surfaces preventing bacterial adhesion by repelling bacterial cells from attaching (antibiofouling) or alternatively, inactivate the bacteria in contact with the surface (bactericidal surfaces) [1]. Colonization of the surface with bacteria has in most cases an adverse effect on the functionality of the interface, such as clogging of industrial pipes and tubing, decreased performance of shipping vessels, contamination of food manufacturing surfaces and medical implants. The strategies to prevent and combat bacterial adhesion and proliferation to abiotic surfaces include chemical modifications with antibacterial agents (antibiotics, antimicrobial peptides, alkyl chains, metals, detergents) and physical modification of the surface topography [2,3]. Because chemical modifications very often lead to toxicity due to the release of the chemical compounds and rapid selection of resistant bacteria, the role of surface topography in creating surfaces with antibiofouling properties is receiving greater consideration [4,5]. The idea is to produce three-dimensional (3D) topographical patterns on the surface that result in reduced contact area so that bacteria are forced to span the distance between structures to generate productive interactions. Obviously, modification of the surface topography can be combined with chemical coating of the surface with antibacterial agents.

Initial approaches to explore the effect of surface topography on bacterial adhesion were carried out by mechanical roughening and polishing techniques, generating random texturized roughness surfaces that modulate bacterial adhesion [6-11]. More recently, micropatterning techniques such as optical lithography, microcontact printing, electron or ion beam lithography that allow the fabrication of periodic microstructures with well-defined and reproducible dimensions and shapes, have been used [12-16]. However, these techniques require multiple steps and long processing times to produce surface geometries, especially if large areas have to be processed. As a complementary alternative to these methods, the Direct Laser Interference Patterning (DLIP) technology provides a new strategy to generate periodic micro- and

nanotopographies on different polymeric and other substrates. This method enables the large-scale fabrication of complex structures by systematically varying the dimensions of the gratings superimposed upon each other. Another significant advantage of DLIP compared with other surface patterning methods is that fairly large areas can be processed within a short period of time (up to several cm^2/s) using single or multiple laser pulses [17].

Biofilms represent the dominant form of bacterial life in natural environments. In biofilms, bacteria grow attached to the inert surface or living tissue and embedded in an extracellular matrix that protects bacteria from environmental stresses, predators, antimicrobials or the immune system [18]. Biofilm formation starts with irreversible attachment of planktonic bacteria to the surface, a process that is mediated by physical forces or specific interactions. Then, sessile bacteria divide and secrete an extracellular matrix that anchors bacteria firmly to the substrate and among them. Finally, single bacteria or cell clusters can actively disintegrate from the biofilm or passively be shed through mechanical disruption [19-21]. *Staphylococcus aureus,* together with *Staphylococcus epidermidis*, are the most important Gram positive bacterial pathogens that can form biofilms on medical devices such as catheters, valves, prostheses and implantable venous access systems (port-A-caths) [20-22]. *S. aureus* from skin and mucous membranes from healthy humans can adhere to the surface via nonspecific interactions based on the physicochemical properties of the cell enveloped or through specific binding between compounds of the cell envelop and proteins of the host serum coating the surface of the implanted material. Living inside the biofilm increases bacterial resistance to the action of the immune system and antimicrobials. As a consequence, staphylococcal biofilm associated infections are difficult to eradicate and in most cases the contaminated implants need to be removed to cure the infection.

In this study, polystyrene polymer surfaces were patterned with periodical line- (1D), pillar-like (2.5D) and a complex combination of lamella- and line-like pattern (3D) by applying the Direct Laser Interference Patterning (DLIP) technique. After patterning, those samples together with

non-patterned substrates of the same materials were used for *Staphylococcus aureus* bacterial adhesion tests *in vitro* under static and continuous flow conditions as well as in an *in vivo* infection model. The results revealed that line- and pillar-like patterns promote *S. aureus* adhesion whereas lamella-like patterns reduce bacterial adhesion in steady state and continuous flow conditions. *In vivo* testing of lamella-patterned polymers demonstrated the potential of this microtopography to reduce staphylococcal biofilm on implanted materials.

2. Experimental Section

2.1 Patterning of polymeric materials

We used commercially available polymeric materials purchased from Goodfellow GmbH (Bad Nauheim, Germany). Two different polystyrene (PS) substrates with a thickness of 125 µm (biaxial orientated) and 1.2 mm were used. The samples were patterned using a high-power pulsed, frequency quadrupled Nd:YAG laser (Quanta Ray, Spectra Physics) emitting a beam with a wavelength of $\lambda = 266$ nm. The samples were irradiated with 10 ns pulses at a frequency of 10 Hz. For obtaining 1D line-like structures the two beam experimental set-up was used which is described elsewhere [23]. The spatial period Λ was varied from 1 to 5 µm by keeping a constant wavelength of $\lambda = 266$ nm and varying the incident angle 2α between the two laser beams following:

$$\Lambda = \frac{\lambda}{2 \sin \alpha}$$

For obtaining a 2D structure (e.g. pillars), the samples were rotated by an angle of 90° between two subsequent laser shots. All experiments were performed at ambient conditions of pressure and temperature. Polyimide (PI, Kapton HN®) and polyethylene terephthalate (PET) with 175 µm thickness were patterned using Nd:YAG laser emitting a linearly polarized beam with wavelengths of $\lambda = 355$ nm (PI) and $\lambda = 266$ nm (PET).

2.2 Bacterial strains and animals manipulation

S. aureus 15981 produces high levels of b1-6 linked poly-N-acetylglucosamine (PIA/PNAG) and it is accepted as a model strain of exopolysaccharide-dependent biofilm formation [24]. Staphylococci were cultured on tryptic soy agar (TSA) or broth (TSB) at 37 °C supplemented with glucose (0.25 %) or with human serum (10 %) when indicated. All animal studies were reviewed and approved by the Comité de Ética, Experimentación Animal y Bioseguridad, of the Universidad Pública de Navarra (approved protocol PI-019/12). The work was carried out at the Instituto de Agrobiotecnología under the principles and guidelines described in European Directive 86/609/EEC for the protection of animals used for experimental purposes.

2.3 Bacterial attachment and biofilm formation

For the analysis of *S. aureus* adhesion under static conditions, an overnight culture of *S. aureus* 15981 strain was diluted with a ratio of 1:100. Two ml of the diluted cultured were added to 6-well microtiter plate. Substrates of 2 x 2 cm^2 from all patterned and non-patterned (reference) polymer surfaces were put in each well and plates, which were incubated for 2 hours at 37 ºC with shaking. After incubation, the substrates with the attached bacteria were removed from the microtiter plate culture with tweezers and gently rinsed three times with sterile PBS to removed non-adherent bacteria.

The biofilm formation under continuous flow conditions, tested on polystyrene surfaces, was performed using 60-ml microfermenters (Pasteur Institute, Laboratory of Fermentation) with a continuous 40 ml h^{-1} flow of medium and constant aeration with sterile compressed air (0.3 bar) [25]. Polystyrene wafers (1 x 1 cm^2) of the patterned surfaces as well as non-patterned surfaces as reference were fixed on glass slides, which were then submerged in the microfermentor. Approximately 10^8 bacteria from an overnight culture of *S. aureus* 15981 were used to inoculate the microfermenters and were then kept at 37 °C for 6 h.

For both analyses, after incubation the substrates with the attached bacteria were removed from the microtiter plate and microfermentor, respectively, gently rinsed three times with sterile PBS and then placed in 1 ml of PBS and vigorously vortexed. Subsequently, the samples were

serially diluted and plated onto TSA plates for enumeration of viable staphylococci (colony forming units, CFU). The relative adhesion was calculated as bacterial counts CFU on patterned surfaces / CFU on non-patterned surfaces.

2.4 Visualization and topographical characterization

A scanning electron microscope (Philips XL30 ESEM-SEG) with an operating voltage of 5 kV was used for visualizing the surface of the patterned sample as well as the attached bacteria. A thin gold coating of several nm was sputtered on the non-conductive samples to avoid charge processes. The topographical analysis (structure quality and depth) was conducted with a confocal microscope (Leica DCM 3D) using a 150x objective with a lateral and z-resolution of 150 nm and 4 nm, respectively. For the epifluorescence analysis polystyrene wafers were incubated with *S. aureus* 15981 expressing the green fluorescence protein (GFP) for 4 h under static conditions. Wide-field fluorescence microscopy was used for imaging of the cells attached to the PS surfaces. Each surface was visualized using a 100x oil immersion lens and 10 fields of view were randomly chosen for statistical analysis.

2.5 *In vivo* model of polymeric-associated biofilm infection.

For the *in vivo* model, patterned and non-patterned substrates with a size of 0.5 x 0.5 cm² were used. Two different analyses were performed: bacterial contamination on the PS substrates (i) prior and (ii) post implantation. For the prior-implantation tests, the substrates were incubated with 0.5 ml of 1:100 overnight dilution of *S. aureus* 15981 culture for 1 hour at 37 ºC with shaking. The *in vivo* tests with post-implantation contamination were performed with sterile PS substrates. For both analyses, CD1 mice (n=6) were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture. After abdominal epilation and antisepsis of the operative field, the animals were operated upon. An incision of 1.5 cm in the skin was performed with displacement of the subcutaneous space and opening of the peritoneal cavity. Then, contaminated and non-contaminated respectively polymeric surfaces were fixed at the abdominal wall. The peritoneal cavity was closed by suture with 6/0 Monosyn®. The animals

were put in a warm environment and when awake placed back in their cages. Within the post-implantation tests, a bacterial suspension containing 10^8 bacteria of *S. aureus* 15981 was injected two days after surgery intraperitoneally at the site of the polymer implantation. After 5 days, all animals were sacrificed and the polymeric substrates were extracted and placed in 1 ml of PBS and vigorously vortexed. The samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci. The relative adhesion was calculated as bacterial counts CFU on patterned surfaces / CFU on non-patterned surfaces.

2.6. Statistical analysis

Statistical analysis was performed by one-way analysis of variance combined with the Bonferroni multiple post-hoc test or by the Mann-Whitney test, with $P \leq 0.05$ considered significant (GraphPad Instat, version 5).

3. Results

3.1. Design of patterned surfaces by DLIP

To analyze whether microstructures generated with Direct Laser Interference Patterning (DLIP) technique on the surface of polystyrene (PS) polymers can modify bacterial adhesion capacity, we generated surfaces with different microtopography geometries. We used spatial grating periods (Λ) varying from 1 to 5 µm and a laser fluence that was adapted to obtain the optimal structure quality (e.g. avoiding collapse of the fabricated array) depending on the spatial period (Table 1). PS wafers with 1.2 mm thicknesses were patterned with periodic line (LN) and pillar (PL) foils with maximal achievable structure depths of $d_{Struc} = 1.63 \pm 0.09$ µm and $d_{Struc} = 1.85 \pm 0.1$ µm respectively (Figure 1A). Scanning electron microscopy analysis of PS surfaces patterning with LN and PL revealed a well-defined, reproducible and homogeneous pattern of lines and pillars with precise edges (Figure 1A and B). A similar laser treatment on thin PS films (125 µm thickness) creates a combination of a lamella microtopography (LA)

with a 2.0 µm spatial period (d_{struc} = 0.47 ± 0.02 µm) and a line-like structure with periodicities of 6 or 8 µm (d_{struc} = 4.33 ± 0.06 µm) (Figure 1A and B). The lamella microtopography results from partially collapsing line-like features due to the lower mechanical stability of the thin PS film compared to the thicker one. The results indicate that DLIP can be used to fabricate 1D to 3D micropatterns on PS polymers.

3.2 Quantitative analysis of S. aureus adhesion to the patterned surfaces.

The clinical strain *S. aureus* 15981 was selected to evaluate the impact of surface microtopography on *S. aureus* adhesion capacity. ~~*S. aureus* 15981 produces high levels of b1-6 linked poly-N-acetylglucosamine (PIA/PNAG) and it is accepted as a model strain of exopolysaccharide-dependent biofilm formation~~ [24]. Polystyrene wafers with patterned and non-patterned surfaces were incubated with bacteria in TSB-gluc media. After 2 hours, the number of bacteria attached to the surface was determined by serial dilution and plating. The results revealed that line- and pillar-like microtopographical patterns enhanced *S. aureus* adhesion to PS polymeric materials (Figure 2A and B). In particular, a spatial period of 1 µm induced higher bacterial attachment ($P<0.05$) than periods of 5 µm. In contrast, the lamella-like topography on the thin PS substrates (LA) caused a significant reduction on the adhesion of *S. aureus* compared to non-patterned PS surfaces (CT) (Figure 2C). These results revealed that microtopographical patterns on PS have a profound impact on *S. aureus* adhesion.

3.3 Qualitative evaluation of bacterial attachment on PS polymers

Because enumeration of bacteria cannot distinguish between monolayers, where most of the bacteria are in contact with the surface, or scattered aggregates, where only few bacteria are in contact with the surface, we used epifluorescence microscopy and scanning electron microscopy to evaluate the adhesion behavior. As it is shown in Figure 3A, large aggregates of bacteria attached to PL (Λ=5 µm) and LN (Λ=5 µm), surfaces were visualized by immunofluorescence. Bacterial aggregates adhered not only to the top of the structure but also

inside the features. ~~These attachment patterns suggest that bacteria respond to the surface topography by maximizing the contact area with the surface.~~ In contrast, few bacteria randomly oriented along the surface were attached on LA (Λ=2 µm) patterned surface. Strikingly, single or small aggregates of bacteria were attached to the non-modified PS surface (CT) (Figure 3A). This behavior was confirmed by SEM analysis. The micrographs of patterned surfaces incubated with *S. aureus* revealed large bacterial aggregates on PL polystyrene surfaces, while only individual bacteria or small bacteria clusters/aggregates scattered on the surface of both the LA and the non-treated materials were observed, respectively (Figure 3B).

3.4 Bacterial attachment to patterned surfaces under flow-continuous conditions

S. aureus 15981 attachment to patterned polystyrene surfaces was assessed under continuous flow conditions using microfermenters [25]. The flow rate of fresh medium (40 ml h^{-1}) imposed in the process was high enough to avoid any significant planktonic growth (Figure 4A). Polystyrene substrates of 1 x 1 cm^2 of PL (Λ=5 µm), LA (Λ=2 µm) and non-patterned surfaces were fixed on the glass slides present inside the microfermenter (Figure 4B). *S. aureus* 15981 strain was inoculated in the microfermenters and incubated for 6 hours. In agreement with the results obtained under static conditions, LA-patterned substrates significantly reduced the adhesion of *S. aureus* compared to the non-patterned surfaces ($P<0.01$) (Figure 4C). Furthermore, PL microtopography increased the adhesion of *S. aureus* to patterned surfaces (Figure 4C).

Once a surface is implanted in a living body and comes into contact with biological fluids, such as blood or serum, the proteins present in the media immediately coat the medical device. Numerous studies indicated that coating of the medical devices with host factors may perturb *S. aureus* attachment and biofilm formation [1,26-28]. To address whether coating of the micropatterned surface with plasma proteins has an impact on the capacity of *S. aureus* to attach irreversibly to the surface, we measured the adhesion capacity of bacteria to LA substrates preincubated with human serum under flow conditions. For that, LA polystyrene

surfaces preincubated with media supplemented with human serum (10 %) for 1 h inside the microfermenter, were inoculated and incubated for 6 h with bacteria. Enumeration of the bacteria attached to the LA surface revealed a small but significant decrease compared to the bacteria attach to the non-patterned surface (Figure 4D). These results indicated that lamella microtopography can efficiently reduce *S. aureus* adhesion under flow continuous conditions even in the presence of serum proteins.

3.5 *In vivo* biofilm formation model on PS surfaces

Although *in vitro* assays have proven effective at identifying mechanisms involved in bacterial attachment and biofilm accumulation, it is important to validate the significance of these assays *in vivo*. Thus, we tested the efficacy of LA microtopography to reduce *S. aureus* attachment and biofilm development using a biofilm infection model in two alternative scenarios. First, LA (Λ=2 μm) and control surfaces pre-coated with 10^4 CFU of *S. aureus* 15981 were implanted in the intraperitoneal cavity of mice (n=6) (Figure 5A). After five days, animals were sacrificed, to aseptically removed the polystyrene wafers and evaluate the bacterial load (Figure 5B). We found that LA surfaces showed a lower degree of colonization than the non-patterned surface ($P<0.05$) (Figure 5C).

Second, sterile LA and non-patterned PS surfaces were implanted into the mice (n=6) and two days after surgery, contaminated with 10^8 CFU of *S. aureus* 15981. Enumeration of *S. aureus* cells attached to the PS wafers 5 days after infection showed that LA surfaces displayed a significantly lower colonization compared to non-patterned surfaces ($P<0.01$) (Figure 5C). Thus, PS-LA wafers displayed a lower level of colonization than non-patterned PS surfaces *in vivo*.

4. Discussion

The establishment of multicellular communities attached to solid surfaces is one of the main persistence strategies of bacteria in the environment, and cause serious problems in industrial

281 settings and in medicine. Recent investigations have focused in modifying surface topography
282 as a technic to repel bacterial adhesion and biofilm formation. In this report we demonstrated
283 that DLIP technology can be used to functionalize polymeric surfaces for the inhibition of *S.*
284 *aureus* adhesion to surfaces. We showed that a lamella microtopography generated by DLIP on
285 polystyrene (PS) polymers reduced *S. aureus* adhesion both in static and continuous flow
286 culture conditions and in a foreign-body associated infection model.
287 Up to date, the methods employed for the fabrication of patterned surfaces with antibacterial
288 properties include laser writing, layer by layer self assembly (LBLSA), structural
289 transformation by electrodeposition on patterned substrates (STEPS) and lithographic
290 techniques [29,30]. However, these techniques present some limitations. LBLSA only allows the
291 production of disordered patterns while lithographic techniques require the used of masks that
292 can only be employed for planar surfaces fabricating patterns with relatively large feature sizes
293 [31]. Besides, it is difficult to implement these techniques for the treatment of large surfaces and
294 for the moment they have been used only to prepare surfaces for demonstration purposes. By
295 contrast, DLIP technology has been used to fabricate periodic structures with micrometer and
296 submicrometer patterned topography on large-area of different polymers, metals, ceramics and
297 coatings [23,32]. To evaluate the antiadhesion properties of the surfaces patterned with DLIP
298 technology, we have selected PS surfaces because it is a widely used biomaterial for a range of
299 medical applications, including fabrication of diagnostic instruments, medical devices,
300 implants, disposable laboratory ware and tissue culture components [24,33]. Our results reveal
301 that adhesion of *S. aureus* (0.6 – 1 μm) to the patterned surfaces showed that pillar (PL) and
302 line (LN) microtopographic features in the range between 1 to 5 micrometers increased
303 bacterial adhesion to not only to PS but also to PI and PET biomaterials compared with non-
304 patterned ones (Figure 2 and supplementary Figure 1). These results agree with previous
305 investigations reporting that surface features in the range of bacterium size allowed for
306 maximization of the bacteria–surface contact area, hence increasing cell attachment

[2,3,10,29,34,35] whereas surface with topographic features smaller than the diameter of bacterial cells display a small accessible surface area [4,5,30]. In the former case, bacterial extracellular appendages such as flagella, pili and fimbriae could assume the responsibility for the adhesion to the sub-micrometer features [6-11,36,37]. Because *S. aureus* does not produce extracellular appendages, one would predict that these bacteria would not adhere efficiently to surfaces with submicrometer patterns. However, many *S. aureus* clinical strains depend on surface proteins such as Bap [38], FnbB [39,40], phenol soluble modulins amyloid fibers [41] to adhere and built the biofilm matrix. Thus, it cannot be excluded that some of these proteins can mediate the adhesion to surfaces pattern with topographic features smaller than *S. aureus* size.

When laser parameters similar to those applied to PS of 1.2 mm thickness were applied on the thin PS polymers (125 µm thickness), a complex topography was obtained. In this case, the first irradiation process generates the characteristic 2.0 µm geometry whereas the second irradiation process causes a partial collapsing of the line and creates the perpendicular lamella features. This topography combines line-like patterns of 2.5 µm feature width and periodicities of 6 or 8 µm with lamella-like patterns of approximately 1.0 µm feature width and periodicities of 2 µm. So far, the LA-microtopography has only been obtained in PS polymers, though efforts are being made in order to obtain the same topographic pattern on PI and PET polymers. In contrast to the PL and LN microtopography, the lamella microtopography (LA) strongly inhibited bacterial adhesion. The reasons why lamella microtextured reduced the capacity of *S. aureus* to establish productive contacts with the surface, both under static and flow condition are not well understood. It is possible that the protruded features of the topographical surface could provide a physical obstacle to prevent the expansion of the bacterial clusters. For instance, physical impediment seems to explain the antibacterial properties of the surface microtopographies inspired in the sharkskin Sharklet AF™. This surface comprised of topographic features designed in diamond geometry (2 µm feature width and spacing, 3 µm feature height) is effective at physically disrupting colonization and subsequent biofilm development [42-44].

Multiple studies have examined the effect of surface topography on bacterial adhesion under static conditions. On the contrary, few studies have explored the effect of fluid flow on bacterial attachment on engineered surfaces [13,24,30]. Our results using flow culture bioreactors with a 40 ml h^{-1} flow rate showed that LA microtopography reduced *S. aureus* adhesion under shear stress conditions significantly more effectively than in steady state conditions [25,30]. One explanation for this observation is that the laminar fluid flow on the smooth surface creates random turbulent flow due to the roughness of the micropattern surface that removes more efficiently the bacteria from the surface. This effect would be amplified due to the reduction of the surface area accessible to bacteria in the LA-microtopography.

Once a biomaterial is implanted in a living body, a layer of blood proteins or other human fluids rapidly adsorbs on the surface and may alter the susceptibility of the material to inhibit bacterial adhesion and biofilm formation. Studies performed *in vitro* have shown that the presence of the serum proteins drastically reduce bacterial adhesion to the surface [26,28]. We showed that lamella-like topography reduces *S. aureus* adhesion to PS surfaces in the presence of human serum, though the reduction was less pronounced. Accordingly, experiments with animal models showed that polystyrene surfaces with lamella microtopography reduced *S. aureus* colonization and biofilm formation on PS surfaces after 5 days independently of whether the infection has occurred during the surgical procedure or post-implantation. These results indicated that surface microtopography showed encouraging efficacy to reduce *S. aureus* attachment and biofilm development *in vivo*.

5. Conclusions

In this paper we illustrate that the flexible DLIP technology can be used to develop engineered microtopographies on polystyrene polymers. The resulting microtopographies have a profound impact on *S. aureus* adhesion capacity indicating that surface topography represents a

promising strategy to reduce *S. aureus* attachment and biofilm development on the surface of indwelling medical devices.

However, because our knowledge about the influence of surface microtopography on bacterial adhesion is still largely empirical, it is necessary to experimentally test every pattern to determine its behavior under both *in vitro* and *in vivo* conditions. In our study, regular line and pillar-like patterns enhance *S. aureus* adhesion whereas a irregular lamella microtopography reduces adhesion both in static and continuous flow culture conditions. Moreover, lamella-like textured surfaces inhibit *S. aureus* adhesion in the presence of human serum proteins and when the material is implanted subcutaneously in a foreign-body associated infection model strongly suggesting that polystyrene surfaces composed of lamella-like texture might provide a promising strategy to reduce *S. aureus* adhesion to biomedical surfaces. Ongoing research is necessary to demonstrate that lamella microtopography on the surface of other polymers, such as polyimide and poly(ethylene terephthalate), also inhibits *S. aureus* adhesion.

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References

[1] J. Hasan, R.J. Crawford, E.P. Ivanova, *Trends Biotechnol.* **2013**, *31*, 295.

[2] M.L.W. Knetsch, L.H. Koole, *Polymers* **2011**, *3*, 340.

[3] S.R. Shah, A.M. Tatara, R.N. D'Souza, A.G. Mikos, F.K. Kasper, *Materials Today* **2013**, *16*, 177.

[4] P. Basak, B. Adhikari, I. Banerjee, T.K. Maiti, *J Mater Sci Mater Med* **2009**, *20 Suppl 1*, S213.

[5] I. Francolini, L. D'Ilario, E. Guaglianone, G. Donelli, A. Martinelli, A. Piozzi, *Acta Biomater* **2010**, *6*, 3482.

[6] S. Abban, M. Jakobsen, L. Jespersen, *Food Microbiol.* **2012**, *31*, 139.

[7] R.D. Boyd, J. Verran, M.V. Jones, M. Bhakoo, *Langmuir* **2002**, *18*, 2343.

[8] N. Mitik-Dineva, J. Wang, V.K. Truong, P. Stoddart, F. Malherbe, R.J. Crawford, E.P. Ivanova, *Curr. Microbiol.* **2009**, *58*, 268.

[9] B. Park, V. Nizet, G.Y. Liu, *J Bacteriol* **2008**, *190*, 2275.

[10] K.A. Whitehead, J. Verran, *Food Bioprod Process* **2006**, *84*, 253.

[11] K.A. Whitehead, D. Rogers, J. Colligon, C. Wright, J. Verran, *Colloid Surface B* **2006**, *51*, 44.

[12] A. Biswas, I.S. Bayer, A.S. Biris, T. Wang, E. Dervishi, F. Faupel, *Adv Colloid Interface Sci* **2012**, *170*, 2.

[13] M.V. Graham, A.P. Mosier, T.R. Kiehl, A.E. Kaloyeros, N.C. Cady, *Soft Matter* **2013**, *9*, 6235.

[14] M. Graham, N. Cady, *Coatings* **2014**, *4*, 37.

[15] P. Kim, A.K. Epstein, M. Khan, L.D. Zarzar, D.J. Lipomi, G.M. Whitesides, J. Aizenberg, *Nano Lett.* **2012**, *12*, 527.

[16] K. Manabe, S. Nishizawa, S. Shiratori, *ACS Appl Mater Interfaces* **2013**, *5*, 11900.

[17] M. Bieda, C. Schmädicke, T. Roch, A. Lasagni, *Adv. Eng. Mater.* **2014**, n.

[18] J.W. Costerton, P.S. Stewart, E.P. Greenberg, *Science* **1999**, *284*, 1318.

[19] G. O'Toole, H.B. Kaplan, R. Kolter, *Annu Rev Microbiol* **2000**, *54*, 49.

[20] L. Hall-Stoodley, P. Stoodley, *Curr Opin Biotechnol* **2002**, *13*, 228.

[21] L. Hall-Stoodley, P. Stoodley, *Trends Microbiol* **2005**, *13*, 7.

[22] F. Götz, *Curr Opin Microbiol* **2004**, *7*, 477.

[23] D. Langheinrich, E. Yslas, M. Broglia, V. Rivarola, D. Acevedo, A. Lasagni, *J. Polym. Sci. B Polym. Phys.* **2011**, *50*, 415.

[24] J. Valle, A. Toledo-Arana, C. Berasain, J.-M. Ghigo, B. Amorena, J.R. Penadés, I. Lasa, *Mol Microbiol* **2003**, *48*, 1075.

[25] J.M. Ghigo, *Nature* **2001**, *412*, 442.

[26] D. Campoccia, L. Montanaro, H. Agheli, D.S. Sutherland, V. Pirini, M.E. Donati, C.R. Arciola, *Int J Artif Organs* **2006**, *29*, 622.

[27] T.J. Foster, J.A. Geoghegan, V.K. Ganesh, M. Höök, *Nat Rev Micro* **2014**, *12*, 49.

[28] J.D. Patel, M. Ebert, R. Ward, J.M. Anderson, *J Biomed Mater Res Part A* **2007**, *80*, 742.

[29] K.A. Whitehead, J. Colligon, J. Verran, *Colloid Surface B* **2005**, *41*, 129.

[30] L.-C. Xu, C.A. Siedlecki, *Acta Biomater* **2012**, *8*, 72.

[31] A. Komaromy, R.I. Boysen, H. Zhang, I. McKinnon, F. Fulga, M.T.W. Hearn, D.V. Nicolau, *Microelectron Eng* **2009**, *86*, 1431.

[32] A.S.F. Lasagni, D. Langheinrich, S. Eckhardt **2012**, Plastic Research Online.

[33] K. Modjarrad, S. Ebnesajjad *Handbook of Polymer Applications in Medicine and Medical Devices*, Amsterdam : Elsevier/William Andrew, **2013**

[34] M. Katsikogianni, Y.F. Missirlis, *Eur Cell Mater* **2004**, *8*, 37.

[35] A.Z. Komaromy, S. Li, H. Zhang, D.V. Nicolau, R.I. Boysen, M.T.W. Hearn, *Microelectron Eng* **2012**, *91*, 39.

[36] L.C. Hsu, J. Fang, D.A. Borca-Tasciuc, R.W. Worobo, C.I. Moraru, *Appl Environ Microbiol* **2013**, *79*, 2703.
[37] R.S. Friedlander, H. Vlamakis, P. Kim, M. Khan, R. Kolter, J. Aizenberg, *Proc Nat Acad Sci USA* **2013**.
[38] C. Cucarella, C. Solano, J. Valle, B. Amorena, I. Lasa, J.R. Penadés, *J Bacteriol* **2001**, *183*, 2888.
[39] M. Vergara-Irigaray, J. Valle, N. Merino, C. Latasa, B. García, I. Ruiz de Los Mozos, C. Solano, A. Toledo-Arana, J.R. Penadés, I. Lasa, *Infect and Immun* **2009**, *77*, 3978.
[40] E. O'Neill, C. Pozzi, P. Houston, H. Humphreys, D.A. Robinson, A. Loughman, T.J. Foster, J.P. O'Gara, *J Bacteriol* **2008**, *190*, 3835.
[41] K. Schwartz, A.K. Syed, R.E. Stephenson, A.H. Rickard, B.R. Boles, *PLoS Pathog* **2012**, *8*, e1002744.
[42] K.K. Chung, J.F. Schumacher, E.M. Sampson, R.A. Burne, P.J. Antonelli, A.B. Brennan, *Biointerphases* **2007**, *2*, 89.
[43] J.F. Schumacher, M.L. Carman, T.G. Estes, A.W. Feinberg, L.H. Wilson, M.E. Callow, J.A. Callow, J.A. Finlay, A.B. Brennan, *Biofouling* **2007**, *23*, 55.
[44] J.F. Schumacher, C.J. Long, M.E. Callow, J.A. Finlay, J.A. Callow, A.B. Brennan, *Langmuir* **2008**, *24*, 4931.

Table 1. Description of polymeric materials modified by DLIP

Polymeric material	Λ = period (μm)	Topography	F = Fluence (J/cm^2)
PS	5	Line (LN)	0.5
PS	5	Pillar (PL)	0.5
PS	3	Line (LN)	0.5
PS	3	Pillar (PL)	0.5
PS	1	Line (LN)	0.5
PS	1	Pillar (PL)	0.5
PS	5	Lamella (LA)	0.5
PS	2	Lamella (LA)	0.5
PS	-	Non-patterned (CT)	-

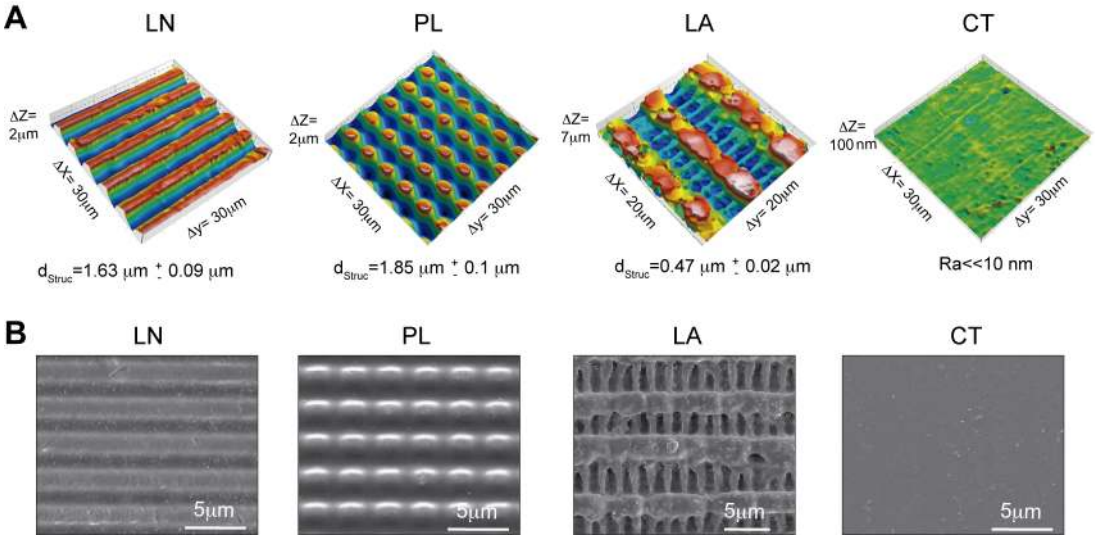

Figure 1: Images from confocal (A) and scanning electron microscopy (B) of PS polymeric surfaces structured by Direct Laser Interference Patterning technique. Periodic arrays of line-like (LN, Λ = 5 μm), pillar-like (PL, Λ = 5 μm), lamella-like (LA, Λ = 2 μm) structures and non-modified surfaces (CT). The laser fluence was kept constant at 0.5 J cm^{-2}.

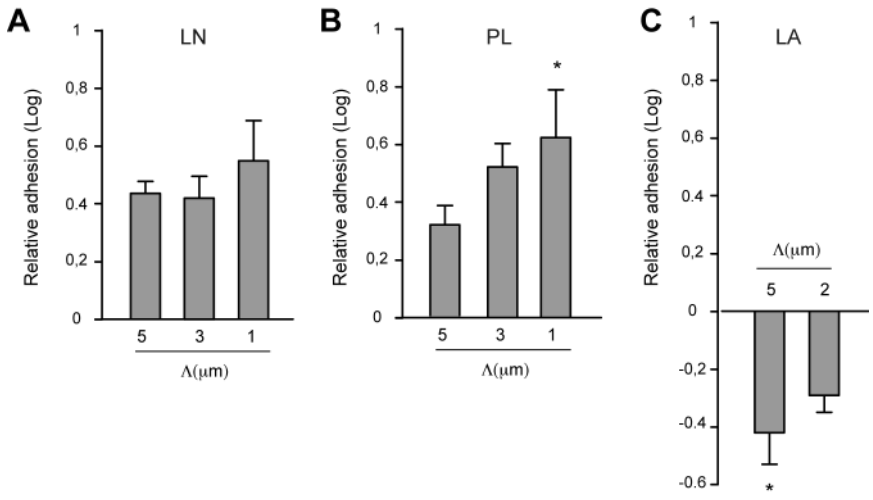

Figure 2. Bacterial adhesion on patterned surfaces under static conditions. Relative adhesion of *S. aureus* on PS patterned surfaces with line (LN) (A), pillar (PL) (B), and lamella-like (LA) (C) structures and with spatial periods (Λ) of 1, 2, 3 and 5 μm. Relative adhesion was calculated as bacterial counts CFU on patterned surfaces / CFU on non-patterned surfaces. Multiple comparisons were performed by one-way analysis of variance combined with the Bonferroni multiple comparison test (GraphPad Instat, version 5). Asterisk indicates significant adhesion differences (*, $P<0.05$ [significant]). ns, non significant differences.

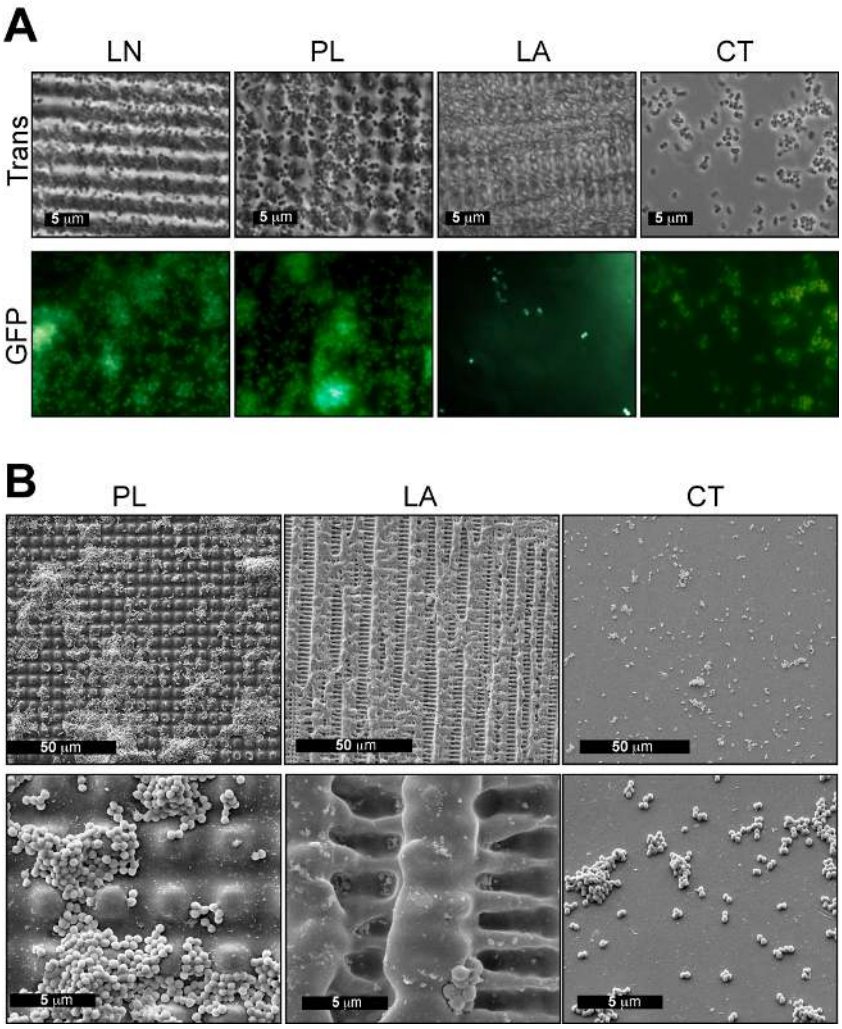

Figure 3: Qualitative evaluation of *S. aureus* attachment to patterned PS wafers. A) Fluorescence microscopic images of *S. aureus* 15981-GFP attached to PL (Λ=5 µm), LN (Λ=5 µm), LA (Λ=2 µm), and non-patterned PS surfaces (CT), showing that bacteria respond to the surface topography. B) Scanning electron micrographs of *S. aureus* cells attached to PL, LA and non-patterned PS surfaces (CT).

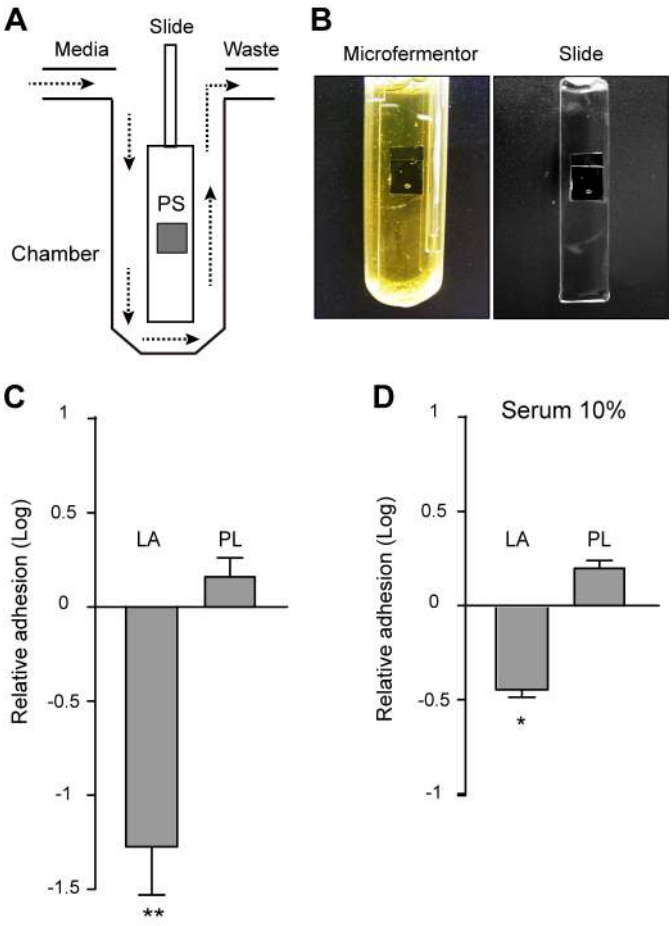

Figure 4: Bacterial adhesion on patterned surfaces in microfermenters. Schematic diagram (A) and photograph image taken from the microfermeters (B). Substrates of 1x1 cm^2 were fixed on the glass slide. Microfermenters were inoculated with *S. aureus* 15981 (OD_{600nm}=1). After 6 hours of incubation, substrates were removed from the glass slide and quantification of adhered *S. aureus* cells was performed by CFU counting of the bacteria removed from the tested surfaces. Graphs show the relative adhesion of *S. aureus* on LA (Λ=2 µm), PL (Λ=5 µm) and non-patterned (CT) PS surfaces under flow culture conditions in the absence (C) or presence of human serum (D). Relative adhesion was calculated as bacterial counts CFU on patterned surfaces / CFU on non-patterned surfaces. Comparisons were performed by one-way analysis of variance combined with the Bonferroni multiple comparison test or Mann-Whitney test (GraphPad Instat, version 5)

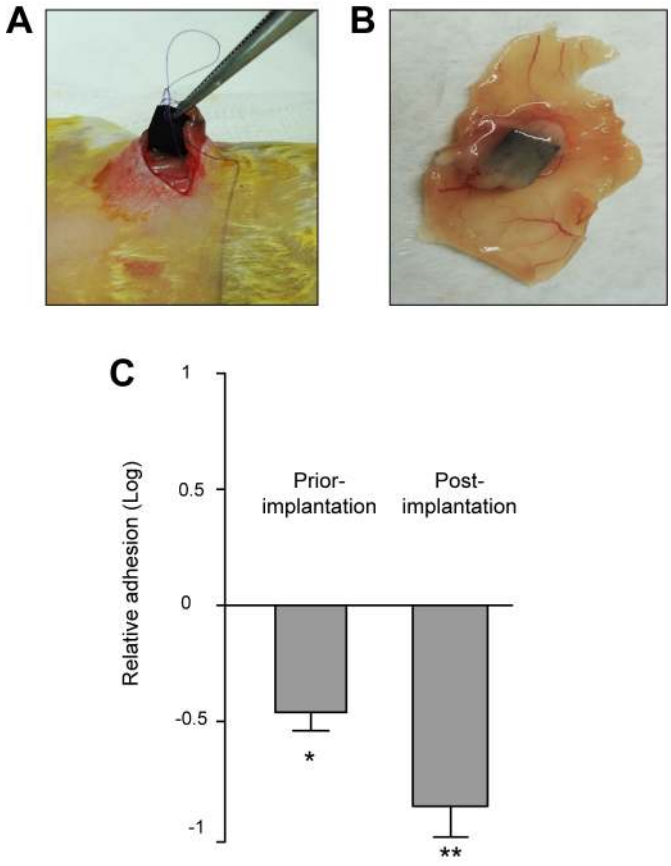

Figure 5: Biofilm formation of *S. aureus* on lamella-like patterned surfaces using an *in vivo* model. A) Implantation of substrates in the intraperitoneal cavity of CD1 mice. B) Biofilm-infected surfaces after 5 days of infection. C) Contamination of PS polymers prior-implantation: PS lamella-like (Λ=2 μm) and control surfaces were first coated with 10^4 cfu of *S. aureus* 15981 and then fixed at the abdominal wall. Contamination of PS polymers post-implantation: PS polymers were first fixed at the abdominal wall before infection. Two day after implantation a 10^8 cfu of *S. aureus* 15981 were injected intraperitoneally at the site of the implant. After 5 days, animals (n=6) of both types of infections were sacrificed and substrates were extracted and placed in 1 ml of PBS. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci.